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1 **Inter and intra-species transfer of a *Clostridium difficile* conjugative transposon**  
2 **conferring resistance to MLS<sub>B</sub>**

3

4

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23 **Running title:** Characterisation of a Tn6194-like transposon

24

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26 **ABSTRACT**

27 Resistance to MLS<sub>B</sub> in *Clostridium difficile* is generally due to *erm*(B) genes. Tn6194, a  
28 conjugative transposon initially detected in PCR-ribotype 027 isolates is an *erm*(B)-  
29 containing element also detected in other relevant *Clostridium difficile* PCR-ribotypes. In  
30 this study, the genome of a *C. difficile* PCR-ribotype 001 strain was sequenced and an  
31 element with two nucleotidic changes compared to Tn6194 was detected. This element  
32 was transferred by filter-mating assays to recipient strains of *C. difficile* belonging to PCR-  
33 ribotype 009 and 027, and to a recipient strain of *Enterococcus faecalis*. Transconjugants  
34 were characterised by Southern blotting and genome sequencing, and integration sites in  
35 all transconjugants were identified by sspPCR. The element integrated the genome of *C.*  
36 *difficile* at different sites and the genome of *E. faecalis* at a unique site. This study is the  
37 first molecular characterisation of a *erm*(B)-containing conjugative transposon in *C.*  
38 *difficile*, and provide an additional evidence of the antibiotic resistance's transmission risk  
39 between pathogenic bacteria occupying the same human intestinal niche.

## 40 INTRODUCTION

41 Resistance to the macrolide-lincosamide-streptogramin B (MLS<sub>B</sub>) group of antibiotics is the  
42 most common phenotype in clinical isolates of *C. difficile* worldwide, and is generally  
43 conferred by *erm*(B) genes encoding 23S rRNA methylases (Huang *et al.*, 2009). These  
44 genes are located on mobile elements showing an important heterogeneity in their genetic  
45 organisation (Farrow *et al.*, 2001; Spigaglia *et al.*, 2005; Spigaglia *et al.*, 2011). The best  
46 known of them is Tn5398 from *C. difficile* 630, a mobilizable non-conjugative element  
47 containing two *erm*(B) copies (Farrow *et al.*, 2001).

48 A putative conjugative transposon belonging to the Tn916-like family of transposons and  
49 carrying an *erm*(B) gene has been recently detected in *C. difficile* 2007855, a strain  
50 belonging to the hyper-virulent PCR-ribotype 027, and designated Tn6194 (He *et al.*,  
51 2010; He *et al.*, 2012). Conjugative transposons related to Tn916 were originally detected  
52 in *Enterococcus faecalis*, which inhabits the same niche of *C. difficile*. Both species are  
53 responsible for nosocomial infections, and horizontal gene transfer between those two  
54 species has been previously proven (Jasni *et al.*, 2010).

55 A recent whole-genome sequencing analysis of *C. difficile* 027/BI/NAP1 isolates  
56 demonstrated that Tn6194 was present in most of the strains belonging to the FQR1  
57 lineage, isolated in the United States and in Asia (He *et al.*, 2012). Moreover, recent  
58 studies showed that this transposon is the most frequently found *erm*(B)-containing  
59 element in different epidemic PCR-ribotypes circulating in European hospitals (Spigaglia *et*  
60 *al.*, 2011; Wasels *et al.*, 2013), and a transposon almost identical has been detected in the  
61 PCR-ribotype 017 strain M68 genome (Brouwer *et al.*, 2012)..

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

## 65 **MATERIALS AND METHODS**

### 66 **Bacterial strains, culture conditions and antibiotic susceptibility**

67 All strains used in this study, listed in Table 1, were grown on Brain Heart Infusion (BHI)  
68 agar plates or in BHI broth (Oxoid Ltd, Basingstoke, UK) supplemented with 0.5% yeast  
69 extract and 0.1% L-cysteine (BHIS). *C. difficile* was grown at 35°C in anaerobic conditions  
70 (85% N<sub>2</sub>, 10% H<sub>2</sub>, 5% CO<sub>2</sub>) and *E. faecalis* at 37°C in aerobic conditions. Erythromycin  
71 and rifampicin were used at a final concentration of 20 mg/L.

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

78

### 79 **Filter-mating assays**

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

86

### 87 **Genomic DNA extraction**

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

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

92

### 93 **Analysis of *C. difficile* CII7 transposon insertion sites**

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

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

103

### 104 **Genome sequencing**

105 Single-end multiplex libraries were created with multiplexed protocol according to the  
106 manufacturer's specification; the sequencing was performed on the Illumina HiSeq 2000  
107 platform. The read length was 110-bp for both *C. difficile* CII7 and *C. difficile*  
108 CII7xCD37(A). Then reads were assembled de novo into contigs using Velvet ([Zerbino &](#)  
109 [Birney, 2008](#)) (Options: Kmer : 71 and 75 respectively for CII7 and CII7xCD37(A) ; -short ;  
110 -fastq ; -unused\_reads ; -read\_trkg). Finally contigs (minimum size, 500 bases) were  
111 reorganized and oriented based on alignment to the finished genome of *C. difficile* strain  
112 630 (CD630) ([Sebahia et al., 2006](#)). Contigs that did not match CD630 were localized at  
113 the end to obtain each genome scaffold. The short reads genomic data have been  
114 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 Tn6194-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.

## 120 RESULTS

121 The entire genome of the PCR-ribotype 001 *C. difficile* strain CII7 that has been previously  
122 shown to contain an element highly similar to Tn6194 was sequenced, and a putative  
123 conjugative transposon of 28014 bp in length with two nucleotide changes compared to  
124 Tn6194 was detected (Fig. 1). Insertion of a guanine at position 7076 (between positions  
125 3152104 and 3152105 in *C. difficile* 2007855; GenBank accession number FN665654)  
126 determines a 207nt-elongation of the CDS coding for a replication initiation factor  
127 homologous to *orf20* of Tn916. The same insertion is also present in the transposon from  
128 the *C. difficile* M68 strain (GenBank accession number FN668375). The replacement of a  
129 guanine by a thymine at position 9691 (positions 3149490 in *C. difficile* 2007855) causes  
130 the substitution of a methionine by an isoleucine within the sequence of a protein with  
131 unknown function. The transposon is located between orthologs of *C. difficile* 630  
132 *CD630\_31060* and *CD630\_31070* in *C. difficile* CII7, while it is integrated within the coding  
133 sequences of the ortholog of *CD630\_28310* and *CD630\_33170* in strains 2007855 and  
134 M68, respectively.

135  
136 Filter-mating assays were carried out to transfer the transposon from CII7 to *C. difficile*  
137 CD37 (MICs = 0.75 mg/L for erythromycin and 3.0 mg/L for clindamycin) and to CD196R,  
138 a rifampicin-resistant derivative of *C. difficile* CD196 (MICs = 1.0 mg/L for erythromycin  
139 and 2.0 mg/L for clindamycin). In this study, transfer was achieved at a frequency of  $1.01 \pm$   
140  $0.36 \times 10^{-8}$  and  $1.43 \pm 0.87 \times 10^{-8}$  per donor for CD37 and CD196R, respectively.  
141 Transconjugants were selected from independent experiments to exclude the possibility of  
142 analysing siblings. Filter-mating assays were also performed using *C. difficile* CII7 or each  
143 one of the four transconjugants CII7xCD37 as donor, and the MLS<sub>B</sub>-susceptible *E. faecalis*  
144 JH2-2 strain as recipient (MICs = 0.38 mg/L for erythromycin and 8.0 mg/L for  
145 clindamycin). Transfer was achieved at a frequency of  $1.74 \pm 1.91 \times 10^{-6}$  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  $\geq$  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<sup>Arg</sup> gene in transconjugants CII7xCD37c and  
169 CII7xCD196Rb. In all *E. faecalis* transconjugants, the transposon was always located  
170 within a copy of the tRNA<sup>Arg</sup> 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

172 number NC\_004668 and CP002621, respectively), its genome contains several copies of  
173 the tRNA<sup>Arg</sup> gene. Interestingly, the element always integrated the same locus (Fig. 2). In  
174 CII7 and in all transconjugants obtained in this study, the transposon was able to excise  
175 from the genome and form a circular intermediate, restoring the original target site (data  
176 not shown).  
177

178 **DISCUSSION**

179 Tn6194 is an *erm*(B)-containing conjugative transposon recently detected in strains  
180 belonging to different PCR-ribotypes, worldwide (He *et al.*, 2010; Spigaglia *et al.*, 2011; He  
181 *et al.*, 2012; Wasels *et al.*, 2013). In this study, the capacity of this element to be  
182 transferred to other strains of *C. difficile* as well as to *E. faecalis* was investigated. While  
183 the conjugative transposon Tn5397 from *C. difficile* 630, which confer resistance to  
184 tetracycline, has been successfully transferred to *E. faecalis* JH2-2 (Jasni *et al.*, 2010), this  
185 is the first evidence of transfer of MLS<sub>B</sub> resistance between these two species, at our  
186 knowledge.

187 There are clear differences of behaviour between conjugative transposons, most likely due  
188 to their respective recombination modules and to the recipient strain used. While Tn916  
189 has a highly preferred insertion site in *C. difficile* CD37, it can integrate the genome of *C.*  
190 *difficile* 630Δ*erm* and R20291 or *E. faecalis* JH2-2 at different sites (Mullany *et al.*, 2012;  
191 Wang *et al.*, 2000). Differently, Tn5397, which contains the *tndX* gene encoding a serine  
192 recombinase, has a preferred integration site in *C. difficile* CD37, two in *C. difficile*  
193 R20291, one in *E. faecalis* JH2-2 and enters the genome of *Bacillus subtilis* CU2189 at  
194 different sites (Jasni *et al.*, 2010; Mullany *et al.*, 1990). Like Tn916, Tn6194 contains  
195 genes encoding for putative tyrosine integrase and excisionase (Brouwer *et al.*, 2012). Our  
196 results suggest that there are a limited number of integration sites of this transposon within  
197 the genome of *C. difficile* CD37 and CD196, and a highly preferential integration site in *E.*  
198 *faecalis* JH2-2.

199 Elements containing *erm*(B) genes show an important heterogeneity in their genetic  
200 structure. Although it has been extensively studied, Tn5398 from *C. difficile* 630 has been  
201 mainly detected in PCR-ribotype 012 isolates. Unlike this mobilizable non-conjugative  
202 element, Tn6194 contains all modules required for its transfer between cells. These  
203 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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214

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

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

<sup>a</sup>in brackets, number of replicates

<sup>b</sup>Ery<sup>S</sup>: erythromycin susceptible; Ery<sup>R</sup>: erythromycin resistant; Cli<sup>S</sup>: clindamycin susceptible; Cli<sup>R</sup>: clindamycin resistant Rif<sup>S</sup>: rifampicin susceptible; Rif<sup>R</sup>: rifampicin resistant.

320

321 **Table 2.** Insertion sites of the Tn6194 and Tn6194-like elements in wild-type strains and transconjugants analysed in this study

Strains	Transposon localisation <sup>a</sup>	Insertion site	
<b><i>C. difficile</i></b>			
2007855	putative adhesin CDS ( <i>CD630_28310</i> )	TGTATCTTATGCTTCAGAATTAGG <b>AG</b> AGAATAGTCAGATTCAAAGTGGTT	
M68	<i>fdhF</i> ( <i>CD630_33170</i> )	TTCATCATCTTCAACTTCCATAGG <b>AG</b> GTCTCCATTGAGCAGCGAACAAGT	
CII7	5'UTR of a transcriptional antiterminator ( <i>CD630_31070</i> )	AATAAATAGTAACTAAGATTTAGG <b>AG</b> GGTTTGCATTGAACAAAAGATTAT	
<b>Transconjugants</b>			
CII7xCD37	a	23S rRNA gene	GAGGAGAGTATCCTAAGGCCAGCG <b>AG</b> AGAACTGTTGTTAAGGAACTCGGC
	b	5'UTR of <i>hfQ</i> ( <i>CD630_19740</i> )	TTAGAAGTTCAATTTAATCTTGGG <b>AG</b> GGTACAAATCTAATGAAAAATACA
	c	tRNA <sup>Arg</sup> gene	CGAACCCCGGACACACGCCTTAGA <b>AG</b> GCGTTGCTCTATCCAGCTGAGCT
	d	5'UTR of a transcriptional antiterminator ( <i>CD630_31070</i> )	AATAAATAGTAACTAAGATTTAGG <b>AG</b> GGTTTGCATTGAACAAAAGATTAT
CII7xCD196R	a	<i>eutL</i> ( <i>CD630_19150</i> )	TTGACGAAGCAACTAAAGCTTCAG <b>AG</b> GTTGATGTTGTATATGCAAAATCA
	b	tRNA <sup>Arg</sup> gene	CGAACCCCGGACACACGCCTTAGA <b>AG</b> GCGTTGCTCTATCCAGCTGAGCT
	c	putative adhesin CDS ( <i>CD630_28310</i> )	TGTATCTTATGCTTCAGAATTAGG <b>AG</b> AGAATAGTCAGATTCAAAGTGGTT
	d	23S rRNA gene	GGGAAAGGTGAAAAGAACCCCGGG <b>AG</b> GGGAGTGAAATAGAACCTGAAACC
<b><i>E. faecalis</i></b>			
All transconjugants	tRNA <sup>Arg</sup> gene	TGAACCAGCGACCTACCGCTTAGG <b>AG</b> GCGTTGCTCTATCCTACTGAGCT	

<sup>a</sup> orthologous CDS in *C. difficile* 630 are indicated in brackets.

**Figure 1:** Tn6194-like element from *C. difficile* CII7.

Grey boxes show homology with Tn916 from *E. faecalis* DS16 and Tn5398 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.