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Clostridium difficile erm(B)-containing elements and the burden on the *in vitro* fitness

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In *Clostridium difficile*, resistance to the macrolide-lincosamide-streptogramin B group of antibiotics generally relies on *erm*(B) genes. In this study, we investigated elements with a genetic organization different from Tn*5398*, the mobilizable non-conjugative element identified in *C. difficile* strain 630. Our results suggested that the elements most frequently found in strains isolated during the European surveillance study in 2005 were related to Tn*6194*, the conjugative transposon recently detected in different *C. difficile* types, including PCR-ribotype 027. We characterized a Tn*6194*-like and a novel element rarely found in clinical isolates. A burden on the *in vitro* fitness of *C. difficile* was observed after the acquisition of these elements as well as of Tn*5398*.

INTRODUCTION

Clostridium difficile is recognized as the leading cause of hospital-acquired diarrhoea. Development of *C. difficile* infection (CDI) is a consequence of antibiotic treatment. Historically, the majority of the epidemic isolates were resistant to the macrolide-lincosamide-streptogramin (MLS_B) family. Resistance to erythromycin and/or clindamycin is still the most common phenotype in *C. difficile* strains isolated in Europe (Huang *et al.*, 2009).

Resistance to MLS_B in this pathogen is usually conferred by *erm*(B) genes. In *C. difficile* 630, the mobilizable non-conjugative element Tn*5398* contains two copies of the gene (Farrow *et al.*, 2001). However, *erm*(B) genes are located on elements different from Tn*5398* in the majority of clinical isolates. In total, 17 different genetic organizations of such elements have been identified so far (Farrow *et al.*, 2001; Spigaglia *et al.*, 2005, 2011). Their genetic organizations were investigated using a PCR-mapping assay based on the sequence of Tn*5398* and named from E1 to E17 (Spigaglia *et al.*, 2005, 2011). In particular, 14 different organizations were identified in 31 clinical strains isolated during the first European surveillance study conducted in 2005 (Barbut *et al.*, 2007; Spigaglia *et al.*, 2011). The most frequent organization detected was E4 (29 %), whereas 9.7 % of the strains contained an element E1, corresponding to Tn*5398*.

In this study, we demonstrate that the *erm*(B)-containing elements with an organization E4 are related to Tn*6194*, a conjugative transposon recently identified in *C. difficile* 2007855, and previously referred as CTn*CD11* (He *et al.*, 2010, 2013). We characterized a Tn*6194*-like transposon and another element, showing the genetic organization E3 that is rarely detected among clinical isolates. The effect of the acquisition of these elements as well as of Tn*5398* on *C. difficile* fitness was investigated.

METHODS

Bacterial strains, culture conditions and antibiotic susceptibility. All strains used in this study are listed in Table 1. The recipient strain *C. difficile* CD13 is a clinical isolate collected in 1993 at the University Hospital of Parma, Italy. *C. difficile* was grown on brain heart infusion (BHI) agar plates or in BHI broth (Oxoid Ltd) at 35 °C in anaerobic conditions (85 % N_2, 10 % H_2, 5 % CO_2). *Escherichia coli* was grown on Luria–Bertani (LB) agar plates or in LB broth at 37 °C (Oxoid Ltd).

Minimal inhibitory concentrations (MICs) to erythromycin, clindamycin and rifampicin were determined using the Etest, as already described (Spigaglia *et al.*, 2011). The breakpoints used for erythromycin and clindamycin were 8 μg ml^{-1}, in accordance with the guidelines established by the Clinical and Laboratory Standards Institute (CLSI, 2007). The breakpoint for rifampicin was 4 mg l^{-1}, in accordance with the CLSI interpretive categories approved for *Staphylococcus aureus*, since no values are provided for anaerobes (CLSI, 2008).

Cloning in *E. coli* and DNA sequencing. Overnight-digested chromosomal DNA was ligated within digested and dephosphorylated plasmid vector pUC19 (Invitrogen). *E. coli* transformants were selected on LB agar supplemented with ampicillin (100 μg ml^{-1})

Abbreviations: BHI, brain heart infusion; CDI, *C. difficile* infection.

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of *C. difficile* F17 and CII7 *erm*(B) regions in this paper are HF678445 and HF678446, respectively.

Three supplementary tables are available with the online version of this paper.

Table 1. Bacterial strains used in this study

Bacterial strain	Relevant characteristics*	PCR-ribotype	Source	Reference
E. coli				
α-select competent cells	F$^-$ *deo*R *end*A1 *rec*A1 *rel*A1 *gyr*A96 *hsd*R17(r_k^-, m_k^+) *sup*E44 *thi*-1 *pho*A Ä (*lac*ZYA-*arg*F) U169 Ö80*lac*ZÄM15 ë$^-$		Bioline	
C. difficile				
630	Em^R (E1) Rif^S	012	Switzerland	Wüst *et al.* (1982)
F17	Em^R (E3) Rif^S	010	Italy	Spigaglia *et al.* (2002)
CII7	Em^R (E4) Rif^S	001	Germany	Barbut *et al.* (2007)
KI11	Em^R (E4) Rif^R	001	Spain	Barbut *et al.* (2007)
KI16	Em^R (E4) Rif^R	020	Spain	Barbut *et al.* (2007)
EI13	Em^R (E4) Rif^S	071	Hungary	Barbut *et al.* (2007)
EII4	Em^R (E4) Rif^S	002	Hungary	Barbut *et al.* (2007)
DI5	Em^R (E4) Rif^S	068	Ireland	Barbut *et al.* (2007)
DII2	Em^R (E4) Rif^S	055	Ireland	Barbut *et al.* (2007)
DI4	Em^R (E4) Rif^S	017	Ireland	Barbut *et al.* (2007)
PIII2	Em^R (E4) Rif^R	017	Greece	Barbut *et al.* (2007)
CD37	Em^S Rif^R	009	VA, USA	Smith *et al.* (1981)
CD13	Em^S Rif^R	039	Italy	This study

*Em^S: erythromycin-susceptible; Em^R: erythromycin-resistant; E1–E4, genetic organization of the *erm*(B)-containing element; Rif^S: rifampicin-susceptible; Rif^R: rifampicin-resistant.

and erythromycin (150 μg ml^{-1}). A primer walking strategy was employed to sequence the inserts using the primers listed in Table S1 (available in JMM Online).

PCR-mapping assay. Strains carrying an *erm*(B)-containing element with a genetic organization E4 were analysed by PCR mapping using overlapping primers designed on the Tn*6194* sequence of *C. difficile* strain 2007855 (accession number FN665654). A total of 13 PCRs, detailed in Table S2, were used to amplify products overlapping for more than 26 kb, internal to the putative conjugative transposon. PCR conditions consisted of an initial denaturation step of 5 min at 94 °C followed by 30 cycles of 30s at 94 °C, 30s at 52 °C, 2 min at 72 °C, and a final elongation step of 10 min at 72 °C.

Transfer of *erm*B determinants. Filter-mating experiments were performed as already described (Farrow *et al.*, 2001). Transconjugants were confirmed by PCR-ribotyping (Bidet *et al.*, 1999) and PCR-detection of *erm*(B) with primers E5 and E6 (Spigaglia & Mastrantonio, 2004). Primers used to detect cotransfer of conjugative transposons from 630 are shown in Table S3.

Pulsed field gel electrophoresis (PFGE). PFGE was performed as previously described (Spigaglia *et al.*, 2001) with the following modifications. After digestion with *Pst*I (Roche Applied Science) overnight at 37 °C, PFGE was performed with a CHEF-Mapper apparatus (Bio-Rad Laboratories) at 6V cm^{-1} for 18 h at 14 °C with an included angle of 120° and linear ramping from 1 to 12 s. When plugs were digested with I-*Ceu*I (NEB), PFGE was run at 6 V cm^{-1} for 14h with an included angle of 120° and linear ramping from 5 to 70 s.

Southern blot hybridization. Digested DNA was transferred to a Hybond N+ membrane (Amersham Biosciences) by Southern blotting. The *erm*(B) internal probe of 268 bp was obtained by PCR using the primers 3140 and E5 (Farrow *et al.*, 2001; Spigaglia *et al.*, 2005). The 23S rRNA probe was obtained by PCR using the primers 6F and 8R (Schmidt *et al.*, 2007). Labelling of the probes, hybridization to the membrane and signal detection were carried out as described in the ECL Direct Nucleic Acid Labelling and Detection System (Amersham Biosciences).

Determination of bacterial fitness *in vitro*. Bacterial fitness was estimated comparing growth rates of resistant and susceptible isogenic strains. Bacterial cultures of 18 h were diluted at OD_{600} 0.05 in 25 ml of BHI. Absorbance was measured every hour for 12 h at OD_{600}. Each growth rate was determined at the beginning of the exponential phase, and the mean values were calculated from at least three independent experiments. Relative growth rates were calculated as the ratio of the growth rates of the resistant versus susceptible strains.

For growth competition assays, susceptible and resistant strains were grown separately in BHI for 24 h, mixed in a 1 : 1 ratio, and diluted 250-fold in 25 ml of BHI. One hundred microlitres were transferred to fresh 25 ml broth every 24 h over three cycles. The number of viable cells was determined at the end of every cycle by plating aliquots on BHI agar plates containing 20 μg erythromycin ml^{-1} or without antibiotic. The competition index (CI) was calculated as the c.f.u. ratio of the resistant and susceptible strains at time t_1 divided by the same ratio at time t_0, and the selection coefficient s was calculated as $s=\ln(CI)/[t \times \ln(2)]$, where t is the number of generations (Foucault *et al.*, 2010). Fitness of the susceptible strain was set to 1, and the relative fitness of the resistant transconjugants was determined as $1+s$.

Statistical analyses. Statistical analyses were performed using GraphPad software.

RESULTS AND DISCUSSION

Characterization of two erm(B)-containing elements

A 7.3 kb *Hin*dIII fragment from CII7 and a 7.3 kb *Nde*I fragment from F17 were cloned in pUC19. *C. difficile*

strains CII7 and F17 contain elements with genetic organization E4 and E3, respectively. A schematic representation of the two cloned fragments and their homology with sequences already deposited in GenBank is shown in Fig. 1.

A region of 6.5 kb of the *Hin*dIII fragment from strain CII7 showed 100 % identity with the 3′ end of Tn*6194* from *C. difficile* 2007855 (GenBank accession number FN665654, position: 3131167–3137705) (He *et al.*, 2010, 2013). Furthermore, 3.2 kb of the fragment, containing the 3′ end of the CII7 Tn*6194*-like element and a partial CDS homologous to *CD630_31070* showed 100 % identity with a region of the genome of *C. difficile* BI-9 (GenBank accession number FN668944, position 3292473–3295633).

Recently, a Tn*6194*-like genetic element has also been identified in *C. difficile* M68 (GenBank accession number FN668375) (Brouwer *et al.*, 2012). To determine the ends of Tn*6194* and its insertion site in strains 2007855, M68, BI-9 and 630 (GenBank accession number AM180355), a genome comparison was performed. We found that this conjugative transposon is located within a gene homologous to *CD630_28310* and *CD630_33170* in strains 2007855 and M68, respectively. Instead, sequence analysis indicates that in BI-9 the ends of a Tn*6194* element are located between *CD630_31060* and *CD630_31070*. The same localization of Tn*6194* found in BI-9 was also observed in CII7. Since both BI-9 and CII7 are PCR-ribotype 001, Tn*6194* seems to integrate into the same genomic region in strains belonging to the same PCR-ribotype.

PCR mapping demonstrated that the elements with a genetic organization E4 were Tn*6194*-like (data not shown), suggesting that these elements are widely distributed among European *C. difficile* clinical isolates.

About 5.2 kb of the 7.3 kb *Nde*I fragment from strain F17, including *erm*(B), showed 99.9 % identity with part of the plasmid pAMβ1 from *E. faecalis* DS5 (GenBank accession number GU128949.1) (Fig. 1). Since reciprocal genetic exchanges between *C. difficile* and *E. faecalis* can occur *in vitro* (Jasni *et al.*, 2010), it is possible that the mosaic element of F17 has been originated by a partial integration of the plasmid pAMβ1 into the genome of *C. difficile*.

Transfer of *erm*B determinants

Conjugation assays were performed to determine if transfer of elements from strain CII7 and F17 to the MLS$_B$-susceptible recipient strains CD37 and CD13 was possible.

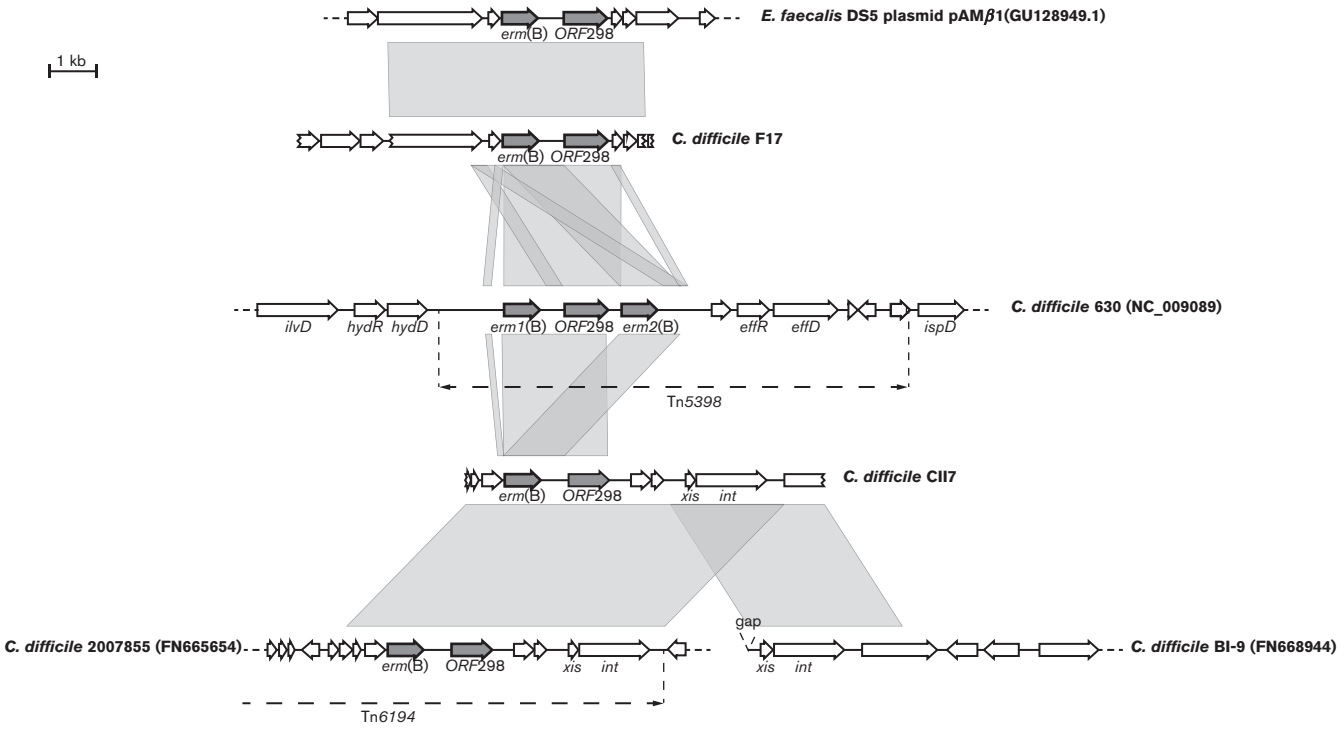

Fig. 1. Organization of *erm*(B)-containing elements in the strains analysed in this study. Top to bottom and left to right: position 3244 to 10 796 from pAMβ1; *erm*(B)-containing *Nde*I fragment from *C. difficile* F17; position 2 325 159 to 2 310 625 from *C. difficile* 630; *erm*(B)-containing *Hin*dIII fragment from CII7; position 3 139 326 to 3 131 167 from *C. difficile* 2007855; position 3 292 473 to 3 299 673 from *C. difficile* BI-9. Accession numbers of deposited sequences are shown within parentheses. Tn*5398* and part of Tn*6194* are indicated on sequences of 630 and 2007855, respectively. Grey boxes indicate regions of homology. Homologous genes found in all sequences are shown in grey.

Table 2. Characterization of transconjugants obtained in this study

Transconjugants (donor x recipient)	Average transfer frequency per donor (SD)*	MIC (μg ml^{-1})†		Progeny saved for this study (no. of independent transconjugants)
		EM	CM	
630xCD13	2.27×10^{-8} (0.31)	⩾256	⩾256	630xCD13 A, B and C (3)
630xCD37	1.15×10^{-8} (0.11)	⩾256	⩾256	630xCD37 A, B and C (3)
CII7xCD13	7.91×10^{-9} (6.60)	⩾256	⩾256	CII7xCD13 A, B and C (3)
CII7xCD37	1.10×10^{-8} (0.42)	⩾256	⩾256	CII7xCD37 A, B and C (3)
F17xCD13	1.06×10^{-9} (0.13)	⩾256	⩾256	F17xCD13 A and B (2)
F17xCD37	4.50×10^{-9} (1.24)	⩾256	⩾256	F17xCD37 A, B and C (3)

*SD, Standard deviation.
†EM, Erythromycin; CM, clindamycin.

Assays were also performed to transfer Tn*5398* from 630 to the same recipient strains.

All elements were successfully transferred to both CD37 and CD13 (Table 2). All transconjugants were obtained from independent filter-mating assays to exclude the possibility of analysing siblings. Three independent transconjugants were selected for each transfer, except when F17 was used as donor and CD13 as recipient. All transconjugants obtained were highly resistant to erythromycin and clindamycin (MIC ⩾256 μg ml^{-1}) (Table 2).

Southern blots and PCR assays showed that Tn*5398* inserts into the genome of all transconjugants (630xCD13 and 630xCD37) into the site previously described in CD37 (Farrow *et al.*, 2001) (data not shown). Transconjugants CII7xCD13 A and B showed the same hybridizing band at 44 kb and CII7xCD13 C at 42 kb. A hybridizing band at 42 kb was also observed when the donor strain CII7 was analysed. Transconjugants CII7xCD37 A, B and C had hybridizing bands at 128, 68 and 61 kb, respectively (Fig. 2a). Transconjugants F17xCD13 and F17xCD37 each showed one hybridizing band at 122 and 128 kb, respectively, while F17 had a 110 kb hybridizing band (Fig. 2b). These results suggested that the Tn*6194*-like element from CII7 could integrate into the recipient genome at different sites, while the element from F17 seems to have a unique integration site.

Localization of *erm*(B) was analysed by Southern hybridization of an *erm*(B) and a 23S probe on I-*Ceu*I PFGE fragments. This enzyme specifically targets genes coding for rRNA (Liu *et al.*, 1993). Hybridization patterns indicated that the resistance gene was located on the chromosome in all transconjugants (data not shown).

C. difficile 630 contains seven conjugative transposons (CTn*1*–CTn*7*) (Sebaihia *et al.*, 2006; Brouwer *et al.*, 2011). Information about the presence of these elements was available only for strain 630 but not for CII7 and F17. To determine if any transposon was cotransferred with Tn*5398*, transconjugants 630xCD13 and 630xCD37 were investigated by PCR. All transconjugants were negative for other elements except 630xCD37B that, in addition to Tn*5398*, acquired CTn*2* (data not shown).

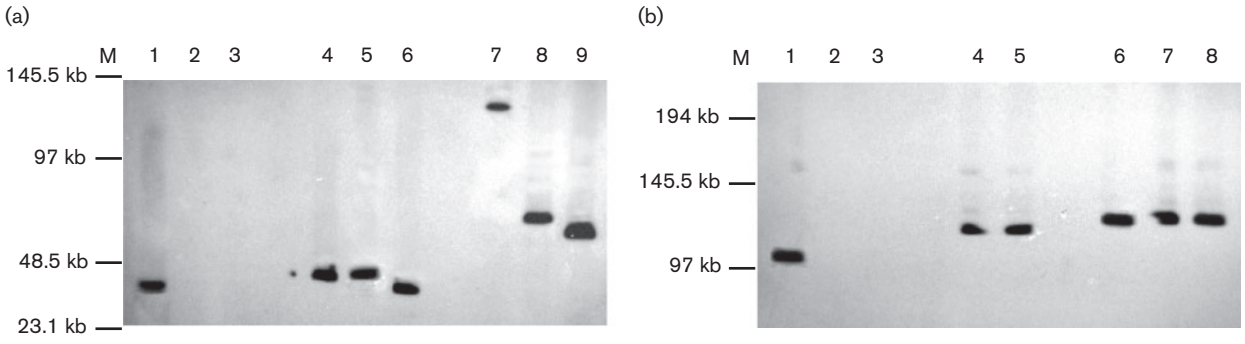

Fig. 2. Detection of *erm*(B) on transconjugants from *Pst*I-digested DNA fragments separated by PFGE. (a) Element from CII7. Lane 1, CII7; lane 2, CD13; lane 3, CD37; lanes 4–6, transconjugants CII7xCD13 A, B and C, respectively; lanes 7–9, transconjugants CII7xCD37 A, B and C, respectively. (b) Element from F17. Lane 1, F17; lane 2, CD13; lane 3, CD37; lanes 4 and 5, transconjugants F17xCD13 A and B, respectively; lanes 6–8, transconjugants F17xCD37 A, B and C, respectively. M, Low range PFG marker (New England Biolabs).

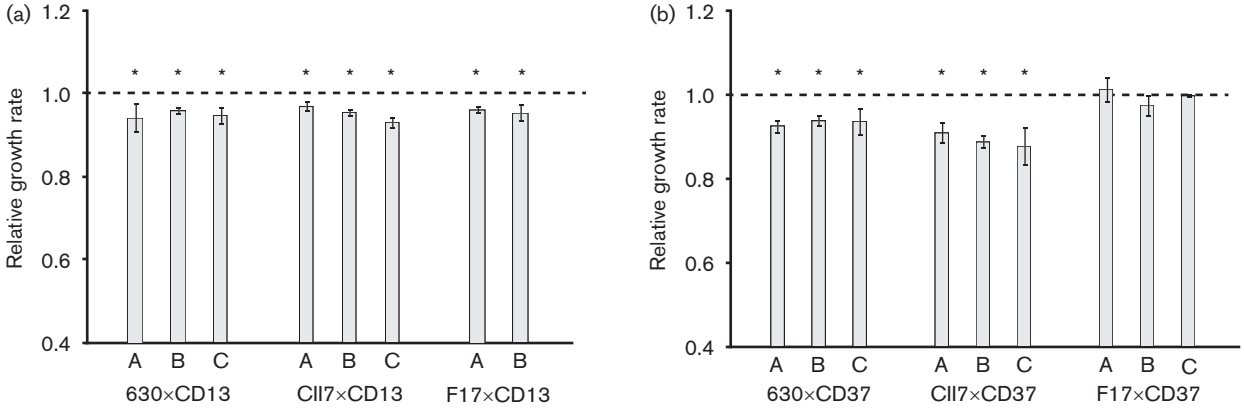

Fig. 3. Relative growth rates of transconjugants derivative of CD13 (a) or CD37 (b). Relative growth rate represents the ratio of growth of each transconjugant compared to that of its wild-type ancestor (set at 1). Values are the means of at least three separate experiments with error deviations. *Significant difference ($P<0.05$) of the mean value.

Fitness cost of *erm*(B)-containing elements

Few studies have investigated the fitness cost associated to transposons (Andersson & Hughes, 2010). It is estimated that their carriage would not be costly, since they are relatively small in size and integrated within the chromosome (Enne *et al.*, 2005). To our knowledge, nothing is known concerning the fitness cost associated with antibiotic resistance in *C. difficile*.

The growth rates of all transconjugants derived from CD13 were significantly reduced, compared to those of their isogenic ancestors, regardless of the element acquired ($P\leqslant0.05$) (Fig. 3). Similarly, all transconjugants derived

Table 3. Competition assays between transconjugants and the susceptible recipient strain CD13 or CD37

Transconjugants tested vs CD13	*s**	Relative fitness per generation (SD)†	*p*‡
630xCD13A	−0.111	0.889 (0.014)	0.002
630xCD13B	−0.113	0.887 (0.008)	<0.001
630xCD13C	−0.093	0.907 (0.012)	<0.001
CII7xCD13A	−0.110	0.890 (0.012)	0.001
CII7xCD13B	−0.136	0.864 (0.012)	0.001
CII7xCD13C	−0.129	0.871 (0.003)	<0.001
F17xCD13A	−0.097	0.903 (0.020)	0.008
F17xCD13B	−0.088	0.911 (0.012)	0.003
Transconjugants tested vs CD37			
630xCD37A	−0.035	0.965 (0.030)	0.299
630xCD37B	0.195	1.195 (0.056)	0.075
630xCD37C	−0.039	0.961 (0.029)	0.134
CII7xCD37A	−0.182	0.818 (0.013)	<0.001
CII7xCD37B	0.042	1.042 (0.032)	0.227
CII7xCD37C	−0.149	0.850 (0.078)	0.054
F17xCD37A	−0.066	0.934 (0.033)	0.048
F17xCD37B	0.029	1.023 (0.055)	0.502
F17xCD37C	−0.020	0.980 (0.038)	0.494

*Selection coefficient.
†Fitness relative to that of the susceptible strain. SD, standard deviation.
‡Statistical significance of difference in fitness.

from CD37 showed a significant reduction in growth rates except those containing the element from F17. The fitness cost for each element acquired was also assessed by competition assays between transconjugants and recipient strains (Table 3). As observed in growth assays, all transconjugants from CD13 were disadvantaged compared to the susceptible ancestor and showed relative fitness between 0.864 ± 0.012 and 0.911 ± 0.012 (Table 3). Different behaviours were observed for transconjugants from CD37 (Table 3). While transconjugants 630xCD37A and 630xCD37C were not significantly disadvantaged, 630xCD37B outcompeted CD37, with a relative fitness of 1.195 ± 0.056. Among transconjugants CII7xCD37, CII7xCD37A was the most disadvantaged with a relative fitness of 0.818 ± 0.013. Finally, in agreement with growth assay results, transconjugants F17xCD37 were not disadvantaged compared to CD37.

Since Tn*5398* has a unique integration site (Farrow *et al.*, 2001), it cannot be ruled out that disruption of the insertion sequence is responsible for the fitness cost observed rather than the carriage of the element itself. Among transconjugants acquiring Tn*5398*, only those from CD13 were disadvantaged. Thus, it can be hypothesized that Tn*5398* imposes a burden depending on the recipient strain. Interestingly, the transconjugant 630xCD37B, containing both Tn*5398* and CTn*2*, prevailed over CD37 in the competition assays. So, it will be very useful to deeply investigate this transconjugant to understand the effect of the CTn*2* acquisition.

While all CII7xCD13 transconjugants were similarly affected by the acquisition of Tn*6194*, CII7xCD37 transconjugants were not, probably because of the different insertion sites of the element in the two recipients. The element from strain F17 imposed a fitness burden only for transconjugants from CD13, underlining the importance of both insertion site and recipient strain type in the fitness cost.

In conclusion, we demonstrated that Tn*6194*-like elements are distributed among European *C. difficile* clinical isolates belonging to different PCR-ribotypes and that the carriage of an *erm*(B)-containing element frequently has a cost on the *in vitro* fitness. Our results also suggest that, independent of the burden that the element imposes on fitness, other factors, such as the capacity of *in vivo* transfer, the different insertion sites and the intrinsic characteristics of the different *C. difficile* types, are involved in the successful spreading of an element among *C. difficile* clinical isolates, although *in vivo* studies will be necessary to confirm these observations.

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REFERENCES

Andersson, D. I. & Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat Rev Microbiol* **8**, 260–271.

Barbut, F., Mastrantonio, P., Delmée, M., Brazier, J., Kuijper, E., Poxton, I. & European Study Group on Clostridium difficile (ESGCD) (2007). Prospective study of *Clostridium difficile* infections in Europe with phenotypic and genotypic characterisation of the isolates. *Clin Microbiol Infect* **13**, 1048–1057.

Bidet, P., Barbut, F., Lalande, V., Burghoffer, B. & Petit, J. C. (1999). Development of a new PCR-ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing. *FEMS Microbiol Lett* **175**, 261–266.

Brouwer, M. S. M., Warburton, P. J., Roberts, A. P., Mullany, P. & Allan, E. (2011). Genetic organisation, mobility and predicted functions of genes on integrated, mobile genetic elements in sequenced strains of *Clostridium difficile. PLoS ONE* **6**, e23014.

Brouwer, M. S. M., Roberts, A. P., Mullany, P. & Allan, E. (2012). In silico analysis of sequenced strains of *Clostridium difficile* reveals a related set of conjugative transposons carrying a variety of accessory genes. *Mobile Genet Elements* **2**, 8–12.

CLSI (2007). *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria*; Approved Standard M11-A7. Wayne, PA: Clinical and Laboratory Standards Institute.

CLSI (2008). *Performance Standards for Antimicrobial Susceptibility Testing*; 18th Informational Supplement M100-S18. Wayne, PA: Clinical and Laboratory Standards Institute.

Enne, V. I., Delsol, A. A., Davis, G. R., Hayward, S. L., Roe, J. M. & Bennett, P. M. (2005). Assessment of the fitness impacts on *Escherichia coli* of acquisition of antibiotic resistance genes encoded by different types of genetic element. *J Antimicrob Chemother* **56**, 544–551.

Farrow, K. A., Lyras, D. & Rood, J. I. (2001). Genomic analysis of the erythromycin resistance element Tn5398 from *Clostridium difficile. Microbiology* **147**, 2717–2728.

Foucault, M. L., Depardieu, F., Courvalin, P. & Grillot-Courvalin, C. (2010). Inducible expression eliminates the fitness cost of vancomycin resistance in enterococci. *Proc Natl Acad Sci U S A* **107**, 16964–16969.

He, M., Sebaihia, M., Lawley, T. D., Stabler, R. A., Dawson, L. F., Martin, M. J., Holt, K. E., Seth-Smith, H. M. B., Quail, M. A. & other authors (2010). Evolutionary dynamics of *Clostridium difficile* over short and long time scales. *Proc Natl Acad Sci U S A* **107**, 7527–7532.

He, M., Miyajima, F., Roberts, P., Ellison, L., Pickard, D. J., Martin, M. J., Connor, T. R., Harris, S. R., Fairley, D. & other authors (2013). Emergence and global spread of epidemic healthcare-associated *Clostridium difficile. Nat Genet* **45**, 109–113.

Huang, H., Weintraub, A., Fang, H. & Nord, C. E. (2009). Antimicrobial resistance in *Clostridium difficile. Int J Antimicrob Agents* **34**, 516–522.

Jasni, A. S., Mullany, P., Hussain, H. & Roberts, A. P. (2010). Demonstration of conjugative transposon (Tn*5397*)-mediated horizontal gene transfer between *Clostridium difficile* and *Enterococcus faecalis. Antimicrob Agents Chemother* **54**, 4924–4926.

Liu, S. L., Hessel, A. & Sanderson, K. E. (1993). Genomic mapping with I-*Ceu* I, an intron-encoded endonuclease specific for genes for

ribosomal RNA, in *Salmonella spp.*, *Escherichia coli*, and other bacteria. *Proc Natl Acad Sci U S A* **90**, 6874–6878.

Schmidt, C., Löffler, B. & Ackermann, G. (2007). Antimicrobial phenotypes and molecular basis in clinical strains of *Clostridium difficile*. *Diagn Microbiol Infect Dis* **59**, 1–5.

Sebaihia, M., Wren, B. W., Mullany, P., Fairweather, N. F., Minton, N., Stabler, R., Thomson, N. R., Roberts, A. P., Cerdeño-Tárraga, A. M. & other authors (2006). The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. *Nat Genet* **38**, 779–786.

Smith, C. J., Markowitz, S. M. & Macrina, F. L. (1981). Transferable tetracycline resistance in *Clostridium difficile*. *Antimicrob Agents Chemother* **19**, 997–1003.

Spigaglia, P. & Mastrantonio, P. (2002). Analysis of macrolide-lincosamide-streptogramin B (MLS_B) resistance determinant in strains of *Clostridium difficile*. *Microb Drug Resist* **8**, 45–53.

Spigaglia, P. & Mastrantonio, P. (2004). Comparative analysis of *Clostridium difficile* clinical isolates belonging to different genetic lineages and time periods. *J Med Microbiol* **53**, 1129–1136.

Spigaglia, P., Cardines, R., Rossi, S., Menozzi, M. G. & Mastrantonio, P. (2001). Molecular typing and long-term comparison of *Clostridium difficile* strains by pulsed-field gel electrophoresis and PCR-ribotyping. *J Med Microbiol* **50**, 407–414.

Spigaglia, P., Carucci, V., Barbanti, F. & Mastrantonio, P. (2005). ErmB determinants and Tn*916*-Like elements in clinical isolates of *Clostridium difficile*. *Antimicrob Agents Chemother* **49**, 2550–2553.

Spigaglia, P., Barbanti, F. & Mastrantonio, P. (2011). Multidrug resistance in European *Clostridium difficile* clinical isolates. *J Antimicrob Chemother* **66**, 2227–2234.

Wüst, J., Sullivan, N. M., Hardegger, U. & Wilkins, T. D. (1982). Investigation of an outbreak of antibiotic-associated colitis by various typing methods. *J Clin Microbiol* **16**, 1096–1101.