Behaviour of a CTn*CD11*-like element in *Clostridium difficile*

Wasels F., Spigaglia P., Barbanti F. and Mastrantonio P.
Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore di Sanità, Rome, Italy.

Introduction

In *Clostridium difficile*, the most common mechanism of resistance to MLS_B involves ribosome methylases encoded by *erm*(B) genes.

These genes are located on putative mobile elements showing a high genetic heterogeneity.
Among them, Tn*5398* from strain 630 is the best known, but it is not the most frequent in *C. difficile* clinical isolates.

In a recent study, it has been observed that the majority of European clinical isolates resistant to MLS_B have an *erm*(B)-containing element with a specific genetic organisation named E4 (Spigaglia *et al*, 2011).

In this study, an element with this organisation was investigated by conjugative assays and partially characterised.

Materials and Methods

Culture conditions

C. difficile clinical strains were grown on Brain Heart Infusion (BHI) agar plates or in BHI broth at 35°C in anaerobic conditions (85% N_2, 10% H_2, 5% CO_2). Erythromycin and rifampicin were used at a final concentration of 20 mg/L.

Filter-mating assays

Filter-mating experiments were performed as already described (Farrow *et al*, 2001).
Assays were performed in presence or absence of 50U of DNAseI (NEB).
For each transfer, at least three independent assays were conduced to avoid analysing siblings. Transconjugants were confirmed by PCR-ribotyping (Bidet *et al*, 1999) and PCR-detection of *erm*(B).

Hybridisation assays

The *erm*(B) gene was detected by Southern blotting conduced on *Pvu*II-digested genomic DNA separated by Pulsed Field Gel Electrophoresis (PFGE) performed as already described (Spigaglia *et al*, 2001).
Labelling of the *erm*(B)-specific probe, hybridisation to the membrane and signal detection were carried out as described in the ECL™ Direct Nucleic Acid Labelling and Detection System (Amersham Biosciences) according to the manufacturer's protocol.

Genomic DNA sequencing

The complete DNA sequence of the transconjugant CII7xCD13 A was obtained by using the 454-Genome Sequencer FLX procedure (Roche Diagnostic, Monza, Milan) on a library obtained using genomic DNA purified by the Nucleobond® buffer set III and the Nucleobond® AXG20 columns (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol.

Transfer of the ErmB determinant

The ErmB determinant from *C. difficile* CII7 was transferred by filter-mating assays to the recipient strains *C. difficile* CD13 and CD37 in presence and in absence of DNAseI.
Transfer frequencies are shown in Table 1. Interestingly, addition of DNAseI did not allow to obtain transconjugants CII7xCD13, while it did not affect transfer to CD37.

Donor strain	Recipient strain	DNAse	Transfer frequency (per donor)
CII7	CD37	-	$6.94 \pm 2.66 \times 10^{-9}$
CII7	CD37	+	$1.04 \pm 0.47 \times 10^{-8}$
CII7	CD13	-	$5.24 \pm 2.58 \times 10^{-9}$
CII7	CD13	+	$< 1 \times 10^{-9}$

Table 1: Transfer frequencies of the ErmB determinant from strain CII7 to CD13 and CD37, in presence and in absence of DNAseI.

Investigation of transconjugants CII7xCD13

Hybridisation assays using an *erm*(B) probe were performed on transconjugants CII7xCD13 and the results are shown in Fig 1. Different hybridising bands were observed, suggesting different integrations sites. Transconjugants CII7xCD13 A and B had the same hybridising band at 35kb, while transconjugant CII7xCD13 C showed a hybridising band at 32kb.

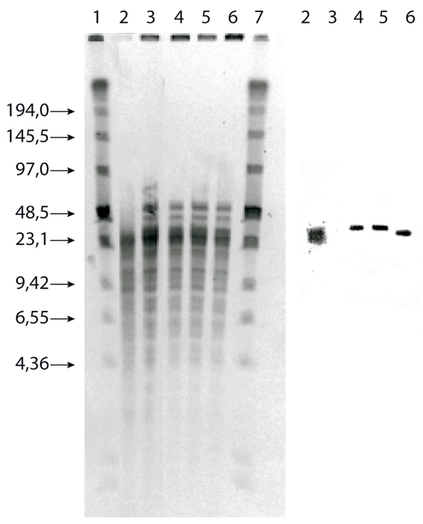

Figure 1: Detection of *erm*(B) on pulsed field gel electrophoresis of *Pvu*II-digested DNA.
Lanes 1,7: Low range PFG Marker (NEB);
Lane 2: *C. difficile* CII7;
Lane 3: *C. difficile* CD13;
Lanes 4-6: transconjugants CII7xCD13 A, B and C, respectively.

Transfer to CD37

Preliminary PCR assays suggest that the CTn*CD11*-like element from CII7 integrated in different regions of the genome of CD37 by a mechanism of integration that does not involve homologous recombination.

Insertion of the CTn*CD11*-like in CII7xCD13 A

The genomic DNA of the transconjugant CII7xCD13 A was sequenced. In this transconjugant the *erm*(B) gene is located on an element that has 100% identity with CTn*CD11*, a putative conjugative transposon found in *C. difficile* 2007855 (He *et al*, 2010), for more than 16kb. The remaining 14kb at the 5' end show a lower identity with this transposon (Fig 2).

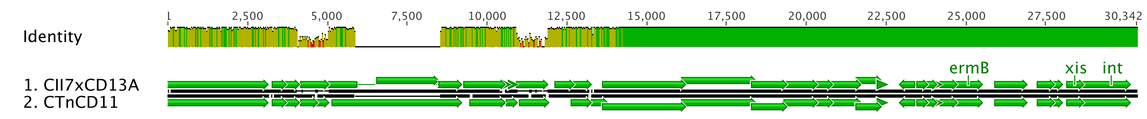

Figure 2: Alignment between CTn*CD11* and the putative conjugative transposon that contains *erm*(B) found in the transconjugant CII7xCD13 A.

A preliminary CD13 genome analysis showed that a putative conjugative transposon is located 2kb downstream of the insertion site of the CTn*CD11*-like element.
The 5' region of the transposon contained in CII7xCD13 A has 100% identity with this transposon, suggesting that a recombination between the element from CII7 and the CD13 putative transposon occurred in this transconjugant (Fig 3).

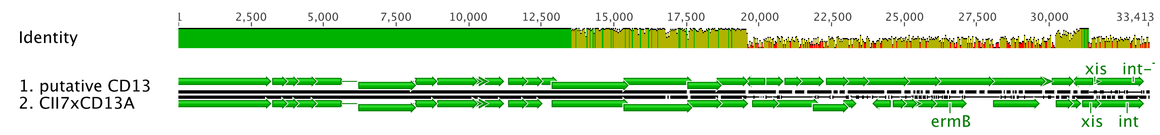

Figure 3: Alignment between the putative conjugative transposon found in CD13 and the element from transconjugant CII7xCD13 A.

Insertion of CTn*CD11*-like in CII7xCD13 C

PCR mapping on the transconjugant CII7xCD13 C suggests that a region of the recipient genome was replaced by a genomic fragment of the donor containing the entire CTn*CD11*-like element, probably by a mechanism of homologous recombination.

Excision of CTn*CD11*-like element from the genome

The capacity of the CTn*CD11*-like element to excise from the genome was evaluated by PCR as previously described (Brouwer *et al*, 2011).
Results shown in Fig 4 indicate that this element is able of excision in CII7 and in the transconjugant CII7xCD13 C. No excision was detected in the transconjugant CII7xCD13 A containing a recombinant element.

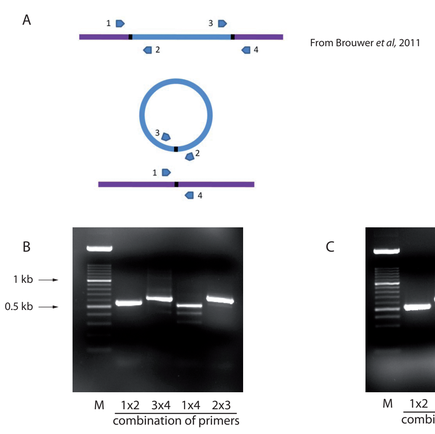

Figure 4: Excision of CTn*CD11*-like elements from the genome.
A: Schematic representation of primers binding;
B: PCR-profile of donor strain CII7 and transconjugant CII7xCD13C;
C: PCR-profile of transconjugants CII7xCD13 A.

Conclusions and perspectives

The CTn*CD11*-like element of strain CII7 can be transferred to other *C. difficile* strains by filter-mating experiments in vitro.

Integration of the element seems to be recipient-dependent.

The mobile element appears to be inserted by homologous recombination when CD13 is used as recipient.

Transconjugants CII7xCD13 can contain the entire CTn*CD11*-like transposon or a recombinant element resulting from a recombination event between this element and another transposon present in the recipient strain.

No transconjugants were obtained after the addition of DNAseI. So, a transformation-like mechanism could be involved in the transfer of the element and a mechanism of homologous recombination in its integration.

The CTn*CD11*-like element from CII7 seems to integrate in CD37 genome by a classical mechanism of conjugative transposition. Further studies will be performed to confirm these preliminary observations.

Acknowledgments

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement No. 237942. We thank Professor Julian Rood for providing us with the C. difficile strain CD37. We are indebted to all the members of the European Study Group on Clostridium difficile (ESGCD) for providing *C. difficile* isolate CII7.

References

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

Brouwer MS, Warburton PJ, Roberts AP, 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.

Farrow KA, Lyras D, Rood JI. 2001. Genomic analysis of the erythromycin resistance element Tn*5398* from *Clostridium difficile*. Microbiology 147: 2717–28.

He M, Sebaihia M, Lawley TD, Stabler RA, Dawson LF, et al. 2010. Evolutionary dynamics of *Clostridium difficile* over short and long time scales. Proc. Natl. Acad. Sci. USA. 107: 7527–7532.

Spigaglia P, Cardines R, Rossi S, Menozzi MG, 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, Barbanti F, Mastrantonio P on behalf of the European Study Group on *Clostridium difficile* (ESGCD). 2011. Multidrug resistance in European *Clostridium difficile* clinical isolates. J. Antimicrob. Chemother. 66: 2227–2234.

Contacts

mail: francois.wasels@iss.it

phone: +39 06 49902822