# Tn*5398* from *Clostridium difficile* 630: new insights in mechanism of transfer, biological burden and expression of *erm*(B)

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## Introduction

Resistance to $MLS_B$ in *C. difficile* is due to the presence of *erm*(B) genes usually located on mobile elements.
The best known of these elements is Tn*5398* from strain 630 (Fig 1).
Tn*5398* contains two copies of *erm*(B) and can be transferred to *C. difficile* CD37, even if it does not contain any gene involved in its mobility. For this reason, it has been previously described as a mobilizable non-conjugative element (Farrow *et al*, 2001).
In this study, conjugation assays were performed between 630 and two different recipient strains of *C. difficile*. The impact of the acquisition of Tn*5398* on the fitness of a recipient strain was also assessed. Finally, regulation of the expression of *erm*(B) genes was analysed in 630 and its derivative sensitive mutant 630Δ*erm* (Hussain *et al*, 2005).

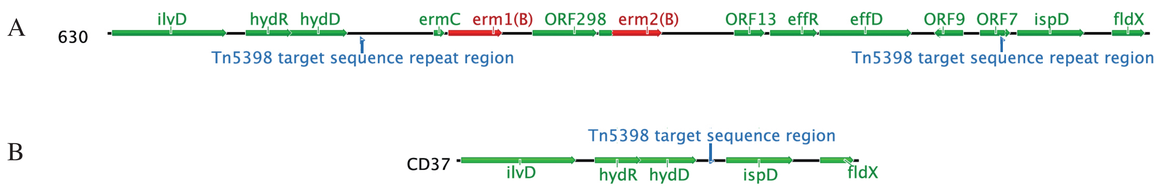


Figure 1: Genetic organisation of Tn*5398*.
A: genetic organisation of the element in *C. difficile* 630. Tn*5398* is delimitated by a straight line. In red, the two copies of *erm*(B); in green, other genes; in blue, repeated sequences at the extremities of the element. The region deleted in the erythromycin-susceptible mutant 630Δ*erm* is delimitated by a dashed line.
B: Genomic region of CD37 containing the insertion site of Tn*5398* (in blue).

## 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).

### Determination of bacterial fitness *in vitro*

Briefly, bacterial growth was monitored by measuring cell density in BHI at $OD_{600}$. Relative growth rates were calculated as the ratio between growth rates of resistant and susceptible strains.
Growth competition assays were conduced as already described (Foucault *et al*, 2010). Briefly, susceptible and resistant strains were grown separately, mixed in a 1:1 ratio, and used to inoculate 25 ml of BHI broth. One hundred microliters were transferred to fresh 25-mL broth every 24 hours over three cycles. Aliquots were plated at the end of every cycle on BHI agar containing 20 mg/L erythromycin or without antibiotic. The competition index (CI) was calculated as the cfu 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. Fitness of the susceptible strain was set to 1, and the relative fitness of the resistant transconjugants was determined as $1 + s$.
Statistical analysis was performed using GraphPad software.

### Expression analysis

RT-PCR were performed using the M-MLV Reverse transcriptase (NEB) and the TaKaRa Taq™ (Takara Shuzo Co., LTD Japan), on total RNA extracted using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany).

## Results

### Transfer of Tn*5398*

Tn*5398* was transferred to both CD13 and CD37. Transfer frequencies are shown in Table 1. Interestingly, addition of DNAseI significantly decreased transfer efficiency when CD13 was used as the recipient (p=0.0003), without affecting transfer to CD37.

| Donor strain | Recipient strain | DNAse | Transfer frequency (per donor) |
|---|---|---|---|
| 630 | CD37 | - | $2.99 \pm 1.56 \times 10^{-8}$ |
| 630 | CD37 | + | $1.22 \pm 0.57 \times 10^{-8}$ |
| 630 | CD13 | - | $3.87 \pm 0.20 \times 10^{-7}$ |
| 630 | CD13 | + | $9.66 \pm 7.46 \times 10^{-9}$ |

Table 1: Transfer frequencies of Tn*5398* from strain 630 to CD13 and CD37, in presence and in absence of DNAseI.

It has been shown that the genome of 630 contains 7 putative conjugative transposons, and the transfer of some of them to CD37 has recently been demonstrated (Sebaihia *et al*, 2006; Brouwer *et al*, 2011) .
In this study, PCR-detection of these transposons indicated that none of them co-transferred with Tn*5398* in all the transconjugants obtained (results not shown).

### Insertion of Tn*5398* into the recipients genome via a mechanism of homologous recombination

Tn*5398* integrates into the genome of CD37 at a specific site (Farrow *et al*, 2011).
In this study, sequencing of the flanking regions of this site indicated that a genomic fragment longer than the only Tn*5398* was transferred. The length of this fragment differed in each transconjugant, suggesting an integration by a mechanism of homologous recombination (Figure 2).
The same observation was done either in absence (e.g. 630xCD37 A and B) or in presence (e.g. 630xCD37 C and D) of DNAseI.

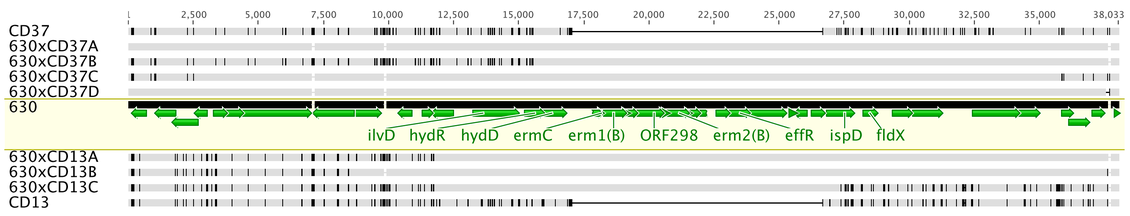


Figure 2: Replacement of the original sequence of CD13 and CD37 by the 630 genomic fragment containing Tn*5398*.
Sequence and genetic organisation of 630 are highlighted. Sequences of the two recipient strains CD37 and CD13 are shown in the first and in last line, respectively. Sequences of the transconjugants are shown between sequences of 630 and their respective recipient.
Sequences of transconjugants and recipient strains are shown in grey. SNPs between them and the sequence of 630 are figured in black.

### Tn*5398* imposes a burden on the fitness of *C. difficile*

Fitness assays were performed on CD13 and its derivative transconjugants 630xCD13. Both comparison of growth rates and competition assays showed that acquisition of Tn*5398* significantly affects the fitness of the bacterium (Fig 3, 4 and Table 2).

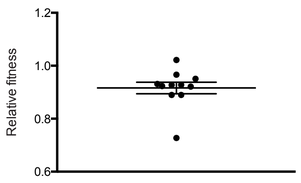


Figure 3: Acquisition of Tn*5398* reduces the growth rate of transconjugants 630xCD13.
Growth rates of transconjugants were compared to growth rate of the wild-type recipient strain CD13.
The relative fitness of 0.916 ± 0.022 indicates that the transconjugants are affected by the acquisition of Tn*5398* (*p*=0.0033). Each independent transconjugant was tested at least 3 times.

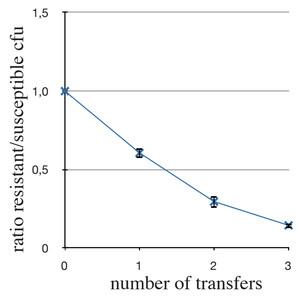


Figure 4: Competition assays between CD13 and its derivative transconjugants 630xCD13.
Evolution of the ratio 630xCD13 / CD13 after successive co-cultures in BHI.

Table 2: Cost of the acquisition of Tn*5398* on the fitness of CD13.

| Strains | Ratio at the end of each transfer [a] | | | *s* [b] | Relative fitness per generation | *p* [c] |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | | | |
| 630xCD13 vs CD13 | 0.61 ± 0.03 | 0.30 ± 0.04 | 0.15 ± 0.02 | -0.083 | 0.917±0.019 | <0.0001 |

[a]: Values represent the $Log_{10}$(cfu) ratios of the transconjugant population vs. the susceptible wild-type population at the end of each transfer (*ca*. 8 generations). [b]: Selection coefficient. [c]: Statistical significance.

### *erm1*(B) expression in 630Δ*erm*

The derivative mutant 630Δ*erm* does not contain the gene *erm2*(B) and is described as susceptible to erythromycin (Hussain *et al*, 2005).
In this study, highly resistant colonies (MIC of erythromycin ≥ 256 g/L) of 630Δ*erm* were isolated after exposure to sub-MIC concentrations of the drug .
*erm1*(B) expression in presence of erythromycin was demonstrated in these colonies by RT-PCR assays. No expression was detected in susceptible colonies, suggesting a regulation at the transcriptional level (Fig 5).
The same result was obtained in three independent assays.

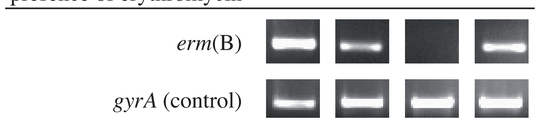


Figure 5: Expression of *erm*(B) in 630 and its derivative mutant 630Δ*erm* in presence and in absence of erythromycin, detected by RT-PCR.
In 630, the product observed can be due to expression of *erm1*(B) and *erm2*(B), while in 630Δ*erm*, only the expression of *erm1*(B) is detected. RT-PCR on *gyrA* was used as a control.

## Conclusions and perspectives

**Tn*5398* was successfully transferred to CD13 and CD37, two different *C. difficile* recipient strains.**

**Our results suggest that Tn*5398* integrates into the genome of the two recipient strains by a mechanism of homologous recombination.**

**Addition of DNAseI to the conjugation mix had an inhibitory effect only when CD13 was used as recipient, suggesting different mechanisms of acquisition for CD13 and CD37.**

**The acquisition of Tn*5398* significantly affects the fitness of the transconjugants 630xCD13 *in vitro*.**

**In this study, transcription of *erm1*(B) was observed in resistant colonies of 630Δ*erm*.**

**Generally, the expression of *erm*(B) is regulated at the post-transcriptional level. Our results suggest the existence of an alternative regulation in *C. difficile* at the transcriptional level.**
**Further studies will be done to better understand the *erm*(B) regulation in 630.**

## Acknowledgments

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