Horizontal gene transfer converts non-toxigenic *Clostridium difficile* strains into toxin producers

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*Clostridium difficile* is a major nosocomial pathogen and the main causative agent of antibiotic-associated diarrhoea. The organism produces two potent toxins, A and B, which are its major virulence factors. These are chromosomally encoded on a region termed the pathogenicity locus (PaLoc), which also contains regulatory genes, and is absent in non-toxigenic strains. Here we show that the PaLoc can be transferred from the toxin-producing strain, 630\(\Delta\text{erm}\), to three non-toxigenic strains of different ribotypes. One of the transconjugants is shown by cytotoxicity assay to produce toxin B at a similar level to the donor strain, demonstrating that a toxigenic *C. difficile* strain is capable of converting a non-toxigenic strain to a toxin producer by horizontal gene transfer. This has implications for the treatment of *C. difficile* infections, as non-toxigenic strains are being tested as treatments in clinical trials.
Clostridium difficile-associated diarrhoea (CDAD) can range from mild to severe and can be fatal due to complications such as pseudomembranous colitis and toxic megacolon. CDAD is almost always associated with antibiotic usage, which disrupts the normal gut microflora allowing C. difficile colonization. The organism produces two potent toxins, A and B, which disrupt the gut epithelium and are the major virulence factors of this organism. Both toxins are encoded on the pathogenicity locus (PaLoc), a 19.6-kb chromosomal region that also encodes potential transcriptional regulators and a holin-like protein. The entire PaLoc is absent from non-toxigenic strains replaced by a highly conserved 115 bp non-coding region at this genomic site.

C. difficile 630Δerm is an erythromycin-sensitive derivative of the toxigenic C. difficile 630 (ref. 7) and contains the PaLoc. This strain also contains at least six active conjugative transposons (CTns), genetic elements that are generally integrated into the genome but are capable of mediating their own transfer to suitable recipients. Conjugative transfer was shown for CTn1, CTn2, CTn4, CTn5, CTn7 and Tn5397, all of which can be transferred to the non-toxigenic C. difficile strain CD37 (refs 9–11). The aim of the current investigation was to determine if any other genetic information is cotransferred with the CTns. We demonstrate that the PaLoc can be transferred to CD37, which results in this non-toxigenic strain being converted to a toxin producer.

Results

The PaLoc transfers to non-toxigenic C. difficile CD37. To determine whether genetic elements were cotransferred in the absence of direct selection when transfer of CTn1 was selected, we initially investigated nine CD37 transconjugants containing CTn1::ermB (an erythromycin-resistant derivative of CTn1 (ref. 9)) by PCR and subsequent DNA sequencing (see Methods section for details). One of these strains had unexpectedly acquired the PaLoc. To determine whether the PaLoc could transfer independently of CTn1, strain 630Δerm tcdB::erm(B) (Table 1) containing a genetically marked PaLoc was used as a donor with CD37 as the recipient. PaLoc-containing transconjugants were obtained at a frequency of 7.5 x 10^-9 transconjugants per donor (s.d. = 4.2 x 10^-5), comparable to transfer frequencies previously reported for other CTns that are transferred between these strains.

It is important to determine whether the newly acquired toxin genes are capable of directing toxin production. To do this, one of the PaLoc-containing transconjugants that had acquired the wild-type PaLoc (that is, one of the transconjugants that contained CTn1 and the PaLoc) was selected for further study and designated PaLoc386 (Table 1). This strain was subject to an in vitro cytotoxicity assay with HFF-1 cells. Filter-sterilized cell supernatants were incubated with a monolayer of HFF-1 cells for 24 h before the cytotoxic effect (CPE) was determined (Fig. 1a). The end point toxin titre was determined for 630Δerm, PaLoc386 and CD37 (Fig. 1c). PaLoc386 produced similar levels of toxin to the donor strain 630Δerm, whereas supernatant from the recipient strain CD37 was negative for toxin production (Fig. 1c). Addition of a commercial TcdB antiserum (see Methods) abolished the CPE of both the 630Δerm and PaLoc386 supernatants indicating that the two strains produced functional toxin B (Fig. 1c).

The PaLoc is transferred on variable-sized DNA fragments. To investigate the mechanism underlying the PaLoc transfer, whole-genome sequences were determined for seven transconjugants containing either the marked or wild-type PaLoc. The sequence of the PaLoc in strain PaLoc386 was identical to that of the donor. In the other transconjugants, the PaLoc contained the clostron insertion, but was otherwise identical to the PaLoc in 630Δerm. To define the horizontally transferred region in the transconjugants, it was necessary to distinguish between donor and recipient genome. To do this, 30 regions of ~10 kb evenly spaced around the 630Δerm genome were selected (shown schematically in Fig. 2) as green bars with the sequences given in SI1) and compared with the corresponding regions of the CD37 genome. These regions were chosen as they did not contain any predicted mobile genetic elements such as CTns, IS trons or prophages. The presence of 10–150 single-nucleotide polymorphisms (SNPs) and indels in these 30 regions allowed discrimination between donor and recipient DNA. Alignment of the genomic DNA sequence of the PaLoc-containing transconjugants in the 30 regions showed that in all cases, regions 1–3 and 8–30 were derived from the 630Δerm genome whereas regions 4 and 7 from the 630Δerm genome. In all of the transconjugants, the PaLoc was located between regions 6 and 7. In the transconjugants, the PaLoc has transferred on variable-sized DNA fragments that include either region 6 or 7 from the donor strain 6 in 6 out of 7 transconjugants, and regions 5 and 6 from the donor in transconjugant PaLoc385. In transconjugant PaLoc26, the PaLoc was transferred from the donor on a relatively small DNA fragment (a maximum size of 67,644 bp) and both regions 6 and 7 in this transconjugant are derived from the recipient. In transconjugants, PaLoc386 and PaLoc37, Tn5397 and the PaLoc have transferred on the same DNA fragment. For example, in transconjugant PaLoc386, CTn5 also transferred but

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### Table 1 | Bacterial strains used in this study.

| Strain (Ribotype) | Properties | Reference or source |
|------------------|------------|---------------------|
| 630 (012)        | Tc<sup>+</sup> Erm<sup>+</sup> Rif<sup>+</sup> | 26 |
| 630Δerm (012)    | Tc<sup>+</sup> Erm<sup>+</sup> Rif<sup>+</sup> | 6 |
| CD37 (009)       | Tc<sup>+</sup> Erm<sup>+</sup> Rif<sup>+</sup> non-toxigenic | 27 |
| CD843            | Positive control for qC630-1 and qC630-2 | 28 |
| 630Δerm CTn1     | ClosTron mutant, donor of CTn1, erm(B) present in CD3086 within CTn1 | 9 |
| CD3086::erm(B) (012) | ClosTron mutant, contains erm(B) | 4 |
| 630Δerm tcdB::erm(B) (012) | inserted within tcdB | |
| OX904 (138)      | Non-toxigenic recent clinical isolate | 13 |
| OX2157 (140)     | Non-toxigenic recent clinical isolate | 13 |
| OX904rif (138)   | Rifampicin-resistant derivative of OX904 | |
| OX2157rif (140)  | Rifampicin-resistant derivative of OX2157 | |
| PaLoc386         | CD37 transconjugant containing the 630Δerm wild-type PaLoc | This study |
| PaLoc12          | CD37 transconjugant containing the ClosTron tcdB::erm(B) PaLoc | This study |
| PaLoc22          | CD37 transconjugant containing the ClosTron tcdB::erm(B) PaLoc | This study |
| PaLoc26          | CD37 transconjugant containing the ClosTron tcdB::erm(B) PaLoc | This study |
| PaLoc35          | CD37 transconjugant containing the ClosTron tcdB::erm(B) PaLoc | This study |
| PaLoc37          | CD37 transconjugant containing the ClosTron tcdB::erm(B) PaLoc | This study |
| PaLoc46          | CD37 transconjugant containing the ClosTron tcdB::erm(B) PaLoc | This study |

erm, erythromycin; Rif, rifampicin; R, resistant; S, susceptible; Tc, tetracycline.
independently of the PaLoc and has inserted near region 16 in the transconjugant (Fig. 2). In contrast to PaLoc transfer, in which the flanking DNA was also derived from the donor, there was no evidence for the transfer of flanking DNA in the case of CTn\textsubscript{5}. The remaining PaLoc-containing transconjugants shown in Fig. 2 did not contain CTn\textsubscript{2}, CTn\textsubscript{4}, CTn\textsubscript{5}, CTn\textsubscript{7} or Tn\textsubscript{5397}.

The approximate length of the transferred DNA in each of the transconjugants was determined by calculating the distance from the first SNP or indel upstream of the PaLoc to the last SNP or indel downstream of the PaLoc that was specific to the donor strain 630\textbackslash{}erm. Because of the high level of sequence identity between CD37 and 630\textbackslash{}erm in the regions flanking the PaLoc, this analysis could only determine the maximum and minimum

**Figure 1 | Cytotoxicity assay comparing toxin production.** Toxin production by the donor, transconjugant and recipient strains were compared using a cytotoxicity assay. (a) HFF-1 cells incubated with the culture supernatant of transconjugant PaLoc386 show rounding of the cells. (b) The cytotoxic effect of PaLoc386 can be neutralized using antibodies against TcdB. Scale bar, 1 mm in a and b. (c) The toxin end point titre was determined for culture supernatants of donor strain 630 Derm, transconjugants PaLoc386 and the recipient CD37. The mean ± s.d. is shown for three independent experiments.

**Figure 2 | Schematic representation of the donor-specific DNA fragments present in the transconjugants.** The circular chromosome of \textit{C. difficile} strain 630 is shown. The boxed region on the left is magnified on the right. The green fragments in the outer ring represent the 10 kb fragments used to differentiate transconjugants from donors; see text for more details. The dark blue fragments in the second ring indicate the mobile genetic elements present in the donor; the red fragment in this ring represents the PaLoc. The donor-specific fragments that are present in the transconjugants are shown in the seven inner-most rings as coloured fragments with the number of each transconjugant indicated. The red fragments in these represent the transferred PaLoc. The dark blue fragments in the track of PaLoc386 depict CTn7 and CTn5 that are both present in this transconjugant.
size of the transferred fragment (Table 2, sequences included in SI2). The PaLoc was transferred on variable-sized DNA fragments ranging from at least 66,034 bp to a maximum of 272,977 bp (Table 2 and Fig. 2).

To exclude transformation as a mechanism of transfer, the mating experiments were repeated in the presence of DNase and no difference in the frequency of transfer was detected. It was also possible that the PaLoc could be transferred by transduction by either of the prophages that are present in strain 630erm (Fig. 2). We think this is improbable given that the transferred DNA is much larger than the phage genomes and is unlikely therefore to be packaged within the phage heads. Nonetheless concentrated phage suspensions were made by mitomycin C induction of 630erm tcdB::erm(B). We attempted to infect strain CD37 with these phage suspensions but did not obtain plaques, indicating that this strain is resistant to these phages. Strain CD843 was used to confirm the presence of infectious phages in SI2). The PaLoc was transferred on variable-sized DNA fragments as shown by transconjugant PaLoc386 in which Ctn5 and Ctnl are transferred and integrated into their preferred genomic sites9 (between regions 15 and 16 for Ctn5, and between regions 3 and 4 for Ctnl (Fig. 2)) with no detectable transfer of flanking DNA. Presumably in this case, Ctn5 and Ctnl transferred by excision to form a circular molecule that was nicked at the origin of transfer (oriT) and transferred as a distinct genetic element.

A striking observation is that although transfer of the PaLoc and the CTns occurs at a low frequency (between 10−7 and 10−9 transconjugants per donor), cotransfer of unselected genetic elements (including the PaLoc) is observed. A probable explanation for this is that when one or more of the CTns initiates a transfer event, other elements present in the same cell are activated by trans-acting regulatory molecules. This type of interaction has been observed between Tn916-like elements15. The first step in transfer by conjugative and mobilizable transposons is excision to form a circular molecule that is nicked at the oriT, and a single strand is transferred to a suitable recipient via a mating pore in a complex process analogous to type IV secretion16. However, the PaLoc is not contained within an obvious mobile element and is transferred on DNA fragments of variable size in different transconjugants. This type of transfer is reminiscent of that mediated by high-frequency recombination (Hfr) in which an oriT within the chromosome can mediate transfer of the chromosome17. In the recipient, the incoming chromosomal DNA is integrated into the chromosome by homologous recombination. In theory, the whole chromosome can be transferred, although this rarely happens due to disruption of the mating pair or nicking of the incoming DNA strand, so that markers proximal to the integrated oriT are transferred at a higher frequency than those located distally. There are examples of CTns mediating Hfr-type transfer, for example, in Vibrio cholerae18 and Bacteroides sp.19 In these cases, the CTns do not excise so that instead of just mobilizing themselves, the chromosome is also mobilized. Three transfer-proficient CTns in the C. difficile genome, CtnJ, Ctn2 and Tn5397 are close to the PaLoc (Fig. 2) and are candidates for mediation of Hfr transfer. The regions flanking the PaLoc in 630erm are homologous to chromosomal DNA of the non-toxigenic strain, CD37, and, consequently, the incoming DNA from the donor could recombine with the recipient chromosome resulting in integration of this region. Whole-genome sequence comparison has indicated that large blocks of DNA have transferred between C. difficile strains in the past, indicating that this form of transfer is an important driver of C. difficile genome evolution20.

A recent study of diversity in the C. difficile S-layer cassette suggests that this cassette is undergoing horizontal gene transfer across genotypes21. The recombination events for the cassette shown in this study were significantly smaller (12–35 kb) than those reported here for the PaLoc, and the recombining loci are located ∼2 Mb apart on the chromosome. Although transfer of the S-layer cassette has not been demonstrated, it is possible that this region can transfer by a mechanism similar to that of the PaLoc, a hypothesis that requires investigation.

### Table 2 | Size of the DNA fragments inserted in the recipient strain.

| Isolate | Minimum size of DNA fragment transferred | Maximum size of DNA fragment transferred |
|---------|----------------------------------------|----------------------------------------|
| PaLoc386 | 271,992 bp | 272,977 bp |
| PaLoc12 | 151,692 bp | 155,024 bp |
| PaLoc22 | 157,688 bp | 170,386 bp |
| PaLoc26 | 66,034 bp | 67,644 bp |
| PaLoc35 | 224,973 bp | 228,497 bp |
| PaLoc37 | 250,027 bp | 252,009 bp |
| PaLoc46 | 141,658 bp | 145,290 bp |

always, occurs with cotransfer of conjugative transposons encoding resistance to antibiotics, resulting in the cotransfer of virulence and antibiotic-resistance genes. This is illustrated in the transconjugants PaLoc386 and PaLoc37, in which Tn5397 (encoding tetracycline resistance) and the PaLoc were transferred on the same DNA fragment from 630erm to CD37. CTns and the PaLoc can also be transferred on separate DNA fragments as shown by transconjugant PaLoc386 in which Ctn5 and Ctnl are transferred and integrated into their preferred genomic sites9 (between regions 15 and 16 for Ctn5, and between regions 3 and 4 for Ctnl (Fig. 2)) with no detectable transfer of flanking DNA. Presumably in this case, Ctn5 and Ctnl transferred by excision to form a circular molecule that was nicked at the origin of transfer (oriT) and transferred as a distinct genetic element.

**Discussion**

This work demonstrates that the PaLoc of C. difficile 630erm is capable of transfer by a conjugation-like mechanism to non-toxigenic strains. In the case of C. difficile CD37, we showed that this resulted in its conversion to a toxin producer. This finding has important clinical implications, as non-toxigenic strains have shown promise as a treatment for CDAD14. Clearly, if the PaLoc is capable of transfer to strains being used for treatment, then we urgently need to understand the conditions under which transfer of the PaLoc is selected. Non-toxigenic strains are found coexisting with toxigenic ones13, providing opportunity for the PaLoc to spread within the C. difficile populations. Also of concern is the finding that transfer of the PaLoc often, but not always, occurs with cotransfer of conjugative transposons encoding resistance to antibiotics, resulting in the cotransfer of virulence and antibiotic-resistance genes. This is illustrated in the transconjugants PaLoc386 and PaLoc37, in which Tn5397 (encoding tetracycline resistance) and the PaLoc were transferred on the same DNA fragment from 630erm to CD37. CTns and the PaLoc can also be transferred on separate DNA fragments as shown by transconjugant PaLoc386 in which Ctn5 and Ctnl are transferred and integrated into their preferred genomic sites9 (between regions 15 and 16 for Ctn5, and between regions 3 and 4 for Ctnl (Fig. 2)) with no detectable transfer of flanking DNA. Presumably in this case, Ctn5 and Ctnl transferred by excision to form a circular molecule that was nicked at the origin of transfer (oriT) and transferred as a distinct genetic element.

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**Methods**

**Strains and growth conditions.** All strains used in this study are listed in SI 3. C. difficile strains were grown in brain–heart infusion (BHI) broth or on BHI agar
(Oxoid Ltd, Basingstoke, UK) supplemented with 5% defibrinated horse blood (E & O laboratories, Bonnibridge, UK). For the cytotoxicity assay, C. difficile was grown in tryptone, yeast (TY) broth consisting of 3% Bact Tryptone (BD Biosciences, Oxford, UK), 2% yeast extract (Oxoid Ltd) and 0.1% thiglycollate (Sigma-Aldrich Company Ltd, UK) at pH 7.4. C. difficile strains were grown at 37 °C under anaerobic conditions (80% N2, 10% H2 and 10% CO2).

Molecular biology techniques. DNA was isolated using the Gentra Puregene Yeast/Bact. Kit (Qiagen, UK). PCR amplification was carried out using the NEB Taq polymerase kit (New England Biolabs, UK) according to the manufacturer’s instructions.

Filter-matings. Donor and recipient strains were grown in BHI broth to an OD600 of ~0.5–0.6. Cells were washed, pellets were taken up into BHI broth and donor and recipient cultures were mixed. The cell mixture was spread onto 0.45 μm pore size cellulose nitrate filters (Sartorius, Epsom, UK) on BHI agar plates. In some cases DNase was added to the mating plates at a concentration of 50 μg ml−1. After 24 h, the filters were placed in 25 ml tubes containing 1 ml BHI broth and were vortexed vigorously. The cell mixture was spread onto selective plates to determine donor, recipient and transconjugant cell numbers on the filters. Putative transconjugants were assessed by PCR for the presence of the erm(8) selection marker using primers ErmRAM-F (5′-AGCGTTATATTGATAAAAAAATAAATATGTTGGG-3′) and ErmRAM-R (5′-AGCGTGGCAGCATTACGTAAGATTATTGCTCCG-3′)23. The presence of the PaLoc was determined by PCR using primers tcpA-F (5′-CATCTAATACGCTTCATCTCC-3′) and tcpA-R (5′-CCACAGAACCATTATGTTGC-3′). The absence of the CDT locus was determined using primers cdtA-F (5′-CTCTAATACGCTTCATCTCC-3′) and cdtA-R (5′-TTGGTITTTGTATTCTTGTTGC-3′). Spontaneous rifampicin-resistant mutants were isolated by plating 105 bacteria on agar plates containing 25 μg ml−1 rifampicin. As the complete genome sequence of strains OX04 and OX215 contained no plasmid, transconjugants were differentiated from spontaneous rifampicin-resistant mutants of the donor by PCR and sequencing of genes encoding the V colicinogenic factor.

Toxin B is essential for virulence of C. difficile. Overnight cultures were diluted 1:10 into tryptone, yeast (TY) broth and incubated without shaking for 48 h. The cultures were centrifuged for 10 min at 4,500 g and the supernatant was filter-sterilized. A dilution series of the supernatant was prepared in PBS. Each dilution was added to confluent HFF-1 cells in duplicate. After 24 h, the cells were assessed for CPE using an inverted microscope.

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Acknowledgements

We thank Dr Sarah Kuehne, Dr Shan Goh and Dr Kate Dingle for providing the bacterial strains. We would like to thank the sequencing team at UCL Genomics for performing the sequencing and with the analysis of the genomic sequences. The research leading to these results has received funding from the European Community’s Seventh Framework Programme (FP7/2007–2013) under grant agreement no. 223585 and the Medical Research Council (grant no. G0601176).
Authors contributions
M.S.M.B., P.M., E.A. and A.P.R. designed the experiments and wrote the manuscript. M.S.M.B., R.W. and H.H. carried out the experiments.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Brouwer, M. S. M. et al. Horizontal gene transfer converts non-toxigenic Clostridium difficile strains into toxin producers. Nat. Commun. 4:2601 doi: 10.1038/ncomms3601 (2013).

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