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Received 4 Feb 2013 | Accepted 12 Sep 2013 | Published 17 Oct 2013

DOI: 10.1038/ncomms3601

OPEN

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 Δ 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.

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C*lostridium difficile*-associated diarrhoea (CDAD) can range from mild to severe and can be fatal due to complications such as pseudomembranous colitis and toxic megacolon¹. CDAD is almost always associated with antibiotic usage, which disrupts the normal gut microflora allowing *C. difficile* colonization². The organism produces two potent toxins, A and B, which disrupt the gut epithelium and are the major virulence factors of this organism^{3,4}. 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 site⁵.

C. difficile 630 Δ erm⁶ is an erythromycin-sensitive derivative of the toxigenic *C. difficile* 630 (ref. 7) and contains the PaLoc^{3,4}. 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 recipients⁸. 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 630 Δ erm tcdB::erm(B) (Table 1)⁴ containing a genetically marked PaLoc was used as a donor with CD37 as the recipient. PaLoc-containing transconjugants were obtained at a frequency of 7.5×10^{-9} transconjugants per donor (s.d. = 4.2×10^{-9}), comparable to transfer frequencies previously reported for other CTns that are transferred between these strains⁹.

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 cytopathic effect (CPE) was determined (Fig. 1a). The end point toxin titre⁴ was determined for 630 Δ erm, PaLoc386 and CD37 (Fig. 1c). PaLoc386 produced similar levels of toxin to the donor strain 630 Δ erm, 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 630 Δ erm 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

Table 1 | Bacterial strains used in this study.

Strain (Ribotype)	Properties	Reference or source
630 (012)	Tc ^R Erm ^R Rif ^S	26
630 Δ erm (012)	Tc ^R Erm ^S Rif ^S	6
CD37 (009)	Tc ^S Erm ^S Rif ^R non-toxigenic	27
CD843	Positive control for ϕ C630-1 and ϕ C630-2	28
630 Δ erm CTn1 CD0386::erm(B)	Clostron mutant, donor of CTn1, erm(B) present in CD0386 within CTn1 (012)	9
630 Δ erm tcdB::erm(B) (012)	Clostron mutant, contains erm(B) inserted within tcdB	4
OX904 (138)	Non-toxigenic recent clinical isolate	13
OX2157 (140)	Non-toxigenic recent clinical isolate	13
OX904rif (138)	Rifampicin-resistant derivative of OX904	This study
OX2157rif (140)	Rifampicin-resistant derivative of OX2157	This study
PaLoc386	CD37 transconjugant containing the 630 Δ erm 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.

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 630 Δ erm. 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 630 Δ erm genome were selected (shown schematically in Fig. 2 as green bars with the sequences given in S11) and compared with the corresponding regions of the CD37 genome¹². These regions were chosen as they did not contain any predicted mobile genetic elements such as CTns, IStons or prophages. The presence of 10–150 single-nucleotide polymorphisms (SNPs) and indels in these 30 regions allowed discrimination between the 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 recipient. In the donor strains, 630 Δ erm and 630 Δ erm tcdB::erm(B), and in all of the transconjugants, the PaLoc is 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 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 (Fig. 2). This contrasts with the other cotransfers where the CTn and the PaLoc transferred on separate DNA fragments. For example, in transconjugant PaLoc386, CTn5 also transferred but

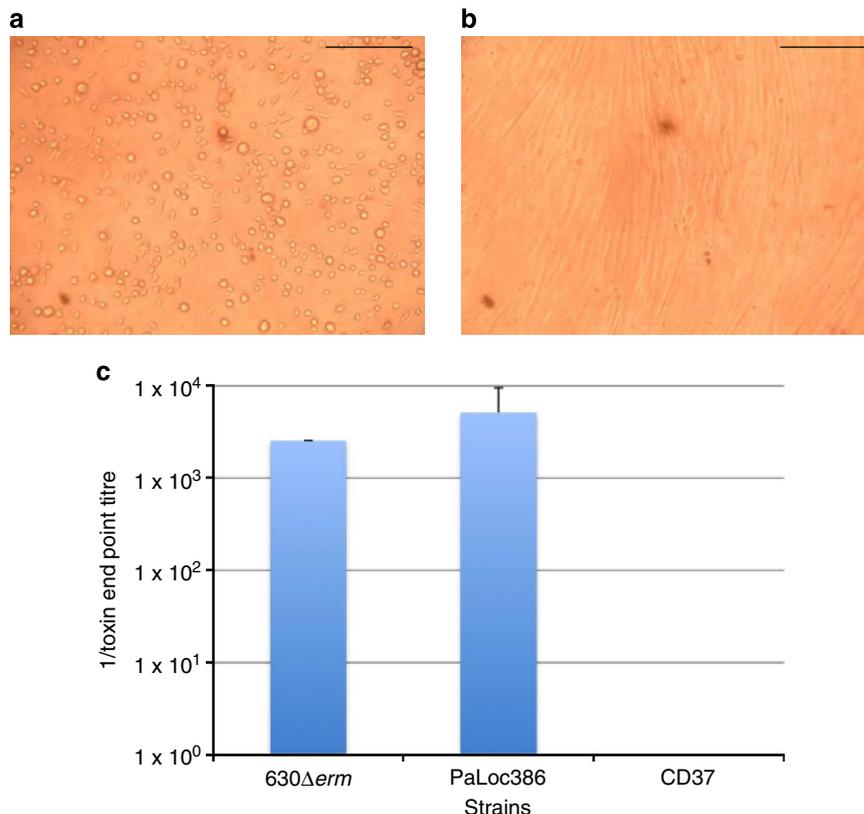


Figure 1 | Cytotoxicity assay comparing toxin production. Toxin production by the donor, transjugant and recipient strains were compared using a cytotoxicity assay. **(a)** HFF-1 cells incubated with the culture supernatant of transjugant PaLoc386 show rounding of the cells. **(b)** The cytotoxic effect 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, transjugants PaLoc386 and the recipient CD37. The mean ± s.d. is shown for three independent experiments.

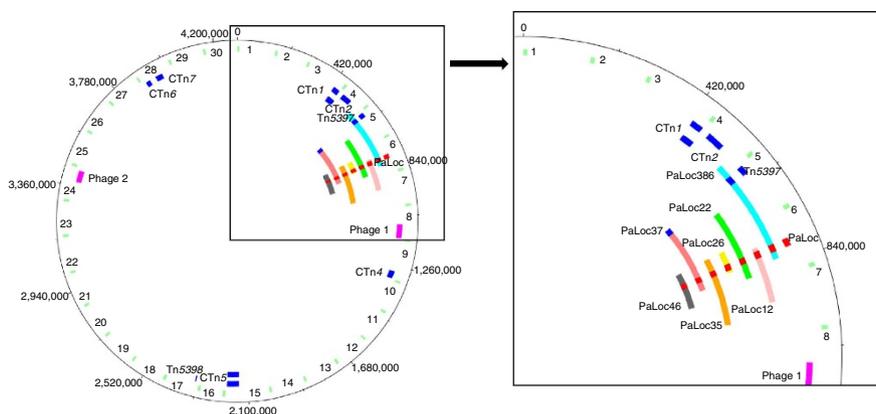


Figure 2 | Schematic representation of the donor-specific DNA fragments present in the transjugants. The circular chromosome of *C. difficile* strain 630 is shown. The boxed region on the left is magnified on the right. The green fragments in the outer ring represent the 10 kb fragments used to differentiate transjugants 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 transjugants are shown in the seven inner-most rings as coloured fragments with the number of each transjugant indicated. The red fragments in these represent the transferred PaLoc. The dark blue fragments in the track of PaLoc386 depict CTn1 and CTn5 that are both present in this transjugant.

independently of the PaLoc and has inserted near region 16 in the transjugant (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 CTn5. The remaining PaLoc-containing transjugants shown in Fig. 2 did not contain CTn2, CTn4, CTn5, CTn7 or Tn5397.

The approximate length of the transferred DNA in each of the transjugants 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 630Δerm. Because of the high level of sequence identity between CD37 and 630Δerm in the regions flanking the PaLoc, this analysis could only determine the maximum and minimum

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

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 630 Δ erm (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 630 Δ erm *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 the suspensions. Although plaques were produced with this host, no lincomycin-resistant transductants were obtained in this phage assay. These results indicate that PaLoc transfer is unlikely to occur via transduction.

The PaLoc can transfer into other non-toxicogenic strains. Recent work by Dingle *et al.*¹³, analysing the molecular epidemiology of a large population of *C. difficile* strains, has suggested that PaLoc transfer is occurring in wild populations. We tested two of the non-toxicogenic isolates from this study (OX904 belonging to clade 4, PCR-ribotype 138 and OX2157 belonging to clade 1, PCR-ribotype 140) to determine whether they could act as recipients for the marked PaLoc in matings using 630 Δ erm *tcdB::erm(B)* as donor. To do this, spontaneous rifampicin-resistant derivatives of these strains were first isolated (see Methods and Table 1). The PaLoc was transferred to OX2157rif at a frequency of 4.4×10^{-8} and to OX904rif at a frequency of 2.3×10^{-9} , indicating that PaLoc transfer is not restricted to particular donor and recipient pairs.

Discussion

This work demonstrates that the PaLoc of *C. difficile* 630 Δ erm is capable of transfer by a conjugation-like mechanism to non-toxicogenic 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-toxicogenic 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-toxicogenic strains are found coexisting with toxicogenic 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 630 Δ erm to CD37. CTns and the PaLoc can also be transferred on separate DNA fragments as shown by transconjugant PaLoc386 in which CTn5 and CTn1 are transferred and integrated into their preferred genomic sites⁹ (between regions 15 and 16 for CTn5, and between regions 3 and 4 for CTn1 (Fig. 2)) with no detectable transfer of flanking DNA. Presumably in this case, CTn5 and CTn1 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 cholerae*¹⁸ 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, CTn1, CTn2 and Tn5397 are close to the PaLoc (Fig. 2) and are candidates for mediation of Hfr transfer. The regions flanking the PaLoc in 630 Δ erm are homologous to chromosomal DNA of the non-toxicogenic 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.

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, Bonnybridge, UK). For the cytotoxicity assay, *C. difficile* was grown in tryptone, yeast (TY) broth consisting of 3% Bacto Tryptose (BD Biosciences, Oxford, UK), 2% yeast extract (Oxoid Ltd) and 0.1% thioglycolate (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 Genra 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*(B) selection marker using primers ErmRAM-F (5'-ACGCGTTATATTGATAAAAA TAATAATAGTGGG-3') and ErmRAM-R (5'-ACGCGTGCGACTCATAGAATT ATTTCTCCCG-3')²². The presence of the PaLoc was determined by PCR using primers tcdA-F (5'-CATCTATAAGTTCTCATATTCCTTC-3') and tcdA-R (5'-CGAGAACTTATATTGTC-3'). The absence of the CDT locus was determined using primers cdtA-F (5'-(C/T)AATACTACTTACAAGGCTCC-3') and cdtA-R (5'-TTTCGTTTTGATTTT(C/T)TGTTTC-3'). Spontaneous rifampicin-resistant mutants were isolated by plating 10¹⁰ bacteria on agar plates containing 25 µg ml⁻¹ rifampicin. As the complete genome sequence of strains OX904 and OX2157 is not available, transconjugants were differentiated from spontaneous rifampicin-resistant mutants of the donor by PCR and sequencing of genes *cwp66* (primers 5'AAACWHAHARYYAATGATACAG and 5'RTWGWYTTTCCCAT CKWGAAC) and gene CD25780 (primers 5'GATGATATAGACTTTTACATC AGAAG and 5'CAGCCGAATTACTTTTAATC) encoding a predicted serine threonine protein kinase.

Cytotoxicity assay. HFF-1 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Life technologies) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin at 37 °C in 5% CO₂. Cells were subcultured by washing twice with sterile PBS and incubating with 0.1% trypsin for 5 min. For the cytotoxicity assay, the cells were seeded at 10⁴ cells per well in 96-well plates and incubated for 24 h in 5% CO₂.

Overnight *C. difficile* 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. The toxin end point titre was recorded as the lowest dilution in the series where the cells were indistinguishable from the negative control. *C. difficile* antitoxin (*C. difficile* test kit, T5003Techlab Inc, Blacksburg, VA, USA) was added to one of the duplicate wells to determine whether the CPE was neutralized.

Genomic sequencing. Genomic sequencing was performed by UCL Genomics as part of a 72-bp paired end run, performed on an Illumina GAI-X. Genome assembly was performed using the Velvet software suite²³ and reads were mapped against strain 630 (AM180355) using xBase²⁴. The transconjugant sequences were compared with the sequence of strain 630 (AM180355) and the shotgun sequence of strain CD37 (AHJJ01000000) using the Artemis Comparison Tool²⁵. SNP analysis was performed by alignment using eBioX version 1.51. Sequence of the chromosomal DNA used to compare the donor- and recipient-specific DNA in the transconjugants is provided in Supplementary Data 1. Sequence at the junctions between the chromosome and the donor-specific fragment is provided in Supplementary Data 2. The ends on either side of the transferred fragment are compared with the donor and recipient sequences.

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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 genomic sequencing and for their help with the analysis of 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>

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



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