**Opinion**

**The evolving epidemic of *Clostridium difficile* 630**

Adam P. Roberts1,2 and Wiep Klaas Smits3

1 Department of Parasitology and 2 Research Centre for Drugs and Diagnostics, Liverpool School of Tropical Medicine, Liverpool, UK. E-mail: [adam.roberts@lstmed.ac.uk](mailto:adam.roberts@lstmed.ac.uk). ORCID: 0000-0002-0760-3088

3 Department of Medical Microbiology, Leiden University Medical Center, Leiden, The Netherlands. E-mail: [w.k.smits@lumc.nl](mailto:w.k.smits@lumc.nl). ORCID: 0000-0002-7409-2847

Abstract

*Clostridium difficile* is a major pathogen responsible for a range of diseases in humans and animals. The genetic tools used to explore *C. difficile* biology are a relatively recent development in comparison to those used to investigate some other pathogens. Consequently, a rapid and haphazard dispersal of strains throughout the scientific community has led to the evolution of different *C. difficile* lineages within strains in different geographical locations and these genotypic differences are likely to affect the phenotype of the organism. Here we review the history of *C. difficile* 630, the first genome-sequenced *C. difficile* isolate and the most widely distributed reference strain, and its derivatives. We also invite researchers to take part in a community wide genome sequencing study to trace the evolution of these strains as they have travelled between laboratories around the world.

Text

On the 24th of February 1988 [1] Richard Lenski began his now famous Long-Term Evolution Experiment (LTEE) [2] in Bruce Levin’s lab at University California, Irvine and later moved it to his own lab at Michigan State University. The LTEE has seen a pair of related founding cultures of *Escherichia coli* each split into 6 parallel cultures and propagated over 60,000 generations with samples being frozen every 500 generations [3]. Genome sequencing has shed light on the molecular evolution occurring within the genomes of these cultures and has demonstrated, with wonderful clarity, remarkable adaptive responses to the laboratory growth media (Davis minimal medium supplemented with limiting glucose at 25 µg/mL) [4] in which it now lives. The most notable adaptation reported to date is the emergence of the ability to use sodium citrate as a sole carbon source [5]; something that traditionally *E. coli* is not able to do in oxic conditions. This work illustrates the adaptive potential of bacteria resulting from systematic sub-culturing in laboratory media; an important aspect of laboratory based research activities worth contemplating as you read on. It is clear that the research community investigating the biology of *Clostridium difficile* has unwittingly carried out our own, rather less well controlled, version of the LTEE.

The first genome sequence of *Clostridium difficile* (strain 630) was published in 2006 [6] (Table 1) and 630 was rapidly adopted as the reference strain for laboratory-based studies. Strain 630 is a clinical isolate responsible for an outbreak of *C. difficile* infection at a Swiss hospital and belongs to PCR ribotype 012 [6]. This ribotype of *C. difficile* is the 8th most common in a recent European hospital-based survey [7] and 1.4% of US strains were found to belong to PCR ribotype 012 [8]. As genetic tools increased in complexity and usefulness requests for the strain 630 grew and it was rapidly disseminated across the planet by individual labs and the strain repositories; NCTC in the UK (NCTC 13307 [9]), DSMZ in Germany (DSM No.: 27543 [10]) and the ATCC in the USA (ATCC® BAA-1382 [11]).

One of the main hurdles to rapid and reproducible genetic manipulation of this strain was our inability to transform it. This lack of a reliable gene transfer system was addressed with the development of conjugative transfer systems which used various donors as the source of DNA. These included *Clostridium perfringens* [12], *Bacillus subtilis* [13-15] and *E. coli* [16] as donors and relied on either conjugative transposons [17] or conjugative plasmids [e.g. 16] as shuttles. The next step in the pathway for useable genetic systems was the development of the ClosTron mutagenesis tool [18], which relied on an erythromycin resistance marker and therefore on the previous, independent generation of a pair of strain 630 derivatives (strains 630Δ*erm* and 630E) which lacked one of the tandemly repeated *erm*(B) genes present in the transposon-like structure designated Tn*5398* [19]*.* These strains originated in different laboratories and were designated 630Δ*erm* (derived in the Mullany laboratory) [20] and 630E (derived in the Rood laboratory) [21]. As for strain 630; 630*erm* and 630E were disseminated through individual laboratories as well as the DSMZ culture collection (DSM No.: 28645) [22]

Parallel, *in vivo* investigations into the role of the toxins in *C. difficile* pathogenicity using strains 630Δ*erm* and 630E [23, 24] revealed between-strain differences which may be explained by changes in the genomes compared to the ancestral erythromycin resistant 630 isolates from which they were derived [25, 26]. The derivatives of *C. difficile* 630 (both *C. difficile* 630Δ*erm* and 630E) are different enough to affect the outcome of experimental investigations, but what then of the original strain?

*C. difficile* 630 has been re-sequenced twice [27, 28] and reannotated three times [29, 30, 31], 630*erm* has been sequenced twice [31, 32], a whole genome comparison of several 630E strains with a 630*erm* strain was published as well [25] (Table 1). Particularly notable differences between the sequences include the presence and chromosomal location of mobile genetic elements [27, 32], as well as the presence of the plasmid pCD630 [31, 33] (see also Figure 1). Certain differences within the genomes, such as the duplication of an rRNA gene cluster [32], might be indicative of laboratory adaptations. Together, these changes indicate that evolution has, and likely is, occurring within the laboratories which these isolates have passed through and strongly suggests independent evolutionary paths are being embarked upon in geographically isolated strains within individual labs. These differences are potentially important for the interpretation of experimental results and standardization of 630 isolates is required as it is often used as a positive control when being compared against mutants constructed in one of its derivatives detailed above. To do this, we need empirical genomic evidence showing which current stock is most closely related to the ancestral strain and likewise to the erythromycin sensitive derivatives now used throughout the community.

With the decreasing cost and increasing ease of full genome sequencing coupled with improved bioinformatics tools we think *C. difficile* researchers are in a position to use the previous dispersal of the 630 and 630-derived strains for a community wide evolutionary analysis. We propose to sequence every 630 strain currently being used by different laboratories. Investigators who wish to take part in this project can send their “lab” version of strain 630 and/or their “wild type” derivatives (630*erm*, 630E) for full genome sequencing to the Smits lab at the Leiden University Medical Center, with details of its provenance, when it was obtained and how it is stored, grown and if and how it has been sub-cultured. We request strains to be sent on dry ice as a frozen stock in Brain Heart Infusion (BHI) broth with 20% glycerol. If this is not possible the Roberts lab will forward a transport swab upon request to swab a 5-day old bacterial culture growing on a BHI agar plate to be sent back to the Smits lab for culturing. Please alert the authors by e-mail prior to sending strains.

Following sequencing, the data will be made publicly available immediately on a dedicated website for analysis by the community, which will also be reflected in the authorship of subsequent reports on the evolutionary adaptation to being a globally used (epidemic) type strain. It is envisaged the outcome will be a detailed understanding of within-laboratory evolution of *C. difficile* 630 highlighting any reproducible evolutionary pathways to laboratory adaptation and an indication and agreement on the most appropriate strain to use.

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**Table 1**

**Sequencing and reannotation projects of *Clostridium difficile* 630 and its derivatives**

|  |  |  |  |
| --- | --- | --- | --- |
| Project description | Template | Accession number(s) for (re)sequence | Reference |
| Sequencing | 630 | AM180355.1 and AM180356.1 | Sebaihia *et al*., 2006 [6] |
| Reannotation | 630 | - | Monot *et al*., 2011 [29] |
| Reannotation | 630 | - | Petit *et al*., 2014 [30] |
| Resequencing | 630 | CP010905.2 | Reidel *et al*., 2015 [27] |
| Sequencing | 630*erm* | LN614756.1 | Van Eijk *et al*., 2015 [32] |
| Resequencing | 630 | <https://www.ebi.ac.uk/ena/data/view/SAMEA2479565> | NCTC, 2016 [28] |
| Resequencing and reannotation | 630 and 630*erm* | CP016318.1 (630*erm*)  CP016319.1 (pCD630)  KX452725 (Tn*5397*) | Dannheim *et al*., 2017 [31] |
| Resequencing | 630*erm* and 630E | PRJNA304508; in particular  SRX1457283 and SRX1477719 (630E *tcdA*+*tcdB*-) and  SRX1477718 (630*erm**pyrE*) | Collery *et al*., 2017 [25] |
| Reannotation | pCD630 | - | Smits *et al*., 2018 [33] |

**Figure 1.**

**Whole genome comparison of *C. difficile* strain 630 and derivatives for which a complete genome sequence is available.** Note that this global overview does not allow discrimination of single nucleotide polymorphisms, but highlights presence/absence and location of certain larger structural variants. 630 genome sequences (black/grey) and 630*erm* sequences (red/orange) are represented as concentric circles. Structural variants are highlighted using symbols. Dots are used to indicate presence(dot)/absence (no dot) or duplications (two dots) of a sequence. Arrows indicate inversions. The dashed arrow between positions 180.4° and 334.8° indicates transposition of the CTn*5* element.

\* Note that in a later study [31] a low number of reads mapping to Tn*5397* was identified in this data. The significance of this is unclear as the coverage is much lower than most chromosomal locations. It is conceivable that the culture from which the DNA was isolated contains a subpopulation of cells that contain the element, whereas the majority has lost it.

\*\* Note that in a recent study [33] plasmid pCD630 was abundantly identified in this isolate; it was not identified in the original study due to size fractionation of the library preparation. No structural variants of pCD630 were identified in either resequencing studies [31,33].

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