Both, toxin A and toxin B, are important in *Clostridium difficile* infection

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The bacterium *Clostridium difficile* is the leading cause of healthcare associated diarrhoea in the developed world and thus presents a major financial burden. The main virulence factors of *C. difficile* are two large toxins, A and B. Over the years there has been some debate over the respective roles and importance of these two toxins. To address this, we recently constructed stable toxin mutants of *C. difficile* and found that they were virulent if either toxin A or toxin B was functional. This underlined the importance of each toxin and the necessity to consider both when developing countermeasures against *Clostridium difficile* infection (CDI). In this article we discuss our findings in the context of previous work and outline some of the challenges which face the field as a result.

**Introduction**

In recent years *Clostridium difficile* infection (CDI) has emerged as the leading cause of healthcare associated diarrhoea in the developed world. The endospores of this Gram-positive anaerobe are widely distributed in the environment and their ingestion by hospitalised patients with a disrupted gut microflora, a common consequence of antibiotic treatment, leads to colonisation and subsequent disease. The clinical symptoms of CDI can range from asymptomatic carriage to a mild self-limiting or severe diarrhoea which may progress to the potentially fatal conditions of pseudomembranous colitis and/or toxic megacolon. In most developed countries, the incidence of CDI continues to increase, concomitant with the emergence of so-called ‘hyper-virulent’ strains, responsible for increased severity of CDI and a rise in fatalities. In England and Wales, for example, where the mortality rates attributable to CDI are apparently falling, *C. difficile* still killed almost 4,000 people in 2009 alone. This upsurge in the incidence of CDI has placed a large financial burden on healthcare systems worldwide.

**Toxin A and Toxin B in Disease**

Two main virulence factors, toxin A and toxin B, have been described for *C. difficile*. The two encoding genes, *tcdA* and *tcdB*, respectively, form part of a well defined pathogenicity locus (PaLoc) (Fig. 1). The locus also encompasses three ancillary genes. The *tcdR* gene encodes an alternative sigma factor (TcdR) responsible for the transcription of *tcdA* and *tcdB*. A second gene, *tcdC*, is suggested to encode an anti-sigma factor (TcdC) which represses toxin production by directly interacting with TcdR or TcdR-containing RNA polymerase. The third gene, *tcdE*, codes for a protein (TcdE) which shares similarity to phage holin proteins, and may be involved in toxin secretion. The entire PaLoc element is 19.6 kb in size and, therefore, represents an investment by the organism in terms of its continued maintenance in the genome (Fig. 1).

The function of the toxins themselves has been studied extensively and their mode of action is now well understood. Both are cytotoxic and belong to the family of large clostridial toxins, capable of inactivating Rho-GTPases and leading to
disorganization of the cytoskeleton and subsequent cell death.\(^6,9\) It is important to understand the relative roles of toxin A and B in CDI, from both a diagnostic and a therapeutic perspective. In the 1980’s the spotlight fell firmly on toxin A, when it was shown that the administration of pure toxin A alone to hamsters caused disease, whereas toxin B alone did not. However, intragastric challenge of hamsters with toxin B did cause disease symptoms if prior damage to the mucosa had occurred through co-administration of sub-lethal concentrations of toxin A.\(^10\) Moreover, co-administration of toxin B with toxin A resulted in more severe disease symptoms. This led to the notion that both toxins acted in concert to bring about disease symptoms, with toxin A bringing about the initial damage to the colon allowing the more potent toxin B access.

The isolation of variant strains which apparently produced no toxin A (A\(^-\)B\(^+\) strains) from the early 1990s onwards presented a challenge to the established dogma that both toxins were required for disease. Whilst some of the early strains isolated were avirulent in the hamster model\(^11\) other independently isolated strains exhibited pathogenic potential.\(^12\) The latter strains, however, were shown to produce variant toxin B, showing similarity to Clostridium sordelli lethal toxin.\(^13,14\) These toxins consist of a TcdB binding domain but have a changed target. They have been shown to be as cytotoxic as ordinary TcdB, however they do show a differential cytopathic effect in cytotoxicity assays.\(^14\) Interestingly, the advent of molecular typing techniques\(^15\) has resulted in the isolation of an increasing number of A\(^-\)B\(^+\) toxinootypes that do not produce detectable toxin A.\(^16-18\) Nonetheless, the vast majority of strains isolated from cases of CDI continued to be found to produce both toxin A and B.

By this stage it was clear that insight into the relative roles of the two toxins required appropriate directed mutagenesis technologies. These would allow the creation of isogenic mutants of A\(^-\)B\(^+\) strains that produced one or other of the two toxins and an assessment of their pathogenic potential in an in vivo model of infection.

### Mutagenesis of C. difficile

The longstanding inability to make directed mutants in C. difficile was solved through the development of two, fundamentally different approaches based either on homologous recombination or on intron retargeting. The creation of insertional mutants by the former approach was achieved using either replication deficient\(^19\) or defective\(^20\) plasmids which are inserted into the chromosome via a single crossover recombination event. As such an event results in duplication of a region of DNA on either side of the inserted plasmid, the mutants generated are predicted to be unstable, as a subsequent recombination event between the duplicated DNA will result in plasmid excision. Indeed, such instability has been observed in a number of studies to date.\(^20-22\)

In the second approach, a system was developed that capitalised on the ability to retarget the specificity of a mobile group II intron to virtually any desired chromosomal locus. Pioneered by the Lambowitz laboratory, this system was marketed by Sigma Aldrich under the brand-name ‘Target\(^\text{\textregistered}\)’.\(^23\) The utility in clostridia of a basic, marker-less targetron was first demonstrated in Clostridium perfringens.\(^24\) The system first applied to C. difficile was termed the ClosTron,\(^25,26\) and incorporated a specialised retrotransposition-activated marker (RAM) within the group II intron, facilitating selection of integrants.

Aside from the extreme rapidity and effectiveness of the ClosTron mutagenesis system, one of its major strengths is that the mutants generated are stable. Thus, excision of the inserted segment of DNA is predicted, and indeed was experimentally verified,\(^25,26\) not to occur.

### Mutants of C. difficile Made by Recombination

The first isogenic toxin mutants of C. difficile were made using homologous recombination.\(^22\) Two mutants were constructed, an A\(^-\)B\(^+\) and a corresponding A\(^+\)B\(^-\). Following the phenotypic characterization of both mutants, their pathogenicity was tested in the hamster infection model. Whilst the parental strain (A\(^+\)B\(^-\)) and the A\(^+\)B\(^-\) single mutant caused disease, the A\(^-\)B\(^+\) single mutant did not. The authors concluded that toxin B alone was essential to disease. This observation caused a paradigm shift in the perception of the relative roles of the two toxins. Crucially, these conclusions had major implications to the development of medical counter-measures, both in terms of diagnosis and therapeutics.

### Mutants of C. difficile Made by ClosTron Technology

Given the major ramifications of the above study, we set out to replicate its findings through the creation of equivalent, stable mutants of the same strain of C. difficile using ClosTron technology.\(^27\) In this instance, we were also able to generate the control of a toxin A\(^-\)B\(^+\) double mutant, the first ever reported for C. difficile, in addition to single A\(^-\)B\(^+\) and A\(^+\)B\(^-\) mutants. The authenticity of the mutants was confirmed at both the DNA and phenotypic level. In the latter respect, the results concurred with those obtained by Lytras et al.\(^21\) Additionally, the double A\(^-\)B\(^+\) mutant was shown to be devoid of the ability to produce cytotoxic activity. Having confirmed the expected phenotypes of the three mutants, we went on to perform in vivo analysis using the hamster model of infection. In agreement with the previous study, all hamsters infected with the A\(^-\)B\(^+\) and A\(^+\)B\(^-\) mutants strains succumbed to CDI within 1–1.3 days. In contrast, however, all of the animals challenged with the A\(^+\)B\(^-\) mutant strain also developed
CDI, albeit over a slightly longer time-frame (mean time to death of 4 days). As expected, animals infected with the double A+B- mutant survived, demonstrating no symptoms of CDI (Fig. 2).

Overall, the findings of our study were largely in agreement with those of Lyras et al.\textsuperscript{21} with one notable exception. Our data clearly showed that an A+B- mutant producing toxin A alone can cause disease in the hamster. This latter finding re-establishes the importance of toxin A in CDI, and suggests that it should remain as a valid target when considering any medical countermeasures.

**The Relative Roles of Toxin A and B**

In comparison to Lyras et al.\textsuperscript{21} our findings created a paradox. How can two studies which generated essentially equivalent A+B- insertion mutants in the same strain of C. difficile, lead to a contradictory outcome in the hamster model of infection? There are a number of factors that need to be considered.

As discussed, we used fundamentally different methods to make our respective toxin B mutants. Nonetheless, both mutants were generated by inserting a segment of DNA into the coding region of tcdB. Whilst not at precisely the same point, the site of insertion is broadly speaking equivalent, residing within the catalytic domain, and resulting in the same phenotype; that is, discernable absence of toxin as measured by cytotoxicity assay and western blots. Therefore, the method of mutant generation is unlikely to explain the differences between the two studies.

Does the answer reside with the in vivo infection model? As in many countries, the UK regulatory authorities do not allow the use of death as an experimental endpoint in studies carried out in vivo, as was used by Lyras et al.\textsuperscript{21} Therefore, we defined terminal CDI based on a clinical scoring system and used this as our experimental endpoint.\textsuperscript{22} Importantly, we are confident that the difference between our two studies does not reside here. As the studies were undertaken in two different locations, subtle differences in animal maintenance and the procedures employed, or the sourcing of animals from disparate commercial suppliers, does introduce other possible variables. Hamsters from different colonies might have different microbiota compositions which could be important in terms of colonisation and susceptibility to CDI. Ideally, the two different mutants would need to be analysed in parallel under identical conditions at the same location. However, given that the differences in execution of the hamster model of infection represent an extremely unlikely explanation for the contradictory outcomes, it is difficult to justify the use of animals for this purpose.

The final difference between the two studies, and perhaps the most telling, relates to the strains used. Whilst the parent strain employed in both cases was C. difficile strain 630, there are fundamental differences between the isolates used. This is because mutant generation to date has relied on the ability to select resistance to erythromycin, a requirement that precludes the use of strain 630 as it carries a functional genomic copy of ermB. In both studies, therefore, an independently isolated derivative strain of 630 was employed that was sensitive to this antibiotic. In our study, we used the strain 630Δerm, isolated in the Mullany laboratory (UCL, London, UK) after 30 repeated subcultures of strain 630 in non-selective media.\textsuperscript{28} Following its isolation, the acquired sensitivity of 630Δerm was shown to be due to a specific deletion of ermB. In parallel, the Lyra laboratory independently isolated another erythromycin sensitive strain,\textsuperscript{20} through an undisclosed number of subcultures of strain 630 in non-selective media, and designated the strain 630E. Interestingly, although the data were not presented, it was reported to have undergone the same specific deletion of ermB as 630Δerm.\textsuperscript{20,28}

It is our hypothesis that during repeated subculture, ancillary mutations have arisen which have impacted on the virulence potential of one or other of the two strains in the presence of different toxin gene alleles. Crude phenotypic analyses support this view, as preliminary side-by-side comparisons of the 630Δerm and 630E have shown clear phenotypic differences. For example, whilst 630Δerm and the parent 630 strain are grossly indistinguishable, strain 630E has become non-motile and lacks any sign of flagella when examined by Electron Microscopy (Baban S, Kuehne S and Minton N, unpublished data). Moreover, unlike 630 and 630Δerm, 630E also exhibits a predilection to flocculate when grown in liquid culture (Kuehne S and Minton N, unpublished data) and has a more severe glucose response, affecting the levels of toxin produced in the presence of this carbon source (Dupuy B, personal communication).

To resolve these differences we have begun a programme of work designed to re-sequence the genomes of all the strains (mutants and progenitor strains) involved in the two studies to identify the nucleotide changes that have arisen, and correlate their presence or absence with observed phenotypes, including virulence/avirulence. At the same time we are in the process of making equivalent Clostron mutants in 630E to those
made in Δerm, together with similar mutants in other strains of \textit{C. difficile}, and in particular hyper-virulent strains. Furthermore the study of toxin mutants in other animal models could be considered. The mouse model is well established to study colonisation but has recently also been developed as an infection model.\textsuperscript{29} The piglet model has also been described as mimicking CDI well.\textsuperscript{30}

Throughout these studies, it should be borne in mind that while extremely useful, animals are only a model of human disease. Findings obtained in animals should, therefore, be treated with a certain amount of caution.

**Future Challenges**

Further work is required to more definitively define the relative role of toxin A and B in CDI. It is noteworthy that the majority of \textit{C. difficile} strains isolated to date carry a functional PaLoc and thus produce both toxin A and B. The notion of continued pressure for its presence in the \textit{C. difficile} population is implicit. What might this selective pressure be? Simplistically, the symptoms of CDI (diarrhoea) lead to more effective dispersal of spores. By implication, this is most efficient in organisms that produce toxin A and B, ensuring both toxins are normally present. Nonetheless, strains which produce only one of the toxins have arisen. This includes an isolated report of an A~B\textsuperscript{+} strain associated with relapse,\textsuperscript{31} as well as the more numerous examples of the isolation of A~B\textsuperscript{+} strains.\textsuperscript{12,16,17,32} The continued pathogenic potential of these variant strains, despite the loss of the capability to produce both toxins, needs to be investigated further to ascertain whether ancillary factors counter the loss of a toxin. For instance, is the toxin B that is produced by naturally occurring A~B\textsuperscript{+} strains equivalent to that produced by A~B\textsuperscript{+} strains, or has the cytotoxic potential of toxin B been altered, as in strains 8,864\textsuperscript{33} and 1,470\textsuperscript{33}? Furthermore, what contribution, if any, does the presence of the binary toxin, CDT, have in naturally occurring A~B\textsuperscript{+} strains?

In the meantime, it remains important to consider both toxin A and toxin B when developing diagnostics and therapeutics alike.

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**References**