The Anti-Sigma Factor TcdC Modulates Hypervirulence in an Epidemic BI/NAP1/027 Clinical Isolate of *Clostridium difficile*

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Abstract

Nosocomial infections are increasingly being recognised as a major patient safety issue. The modern hospital environment and associated care health practices have provided a niche for the rapid evolution of microbial pathogens that are well adapted to surviving and proliferating in this setting, after which they can infect susceptible patients. This is clearly the case for bacterial pathogens such as Methicillin Resistant Staphylococcus aureus (MRSA) and Vancomycin Resistant Enterococcus (VRE) species, both of which have acquired resistance to antimicrobial agents as well as enhanced survival and virulence properties that present serious therapeutic dilemmas for treating physicians. It has recently become apparent that the spore-forming bacterium *Clostridium difficile* also falls within this category. Since 2000, there has been a striking increase in *C. difficile* nosocomial infections worldwide, predominantly due to the emergence of epidemic or hypervirulent isolates that appear to possess extended antibiotic resistance and virulence properties. Various hypotheses have been proposed for the emergence of these strains, and for their persistence and increased virulence, but supportive experimental data are lacking. Here we describe a genetic approach using isogenic strains to identify a factor linked to the development of hypervirulence in *C. difficile*. This study provides evidence that a naturally occurring mutation in a negative regulator of toxin production, the anti-sigma factor TcdC, is an important factor in the development of hypervirulence in epidemic *C. difficile* isolates, presumably because the mutation leads to significantly increased toxin production, a contentious hypothesis until now. These results have important implications for *C. difficile* pathogenesis and virulence since they suggest that strains carrying a similar mutation have the inherent potential to develop a hypervirulent phenotype.

Citation: Carter GP, Douce GR, Govind R, Howarth PM, Mackin KE, et al. (2011) The Anti-Sigma Factor TcdC Modulates Hypervirulence in an Epidemic BI/NAP1/027 Clinical Isolate of Clostridium difficile. PLoS Pathog 7(10): e1002317. doi:10.1371/journal.ppat.1002317

Editor: Theresa M. Koehler, The University of Texas-Houston Medical School, United States of America

Received May 10, 2011; Accepted August 30, 2011; Published October 13, 2011

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Funding: This work was supported by Project Grants from the Australian National Health and Medical Research Council and the Australian Research Council (Monash University), Grant AI057637 from the United States National Institute of Allergy and Infectious Diseases (Monash University and Institut Pasteur) and by Project and Programme Grants from Institut Pasteur, The Wellcome Trust and a personal fellowship for GRD from the Royal Society of Edinburgh (Glasgow University). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

*C. difficile* is the causative agent of a spectrum of gastrointestinal diseases, collectively known as *C. difficile* infections, or CDI, that are induced by treatment with antibiotics that disrupt the normal gastrointestinal microbiota. CDI can range from mild diarrhoea, through moderately serious disease, to severe life-threatening pseudomembranous colitis, a chronic, often fatal, gastrointestinal disease [1]. During the past decade, there has been an astonishing increase in the rate and prevalence of *C. difficile* infections in many parts of the world, including the UK, USA, Canada and Europe, largely due to the emergence of a “hypervirulent” or epidemic group of isolates belonging to the BI/NAP1/027 category [2,3]. These strains are highly resistant to fluoroquinolones [3] and are associated with more severe disease and higher mortality rates [4–7]. *C. difficile* now also causes disease in those previously not at risk, such as children and pregnant women, with community-associated *C. difficile* disease being increasingly common [8–10].

The reasons for the emergence of these strains, and for their increased virulence, remain largely speculative. The use of fluoroquinolones, and the emergence of fluoroquinolone resistant strains, are undoubtedly driving factors in these new epidemics [11], however, the reasons for the heightened virulence and persistence of these strains are unknown. Genotypic and phenotypic comparison of the hypervirulent BI/NAP1/027 isolates to historical strains has identified numerous differences that may contribute to hypervirulence. Phenotypically, these differences may include the production of a toxin known as binary toxin, or CDT [3], and a higher sporulation rate [12]. Whole genome comparisons have identified numerous genetic differences with BI/NAP1/027 strains having an additional 234 genes compared to the well characterised strain 630 [13].
TcdC Modulates Hypervirulence in C. difficile

Author Summary

Hospital infections are increasingly being recognised as a major patient safety issue with the hospital environment providing a niche for the rapid evolution of microbial pathogens that are well adapted to infecting susceptible patients. The spore-forming Clostridium difficile is one such bacterium, which causes disease in patients undergoing antibiotic therapy. Since 2000, there has been a striking increase in C. difficile infections due to the emergence of hypervirulent isolates that appear to possess extended antibiotic resistance and virulence properties. Here we use a genetic approach to identify a factor linked to the development of hypervirulence in C. difficile. This study shows that a naturally occurring mutation in a negative regulator of toxin production, the anti-sigma factor TcdC, is an important factor contributing to the development of hypervirulence in epidemic isolates, presumably because it leads to significantly increased toxin production. These results have important implications for C. difficile pathogenesis since they suggest that strains carrying a similar mutation have the inherent potential to develop a hypervirulent phenotype. This study has increased our understanding of how these new variant strains cause disease and why they are more harmful, which is critical for the development of improved strategies for preventing and treating these infections.

including five unique genetic regions that are absent from both strain 630 and non-epidemic 027 strains [4]. Fundamentally, however, the factors directly resulting in the development of hypervirulence by these strains remain unknown.

The major virulence factors of C. difficile are two members of the large clostridial cytotoxin family, toxin A and toxin B, encoded by the tcdA and tcdB genes, respectively, which are potent monoglucosyltransferases that irreversibly modify members of the Rh family of host regulatory proteins [14]. Two recent studies definitively showed that toxin B plays a major role in the virulence of C. difficile [15,16]. The role of toxin A in disease was less clear however, with conflicting data concerning toxin A reported [15,16].

Epidemic strains are reported to produce significantly more toxin A and toxin B than other strains [2]. The tcdA and tcdB genes are located on the chromosome within a region known as the pathogenicity locus or PaLoc [17]. In addition to tcdA and tcdB, the PaLoc encodes three additional genes designated tcdR, tcdE and tcdG, which encode an alternative sigma factor, TcdR [18], a putative holin, TcdE [19], and an anti-sigma factor, TcdC [20], respectively. The expression of toxins A and B is controlled in a complex manner by several factors, including TcdR and TcdC. TcdG is thought to negatively regulate toxin production by interacting with TcdR or with TcdR-containing RNA polymerase holoenzyme or both [20]. TcdR is essential for toxin production [18]. BI/NAP1/027 C. difficile strains have a nonsense mutation in tcdG, which results in the production of a truncated protein that no longer negatively regulates TcdR. This mutation is postulated to be responsible for the increased toxin production observed in vitro in these strains [2]. Accordingly, this observation has prompted debate over the importance of the tcdG mutation in the hypervirulent phenotype. However, there is currently a lack of experimental evidence to support this hypothesis, with inconsistent reports in the published literature [20–22].

Despite their important impact worldwide on public health little is known about the virulence factors of BI/NAP1/027 strains and many important questions about the pathogenesis of disease caused by these strains remain to be answered, especially the role played by TcdC. BI/NAP1/027 isolates have proven difficult to genetically manipulate, which has hampered our ability to study these strains at the molecular level. To address these questions, here we use a novel Tn916-based plasmid conjugation system to facilitate the efficient transfer of plasmids into BI/NAP1/027 strains of C. difficile. Using this system, we have demonstrated conclusively the role of TcdC as a negative regulator of toxin production in C. difficile. Furthermore, using the hamster model of infection, we provide evidence to show that the tcdC mutation found in BI/NAP1/027 strains is an important factor in the development of hypervirulence by these strains. This study is the first to use isogenic strains to identify a factor involved in the development of a hypervirulent phenotype in C. difficile, and also represents the first in vivo demonstration of the role of TcdC in the pathogenesis of C. difficile disease.

Results

Complementation of the tcdC mutation in a BI/NAP1/027 epidemic isolate in trans

To determine if mutation of the tcdC gene in C. difficile BI/NAP1/027 isolates leads to the development of a hypervirulent phenotype it was necessary to construct isogenic BI/NAP1/027 strains that only differed in their ability to produce a functional TcdC protein. To construct the isogenic strains required for this analysis, genetic manipulation of BI/NAP1/027 isolates was required. The genetic manipulation of these strains has proved difficult and attempts to transfer plasmids into BI/NAP1/027 strains using published methods, which rely on RP4-mediated conjugation from Escherichia coli [23–27], were not successful, even though transfer of plasmids into the genetically amenable strains JIR8094, an erythromycin sensitive derivative of strain 630 [24], and CD37 was readily achieved (Table S1). To overcome this barrier and to facilitate DNA transfer into the strains of interest, we developed a novel plasmid transfer system that exploits the conjugation apparatus encoded by the broad-host range transposon Tn916.

The oriT region of Tn916 (oriT, trans) [28] was cloned into the catP-containing C. difficile shuttle plasmid pMTL9361Cm [29], generating pDLL4. This plasmid was introduced into C. perfringens strain JIR4225, which contains five copies of Tn916 [30] and plate matings were performed between this donor strain and several C. difficile strains, including a BI/NAP1/027 strain, M7404, which is a Canadian epidemic isolate [29]. Transconjugants from these matings were isolated on medium supplemented with thiamphenicol and cefoxitin. The efficiency of plasmid transfer into strain M7404 was $1.2 \times 10^7$–$4 \times 10^8$ transconjugants/ml of plated culture. Analysis of transconjugants using PCR specific for the catP gene together with restriction analysis confirmed that all putative colonies carried pDLL4 (data not shown), verifying successful plasmid transfer into the BI/NAP1/027 strain M7404. Similar plasmid transfer efficiencies were obtained for numerous other C. difficile strains (Table S1), highlighting the utility of this methodology for the genetic manipulation of clinically relevant strains.

To complement the tcdC mutation in a BI/NAP1/027 strain the intact tcdC gene from strain VP10463, together with 300 bp of its upstream region, was cloned into the shuttle plasmid pDLL4, generating pDLL17. This plasmid was transferred by Tn916-mediated conjugation from C. perfringens strain JIR4225 to C. difficile strain M7404 as before. PCR was subsequently used to confirm the presence of plasmid pDLL17 in representative transconjugants (data not shown).
To determine whether the presence of pDLL17 complemented the TcdC deficiency of M7404, Western immunoblots using TcdC-specific antibodies were performed. Lysates were collected from the wild-type M7404, the pDLL4-carrying vector control strain M7404(VC) and the pDLL17-tcdC strain M7404(tcdC), as well as strains VPI10463 and the PaLoc-deficient strain VPI11186, which served as positive and negative controls, respectively. An additional control strain, M7404(cured), was generated by serially passaging strain M7404(tcdC) on non-selective growth medium and curing the plasmid from this strain. Loss of the plasmid was confirmed by sensitivity of the strain to thiamphenicol followed by PCR analysis to verify the absence of several plasmid encoded genes (data not shown). As Figure 1A shows, whilst no TcdC could be detected in the lysates of the negative control strain, the wild-type M7404, M7404(VC) and the plasmid-cured strain M7404(cured), a 34-kDa protein that reacted with TcdC-specific antibodies was detected in lysates of the tcdC-complemented strain M7404(tcdC). This band was the same size as the immunoreactive TcdC protein produced by the positive control strain VPI10463, confirming that the tcdC mutation in the BI/NAP1/027 epidemic isolate M7404 was efficiently complemented in trans. Since complementation was performed using a multicopy plasmid, we also quantified TcdC production levels from strain M7404(tcdC) in comparison to strain VPI10463 using a time-course assay. Previous studies involving transcriptional analysis of PaLoc genes during different growth phases showed that tcdC is expressed in early exponential phase but not in stationary phase, whereas the other PaLoc genes show the opposite expression pattern [31]. VPI10463 (Figure 1B) and M7404(tcdC) (Figure 1C) exhibited similar TcdC expression patterns, with higher levels of TcdC observed in early exponential phase and negligible amounts detected beyond 16 hours, suggesting that the regulatory regions governing tcdC expression have been retained on the tcdC-carrying fragment used to construct pDLL17. In addition to the kinetics of TcdC expression in strain M7404(tcdC) mirroring that of VPI10463, a similar amount of protein was also detected at each time point with VPI10463 producing 1.3- to 1.6-fold more protein (Figure 1B) than M7404(tcdC) (Figure 1C). Therefore, although tcdC complementation was achieved using a multicopy plasmid vector, a physiologically relevant amount of TcdC protein was expressed during the appropriate growth phases in strain M7404(tcdC).

TcdC-mediated repression of toxin production in C. difficile

To define the role of TcdC in the virulence of a BI/NAP1/027 C. difficile isolate, female Golden Syrian hamsters were infected with spores of strain M7404 carrying either the vector control or the tcdC plasmid [n = 10 and n = 12, respectively]. For comparative purposes, a group of hamsters (n = 14) was also infected with strain 630, a strain previously characterised as being less virulent than other clinical isolates [32]. Following infection, all C. difficile strains were found to be equally efficient at colonising the hamsters (data not shown). Infection of colonised hamsters was allowed to proceed and animals were monitored by telemetry. The end point of infection was achieved when the core body temperature of the hamsters dropped to 35°C. This parameter has previously been shown to be a reliable indicator of non-recoverable disease [33]. At this point, the animals were immediately culled for animal ethics reasons. Bacteria were then isolated from the culled hamsters, the bacterial load quantified and isolates subjected to MVLA analysis [33] to confirm that these isolates were the same strain as originally used for infection.

Hamsters infected with the M7404(tcdC) derivative showed a significant delay (p = 0.0003; Logrank (Mantel-Cox) test; 95% confidence interval) in the mean time taken to reach non-recoverable disease (2570 minutes or 42.9 hours) in comparison to the vector-carrying M7404 group (M7404(VC)), with a mean time of 1869 minutes or 31.15 hours (Figure 3). In one of the hamsters colonised with the M7404(VC) strain, the time taken to reach the end point of infection was substantially longer than the other hamsters in this group (2814 minutes or 46.9 hours). This hamster was shown by statistical analysis (p = 0.0405; Grubbs test; 95% confidence interval) to be an outlier and was therefore excluded from the experimental analysis. Note that statistical significance would be retained upon inclusion of this outlier. Interestingly,
whilst the mean time to the end point of infection in the strain 630 group of hamsters (2701 minutes or 45.02 hours) was significantly longer than that of hamsters infected with M7404(VC) \((p = 0.0001; \text{Logrank (Mantel-Cox) test; 95\% confidence interval})\), there was no significant difference in the mean time taken to achieve non-recoverable disease in the 630 group compared to the M7404(tcdC\(^+\)) derivative, indicating that the virulence of the TcdC-complemented strain was equivalent to that of strain 630.

It is apparent from these virulence experiments that the expression of TcdC in a BI/NAP1/027 isolate has an important effect on virulence, resulting in a significant delay in the time needed to reach non-recoverable disease. These data therefore provide compelling evidence that the naturally occurring mutation of \(tcdC\) in BI/NAP1/027 isolates is an important factor in the development of a hypervirulent phenotype by these strains.
TcdC status of clinical isolates does not predict toxin production level

The results presented here show that a BI/NAP1/027 strain complemented with \textit{tcdC} is not as virulent as its isogenic vector-carrying control, suggesting that any \textit{Clostridium difficile} strain that acquires a null TcdC phenotype has the potential to develop a hypervirulent phenotype. Furthermore, phylogenetic studies have shown \textit{C. difficile} to be a genetically diverse species, with disease-causing isolates seemingly arising from multiple lineages, suggesting that virulence in these strains may have evolved independently \[4,34\]. The \textit{tcdC} status of a diverse group of clinical isolates was therefore determined in parallel to the genetic studies described.
higher levels that were approximately 10-fold more than strain DLL3053 even though both strains have identical tcdC alleles.

In comparison to the TcdC-positive reference strains, all of the tcdC-deficient clinical isolates produced more toxin than strain JIR8094 apart from strain DLL3053 (Figure 4A). Conversely, however, all strains produced significantly less toxin than VPI10463 (p = 0.0099–0.0202; unpaired t-test, 95% confidence interval), including the BI/NAP1/027 strain KI. VPI10463 produced over 100-fold more toxin than strains JIR8094 and DLL3053 and over 30-fold more than KI (Figure 4B). This observation is in agreement with recently published findings, which showed that VPI10463 produced significantly more toxin than other strains [12]; however, in that study, strains were grown using glucose-rich BHI medium so differential effects of glucose on toxin production by each strain could not be ruled out. In agreement with other studies [12,22,37], our data therefore suggest that the tcdC-status alone of C. difficile isolates is not an accurate predictor of high-level toxin production.

**Discussion**

The hypothesis that the naturally-occurring tcdC mutation in epidemic BI/NAP1/027 isolates contributes to hypervirulence is widely accepted, despite a lack of supportive experimental evidence. Indeed, the exact role of TcdC in the pathogenesis of C. difficile disease has remained controversial with conflicting findings reported in the literature [20–22]. As a result, several published studies have suggested that there is a need to assess isogenic tcdC strains in order to conclusively determine the role of this gene in the virulence of C. difficile [12,22,40]. We have now constructed such isogenic strains and compared them in an animal model. The results conclusively show that TcdC negatively regulates toxin production in C. difficile. Most importantly, complementation of the tcdC mutation in the BI/NAP1/027 epidemic isolate M7404 clearly showed that this mutation is a less important factor in the development of hypervirulence by this strain since the genetic complementation of tcdC reduced virulence in comparison to the wild-type strain.

To elucidate the role of TcdC in hypervirulence, it was necessary to construct an isogenic panel of BI/NAP1/027 strains

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**Figure 3. Virulence of C. difficile wild-type and tcdC-complemented strains in hamsters.** Kaplan-Meier survival curve demonstrating time from infection with C. difficile to death. M7404(VC), wild-type M7404 carrying shuttle plasmid pDLL4 (●); M7404(tcdC +) (□) and strain 630, a C. difficile isolate with known low virulence (▲). Hamsters were infected intragastrically with 10,000 spores from each strain; M7404(VC) (n = 9), M7404(tcdC +) (n = 12) and strain 630 (n = 14).

doi:10.1371/journal.ppat.1002317.g003
that were identical except for the presence or absence of the wild-type tcdC gene. Despite the publication of studies describing the successful transfer of plasmids into the BI/NAP1/027 isolate R20291 [25,26,41], this group of strains has remained difficult to work with at the molecular genetic level. As such, a new system that utilised the conjugation apparatus of Tn916 was developed in this study and used successfully to genetically manipulate a number of clinically relevant isolates, including a BI/NAP1/027 strain of C. difficile. Tn916 is a broad host-range conjugal transposon that was recently used to transfer plasmids into genetically intractable strains of Enterococcus faecium [28] and has been shown to transfer into C. difficile [42,43]. C. perfringens was chosen for use as a donor strain in anticipation that it may be more proficient for the transfer of plasmids into C. difficile in comparison to the more distantly related E. coli. The addition of oriT916 onto the shuttle vector pMTL9361Cm facilitated the efficient transfer of this plasmid into strain M7404 from a Tn916-carrying C. perfringens strain. Furthermore, this system has been successfully used to transfer shuttle plasmids into every C. difficile isolate tested so far (Table S1). Most importantly, this new technology facilitated the complementation of the tcdC mutation in strain M7404 enabling the role of TcdC in the virulence of BI/NAP1/027 strains of C. difficile to be investigated.

Previous in vitro studies have shown that TcdC is able to sequester the TcdR sigma factor, preventing its association with core RNA polymerase and blocking toxin gene expression [20]. These experiments suggested that TcdC was important in the regulation of toxin production by C. difficile, but the in vivo role of this protein was not determined. Conversely, several studies on C. difficile clinical isolates [22,37] showed that the absence of a functional tcdC gene was not an accurate predictor of high level toxin production or increased disease severity, indicating that TcdC may not play an important role in virulence in these strains [22,37]. The analysis of Australian clinical isolates in the present study is in accordance with these latter studies in that isolates with naturally occurring tcdC mutations were found to produce toxin at a range of different levels that were not necessarily high. However, since these strains, and those in the other studies [22,37], are not isogenic it is not possible to draw conclusions about the importance of tcdC in the context of toxin yield or virulence. By contrast, the isogenic tcdC strains studied here clearly show that TcdC is a negative regulator of toxin production since the tcdC complemented BI/NAP1/027 C. difficile strain produced significantly less toxin A and B than the non-complemented control strains. The finding that TcdC-status is not correlated with toxin production in clinical isolates highlights the limitation of accurately assigning gene function by studying non-isogenic strains, particularly in a highly heterogeneous species such as C. difficile. In this context, it might be of interest to study the function of tcdC in isogenic strains generated in a different genetic background such as a ribotype 078 isolate.

Analysis of PaLoc gene expression by qRT-PCR demonstrated that TcdC exerts regulatory control of toxin production at the transcriptional level, and this is in keeping with its proposed role as an anti-sigma factor [20]. The observation that the expression of tcdR and tcdE is reduced in the tcdC-complemented strain, together with tcdA and tcdB, is probably because of autoregulation of tcdR since TcdR upregulates its own expression and that of the other PaLoc genes [23].

The virulence of strain M7404 was reduced upon complementation of tcdC, clearly demonstrating that the tcdC mutation in BI/NAP1/027 strains has a significant impact on virulence and is likely to be an important factor in the development of hypervirulence by these strains. Surprisingly, the virulence of M7404(tcdC+) was found to be equivalent to that of strain 630, which has been shown in other studies to be reduced in virulence in comparison to other isolates, including three other BI-type strains [32]. These findings have important implications for C. difficile.
C. difficile virulence since they suggest that strains carrying tcdC mutations have the inherent potential to develop hypervirulence. The recent emergence of a new class of hypervirulent strains, ribotype 078 [44], may be one such example. These isolates encode a non-functional TcdC protein [37], produce significantly more toxin than non-epidemic strains, are associated with more severe disease as well as higher rates of mortality and are increasingly being identified as the causative agent of CDI [44,45]. Although these experiments show that TcdC-status alone can modulate virulence it is probable that multiple factors working synergistically are necessary for the development of hypervirulence in the BI/NAP1/027 strains. It is likely that the accumulation of multiple genetic changes in addition to the tcdC mutation has enabled BI/NAP1/027 strains to become the predominant disease-causing isolates in numerous countries. Of particular importance might be variations in the functional activity of the encoded toxins since these isolates were recently shown to produce a toxin B that shows variation across the C-terminal receptor binding domain of the protein [46], resulting in more potent activity across a wider range of cell lines in comparison to toxin B from the historical, non-epidemic strain 630 [4]. Furthermore, using the zebrafish embryo model of intoxication, the BI/NAP1/027 toxin B was recently shown to have pronounced in vivo cytotoxic activity in comparison to toxin B from VPI10463, another historical non-epidemic isolate, with greater tissue tropism and more extensive tissue destruction observed [47]. Since toxin B is thought to be one of the major virulence factors of C. difficile [15,16] these observations suggest that TcdB variations might play an important role in the hypervirulent phenotype.

There are other factors that may influence C. difficile hypervirulence. The BI/NAP1/027 strains encode an additional toxin known as binary toxin or CDT [2]. The role of this toxin in CDI remains to be elucidated but a recent study showed that CDT toxin known as binary toxin or CDT [2]. The role of this toxin in disease-causing isolates is important since they suggest that strains carrying tcdC mutations have the inherent potential to develop hypervirulence. The recent emergence of a new class of hypervirulent strains, ribotype 078 [44], may be one such example. These isolates encode a non-functional TcdC protein [37], produce significantly more toxin than non-epidemic strains, are associated with more severe disease as well as higher rates of mortality and are increasingly being identified as the causative agent of CDI [44,45]. Although these experiments show that TcdC-status alone can modulate virulence it is probable that multiple factors working synergistically are necessary for the development of hypervirulence in the BI/NAP1/027 strains. It is likely that the accumulation of multiple genetic changes in addition to the tcdC mutation has enabled BI/NAP1/027 strains to become the predominant disease-causing isolates in numerous countries. Of particular importance might be variations in the functional activity of the encoded toxins since these isolates were recently shown to produce a toxin B that shows variation across the C-terminal receptor binding domain of the protein [46], resulting in more potent activity across a wider range of cell lines in comparison to toxin B from the historical, non-epidemic strain 630 [4]. Furthermore, using the zebrafish embryo model of intoxication, the BI/NAP1/027 toxin B was recently shown to have pronounced in vivo cytotoxic activity in comparison to toxin B from VPI10463, another historical non-epidemic isolate, with greater tissue tropism and more extensive tissue destruction observed [47]. Since toxin B is thought to be one of the major virulence factors of C. difficile [15,16] these observations suggest that TcdB variations might play an important role in the hypervirulent phenotype.

There are other factors that may influence C. difficile hypervirulence. The BI/NAP1/027 strains encode an additional toxin known as binary toxin or CDT [2]. The role of this toxin in CDI remains to be elucidated but a recent study showed that CDT induces the formation of microtubule-based protrusions on the host cell surface thereby increasing C. difficile adherence to epithelial cells. Moreover, intestinal colonisation of gnotobiotic mice with a BI/NAP1/027 C. difficile strain was significantly reduced in mice treated with CDT-neutralising antibodies in comparison to control mice [48]. These findings suggest that CDT may be an important colonisation factor, enhancing the ability of BI/NAP1/027 strains to initiate infection as well as causing adjunctive tissue damage during later stages of infection, potentially leading to more severe disease. Many BI/NAP1/027 strains are also more proficient at sporulation than non-epidemic C. difficile strains [12,49]. C. difficile spores are highly infectious [50] and play a critical role in the transmission of CDI and perhaps in disease relapse, which is a serious problem in patients with CDI [51]. In this context, enhanced sporulation is ostensibly an important adaptation by BI/NAP1/027 isolates, which would result in larger numbers of spores being shed from infected patients and an increased environmental spore load, ultimately leading to higher transmission rates. Finally, the development of florquinolone resistance, in particular to moxifloxacin and gatifloxacin, is unquestionably a major factor in epidemics caused by BI/NAP1/027 strains [3,11]. In this regard, the hypothetical co-evolution of enhanced virulence traits and antibiotic resistance in C. difficile mirrors trends seen with other significant nosocomial pathogens such as Methicillin Resistant Staphylococcus aureus (MRSA) [52] and Vancomycin Resistant Enterococcus (VRE) species [53]. In summary, it is clear that our findings represent an important breakthrough in our understanding of the development of hypervirulence in prevailing C. difficile isolates and will provide a significant reference point for future studies on epidemic strains and their control.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the United Kingdoms Home Office Animals (Scientific Procedures) Act of 1986 which outlines the regulation of the use of laboratory animals for the use of animals in scientific procedures. The experiments were subject to approval by the University of Glasgow Ethics Committee and by a designated Home Office Inspector (Project Number 60/4218). All experiments were subject to the 3 R consideration (refine, reduce and replace) and all efforts were made to minimize suffering.

Bacterial strains and growth conditions

The characteristics and origins of all recombinant strains and plasmids are shown in Table 1 and Table S2, respectively. All bacteriological culture media were obtained from Oxoid. C. difficile strains were cultured in BHIS [54] or TY medium [15], unless otherwise stated, in an atmosphere of 10% H2, 10% CO2, and 80% N2 at 37°C in a Coy anaerobic chamber. Escherichia coli was cultured in 2×YT medium aerobically at 37°C, with shaking for broth cultures. All antibiotics were purchased from Sigma-Aldrich and were used at the following concentrations: cycloserine (Cs, 250 μg/ml), cefoxitin (Cf, 5 μg/ml), thiamphenicol (Tm, 10 μg/ml) or tetracycline (Tc, 10 μg/ml), chloramphenicol (Cm, 25 μg/ml).

Molecular biology and PCR techniques

Plasmid DNA was isolated using a QIAprep spin miniprep kit (Qiagen). Genomic DNA was prepared using a DNaseasy tissue kit (Qiagen). Standard methods for the digestion, modification, ligation, and analysis of plasmid and genomic DNA were used [55]. Nucleotide sequence analysis was carried out using a PRISM BigDye Terminator cycle sequencing kit (Applied Biosystems) and detection was performed by Micromon at Monash University. Oligonucleotide primer sequences are listed below. Unless otherwise stated, all PCR experiments were carried out with Phusion DNA polymerase (New England Biolabs) and the 2× FailSafe PCR buffer E (Epicentre) according to the manufacturer’s instructions.

Construction of recombinant plasmids

For construction of the Tn916 transferable clostridial shuttle vector, PCR was performed using primers DLPS3 (5′-GAATTCCGCGCTTTTTTTTTATACGCGGTTG-3′) and DLPS4 (5′-GAATTCCGCGGAAAAGGACGATATTGTCGG-3′) and chromosomal DNA extracted from Clostridium perfringens strain JIR4225 [30]. The resulting 700 bp DNA fragment, which contained the onT region of Tn916, was TOPO-cloned into pCR-Blunt II-TOPO according to the manufacturer’s instructions (Invitrogen). The fragment was then excised from pCR-Blunt II-TOPO using EcoRI and cloned into the equivalent sites of plasmid pMTL9361Cm [29], resulting in plasmid pDLL4.

For construction of the tcdC-carrying plasmid, PCR was performed using primers DLPS5 (5′-GAGAGGAGCTGCAGCC-3′) and DLPS6 (5′-GAATTCTGCAGGAGAGC-3′) and chromosomal DNA extracted from C. difficile strain VPI10463 genomic DNA in order to amplify a 1085 bp fragment encompassing the tcdC gene and upstream region. This fragment was then TOPO-cloned into pCR-Blunt II-TOPO, before being excised with PstI and subcloned into the equivalent site of plasmid pDLL4, resulting in the final construct pDLL17.

PLoS Pathogens | www.plospathogens.org
October 2011 | Volume 7 | Issue 10 | e1002317
Table 1. Bacterial strains.

| Strain | Characteristics | Source/Reference |
|--------|-----------------|------------------|
| E. coli |                 |                  |
| DH5α   | F-<em>q</em>R<sup>−</sup> Δ<i>lacZ</i>ΔM15Δ<i>lacZY</i>-argF<i>U169</i> endA1 recA1 <i>hisd</i>17(λ<sup>−</sup> m<sup>−</sup> <i>−</i>)deoR thi-1 supE44 gyrA96 relA1 | Life Technologies |
| TOP10  | F-<em>met</em>A Δ<em>mini</em>hisΔRMS-mcr<sup>B</sup>Δ<i>q</i>80Δ<i>lacZ</i>ΔM15 Δ<i>lacX</i>74 <i>nupG</i> recA1 araD139 Δ<em>ara-leu</em>7697 galU galK <i>ramL</i>(<sup>S</sup>)<sup>−</sup> endA1 5<sup>−</sup> | |
| HB101  | thi-1 <i>his</i>5209(<sup>−</sup> m<sup>−</sup> <i>−</i>) supE44 recA8 ara-14 leuB5 proA2 lacY1 galK rpsL20 (<sup>S</sup>)<sup>−</sup> ytr-5 mtl-1 | [60] |
| C. perfringens |                 |                  |
| JIR4225 | <i>C. perfringens</i> strain JIR325 with 5 chromosomal copies of <i>Tn</i>916 | [30] |
| C. difficile |                 |                  |
| M7404  | Canadian BI/NAP1/027 isolate | [29] |
| 630    | Wild-type <i>C. difficile</i> strain; first <i>C. difficile</i> genome available | [61] |
| JIR8094| Erythromycin sensitive derivative of strain 630 | [24] |
| VPI10463 | PaLoc-positive <i>C. difficile</i> isolate | [38] |
| VPI11186 | PaLoc-negative <i>C. difficile</i> isolate | [38] |
| CD37   | PaLoc-negative <i>C. difficile</i> isolate | [62] |
| KI     | Australian BI/NAP1/027 isolate | [36] |
| DLL3053| Australian toxinotype III clinical isolate | This study |
| DLL3054| Australian toxinotype V clinical isolate | This study |
| DLL3055| Australian toxinotype V clinical isolate | This study |
| DLL3056| Australian toxinotype XIV clinical isolate | This study |
| M7404(VC) | Dll3001 (M7404 carrying shuttle plasmid pDLL4) | This study |
| M7404(tcdC<sup>+</sup>) | Dll3002 (M7404 carrying tcdC expression plasmid pDLL17) | This study |
| M7404(cured) | Dll3003 (M7404(tcdC<sup>+</sup>) cured of plasmid pDLL17) | This study |

doi:10.1371/journal.ppat.1002317.t001

Transfer of plasmid DNA into <i>C. difficile</i> by conjugation

The conjugation procedure utilizing <i>E. coli</i> HB101(pVS520) as the conjugative donor was carried out as previously described [29]. Recombinant plasmids were introduced into <i>C. perfringens</i> strain JIR4225 as before [56]. Conjugations utilizing <i>C. perfringens</i> strain JIR4225 were then performed as follows: separate 90 ml BHIS broth cultures were inoculated with 1 ml aliquots from an overnight starter culture and grown to mid-exponential phase. Approximately overnight, broth cultures were inoculated with 1 ml aliquots from an overnight <i>C. difficile</i> JIR325 was then performed as follows: separate 90 ml BHIS agar supplemented with cefoxitin and thiamphenicol or tetracycline, and the plates incubated under anaerobic conditions for 24 to 72 h.

Toxin A-specific Western blots

The toxins were partially purified by ammonium sulphate precipitation from culture supernatants harvested after growth for 72 hours and toxin A was then detected by Western blotting as described previously [15].

TcdC-specific Western blots

For non-quantitative TcdC-specific Western Blots, crude extracts of <i>C. difficile</i> were prepared by sonication of samples taken from cultures that had been grown for 12 hours under anaerobic conditions. The crude extracts from each strain were then subjected to electrophoresis in a 15% SDS-PAGE gel and transferred to a nitrocellulose membrane using standard methods [55]. Membranes were treated with anti-TcdC antibody [37] and detected following treatment with goat anti-mouse IgG-alkaline phosphatase conjugated secondary antibody using standard procedures. For quantitative TcdC-specific Western blots, cultures of <i>C. difficile</i> VPI10463 and <i>C. difficile</i> M7404(tcdC<sup>+</sup>) were grown under anaerobic conditions and samples were removed every 4 hours for 24 or 28 hours respectively. Each sample was normalized to an optical density (600 nm) of 0.9 prior to lysis, to ensure that the same number of cells was present. Lysates were then prepared by sonication prior to SDS-PAGE gel electrophoresis, transfer and detection, as described above. Following detection, the amount of TcdC in each lysate was quantified by densitometric analysis, using purified recombinant TcdC protein (rTcdC) as the standard and the ImageJ software package, according to published methods [58].

Vero cell cytotoxicity assays

Toxin B was detected in <i>C. difficile</i> culture supernatants harvested after growth for 72 hours by Vero cell cytotoxicity assays as described previously [15], except that each well was seeded with 1 x 10<sup>5</sup> cells.

RNA extraction and reverse transcription

Total RNA was extracted from <i>C. difficile</i> cultures grown for 12 h in TY media. Reverse transcription was performed using
AMV Reverse Transcriptase (Promega) using random hexamer oligonucleotides primers and 2 μg template RNA. The cDNA samples were then purified using Qiaquick Columns (Qiagen).

qRT-PCR assay design and real time PCR
PaLoc gene specific primers were designed using Primer 3 software (Genious Software). qRT-PCR was performed using an AB7500 real-time PCR instrument (Applied Biosystems). Reactions were carried out using the FastStart Universal SYBR Green Master Mix (Roche) with 40 ng of cDNA as template. Standard curves were generated for each primer pair using C. difficile genomic DNA, and melt curve analysis was performed following each qRT-PCR reaction to verify amplification specificity. Samples were normalised using the C. difficile 16S gene.

Preparation of spores for animal infection
Spores were prepared from C. difficile cultures grown in 500 ml of BHI broth. Cultures were pelleted by centrifugation for 10 mins and re-suspended in 50% ethanol. The material was then vortexed every 10 min for 1 h before centrifugation for 10 mins. The pellet was then treated with 1% Sarkosyl in PBS for 1 h at room temperature and again pelleted by centrifugation, followed by incubation overnight at 37°C with lysozyme (10 mg/ml) in 125 mM Tris-HCl buffer (pH 8.0). The sample was treated in a sonicating water bath (3 pulses of 3 min each; 1510 Branson) before centrifugation through a 50% sucrose gradient for 20 mins. The pellet was incubated in 2 ml of PBS containing 200 mM EDTA, 300 ng/ml proteinase K and 1% Sarkosyl for 30 mins at 37°C before centrifugation through a 50% sucrose gradient for 20 mins. The final pellet was then washed twice in sterile distilled water before finally being resuspended in 1 ml of sterile water. Spore preparations were stored at −80°C prior to use.

Hamster experiments
Female Golden Syrian hamsters purchased from Harlan Olac UK were used for all animal experiments. Telemetry chips (Vitalview Emitter) were inserted by laparotomy into the body cavity of the animals at least 3 weeks before infection with C. difficile. Animal experiments were then carried out as described previously [33], except that animals received 1×10^8 spores of C. difficile. Animals were culled when core body temperature dropped below 35°C. This study was carried out in strict accordance with the recommendations in the United Kingdoms Home Office Animals (Scientific Procedures) Act of 1986 which outlines the regulation of the use of laboratory animals for the use of animals in scientific procedures. The experiments described were subject to approval by the University of Glasgow Ethics Committee and by a designated Home Office Inspector (Project Number 60/4219). All experiments were subject to the 3 R consideration (refine, reduce and replace) and all efforts were made to minimize suffering.

Quantification of bacterial load
To estimate colonisation, hamsters were sacrificed and the gut region from the caecum to the anus removed. The tissues were homogenised in PBS using a Stomacher and viable counts were performed on the homogenate as described previously [33].

Confirmation of infecting strains
To confirm that the bacteria isolated from the hamster were the same strain as originally used for infection, genomic DNA was isolated and subjected to MVLA as described previously [59]. Plasmid rescue was performed as previously described [29] followed by restriction digest analysis to confirm plasmid integrity.

Supporting Information
Table S1 Efficiency of RP4 or Tn916-mediated plasmid transfer to C. difficile strains. The efficiency of RP4 or Tn916-mediated plasmid transfer from E. coli or C. perfringens donors, respectively, to C. difficile recipient strains is shown, calculated as described in Materials and Methods and expressed as transconjugants per ml.
(LOC)

Table S2 Bacterial plasmids. Bacterial plasmids used in this study, together with the genetic features relevant to this work, are shown.
(LOC)

Acknowledgments
We thank Christoph von-Eichel Streiber for kindly providing toxin A-specific antibodies and Rachael Poon and Tongted Phumooma-Das for assistance with Vero cell cytotoxicity assays.

Author Contributions
Conceived and designed the experiments: GPC BD G AJ JIR GRD DL. Performed the experiments: GPC PMH KEM DK RG GRD JS AMB AA. Contributed reagents/materials/analysis tools: GPC BD GAJ JIR PMH KEM DK RG AA GRD JS AMB DL. Performed the paper: GPC DL.

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Title: 
The Anti-Sigma Factor TcdC Modulates Hypervirulence in an Epidemic BI/NAP1/027 Clinical Isolate of Clostridium difficile

Date: 
2011-10-01

Citation: 
Carter, G. P., Douce, G. R., Govind, R., Howarth, P. M., Mackin, K. E., Spencer, J., Buckley, A. M., Antunes, A., Kotsanas, D., Jenkin, G. A., Dupuy, B., Rood, J. I. & Lyras, D. (2011). The Anti-Sigma Factor TcdC Modulates Hypervirulence in an Epidemic BI/NAP1/027 Clinical Isolate of Clostridium difficile. PLOS PATHOGENS, 7 (10), https://doi.org/10.1371/journal.ppat.1002317.

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