Variant forms of the binary toxin CDT locus and *tcdC* gene in *Clostridium difficile* strains

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Variability in the genes for toxin A, toxin B and other pathogenicity locus regions is well known and is the basis for the distribution of *Clostridium difficile* strains into variant toxinotypes. Previous data have indicated that some *C. difficile* strains have a non-functional truncated form of the binary toxin (CDT) locus. This study analysed variability in the CDT locus and the presence of deleted *tcdC* genes in *C. difficile* strains. A total of 146 strains were screened, including known variant toxinotypes and non-variant A⁺B⁺ (toxinotype 0) and A⁺B[−] *C. difficile* strains. In all of the strains studied, only two forms of the CDT locus were found: a full-length 4.3 kb fragment encoding the functional binary toxin or a truncated 2.3 kb fragment. Whilst the full-length CDT locus was found almost exclusively in variant toxinotypes, the truncated form was detected in 79 % of toxinotype 0 strains. Non-toxinogenic A[−]B[−] strains with a truncated version were not found and only rarely possessed the full-length CDT locus (A[−]B[−]CDT⁺ strains). Four different forms of the *tcdC* gene were found; three represented deleted versions and typically were found in toxinotypes III–VII, XI, XIV–XVI and XXIV.

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INTRODUCTION

Over the last decade, *Clostridium difficile* has developed into one of the most important nosocomial pathogens. This Gram-positive, sporulating micro-organism is associated with post-antibiotic intestinal infections, and the prevalence rate and disease severity seem to be rising (McDonald *et al.*, 2006). Additionally, a new highly virulent and multi-resistant type (BI/NAP1/027) has emerged recently and is spreading in Canada, the USA, the UK, the Netherlands, Belgium and France (Loo *et al.*, 2005; Pepin *et al.*, 2004; McDonald *et al.*, 2005; Smith, 2005; Joseph *et al.*, 2005; Kuijper *et al.*, 2006; Tachon *et al.*, 2006). In addition to high fluoroquinolone resistance, this epidemic type has some features previously known but uncommon among *C. difficile* isolates: variant genes for toxins A (TcdA) and B (TcdB), production of the binary toxin (CDT) and deletions in the *tcdC* gene.

Variability in the *tcdA* and *tcdB* genes has been well studied and the strains with changed genes have been grouped into toxinotypes (Rupnik *et al.*, 1998). Major changes are defined as deletions and RFLPs in all six PCR fragments covering *tcdA* and *tcdB*, whilst minor changes are limited to only one

of the PCR fragments (usually the A3 fragment) covering the toxin genes or other pathogenicity locus (PaLoc) regions. To date, 22 toxinotypes have been described and two additional types are reported in this paper. The best-studied and the most widespread are toxinotypes III (including ribotypes 027, 034, 075 and 080) and VIII (including ribotypes 017 and 047) (Rupnik *et al.*, 2001).

Binary toxin CDT is detected in some strains in addition to TcdA and TcdB, the primary virulence factors of *C. difficile* (Popoff *et al.*, 1988; Rupnik *et al.*, 2003a). Binary toxin CDT is composed of two unlinked components, the enzymic (CDTa) and binding (CDTb) components, and acts as an actin-specific ADP-ribosyltransferase (Popoff *et al.*, 1988; Barth *et al.*, 2004). CDT has been shown to have a cytotoxic effect on Vero cells in culture (Perelle *et al.*, 1997) and an enterotoxic effect in a rabbit ileal loop assay (Geric *et al.*, 2006). The role of CDT in the development of human disease is not currently well understood. Interestingly, binary toxin-producing strains often seem to be associated with community-acquired *C. difficile* infections (Terhes *et al.*, 2004; Barbut *et al.*, 2005). The prevalence of binary toxin-producing strains has been estimated to be between 1.6 and 6 % (Stubbs *et al.*, 2000; Rupnik *et al.*, 2001, 2003b; Geric *et al.*, 2003; Goncalves *et al.*, 2004; Terhes *et al.*, 2004; Alonso *et al.*, 2005), although much higher percentages have been reported in other studies (Braun *et al.*, 2000; Perelle

Abbreviations: LCT, large clostridial toxin; PaLoc, pathogenicity locus; TcdA, toxin A; TcdB, toxin B.

et al., 1997; Spigaglia & Mastrantonio, 2002; McEllistrem *et al.*, 2005) and the incidence seems to have increased in recent years (Spigaglia & Mastrantonio, 2004).

CDT is encoded on a 4.3 kb chromosomal locus, composed of the genes *cdtA* and *cdtB* (Perelle *et al.*, 1997). Within the fully sequenced genome of A⁺B⁺CDT⁻ *C. difficile* strain 630 (Sebaihia *et al.*, 2006), regions with significant similarity to binary toxin have been found to be present, representing a truncated CDT locus (Fig. 1). The regions at the 5' end of the *cdtA* gene and the 3' end of the *cdtB* gene were highly conserved, but approximately 2 kb of sequence was missing in this strain (Fig. 1a) (Rupnik *et al.*, 2003a). A similar 2 kb deletion has been reported for the reference *C. difficile* strain VPI 10463 (Chang & Song, 2001).

Another marker for *C. difficile* strains that differ from the reference strain VPI 10463 is a deletion in the *tcdC* gene. The *tcdC* gene is part of the PaLoc (encoding the toxins TcdA and TcdB and three accessory genes, *tcdR*, *tcdE* and *tcdC*) and encodes a putative negative regulator of TcdA and TcdB (Hundsberger *et al.*, 1997). Truncated forms of this gene have been described previously (Spigaglia & Mastrantonio, 2002), but the presence of a deletion in the epidemic strain BI/NAP1/027 has again focused attention on the importance of such deletions and their potential role in

enhanced virulence (McDonald *et al.*, 2005; MacCannell *et al.*, 2006).

The aim of this study was to determine the distribution of the truncated and full-length CDT locus in *C. difficile* strains with different large clostridial toxin (LCT) production status and from different toxinotypes and to assess the correlation of the truncated *tcdC* gene with different variant toxinotypes.

METHODS

Strains. We screened 146 *C. difficile* isolates, including variant and non-variant toxinotypes, as well as non-toxinogenic strains (A⁻B⁻).

Eighty strains were from toxinotype 0 (A⁺B⁺ strains producing toxin TcdA and TcdB, identical to the reference strain VPI 10463), whilst 25 did not produce TcdA and TcdB (A⁻B⁻). The majority of these strains were from the collection of M. Delmée at UCL Brussels and belonged to different serogroups (A–I, K and X). Additional strains from various origins (Sweden, Germany and Japan) previously sent to our laboratory for toxinotyping were also included and were subsequently serotyped as described by Delmée *et al.* (1985).

The remaining 41 strains were representatives of all 24 known toxinotypes (<http://www.mf.uni-mb.si/mikro/tox>; 22 toxinotypes published so far, with two new ones described in this paper). For each toxinotype, the reference strain was tested. For toxinotypes IV, V, VI, IX, XIa, XIb and XII we tested one additional strain, for toxinotype

Fig. 1. (a) Comparison of the region encoding the binary toxin CDT in *C. difficile* CDT-positive strain CD196 and the fully sequenced strain *C. difficile* 630. In strain 630, a truncated form of the CDT is present. Three regions (boxes 1–3) show high homology with strain CD196, and 2 kb between boxes 2 and 3 is deleted (Δ). Numbers indicate positions in the nucleotide sequence. The positions of two PCR fragments are indicated with solid lines. The CDTc fragment amplifies the entire binary toxin locus, whilst the BTb fragment is generally used for *C. difficile*-specific detection of binary toxin genes. (b) Amplification of the CDTc PCR fragment in representative toxinotypes, revealing the presence of the complete or truncated CDT locus. M, 500 bp DNA ladder (Gibco Invitrogen); XIa, positive control with functional genes for the binary toxin; 630, positive control with a truncated CDT locus.

III two additional strains and for toxinotype VIII seven additional strains. Three strains of toxinotype III were representatives of subtypes IIIa, IIIb and IIIc, which differed in B2s fragment RFLPs (Geric *et al.*, 2004). Two representatives of the so far unpublished toxinotypes determined in this study (XXIII and XXIV) were also included. Strain 8785 originated from the collection of M. Delmée, Brussels, Belgium, and was the type strain of serogroup A9. It had a novel combination of restriction patterns in fragments B1 (restriction pattern 5) and A3 (restriction pattern 2) and therefore represents a novel toxinotype, XXIII. Strain 597B, originally isolated in Kuwait, is the type strain of toxinotype XXIV. It showed no differences in the *tcdB* and *tcdA* genes when compared with *C. difficile* reference strain VPI 10463, but was distinguished from toxinotype 0 by a small deletion (~20 bp) in the *tcdC* gene and by the presence of the binary toxin genes *cdtA* and *cdtB* (see Results and Discussion).

Characterization of truncated types of *tcdC*. The *tcdC* gene was amplified by PCR for the PLC fragment using primers Tim2 and Strupp2, as described previously (Braun *et al.*, 1996), and was digested with *NcoI* to reveal short deletions within the *tcdC* gene.

CDT locus amplification. Strains were grown on Columbia 5% sheep blood agar (BBL; BD). For crude DNA preparation, 12–24 h cultures were suspended in 5% Chelex 100 (Bio-Rad), vortexed and boiled for 10 min. After centrifugation for 10 min at 10 000 g, the supernatant was transferred into a fresh tube and stored at 4 °C until use.

PCR for the BTb fragment corresponding to a shorter part of the *cdtB* gene encoding the binding component of the binary toxin (Fig. 1a) was performed as described previously (Stubbs *et al.*, 2000).

Primers BIN5 (5'-AATATTGGGAGGGAGAATAAATG-3') and BIN8 (5'-AATATATATTGTATTGAGGGGAC-3'), which are positioned outside the CDT locus (Spigaglia & Mastrantonio, 2002), were used for detection of the complete CDT locus sequence. PCRs were performed in a total volume of 50 µl using 3 µl crude DNA extract, 200 µM each dNTP and 15 pM each primer. The final concentration of MgCl₂ in the reaction mixture was 3 mM. The hot-start PCR protocol started with denaturation at 93 °C for 3 min after which 2 U *Taq* polymerase (Roche) was added per reaction, followed by 35 cycles of denaturation at 93 °C for 4 s and annealing and extension at 47 °C for 5 min. The reaction was terminated by a 10 min incubation at 47 °C. Fragments were visualized on a 1% agarose gel.

Similarity dendrogram of the PaLoc regions. A dendrogram of the PaLoc region for the different toxinotypes was constructed based on PCR RFLP patterns in the reference strains of toxinotypes 0–XXIV. For each toxinotype, data on amplification and RFLPs for seven DNA fragments covering the PaLoc region (Braun *et al.*, 1996; Rupnik *et al.*, 1998; Geric *et al.*, 2004) were included in the matrix: B1 cut with *HincII* and *AccI*, B2s cut with *HindIII* and *RsaI*, B3s cut with *HindIII* and *RsaI*, A1 cut with *NsiI* and *PstI*, A2 cut with *HaeIII*, A3 cut with *EcoRI* and PLC cut with *NcoI*.

For successful amplification of a particular fragment as well as the presence of each possible restriction fragment, '1' was entered in the matrix and for its absence '0' was recorded. Jaccard's similarity coefficients were calculated using the computer software Free-Tree version 0.9.1.50 (Pavlycek *et al.*, 1999) and analysed by the unweighted pair group method using arithmetic averages (UPGMA). The resulting PaLoc similarity tree of discrete characters was created using MEGA3 (Kumar *et al.*, 2004).

RESULTS AND DISCUSSION

Of the 146 strains studied, 81 had been typed previously as toxinotype 0. However, when all strains were retested for the

short BTb PCR fragment (Fig. 1a) used for routine detection of functional CDT genes, one strain (597B) was positive and subsequently was found to contain the full-length CDT locus. The same strain also had an altered *tcdC* gene (Fig. 2; type 2). As toxinotypes are defined as groups of strains with changes in the PaLoc region (and not solely changes in the *tcdA* and *tcdB* genes), we included this strain as a novel toxinotype, XXIV. Characteristics of this toxinotype were a deletion in the *tcdC* gene and the presence of binary toxin genes, with the *tcdA* and *tcdB* genes being identical to toxinotype 0. To date, only one other similar strain has been described that was typed as toxinotype 0 using A3 and B1 fragment PCR, but had the binary toxin genes and a *tcdC* deletion (Spigaglia & Mastrantonio, 2002).

The distribution of the studied strains into known and new toxinotypes as described above was implemented in all further analyses of the binary toxin locus and *tcdC* gene deletions.

Forty-one strains from variant toxinotypes were tested for deletions in *tcdC*. After restriction with *NcoI*, four restriction types could be distinguished: type 1 was the full-length gene and types 2–4 represented three different deletions (Fig. 2). So far in the literature, *tcdC* deletions have been described in toxinotypes V and VI (Spigaglia & Mastrantonio, 2002) and toxinotype III (Warny *et al.*, 2005; McDonald *et al.*, 2005; Loo *et al.*, 2005). Some toxinotype III strains have an

Fig. 2. Types of *tcdC* deletion found in the variant toxinotypes. Although the length differences in the *tcdC* products are visible in the amplified products (not shown), the types of deletion can be differentiated more readily following *NcoI* restriction.

additional nonsense mutation in the *tcdC* gene (MacCannell *et al.*, 2006). As shown in Fig. 3, the *tcdC* deletions were not specific for toxinotypes III, V and VI, but were present in most of the variant toxinotypes. Truncated versions 2 and 3 were prevalent, whilst version 4 was detected only in toxinotype IV. In two toxinotypes with large deletions in the *tcdA* gene (X and XVII), *tcdC* could not be amplified with the primers used.

When amplifying the binary toxin CDT locus in the 146 strains studied, only two types of fragment were obtained: the full-length 4.3 kb fragment encoding the functional binary toxin or a truncated 2.3 kb fragment (Fig. 1b). The short 2.3 kb version of the CDT locus has been reported previously in strains VPI 10463 and 630 (Chang & Song, 2001; Rupnik *et al.*, 2003a) and does not lead to expression of functional binary toxin. Our data suggested that the deletion in the truncated CDT locus is always of the same size (Fig. 1b). Some minor sequence differences in CDT genes have been published (Stubbs *et al.*, 2000; Pituch *et al.*, 2005), but whether they affect the function of the toxin is not known. The truncated versions have not been described so far for other species known to produce related binary toxins (*Clostridium perfringens* type E and *Clostridium spiroforme*).

It has been recognized previously that the presence of functional binary toxin genes encoded by the 4.3 kb CDT locus correlates well with variant toxinotypes including A⁺B⁺, A⁻B⁺ and A⁻B⁻ strains (Stubbs *et al.*, 2000; Rupnik *et al.*, 2001, 2003a, b; Geric *et al.*, 2004; Spigaglia & Mastrantonio, 2002; Goncalves *et al.*, 2004). This study presents an overview of all of the known variant toxinotypes and has confirmed that the full-length CDT locus is found in variant toxinotypes with significantly changed LCT toxin genes (shown in bold in Table 1), whereas the truncated version of the CDT locus is predominant in variant toxinotypes with minor changes in the LCT toxin genes (Table 1). Only three toxinotypes did not contain any CDT-related sequences using the primers and amplification conditions described: II, VIII and XXI. Interestingly, two of them (VIII and XXI) are major toxinotypes. For type XXI, only a single representative is known. However, toxinotype VIII represents the most widespread variant toxinotype (A⁻B⁺ strains of serogroups F and X); therefore, a larger selection of strains could be tested. However, all were found to be negative for CDT amplification.

To address the correlation of the variant PaLoc region and the presence of the CDT locus, we constructed a tree

Fig. 3. Similarity tree of the PaLoc region in the different toxinotypes based on Jaccard's similarity indices using UPGMA. The presence of the complete binary toxin CDT locus (+) or the truncated CDT locus (Δ) or absence of the CDT locus (-) is indicated. The presence of a *tcdC* form (1-4) related to deletion size is shown for *tcdC* (see Fig. 2). NA, Not amplified.

Table 1. Presence and composition of the CDT locus in the *C. difficile* strains tested

Toxinotype	Toxin production status	CDT locus			No. of strains tested
		None	Truncated	Full-length	
0	A ⁺ B ⁺	17	63	0*	80
PaLoc absent	A ⁻ B ⁻	25	0	0†	25
Variant toxinotypes‡	A ⁺ B ⁺	2 (II, XXI)	7 (I, XII, XIII, XVIII-XX)	18 (III-VII , IX, X, XIV, XV, XXII , XXIII , XXIV)	27
	A ⁻ B ⁺	8 (VIII)	0	2 (XVI , XVII)	10
	A ⁻ B ⁻	0	0	4 (XIa , XIb)†	4

*Two strains have been determined with a PaLoc region almost identical to toxinotype 0 except for a small deletion in *tcdC* (Spigaglia & Mastrantonio, 2002; and this work, toxinotype XXIV).

†Three non-toxinogenic (PaLoc-negative) strains with binary toxin genes have been reported previously (Geric *et al.*, 2003). Toxinotype XI strains do not produce TcdA and TcdB, but have the remnants of the PaLoc region present.

‡Variant toxinotypes with major changes in the PaLoc region (RFLPs and/or deletions in all three domains of the LCT toxin genes: B1, B2 and B3, and A1, A2 and A3) are shown in bold.

reflecting similarities between the PaLoc regions in different toxinotypes (Fig. 3). This tree did not show the phylogenetic relationships among toxinotypes, but clearly distinguished among toxinotypes with minor changes (branch 1) and major changes (branches 2 and 3) in the PaLoc region. Toxinotypes with similar changes in the PaLoc region grouped together, e.g. V–VII, XVI and XI, and X and XVII. Interestingly, toxinotypes XXI and VIII, the only major variant toxinotypes without the CDT locus, were positioned separately and did not belong to any of the major branches. The evolution of the *C. difficile* CDT locus needs further investigation, especially with regard to the striking co-existence of binary toxin genes and changes in the PaLoc region in variant strains, and it has been suggested that different toxinotypes do not have a common ancestor (Wozniak *et al.*, 2000).

In non-variant strains (toxinotype 0), the truncated CDT locus was found in 63/80 strains (79%) (Table 1). In several cases where more than one strain per serogroup was tested (e.g. serogroups K and A14), there was no correlation between serogroup and the type of CDT locus (data not shown). Neither the full-length nor the truncated form of the CDT locus was amplified in any of the 25 PaLoc-negative A⁻B⁻ strains (Table 1). However, A⁻B⁻CDT⁺ strains have been described previously and two subgroups have been differentiated. The first group is A⁻B⁻CDT⁺ strains in which the PaLoc is replaced by 115 bp, as has been found for non-toxinogenic strains (Geric *et al.*, 2003). The second group of A⁻B⁻CDT⁺ strains is represented by toxinotypes XIa and XIb, known to produce binary toxin but not toxins TcdA and TcdB, although a short part of the non-functional PaLoc is still present (Table 1) (Stubbs *et al.*, 2000; Geric *et al.*, 2003).

The presence of the full-length or truncated CDT locus in A⁺B⁺ strains and its predominant absence in A⁻B⁺ and

A⁻B⁻ strains could suggest a possible positive selection of strains with numerous virulence factors.

In summary, we have presented data showing the heterogeneity of the *tcdC* gene and the binary toxin locus in *C. difficile* strains. Both features – deletions in *tcdC* and the presence of a functional full-length CDT locus – are characteristic of the majority of variant toxinotypes, the emerging epidemic type BI/NAP1/027 (toxinotype III) being one of the recent well-characterized examples. Interestingly, toxinotype VIII, one of the most widespread variant *C. difficile* types, characterized by changes in *tcdA* and *tcdB* and with an A⁻B⁺ phenotype, possessed neither a *tcdC* deletion nor any form of the CDT locus.

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