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tcdC Genotypes Associated with Severe TcdC Truncation in an Epidemic Clone and Other Strains of *Clostridium difficile*▽

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Severe *Clostridium difficile* associated disease is associated with outbreaks of the recently described BI/NAP1 epidemic clone. This clone is characterized by an 18-bp deletion in the *tcdC* gene and increased production of toxins A and B in vitro. TcdC is a putative negative regulator of toxin A&B production. We characterized *tcdC* genotypes from a collection of *C. difficile* isolates from a hospital that experienced an outbreak caused by the BI/NAP1 epidemic clone. Sequence analysis of *tcdC* was performed on DNA samples isolated from 199 toxigenic *C. difficile* isolates (31% BI/NAP1) from 2001 and 2005. Sequences obtained from 36 (18.6%) isolates predicted wild-type TcdC (232 amino acid residues), whereas 12 (6.1%) isolates had *tcdC* genotypes with previously described 18- or 39-bp deletions. The remaining isolates comprised 15 unique genotypes. Of these, 5 genotypes contain 18- or 36-bp deletions. Of these five genotypes, one is characterized by a single nucleotide deletion at position 117 resulting in a frameshift that introduces a stop codon at position 196, truncating the predicted TcdC to 65 amino acid residues. All 62 of the isolates in this collection comprising the epidemic clone are characterized by this genotype. This result suggests that severe truncation of TcdC is responsible for the increased toxin production observed in strains belonging to the BI/NAP1 clone and that the 18-bp deletion is probably irrelevant to TcdC function. Further investigations are required to determine the effect of this and other *tcd*C genotypes on toxin production and clinical disease.

Clostridium difficile is the etiologic agent responsible for human diseases ranging from mild diarrhea to severe pseudomembranous colitis, which are collectively referred to as *C. difficile*-associated disease (CDAD) (1, 2, 9). A hospital outbreak with increased rates of severe CDAD was noted at the University of Pittsburgh Medical Center-Presbyterian Hospital (UPMC-P) in 2000 to 2001, with 26 colectomies and 18 deaths in that period (3, 15). Similar outbreaks of severe CDAD have since been reported throughout the United States and Canada (8, 12). An epidemic *C. difficile* clone, designated BI by restriction endonuclease typing (REA), NAP-1 by pulsed field gel electrophoresis, and toxinotype III by the toxinotyping method of Rupnik et al. has emerged and is in part responsible for these outbreaks (12, 19–21). Whereas BI/NAP1–toxinotype III isolates comprised only 0.3% of >6,000 pre-2001 *C. difficile* isolates maintained in the reference collection at the Hines Veterans Affairs Hospital (HVA), 10 to 75% of post-2001 isolates in U.S. hospitals and 51% of 2001 UPMC-P hospital-acquired *C. difficile* isolates belong to the BI/NAP1 clone (12). Thus, the increased incidence of severe CDAD closely parallels the emergence of the BI/NAP1 clone.

Recent epidemic BI/NAP1 isolates have been found to hyperproduce toxins A and B in vitro, the organism's major virulence factors (24). These toxins are encoded on the 19.6-kb pathogenicity locus (PaLoc) by the *tcdA* and *tcdB* genes. In addition, three accessory genes *tcdC*, *tcdR* (formerly *txeR* or *tcdD*), and *tcdE* which encode proteins thought to be involved in toxin regulation and secretion are located on the PaLoc (5). In reference strain VPI10463, transcriptional analysis of the PaLoc demonstrates that *tcdC* is expressed during logarithmic growth phase in contrast to *tcdA*, *tcdB*, and *tcdR*, which are most highly expressed during the stationary phase (6, 7). These data suggest that *tcdC* functions as a negative regulator of toxin synthesis.

Spigaglia and Mastrantonio have described three *tcdC* genotypes from nonepidemic *C. difficile* isolates. Genotype *tcdC-A* is characterized by a nonsense mutation (C184T) that is predicted to result in severe truncation of the TcdC protein from 232 to 61 amino acid residues. In addition, this genotype contains a 39-bp deletion from nucleotides 341 to 379. Genotype *tcdC-A* was identified in *C. difficile* isolates of toxinotypes V and VI. In the same study, genotypes *tcdC-B* and *tcdC-C* were identified. These *tcdC* alleles were characterized by 18-bp deletions at nucleotides 330 to 347 (23). Of note, genotype *tcdC-C* was derived from a *C. difficile* isolate of an asymptomatic patient. On the other hand, similar 18-bp deletions have been detected by fragment size discrimination and sequencing of partial *tcdC* amplicons (G. Killgore, Centers for Disease Control and Prevention, unpublished data) among BI/NAP1 isolates and are proposed to result in a loss of toxin regulation accounting for the more severe disease attributed to the epidemic clone (12, 24). Six epidemic clone isolates from Canada

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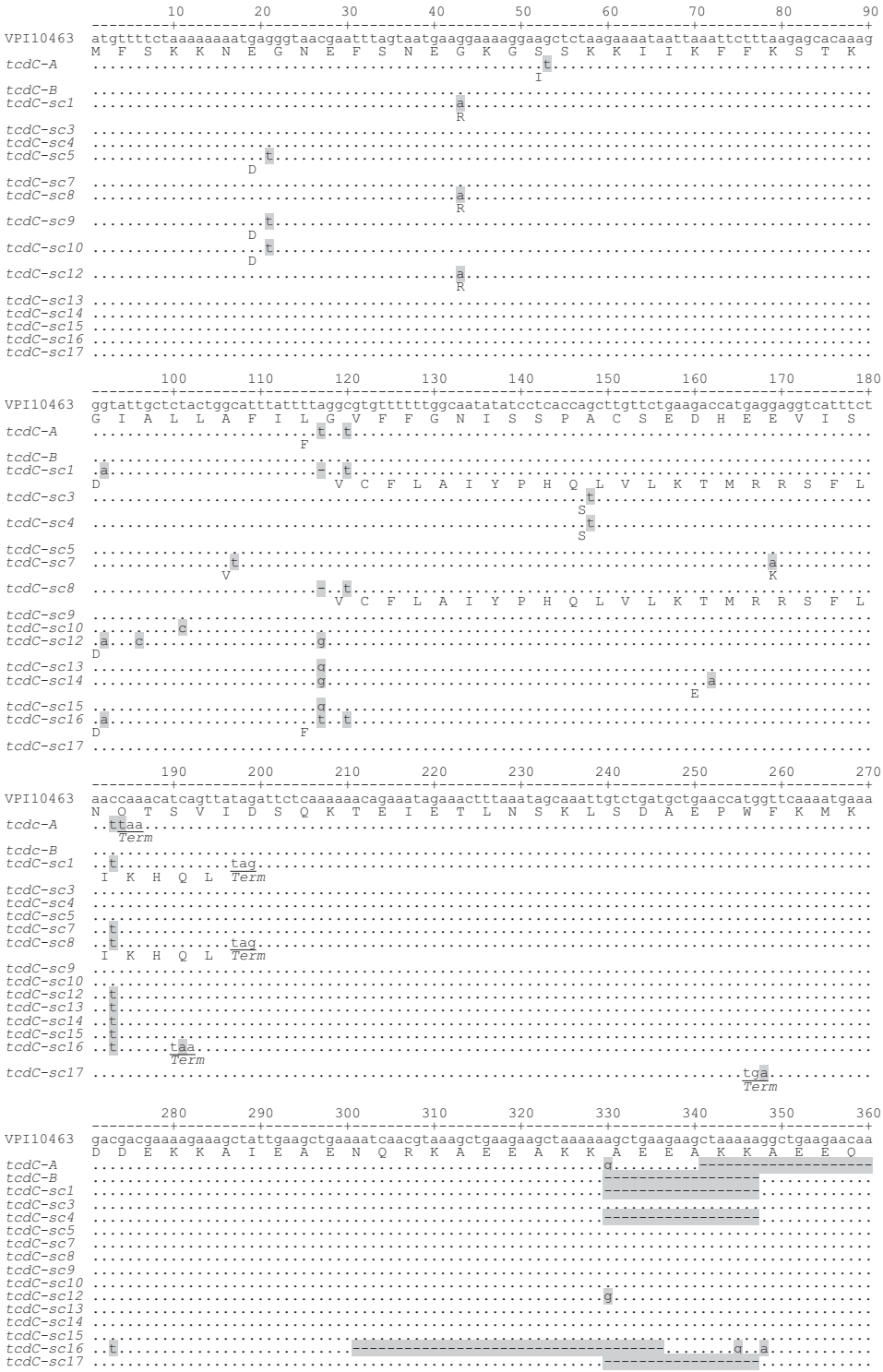

FIG. 1. Comparison of TcdC nucleotide and amino acid sequences to the published sequence for the reference strain VPI10463. Dots and dashes indicate identical bases and deletions, respectively. Only amino acid changes are depicted. Stop codons are underlined. Genotype *tcdC-sc2* is not depicted but is identical to *tcdC-sc1* with the exception of an inserted nucleotide (t) in the untranslated region at position 212.

and one reference strain from the United Kingdom have recently been shown to have *tcdC* genotypes containing both 18-bp deletions and a single nucleotide deletion at position 117 that introduces a frameshift mutation that truncates the predicted TcdC product to 65 amino acid residues (10).

We describe *tcdC* genotypes from a collection of clinical *C. difficile* isolates from UPMC-P by sequence analysis of the entire *tcdC* gene to determine (i) the prevalence of previously reported and novel *tcdC* genotypes and (ii) the association between *tcdC* genotype and the epidemic BI/NAP1 clone.

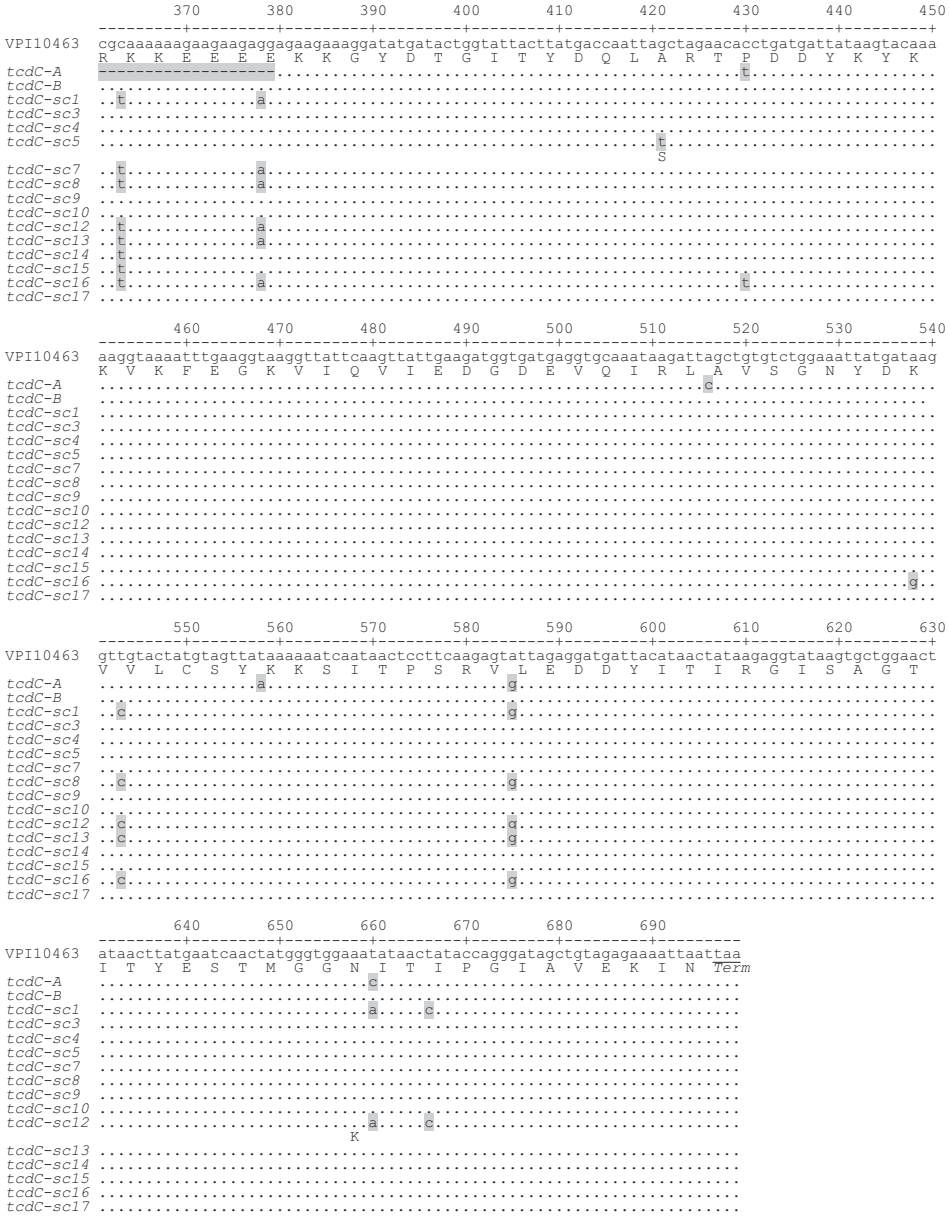

FIG. 1—*Continued.*

MATERIALS AND METHODS

Setting. All isolates of *C. difficile* in the present study originated from diagnostic specimens sent for *C. difficile* toxin testing for suspected CDAD from patients at UPMC-P, an 834-bed tertiary care teaching facility affiliated with the University of Pittsburgh Schools of the Health Sciences. This facility serves as a referral center for patients from institutions throughout western Pennsylvania, eastern Ohio, and northern West Virginia. Isolates from UPMC-P inpatients, affiliated outpatient clinics, and the UPMC-P emergency department and from non-hospital-acquired cases of CDAD were included.

Isolation of *C. difficile*. Diagnostic testing for CDAD was carried out using a cell culture cytotoxicity assay for *C. difficile* toxins (CDT) (Diagnostic Hybrids; Athens, OH). Testing for CDAD was done at the discretion of the patients' healthcare providers. During the study period, all stools sent for CDAD testing were also cultured for *C. difficile* on Remel CCFA (cefoxitin, cycloserine, fructose agar) at 37°C after 4 h of anaerobic preincubation as previously described (13). Presumptive *C. difficile* isolates were identified by odor and colony morphology, subcultured on prereduced CDC anaerobe 5% sheep blood agar (Becton Dickinson, Franklin Lakes, NJ), and biochemically identified with a RapID ANA II panel (Remel, Lenexa, KS). *C. difficile* isolates were suspended in prereduced chopped meat broth (Anaerobe Systems, Morgan Hill, CA) and incubated for 48 h at 37°C. Then, 1.5 ml of the broth culture was centrifuged at 10,000 × *g* at room temperature. The supernatant was inoculated onto cell culture using the same CDT cytotoxicity assay used for CDT detection in stool specimens. CDT-positive isolates were stored in the remaining chopped meat broth at room temperature and were available for *tcdC* genotyping.

Selection of isolates. A total of 199 UPMC-P toxigenic *C. difficile* isolates were genotyped with contributions from two time periods. The first group comprised 126 *C. difficile* isolates, a subset of CDT-positive stool specimens collected between 21 March and 19 December 2001. The second group comprised 73 *C. difficile* isolates, a subset of CDT-positive stool specimens collected between 2 April and 30 May 2005.

Sequencing of *tcdC*. Isolates were subcultured onto prereduced TSA II with 5% sheep blood plates (Becton Dickinson, Franklin Lakes, NJ). DNA was extracted by QIAGEN DNeasy kit (Valencia, CA) according to the manufactur-

TABLE 1. Characteristics of *tcdC* genotypes of isolates from 199 cases of CDAD at UPMC 2001 and 2005

tcdC genotype[a]	No. of cases (%)	Predicted truncating mutations	Nucleotide deletions (>1 bp)	Deletion (>1 bp) beyond stop codon?	Predicted TcdC length (amino acid residues)	No. of residue changes in TcdC (no. deleted)	REA type(s) within group (no. typed)
Wild type (VPI10463)	36 (18.1)				232	0 (0)	K (1)
tcdC-A	3 (1.5)	C184T	39	Yes	61	2 (171)	
tcdC-B	9 (4.5)	None	18	No	226	0 (6)	
tcdC-sc1	91 (45.7)	Δ117A	18	Yes	65	27 (167)	BI 9 (3), BI10 (2)
*tcdC-sc2**	1 (0.5)	Δ117A	18	Yes	65	27 (167)	BR2 (1)
tcdC-sc3	23 (11.9)	None	None		232	1 (0)	J9, 28-31 (6)
tcdC-sc4	1 (0.5)	None	18	No	226	1 (6)	
tcdC-sc5	5 (2.5)	None	None		232	2 (0)	
tcdC-sc7	6 (3.0)	None	None		232	2 (0)	
tcdC-sc8	1 (0.5)	Δ117A	None		65	26 (167)	
tcdC-sc9	9 (4.5)	None	None		232	1 (0)	CL (1)
tcdC-sc10	1 (0.5)	None	None		232	2 (0)	
tcdC-sc12	2 (1.0)	None	None		232	2 (0)	
tcdC-sc13	1 (0.5)	None	None		232	0 (0)	
tcdC-sc14	3 (1.5)	None	None		232	1 (0)	BM (1)
tcdC-sc15	5 (2.5)	None	None		232	0 (0)	
tcdC-sc16	1 (0.5)	C191A	36	Yes	63	2 (169)	
tcdC-sc17	1 (0.5)	G258A	18	Yes	85	0 (147)	

[a] *, Genotype *tcdC-sc2* is identical to genotype *tcdC-sc1* except for the insertion of a single nucleotide (t) at position 212.

er's instructions. The *tcdC* gene was PCR amplified with primers C1 and C2 (IDT, Coralville, IA) as previously described (23). These primers amplify a 718-bp fragment of the PaLoc encompassing the entire *tcdC* gene (23). The reaction mixture contained AmpliTaq 10× PCR buffer, 125 nmol of $MgCl_2$, 10 pmol of C1 and C2 primers, 10 nmol of each deoxynucleotide triphosphate, and 1.5 U of AmpliTaq gold (Applied Biosystems, Foster City, CA). The template was denatured at 95°C for 5 min, and DNA was amplified for 35 cycles consisting of 1 min at 95°C, 1 min at 50°C, and 1 min at 72°C. Sequencing was performed with the amplification (C1 and C2) primers with a BigDye terminator 3.1 kit (Applied Biosystems) according to the manufacturer's instructions. Capillary sequence analysis was performed with a 3730 DNA sequencer (Applied Biosystems) at the University of Pittsburgh's Genomics and Proteomics Core Laboratories facility. Sequences were analyzed and amino acid sequences deduced with the Lasergene 7.0.0 software package (DNAStar, Madison, WI) and compared to the published *tcdC* sequence for strain VPI10463. Isolates with sequences that differed from those previously published genotypes were named sequentially with the prefix *sc* (Fig. 1 and Table 1).

***C. difficile* genotyping methods.** All isolates tested had previously undergone molecular genotyping by MLVA (for multilocus, variable number of tandem repeats analysis) (11). Briefly, *C. difficile* isolate genomic DNA was PCR amplified and sequenced at seven tandem repeat loci. Tandem repeat copy numbers were manually counted and concatenated to generate an MLVA type for each isolate. Minimum spanning tree (MST) analysis of MLVA types was performed to determine the genetic distance between isolates using the summed tandem repeat difference (STRD) as a coefficient of genetic distance in the BioNumerics software (v3.0; Applied Maths, Austin, TX). This method was validated against HVA reference strains including isolates belonging to the epidemic BI/NAP1 clone (11). Fifteen isolates in the present study had been REA typed at the HVA reference lab as part of a previous study (11).

In the present study, MST analysis of MLVA data was used to attribute *tcdC* genotypes to genetically related isolates. Clonal complexes were defined by an STRD of ≤2 (shaded areas in Fig. 2). Based on this definition, isolates were considered to be part of the BI/NAP1 epidemic population if they clustered with REA-typed HVA BI reference strains.

Nucleotide sequence accession numbers. The unique nucleotide sequences of the *tcdC* genes of representative *C. difficile* strains for genotypes *tcdC-sc1*, *tcdC-sc2*, *tcdC-sc3*, *tcdC-sc4*, *tcdC-sc5*, *tcdC-sc7*, *tcdC-sc8*, *tcdC-sc9*, *tcdC-sc10*, *tcdC-sc12*, *tcdC-sc13*, *tcdC-sc14*, *tcdC-sc15*, *tcdC-sc16*, and *tcdC-sc17* were assigned GenBank accession numbers DQ861412, DQ861414, DQ861415, DQ861416, DQ861417, DQ861418, DQ861419, DQ861420, DQ861421, DQ861422, DQ861423, DQ861424, DQ861425, DQ861426, and DQ861413, respectively.

RESULTS

Frequency of *tcdC* genotypes and new genotypes identified. The frequencies of the genotypes within this collection of 199 *C. difficile* isolates are summarized in Table 1. Wild-type *tcdC* sequences corresponding to that of toxigenic strain VPI10463 comprised 36 of 199 (18.1%) isolates. Genotypes previously described, *tcdC-A* and *tcdC-B*, were present in 3 of 199 (1.5%) and 9 of 199 (4.5%) isolates, respectively.

The remaining 151 isolates belonged to one of 15 unique *tcdC* genotypes (Fig. 1). Of these, genotype *tcdC-sc1* represented the greatest proportion (91 of 199 isolates [45.7%]), followed by *tcdC-sc3* (23 isolates [11.6%]). Three of these *tcdC* genotypes (*tcdC-sc1*, *tcdC-sc2*, and *tcdC-sc8*) are characterized by a single nucleotide deletion at nucleotide position 117 (Δ117). This deletion results in a frameshift that introduces a nonsense mutation at position 196 and a radical alteration in the preceding 26 deduced amino acid residues. The predicted *tcdC* gene product is truncated from 232 to 65 amino acid residues. Both *tcdC-sc1* and *tcdC-sc2* contain 18-bp deletions at nucleotides 330 to 347, which are identical to the previously described 18-bp deletions characteristic of the BI/NAP1 clone (11, 12, 24). Because the 18-bp deletion is located downstream of the mutated nucleotide at position 117 this region of *tcdC* is predicted to be untranslated. Unlike *tcdC-sc1* and *tcdC-sc2*, *tcdC-sc8* lacks the downstream 18-bp deletion. *tcdC-sc2* contains an insertion of a single nucleotide (t) at position 212, i.e., in an untranslated region.

Two genotypes, *tcdC-sc16* and *tcdC-sc17*, were characterized by single-nucleotide nonsense mutations (C191A and G258A, respectively) that are predicted to truncate the TcdC protein to 63 and 85 amino acid residues, respectively. In addition, these genotypes were remarkable in that a 36-bp deletion was observed at nucleotides 301 to 336 in the *tcdC-sc16* genotype and an 18-bp deletion was observed at nucleotides 330 to 347 in the

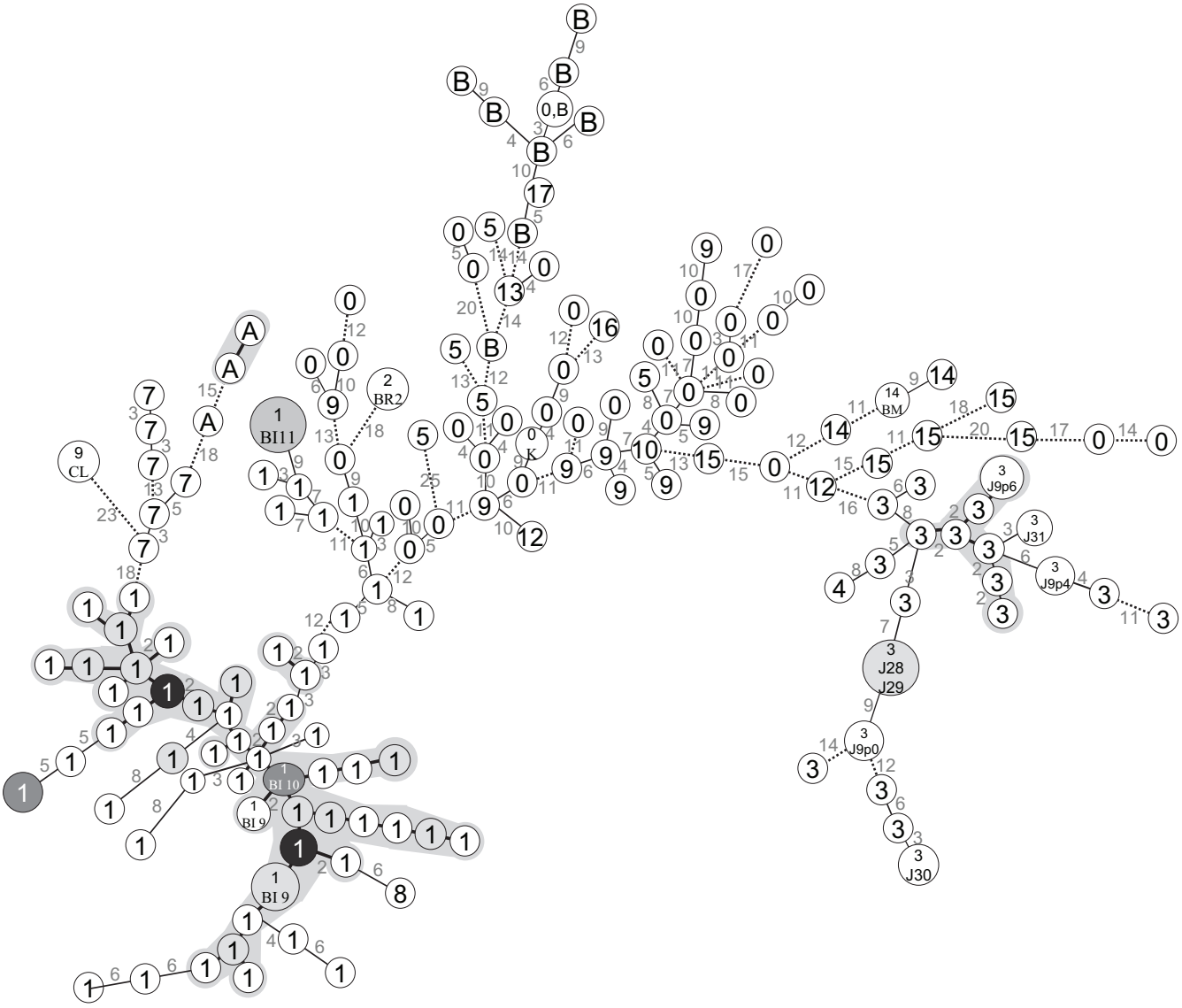

FIG. 2. Minimum-spanning tree of MLVA data depicting genetic relatedness of 199 isolates of *C. difficile* from cases of CDAD at UPMC-P in 2001 and 2005. Each circle depicts one MLVA genotype, with the STRD between adjacent isolates displayed in gray. Where no numeral is shown, the STRD equals 1. STRDs of ≥11 are depicted with dashed lines. Clouds of gray surround isolates with an STRD of ≤2; these complexes represent closely related isolates. White circles depict genotypes represented by one isolate; light gray circles with black numerals, dark gray circles with white numerals, and black circles with white numerals depict genotypes represented by 2, 3, and ≥5 isolates, respectively. The circles are labeled with the *tcdC* genotype described in Fig. 1 (wild-type *tcdC* = 0, *tcdC-A* = A, *tcdC*-B = B, *tcdC*-sc1 = 1, *tcdC-sc2* = 2, *tcdC-sc3* = 3, etc.); available REA types are displayed below the *tcdC* genotype. The tree has been redrawn for ease of viewing and is not to scale.

*tcdC-sc17*genotype, the latter similar to the *tcdC-B* and *-C* alleles described by Spigaglia and Mastrantonio (23). In each case, the 18- and 36-bp deletions in *tcdC-sc16* and *tcdC-sc17* reside downstream of the nonsense mutation in a region of the *tcdC* gene that is predicted to be untranslated. Neither genotype was associated with more than one case of CDAD (Table 1), nor was either genotype genetically related to epidemic clone isolates by MLVA (Fig. 2). Genotype *tcdC-sc16* was most closely related to isolates with wild-type *tcdC* genotypes, whereas genotype *tcdC-sc17* was closely related to isolates with *tcdC-B*, a *tcdC* genotype characterized by an 18-bp deletion only.

Genotype *tcdC-sc4* has an 18-bp deletion that, like the previously described *tcdC-B* allele, is in frame and predicted to result in a 6-amino-acid residue deletion. Unlike *tcdC-sc17*, the *tcdC-sc4* genotype does not harbor upstream mutations that disrupt the reading frame and result in truncation of TcdC. A single, polar amino acid substitution (A50S) caused by the single-nucleotide mutation G148T distinguishes this genotype from *tcdC-B.*

Ten *tcdC* genotypes had silent mutations that are predicted to generate either wild-type TcdC (*tcdC-sc13* and *tcdC-sc15*) or TcdC containing minor residue changes (Fig. 1). The salient features of all genotypes occurring in this collection are summarized in Table 1. There were no statistically significant differences in the proportions of the genotypes from 2001 to 2005

with the exception of two genotypes, *tcdC-sc3* and wild-type *tcdC*. *TcdC-sc3* occurred in 22 of 126 (17.5%) 2001 isolates and 1 of 73 (1.4%) 2005 isolates ($P = 0.001$). Wild-type isolates occurred in 12 of 126 (9.5%) 2001 isolates and 24 of 73 (32.9%) 2005 isolates ($P = 0.001$).

Relationship between *tcdC* genotype, the epidemic clone, and other REA/MLVA types. MST analysis of MLVA genotypes from the study isolates and their corresponding *tcdC* genotypes are shown in Fig. 2. In general, *tcdC* genotypes correlated with the genetic relationships depicted by MLVA genotyping even though this genotyping method relies on VNTRs in loci distinct from the *tcdC* gene. Genetically related isolates had identical *tcdC* genotypes. For example, most isolates bearing the *tcdC-sc1* genotype are highly related by MLVA. Similarly, isolates bearing the *tcdC-sc3* genotype belong to another genetically related population, and isolates with the wild-type (labeled "0" in Fig. 2) tend to cluster together by MST of MLVA genotypes. There was only one example of isolates with identical MLVA genotypes containing more than one *tcdC* genotype (Fig. 2). In this instance, an isolate characterized by wild-type *tcdC* was identical by MLVA to an isolate with the previously identified genotype, *tcdC-B*.

Significantly, all 62 (100%) isolates that were identified as belonging to the epidemic clone (BI/NAP1) by MLVA (*sc1* isolates contained in the shaded area in Fig. 2) have the *tcdC-sc1* genotype characterized by the nucleotide 117 deletion predicted to truncate the TcdC protein. Isolates bearing *tcdC-A* or *tcdC-B* genotypes were not genetically related to the epidemic (BI/NAP1) clone by MLVA despite containing 39- and 18-bp deletions in *tcdC*.

Genotype *tcdC-sc1* was found in 91 of 199 (45.7%) of *C. difficile* isolates examined. Of these isolates, 62 were from 2001 (62 of 126 [49.2%]) and 29 were from 2005 (29 of 73 [39.7%]). Thirty nonepidemic clone isolates were genotype *tcdC-sc1* but were nonetheless closely related to the BI/NAP1 epidemic clone (Fig. 2). Of these 30 isolates, 11 (36.7%) are from 2001 and 19 (63.3%) are from 2005, and the 3 isolates most distantly related to the epidemic clone depicted in Fig. 2 by STRD of 24 are themselves typed as BI 11 by the HVA REA genotyping system.

Genotype *tcdC-sc8*, which shares the deletion of nucleotide 117 found in *tcdC-sc1* (Table 1), is related to the epidemic clone isolates cluster by MLVA (Fig. 2). Genotype *tcdC-sc2*, however, which also shares the deletion of nucleotide 117, is more distantly related to the epidemic clone by MLVA, suggesting that potential de novo mutations or lateral transfers of *tcdC* in this strain may have occurred.

Association of 18-, 36-, and 39-bp deletions in *tcdC* genotypes with the epidemic clone. Two of the previously described *tcdC* genotypes and five of the genotypes described in the present study contain deletions of 18, 36 or 39 bp, but in only two of these genotypes (*tcdC-B* and *tcdC-sc4*) are these deletions predicted to alter the predicted TcdC protein in the form of deletions of three amino acid residues. In the other six genotypes (*tcdC-A*, *tcdC-sc1*, *sc2*, *sc16*, and *sc17*), upstream mutations predict truncations of 147 to 171 amino acid residues of the TcdC protein in addition to radically altering 2 to 27 amino acid residues prior to the truncations due to frameshifts (Table 1).

If one defines a highly mutated TcdC protein as one having >6 amino acid deletions, as is the case with *tcdC* genotypes *tcdC-A*, *tcdC-sc1*, *tcdC-sc2*, *tcdC-sc8*, *tcdC-sc16*, and *tcdC-sc17*, 18-, 36-, or 39-bp deletions accompany 97 of 98 (99.0%) isolates with *tcdC* genotypes predicted to have highly mutated TcdC proteins. Using 18-, 36-, or 39-bp deletion analysis as a predictor of such high degrees of *tcdC* mutation is therefore 99.0% sensitive and 90.1% specific for genotypes resulting in highly mutated TcdC proteins. The only false negative in this regard is genotype *tcdC-sc8*, which lacks an 18-bp deletion and yet has a highly mutated TcdC protein predicted by its single nucleotide deletion, Δ117A.

DISCUSSION

The key finding of the present study was the identification of a genotype, *tcdC-sc1*, that was present in all of our epidemic BI/NAP1 isolates and related isolates by MLVA. This genotype is characterized by an upstream deletion at nucleotide 117 that causes severe truncation of the predicted TcdC protein. A similar genotype, *tcdC-sc2*, shared this deletion but was more distantly related by MLVA to the BI/NAP1 epidemic clone isolates. Genotypes *tcdC-sc1* and *tcdC-sc2* share the Δ117A mutation together with 18-bp deletions, features identified by MacCannell et al. in six epidemic strains of *C. difficile* from sites in Canada and one reference strain from the United Kingdom (10).

The emergence of a strain with dysfunctional suppression of log-phase toxin elaboration may explain recently reported CDAD outbreaks with fulminant presentations and increased severity of disease. These strains have been demonstrated to produce increased levels of toxins A and B during both phases of growth (logarithmic and stationary) (24). Therefore, the apparent decreased responsiveness to appropriate antimicrobial therapies may be due to an increased *C. difficile* toxin burden prior to CDAD recognition (14, 16–18).

Other evidence that *tcdC* mutations might be the causal link to severe disease is also tantalizing. Strain 8864, which contains a rearrangement of its PaLoc that leaves it with no functional *tcdA* or *tcdC* but which is nonetheless TcdB positive, is known to be exceptionally more cytotoxic in vitro than strain VPI10463 and more pathogenic in animal models (22). Recently, TcdC has been demonstrated to be membrane associated, leading to speculation that if it does perform a role in transcriptional regulation, it may also serve as a sensor for metabolite deprivation or have some other signaling function (4). Ultimately, determination of causality for *tcdC* mutations and severe CDAD will require development of the necessary molecular tools for genetic manipulation of *Clostridium* spp.

The present findings should be interpreted with caution, however, as there is as yet no definitive evidence that TcdC functions as a transcriptional regulator. Whether the predicted shortened TcdC's are responsible for the increase in CDAD incidence and severity or are simply a marker of increased virulence cannot be ascertained from the present study. Even if TcdC is a transcriptional regulator of *tcdA* and *tcdB*, the impact of the various predicted changes in the protein that we observed are not known. Ten *tcdC* genotypes with silent or conservative predicted mutations were also identified in the present study, and the effects of the latter on *tcdC* phenotype are unknown. Mutations such as the A50S amino acid substi-

tution present in the predicted TcdC in the modest outbreak of *tcdC-sc3*/REA type J isolates in 2001 are of unknown significance and merit further investigations of toxin phenotypes in vitro. Moreover, the existence of genotypes with severe TcdC truncation (*tcdC-sc2*, *tcdC-sc16*, and *sc17*) falling far outside the BI/NAP1 epidemic clone are intriguing. Further studies are required to determine whether these *tcdC* genotypes are associated with either increased toxin production and more severe disease or disease in low-risk populations.

Regardless of its impact on *C. difficile* pathogenesis, *tcdC* genotyping in conjunction with other methods such as MLVA could provide additional information regarding the clonality and pathogenic potential of *C. difficile* populations. The finding that *tcdC* genotypes were reasonably concordant with MLVA and REA types (Fig. 2) supports this view.

The presence of 30 isolates with genotype *tcdC-sc1* outside of the main epidemic clone outlined by MLVA but within the BI designation by REA as depicted in Fig. 2 suggests clonal expansion and evolution of the epidemic clone rather than de novo generation of or lateral gene transfer to a different strain. Alternatively, these 30 isolates may represent a clonal population that is closely related yet distinct from BI/NAP1.

We found that the 18- and 39-bp deletions previously described as an indicator of *tcdC* mutation are for the most part silent deletions except in the case of *tcdC-B* and *tcdC-sc4* (12). Thus, detection of these deletions in *tcdC* by conventional PCR is only 90% specific for severe TcdC truncation. Future molecular epidemiologic studies of CDAD should therefore focus on sequencing the entire *tcdC* gene to establish the presence or absence of single-nucleotide nonsense and frameshift mutations rather than strictly upon 18-, 36-, or 39-bp deletions. Although *tcdC-sc8* has only been found in one isolate thus far, detection of similar genotypes would further reduce the sensitivity of an assay for severe *tcdC* mutation based solely on PCR amplicon size.

The present study demonstrates that clinical isolates of *C. difficile* frequently harbor *tcdC* genetic variants. Furthermore, the identification of a single mutant *tcdC* genotype strongly associated with the epidemic BI/NAP1 clone supports the hypothesis first proposed by Spigaglia and Mastrantonio that TcdC variants may produce different levels of toxin that results in *C. difficile* strains of unique pathogenic potential. This inference can be tested by studying the association of various *tcdC* genotypes with CDAD severity.

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AUTHOR'S CORRECTION

tcdC Genotypes Associated with Severe TcdC Truncation in an Epidemic Clone and Other Strains of *Clostridium difficile*

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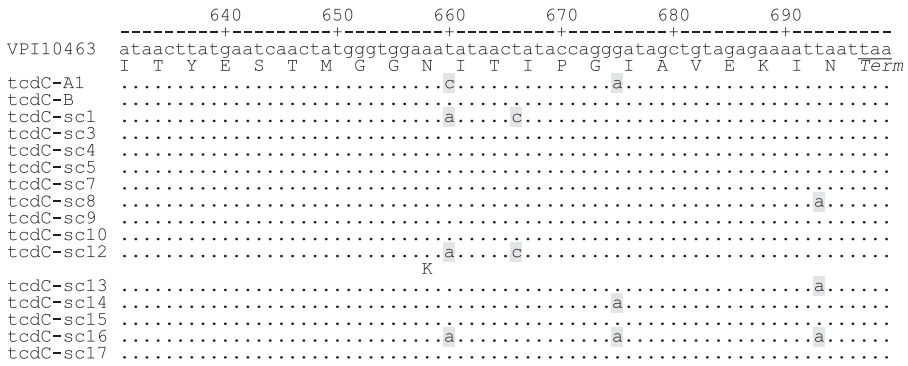
Volume 45, no. 1, p. 215–221, 2007. In our paper, we originally described 14 novel *tcdC* genotypes in *C. difficile* using primers published by P. Spigaglia and P. Mastroantonio (J. Clin. Microbiol. **40:**3470–3475, 2002). Subsequently, we discovered sequencing errors at the 3′ end of *tcdC* in five of these genotypes due to the location of primer C1, which overlaps the last 26 nucleotides of *tcdC*. Sequence analysis with new primers that reside outside *tcdC* identified nucleotide changes in *tcdC-sc2*, *tcdC-sc8*, *tcdC-sc13*, *tcdC-sc14*, and *tcdC-sc16*. Additionally, all three isolates bearing the previously published *tcdC-A* genotype from our collection consistently generated a distinct genotype when sequenced with these primers. Therefore, we are designating this allele *tcdC-A1* (GenBank accession number EF470292). The nucleotide changes described either are untranslated (*tcdC-A1*, *tcdC-sc2*, *tcdC-sc8*, and *tcdC-sc16*) or result in silent mutations (*tcdC-sc13* and *tcdC-sc14*). The GenBank entries for our novel genotypes (GenBank accession numbers DQ861426, DQ861417, DQ861421, DQ861422, and DQ861424) have been corrected accordingly. These revisions illustrate 15 new *tcdC* genotypes.

Page 216: The legend to Fig. 1 should read as follows. "Comparison of TcdC nucleotide and amino acid sequences to the published sequence for the reference strain VPI10463. Dots indicate identical bases, dashes indicate deletions, and shaded letters correspond to nucleotide changes. Only amino acid changes are depicted. Genotype *tcdC-sc2* is not depicted but is identical to *tcdC-sc1* with the exception of an inserted nucleotide (t) between positions 212 and 213 and a nucleotide (t) at position 660."

Page 217: The bottom panel of Fig. 1 should appear as follows.



Page 219, Fig. 2: In the portion of the figure branching up from genotype 17 near the top, the genotype given as "0, B" should be "B." This correction strengthens our original conclusion that *tcdC* genotypes are concordant with MLVA genotypes.

Page 220, column 1: Lines 18 to 22 should read ". . . together by MST of MLVA genotypes. All isolates with identical MLVA genotypes contained identical *tcdC* genotypes."