Analysis of TcdB Proteins within the Hypervirulent Clade 2 Reveals an Impact of RhoA Glucosylation on Clostridium difficile Proinflammatory Activities

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Clostridium difficile strains within the hypervirulent clade 2 are responsible for nosocomial outbreaks worldwide. The increased pathogenic potential of these strains has been attributed to several factors but is still poorly understood. During a C. difficile outbreak, a strain from this clade was found to induce a variant cytopathic effect (CPE), different from the canonical arborizing CPE. This strain (NAP1, v) belongs to the NAP1 genotype but to a ribotype different from the epidemic NAP1/RT027 strain. NAP1, v and NAP1 share some properties, including the overproduction of toxins, the binary toxin, and mutations in tcdC. NAP1, v is not resistant to fluoroquinolones, however. A comparative analysis of TcdB proteins from NAP1/RT027 and NAP1, v strains indicated that both target Rac, Cdc42, Rap, and R-Ras but only the former glucosylates RhoA. Thus, TcdB from hypervirulent clade 2 strains possesses an extended substrate profile, and RhoA is crucial for the type of CPE induced. Sequence comparison and structural modeling revealed that TcdBNAP1 and TcdBNAP1V share the receptor-binding and autoprocessing activities but vary in the glucosyltransferase domain, consistent with the different substrate profile. Whereas the two toxins displayed identical cytotoxic potencies, TcdBNAP1 induced a stronger proinflammatory response than TcdBNAP1V as determined in ex vivo experiments and animal models. Since immune activation at the level of intestinal mucosa is a hallmark of C. difficile–induced infections, we propose that the panel of substrates targeted by TcdB is a determining factor in the pathogenesis of this pathogen and in the differential virulence potential seen among C. difficile strains.

Clostridium difficile, a Gram-positive spore-forming anaerobe, is the leading cause of antibiotic-associated diarrhea in hospitalized patients (1). Antibiotic treatment modifies the balance of commensal microbiota, allowing C. difficile to extensively colonize the gut. The resulting C. difficile infection (CDI) leads to a variety of clinical outcomes that range from mild diarrhea to potentially fatal pseudomembranous colitis (2).

The main virulence factors associated with CDI are two large exotoxins, TcdA and TcdB. The toxins are encoded by the tcdA and tcdB genes, respectively, which are located in a 19.6-kb pathogenicity locus (PaLoc) together with the tcdE (holin-like), tcdC (putative negative regulator), and tcdR (sigma factor) genes (3, 4). The toxins glucosylate small GTPases (5), and their combined action results in colonic tissue inflammation and massive colonic fluid secretion (2). In cell cultures treated with C. difficile toxins, monoglucosylation of RhoA, Rac1, and Cdc42 disrupts the actin cytoskeleton and causes an arborizing cytopathic effect (CPE) (5).

TcdB is a 270-kDa cytotoxin, and its mechanism of action involves host cell receptor binding (6), uptake by endocytosis (7), pH-dependent pore formation (8), translocation across the endosomal membrane (9), host factor-dependent autoprocessing (10), and release of the glucosyltransferase domain (GTD) into the host cell (11). The C-terminal domain of the holotoxin contains a number of short, homologous regions with combined repetitive oligopeptides (CROPs) and is thought to be important for binding host cell receptor(s) (11). The middle part of the toxin represents the translocation region with autoprocessing activity mediated by an autoprotease domain (9). The GTD located in the N-terminal region is composed of a catalytic and a substrate recognition subdomain; this region is responsible for the cytotoxic activity in the host cell cytosol (12).

C. difficile strains producing a variant TcdB have been previously reported, mainly in TcdA-negative strains (13–15). In cultured cells, these TcdB variants induce a CPE characterized by the
collapse of the actin cytoskeleton with complete rounding of the cell body and detachment from the surface in contrast to the classic arborizing effect (13). This variant CPE is due to a different pattern of glucosylated GTPases since classic TcdB modifies RhoA, Rac, and Cdc42 whereas variant TcdB targets Rac, Cdc42, Rap, Ral, and R-Ras (5, 13, 14, 16). Furthermore, variations based on the PaLoc sequence have classified these groups of strains in separate toxino types (17).

The epidemic NAP1/RT027 C. difficile strains have rapidly spread and have been responsible for epidemic outbreaks world-wide (18, 19). Among the factors that have been proposed to contribute to the increased virulence of these strains are resistance to fluoroquinolones, higher sporulation capacity, and increased production of toxins (20–22). It has been demonstrated that TcdB from epidemic NAP1/RT027 strain, TcdB_{nAP1V} does not glucosylate Rho and partially targets Cdc42. Whereas the cytopathic potency of this TcdB_{nAP1V} is similar to that of TcdB purified from classic NAP1 strains, it induces a significantly lower quantity of proinflammatory mediators in the ligated loop model, suggesting that the panel of glucosylated small GTPases determines the biological outcome induced by C. difficile toxins.

**Materials and Methods**

**Isolation and characterization of C. difficile strains and fluoroquinolone resistance.** The NAP1 strains were isolated from stool samples according to the protocols previously described (24). Fragments of tcdA, tcdB, cipA, and tcdC were amplified by PCR using primers and conditions previously reported (25, 26). MICs of ciprofloxacin, moxifloxacin, and levofloxacin resistance breakpoints were >4 µg mL⁻¹. Mutations in the fluoroquinolone resistance-determining region of gyrA and gyrB and in the tcdC genes were identified using Artemis (27) and BLAST tools.

**PFGE typing.** The pulsed-field gel electrophoresis (PFGE) procedure was derived from published protocols (28, 29). Bacteria from 6- to 8-h cultures in brain heart infusion (BHI) were disrupted in lysis buffer. Agarose plugs were prepared by mixing equal volumes of bacterial suspensions and SeaKem Gold agarose (Lonza) in Tris-EDTA (TE) buffer containing SDS. After overnight digestion with Smal (Roche), DNA fragments were separated on 1% agarose (Bio-Rad) gels. Images were analyzed with the BioNumerics software, v5.1 (Applied Maths), and the patterns were compared to those deposited in the database of the National Microbiology Laboratory, Public Health Agency of Canada (Michael R. Mulvey).

**PCR-restriction fragment length polymorphism (RFLP) analysis and ribotyping.** For toxino typing, C. difficile VPI 10463 was used as a control according to the published protocols (30). For ribotyping, primer sequences and reaction conditions were taken from the work of Biet et al. (31).

**Whole-genome sequencing, MLST, and PaLoc/TcdB comparison.** Whole-genome sequences were obtained using multiplexed paired-end libraries and the sequencing-by-synthesis HiSeq platform (Illumina). Reads were assembled using Velvet (32), contigs of >300 bp were scaffolded with SSPACE (33), and gaps were filled using GapFiller (34). The resulting scaffolds were ordered using MaGe (35) and the genomes of reference strain R20291 (NAP1/RT027/ST01) or M68 (NAP9/RT017/ST37). For automatic annotation, we used Prokka (36) and custom C. difficile databases. For core gene multialignment, variant calling, and core genome phylogeny, we used the Harvest suite (37) and FigTree (http://tree.bio.ed.ac.uk/software/figtree/). For multilocus sequence typing (MLST), we used the MLST 1.7 tool maintained by the Center for Genomic Epidemiology at the Danish Technical University (38). PaLoc and TcdB sequences were extracted manually and aligned with MAFFT (39) or MUSCLE (40). For these sequences, phylogenetic tree estimation through maximum likelihood was done using Fasttree (41). TcdB recombination was detected using DualBrothers (42).

**Quantification of secreted toxins.** The NAP1V and NAP1 strains were grown in TYT broth (3% Bacto tryptose, 2% yeast extract, and 0.1% thioglycolate, pH 6.8) for 24 h, as described previously (29). Decimal dilutions of these supernatants were added to HeLa cell monolayers. The cells were monitored for appearance of CPE. Specific TcdB antisera (TechLab) was used to neutralize the effect of the toxin. Nontoxigenic C. difficile ATCC 700057 was used as a negative control. Cytotoxicity was expressed as the inverse of the dilution of the supernatants that caused 50% cell rounding in the monolayers (CPE₃₀). The amount of toxins was quantified by Western blotting, for which the proteins from bacterium-free supernatants at 24 h were concentrated by methanol-chloroform precipitation (43). Proteins were separated in SDS-PAGE gels and electrotransferred to polyvinylidene difluoride (PVDF) membranes. The membranes were probed with monoclonal anti-TcdA (TTC8) or anti-TcdB (2CV) antibody (tgcBIOMICS) (43). Chemiluminescent signals emitted by a goat anti-mouse IgG-horseradish peroxidase conjugate (Invitrogen) in the presence of the Lumi-Light Plus Western blotting substrate (Roche) were recorded with a ChemiDoc XRS documentation system (Bio-Rad). Transcripts of tcdA and tcdB were quantified by real-time quantitative PCR (qRT-PCR) as described previously (44). The amplification followed conditions previously reported (44). The relative expression of genes was calculated by the threshold cycle (ΔΔC_{T}) method using rpoA transcript as the endogenous control (45).

**Toxin purification.** TcdB proteins were obtained from supernatants of NAP1 strains grown in a dialysis system culture and purified as described previously (46). The purity of the toxins was determined by SDS-PAGE and mass spectrometry which indicated the presence of peptides derived from TcdB only and not TcdA (data not shown).

**Cytotoxic effect produced by NAP1 toxins.** Confluent 3T3 fibroblasts, Vero cells, and HeLa cells grown in 12-mm glass slides were intoxicated with 0.2 nM TcdB_NAP1, and TcdB_{nAP1V}. The cells were immobilized and fixed according to previously described protocols (47). The CPE was evaluated by phase-contrast, fluorescence, and scanning electron microscopy as indicated in the figure legends.

**In vitro glycosyltransferase activity.** The TcdB ability to glycosylate different monomeric GTPases was examined through a radioactivity assay, as previously described (48, 49), and Western blot assays. Briefly, for the radioactive test, UDP-[¹⁴C]glucose (250 mCi/mmol; PerkinElmer), GTP, each recombinant GTPase–glutathione S-transferase (GST), and each TcdB were mixed in a reaction buffer. After 1 h of incubation at 37°C, the proteins were separated by SDS-PAGE. Glycosylation of GTPase was analyzed by phosphorimaging. For graphical representation, band density was measured with ImageQuant TL. For the Western blotting, the same reactions and conditions were used and assays were performed using UDP-glucose (Sigma). After protein separation by SDS-PAGE, the proteins were transferred to PVDF membranes. The GTPase was detected with monoclonal anti-RhoA antibody (Abcam; ab54835) by Western blotting. The control RhoA-GST proteins were stained with Coomassie blue.

**RhoA, Rac1, and Cdc42 GTPase activation assays.** The TcdB ability to inactivate GTPases was determined on confluent 3T3 fibroblast grown in Dulbecco modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (Sigma). For the pulldown steps, GTP-RhoA was precipitated with GST-tagged Rho binding domain (RBD) and GTP–Rac1 and GTP–Cdc42 were precipitated with GST–p21 binding domain (pBD).
Confluent 3T3 fibroblasts cultured in 6-well plates were intoxicated with 0.2 nM TcdB of NAP1v and NAP1 strains under the conditions indicated in the figure legends. After the intoxication, the cells were treated as previously described (47). Briefly, cells were washed with phosphate-buffered saline (PBS) and lysed with precipitation buffer. Lysates were centrifuged and incubated with Rho binding domain (RBD) of the human Rhotekin protein, which had been expressed as a GST fusion protein (RBD-GST), or Rac/Cdc42 (p21) binding domain (PBD) of the human p21-activated kinase 1 protein, which had been expressed as a GST fusion protein (PBD-GST). Active proteins were pulled down by centrifugation, resolved by SDS-PAGE, and transferred to PVDF membranes. GTPases were detected using anti-RhoA (Abcam; ab54835), anti-Rac1 (Abcam; ab33186), or anti-Cdc42 (Abcam; ab41429) antibody by Western blotting. For detection of RhoA glucosylation using the monoclonal antibody, HeLa cells, Vero cells, and 3T3 fibroblasts were intoxicated with 0.2 nM TcdB from NAP1 or NAP1v strains for 6 and 24 h. After intoxication, cells were lysed in 2% SDS and 20 μg of each lysate was separated by 10% SDS-PAGE, electrophoresed to PVDF membranes, and probed with the anti-RhoA monoclonal antibody.

Structural analysis and TcdB GTD modeling. The homology models were made using Modeler and Chimera bioinformatics tools, as described previously (49), based on the tcdB sequences of NAP1, NAP1v, and VPI 10463 strains. Adjustments to the multiple-sequence alignment constructed by using ClustalW (50) were made based on the structure-based alignment performed by superimposing the structures of TcdB proteins.

Kinetics of CPE induced by toxins. Confluent HeLa cells were intoxicated with 10 pM TcdB\textsubscript{NAP1} and TcdB\textsubscript{NAP1v}. The percentage of round cells in each well was evaluated every hour for a period of 12 h and then at 24 h.

Determination of TNF-α induction. Confluent RAW 264.7 cells were intoxicated with 0.5 nM TcdB\textsubscript{NAP1} and TcdB\textsubscript{NAP1v} for 6 h. The concentration of tumor necrosis factor alpha (TNF-α) in the supernatants was determined by commercial enzyme-linked immunosorbent assay (ELISA) according to the instructions of the manufacturer (R&D Systems).

Murine ileal loop model. Animal experimental procedures were approved (CICUA-38-14) by the University of Costa Rica Animal Care and Use Committee. Male Swiss mice of 20 to 25 g were subjected to fasting 24 h before use. The length and weight of the intestinal loops were recorded (51). The control solution was injected. Mice were sacrificed 4 h after inoculation, and the ileal loop was ligated, and 10 μg of each toxin or the corresponding control solution was injected. Mice were sacrificed 4 h after inoculation, and the length and weight of the intestinal loops were recorded (51). The neutrophil accumulation in homogenized ileal tissue was evaluated through determination of myeloperoxidase (MPO) activity with the histochemical method.

RESULTS

A NAP1 strain inducing a variant cytopathic effect. In a preliminary study performed on a collection of clinical isolates from tertiary care hospitals, the presence of the NAP1 genotype was reported (24). Among 33 NAP1 isolates analyzed, we found a strain whose supernatant induced a cytopathic effect (CPE) different from the classic arborizing CPE observed for the other NAP1 strains (data not shown). PFGE analysis indicated that the Smal macrorestriction pattern of this particular variant strain was 279, while the pattern of the other NAP1 strains was 001 (Fig. 1A). This strain, here designated NAP1\textsubscript{v}, was analyzed by whole-genome sequencing and comparative genome analyses. A phylogenetic reconstruction based on core single nucleotide polymorphisms (SNPs) revealed that NAP1\textsubscript{v} is more closely related to historical and epidemic NAP1/RT027/ST01 strains than to TcdA-negative NAP9/RT017/ST37 strains with genes encoding variant TcdB (53) (Fig. 1B). This relationship to the clade 2 of hypervirulent lineages postulated by Griffiths et al. (54) was confirmed through ribotyping and MLST, as both the NAP1\textsubscript{v} (RT019/ST67) and the NAP1 (RT027/ST01) strains belong to this clade (54). In agreement with this finding, the NAP1\textsubscript{v} strain carries the tcdA, tcdB, and cdtB genes and presents an 18-bp deletion and a single-base-pair deletion at position 117 in tcdC, characteristic of NAP1/RT027 strains. On the other hand, NAP1\textsubscript{v} was not resistant to fluoroquinolones and did not present the amino acid transition from Thr82 (ACT) to Ile (ATT) in GyrA as observed in classic NAP1/RT027 strains (55).

The levels of secreted TcdA and TcdB and the expression of tcdA and tcdB transcripts were measured to determine whether the NAP1\textsubscript{v} strain produces increased amounts of toxin relative to the classical epidemic NAP1/RT027 strain. Titration of toxin activity in bacterium-free supernatants indicated that the NAP1 and NAP1\textsubscript{v} strains induced similar CPE\textsubscript{50} liters (Fig. 2A), and in agreement, the levels of secreted toxins were similar for the two strains (Fig. 2B). tcdA and tcdB mRNAs were quantified by real-time quantitative PCR. The level of both transcripts was signifi-
significantly higher in both the NAP1 and NAP1V strains than in control strains at all times tested (Fig. 2C). Interestingly, the NAP1V strain produces even more toxin transcripts than does the NAP1 counterpart, a detail that should be considered in future experiments dealing with the regulation of these genes. Altogether, these results demonstrate that the NAP1V strain is closely related to the epidemic NAP1/RT027 strains but displays distinctive genotypic and phenotypic characteristics associated with TcdB that we further explored.

**TcdBNAP1V induces a variant CPE related to a distinct GTPase glucosylation pattern.** To analyze the cytopathic characteristics of toxin B from NAP1V (TcdBNAP1V) and compare the toxin with those from a classic NAP1 strain (TcdBNAP1), both toxins were purified. After intoxication of HeLa cells, Vero cells, and 3T3 fibroblasts with TcdBNAP1, the classical arborizing CPE was observed (Fig. 3). In contrast, TcdBNAP1V induced cell rounding and detachment but no protrusions or arborizing effects (Fig. 3). Hence, TcdBNAP1V was responsible for the variant CPE produced by NAP1V supernatants.

Next, we determined the glucosylation pattern of TcdBNAP1 and TcdBNAP1V using a panel of small GTPases and a radioactive in vitro assay. TcdBNAP1 modified a panel of substrates characteristic of classic TcdB proteins, with RhoA, Rac1, and Cdc42 being readily glucosylated (Fig. 4A). Interestingly, we observed modification to a lesser extent of Rap1, Rap2, and R-Ras, which has not been reported previously for classic TcdB proteins inducing arborizing CPE. On the other hand, TcdBNAP1V glucosylated Rac1, but the glucosylation of RhoA and Cdc42 was significantly diminished (Fig. 4B). As with TcdBNAP1, TcdBNAP1V was able to glucosylate Rap1, Rap2, and R-Ras at low levels.

To confirm the panel of substrates modified by the toxins, we monitored the ex vivo glucosylation of RhoA, Rac1, and Cdc42 after intoxication of cultured cells by pulldown assays. When 3T3 cells were incubated with TcdBNAP1, Rho-GTP was undetectable at 6 h (Fig. 4C). In contrast, Rho-GTP was detected in cells intoxicated with TcdBNAP1V for up to 24 h, confirming the lack of modification of this small GTPase in the in vitro assay (Fig. 4C). Both TcdB proteins inactivated Rac1 after 6 and 24 h of treatment, again confirming the results of the in vitro glucosylation assay (Fig. 4C). Additionally, the level of Cdc42-GTP exhibited a significant and...
experiments are shown. (C) Effect of TcdBNAP1 and TcdBNAP1V on the activa-
tion state of small GTPases. 3T3 fibroblasts were intoxicated with TcdBNAP1 and TcdBNAP1V, for the indicated times. After treatment, cells were lysed. One part of the lysates was used as a control for total amount of GTPases, and the other one was incubated with PBD-GST or RBD-GST-Sepharose beads. Active proteins were pulled down and analyzed by Western blotting. GTPases were detected using anti-RhoA, anti-Rac, and anti-Cdc42, respectively. Cells treated with TcdBNAP1 show inactivation of RhoA, whereas cells intoxicated with TcdBNAP1V do not. Cytotoxic necrotizing factor 1 (CNF) from Escherichia coli was used as a positive control for GTase activation. Negative-control cells were left untreated.

FIG 4 The NAP1V strain does not glycosylate RhoA. (A) TcdBNAP1 and TcdBNAP1V were tested for their ability to glycosylate a panel of recombinant GTases using UDP-[14C]glucose as a cosubstrate. Labeled bands were detected by phosphorimaging analysis. (B) The band intensities of the GTase glycosylation were quantified by densitometry. Each experiment was normalized to Rac1 signal. Means ± standard deviations from three independent experiments are shown. (C) Effect of TcdBNAP1 and TcdBNAP1V on the activation state of small GTPases. 3T3 fibroblasts were intoxicated with TcdBNAP1 and TcdBNAP1V, for the indicated times. After treatment, cells were lysed. One part of the lysates was used as a control for total amount of GTPases, and the other one was incubated with PBD-GST or RBD-GST-Sepharose beads. Active proteins were pulled down and analyzed by Western blotting. GTPases were detected using anti-RhoA, anti-Rac, and anti-Cdc42, respectively. Cells treated with TcdBNAP1 show inactivation of RhoA, whereas cells intoxicated with TcdBNAP1V do not. Cytotoxic necrotizing factor 1 (CNF) from Escherichia coli was used as a positive control for GTase activation. Negative-control cells were left untreated.

consistent decrease at 6 h after intoxication with TcdBNAP1 and Cdc42-GTP completely disappeared after 24 h of treatment (Fig. 4C). However, TcdBNAP1V was able to decrease the level of Cdc42-GTP only after 24 h of intoxication, indicating that this small GTase is not a preferred substrate of this variant TcdB (Fig. 4C). Interestingly, no signal was detected in the control for total Rho (loading control) in cells treated with TcdBNAP1, indicating either that the protein is degraded after glucosylation or that, alternatively, the antibody to Rho that we used in this work does not recognize the glucosylated isofrom (Fig. 4C). To distinguish between these two possibilities, recombinant Rho was incubated with either TcdBNAP1 or TcdBNAP1V in the presence of UDP-glucose. Rho was detected by Coomassie blue staining after treatment with both toxins but was not detected by Western blotting after treatment with TcdBNAP1 (Fig. 5A). This result indicates that the monoclonal antibody used does not interact with glucosylated Rho and confirms the fact that TcdBNAP1V does not modify this small GTase. Thus, this monoclonal antibody represents a valuable tool to monitor Rho modification by large clostridial cytotoxins. To further explore this concept, different cell lines (HeLa cells, 3T3 fibroblasts, and Vero cells) were intoxicated for 6 and 24 h with either TcdBNAP1 or TcdBNAP1V. RhoA was detected only in lysates prepared from cells intoxicated with TcdBNAP1V (Fig. 5B), confirming that the ability to target this small GTase is the main difference at the level of substrates between the two toxins.

TcdBNAP1V sequence combines the enzymatic domain of variant toxins with the receptor-binding domain of TcdB from the hypervirulent clade 2. Since TcdBNAP1V clearly presents different phenotypic behavior than TcdBNAP1, we focused on differences at the sequence level. Phylogenetic analysis indicates that the PaLoc of the NAP1V strain is more closely related to TcdA-positive strains carrying variant TcdB proteins (Fig. 6). Next, we determined the toxontype of the NAP1V strain. The tcdB polymorphisms of the B1 fragment (containing the coding region for the TcdB glucosyltransferase domain) (Fig. 7A) from NAP1V were identical to those of TcdA-negative strain NAP9/RT017 and different from that of NAP1/RT027 (Fig. 7B). The restriction patterns for tcdA were the same for the two strains (Fig. 7C). Thus, the toxontype of the NAP1V strain (toxontype XXIII) is not the classic one found in NAP1 strains (toxontype II) and rather coincides with the toxontype present in TcdA-positive strains carrying variant TcdB proteins. A detailed analysis and comparison of the sequences from the different TcdB proteins indicates that the primary sequence of the glucosyltransferase domain of TcdBNAP1V is more closely related to the corresponding region of TcdB proteins inducing a variant CPE than to that of TcdB proteins inducing a classic arborizing CPE (Fig. 8A). Indeed, the identity in the first 546 amino acid residues between TcdBNAP1V and TcdBNAP1 is 100%, whereas that
between TcdBNAP1 and TcdBNAP1V is 80%. Furthermore, the identity in the substrate specificity domain (amino acids 365 to 516) between TcdBNAP1 and TcdBNAP1V is only 62%. The glucosyltransferase domain (GTD) sequences of TcdB NAP1V and TcdB NAP1 were analyzed in the context of the VPI 10463 reference strain (identical to the 630 reference strain). Although the core residues of the GDTS are conserved between TcdBNAP1V and TcdBNAP1, the surface residues are divergent (Fig. 8B). These divergent residues are predicted to be involved in the substrate affinity of the GDTS. In contrast, the GDTS of TcdBNAP1V and TcdB NAP1/RT027 are very similar (Fig. 8B). The CROPs domain of TcdBNAP1V is highly similar to the corresponding region of TcdB proteins from classic NAP1 strains (Fig. 8A). The identity in this region (amino acids 1645 to 2366) between TcdBNAP1V and TcdBNAP1 is 99%.

These data indicate the possibility of a recombination event that led to the sequence encoding TcdB NAP1V. To explore this, we applied a Bayesian approach to infer changes in tree topologies and evolutionary rates using TcdB sequences from strains NAP1V, R20291 (NAP1/RT027), M120 (NAP7/RT078), and NAP9/RT017 (Fig. 9C). The increase in rate and severity of CDI has been linked to the emergence and spread of the epidemic NAP1 strain (19, 56). This genotype has acquired several genetic determinants that contribute to its increased virulence; among these, the overproduction of toxins (linked to mutations in the tcdC gene), the presence of a binary toxin, and resistance to fluoroquinolones have been considered to play an important role (21, 22, 56). It has been shown that TcdB is essential for C. difficile virulence and that its glucosyltransferase activity is required for activity in an ileal loop model (57, 58). In this context, previous studies have concluded that variations in the sequence of this toxin lead to an augmented cytotoxicity. TcdBNAP1 caused a statistically significant increase in MPO activity, whereas TcdBNAP1V elicited a reaction undistinguishable from that of the control (Fig. 9C). The levels of IL-1β and IL-6 were significantly increased by TcdBNAP1, and again, TcdBNAP1V was unable to induce any reaction (Fig. 9C).

**DISCUSSION**

The increase in rate and severity of CDI has been linked to the emergence and spread of the epidemic NAP1 strain (19, 56). This genotype has acquired several genetic determinants that contribute to its increased virulence; among these, the overproduction of toxins (linked to mutations in the tcdC gene), the presence of a binary toxin, and resistance to fluoroquinolones have been considered to play an important role (21, 22, 56). It has been shown that TcdB is essential for C. difficile virulence and that its glucosyltransferase activity is required for activity in an ileal loop model (57, 58). In this context, previous studies have concluded that variations in the sequence of this toxin lead to a augmented cytotoxicity. TcdBNAP1 caused a statistically significant increase in MPO activity, whereas TcdBNAP1V elicited a reaction undistinguishable from that of the control (Fig. 9C). The levels of IL-1β and IL-6 were significantly increased by TcdBNAP1, and again, TcdBNAP1V was unable to induce any reaction (Fig. 9C).
a variant TcdB. Our goals were to understand the differences in the CPEs induced by this toxin and the potential role of the panel of modified substrates in the biological effects induced by TcdB proteins secreted by strains from the hypervirulent clade 2 and to elucidate the emergence of this NAP1 variant strain.

Due to the PFGE classification and the particular CPE induced by bacterium-free supernatants derived from the NAP1V strain, we reasoned that this isolate would have phenotypic characteristics associated with important differences at the level of TcdB. The NAP1V strain is, in fact, a toxin-overproducing isolate, harboring deletions in \( \text{tcdC} \) and carrying the binary toxin gene. Nonetheless, it is not resistant to fluoroquinolones, since it does not harbor the typical mutation in \( \text{gyrA} \) found in NAP1 strains (60). Despite the strain belonging to the NAP1/RT027 genotype, the NAP1V/RT019 macrorestriction pattern differs from that of the classical NAP1/RT027 strains and its toxidentotype differs due to variations within the tcdB-encoded N-terminal region. Indeed, the digestion pattern of the amplified B1 fragment coding for the catalytic region of TcdBNAP1V was indistinguishable from the corresponding one presented in TcdA-negative strains. This effect, referred to as variant CPE, was

**FIG 8 TcdBNAP1V shares with TcdBNAPI the receptor-binding domain but not the enzymatic domain.** (A) Sequence alignment of (i) variant toxins B from NAP9_M68, CD_1470, and CD_8864 reference strains inducing a variant (V) CPE; (ii) TcdBNAP1 from a clinical isolate and a reference epidemic NAP1/RT027 strain (R20291) inducing a classic (C) CPE; and (iii) TcdBNAP1V. Black lines represent disagreements in the sequence of TcdBNAPI0463, which was selected as a reference for the alignment. The blue box highlights a distinct glycosyltransferase region shared between NAP1V and other variant strains. The green box shows sequence stretches in the repetitive CROPs domains shared between TcdBNAPI, TcdB20291, and TcdBNAP1V. (B) Comparison of the TcdBNAP1 and TcdBNAP1V sequences in the context of the TcdB GTD structure (PDB 2BVM, VPI 10463 sequence). Sequence conservation on the putative GTPase-binding face compared to the GTD from \( \text{C. difficile} \) VPI 10463 with NAP1 and NAP1V. TcdB GTD structures is shown (red, conserved; blue, not conserved). UDP-glucose is depicted in white in the GTD active site. (C) Resulting recombination detection graphs using TcdB sequences from strains NAP9 (M68, RT017 reference strain), R20291 (epidemic NAP1/RT027 reference strain), NAP7 (epidemic M120, RT078 reference strain), and NAP1V. Signs of possible recombination events are represented as changes in the topology graphs (first row) that appear at the most probable topology between the segments. The cross of the topological lines (green and red lines) indicates recombination breakpoints. Resulting trees are compatible with a scenario in which TcdBNAP1V emerged through recombination of tcdB sequences from NAP9 and NAP1 strains.
While it has now been described in a wider range of strains (13, 17), variance with Bonferroni’s correction.* There is a clear correlation between the small GTPases modified and the type of CPE induced by TcdBNAP1V and variant TcdB proteins from TcdA-negative strains. This concordance is in agreement with the primary sequence of the toxins since the GTD of TcdBNAP1V has a high identity to the corresponding domain found in variant TcdB proteins. On the other hand, the autoprocessing domain and the carboxy-terminal region of TcdBNAP1V are almost identical to the corresponding regions from TcdB. These results, along with sequence comparison, reveal that TcdBNAP1V is a toxin of the classical NAP1/RT027 genotype but with modifications within the enzymatic domain.

Recently, a strain belonging to clade 2, RT244/ST41, was reported to display an increased virulence (62). As a NAP1 strain, RT244/ST41 harbors a binary toxin; however, it does not produce increased amounts of toxins and is fluoroquinolone susceptible. These data indicate that the NAP1/RT019 strain shares more features with the classic NAP1/RT027 than RT244/ST41 and would then be more closely related to the epidemic strain. Interestingly, the strain RT244/ST41 genome also seems to encode a variant TcdB. A comparative and detailed assessment of the virulence potential of these three strains would allow one to determine the relative contribution of factors such as the presence of binary toxin, overproduction of toxins, fluoroquinolone resistance, and type of toxin produced to the increased virulence displayed by members of this clade.

Nosocomial outbreaks caused by TcdA-negative strains have increased in the last decade (53, 64, 65). Interestingly, all these strains have been reported to harbor variant TcdB proteins. This might be an indication that the biological effects induced by classic TcdB proteins differ from those induced by variant TcdB proteins and that TcdA-negative strains compensate for the lack of this toxin by using a TcdB with a different panel of substrates. When we compared the responses to TcdB on the ligated loop model, we could indeed find a significant biological difference between the two toxins. Whereas TcdBNAP1 induced an immune activation, TcdBNAP1V induced a much milder and almost undetectable response. Secretion of TNF-α by macrophages has been associated with the glucosyltransferase activity of C. difficile toxins (66), and since TcdBNAP1V and TcdBNAP1 have a high degree of identity in the regions determining receptor binding and entrance to the cell and the two toxins have similar cytopathic potencies, we assume that the biological differences detected in our assays are due to a differential panel of substrates glucosylated. Since the main difference in substrates is at the level of RhoA modification, we hypothesize that glucosylation of this small GTPase enhances the proinflammatory response induced by C. difficile toxins. The use of a panel of purified toxins with differing substrate panels like the ones indicated in this article in a wide range of experimental models would allow the dissection of the relevance of the modification of different small GTPases in the outcome of CDI.

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