

Binary Toxin Production in *Clostridium difficile* Is Regulated by CdtR, a LytTR Family Response Regulator[∇]

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Clostridium difficile binary toxin (CDT) is an actin-specific ADP-ribosyltransferase that is produced by various *C. difficile* isolates, including the “hypervirulent” NAP1/027 epidemic strains. In contrast to the two major toxins from *C. difficile*, toxin A and toxin B, little is known about the role of CDT in virulence or how *C. difficile* regulates its production. In this study we have shown that in addition to the *cdtA* and *cdtB* toxin structural genes, a functional *cdt* locus contains a third gene, here designated *cdtR*, which is predicted to encode a response regulator. By introducing functional binary toxin genes into *cdtR*⁺ and *cdtR*-negative strains of *C. difficile*, it was established that the CdtR protein was required for optimal expression of binary toxin. Significantly increased expression of functional binary toxin was observed in the presence of a functional *cdtR* gene; an internal deletion within *cdtR* resulted in a reduction in binary toxin production to basal levels. Strains that did not carry intact *cdtAB* genes or *cdtAB* pseudogenes also did not have *cdtR*, with the entire *cdt* locus, or CdtLoc, being replaced by a conserved 68-bp sequence. These studies have shown for the first time that binary toxin production is subject to strict regulatory control by the response regulator CdtR, which is a member of the LytTR family of response regulators and is related to the AgrA protein from *Staphylococcus aureus*.

Clostridium difficile is an anaerobic, gram-positive spore-forming bacterium that is recognized as the causative agent of a broad spectrum of intestinal diseases ranging from mild self-limiting diarrhea to more serious and potentially life threatening manifestations such as pseudomembranous colitis and toxic megacolon (4). Collectively, these diseases are referred to as *C. difficile*-associated disease (CDAD). The onset of CDAD is typically associated with the use of antibiotics or other therapeutics such as cytotoxic anticancer drugs, which disrupt the normal intestinal microflora (50). Once this colonization resistance (51) is removed, it is proposed that ingested *C. difficile* spores germinate, colonize the gastrointestinal tract, and elaborate toxins, which results in an acute inflammatory response and severe damage to the intestinal epithelium.

C. difficile is a significant and increasing source of morbidity and mortality and is a very significant financial burden in hospitals throughout the developed world (9, 22, 41, 59). More recently, a new, highly virulent group of strains, known as NAP1/027, has emerged. These hypervirulent strains are reported to produce significantly more toxin than other strains and are associated with more severe disease and high mortality rates (59). The hypervirulent strains are highly resistant to fluoroquinolones and are responsible for epidemics seen in Canada, the United States, and the United Kingdom and several other European countries (21, 25, 28, 48).

The major virulence factors of *C. difficile* are two members of the large clostridial cytotoxin family, toxin A and toxin B,

which are encoded by the genes *tcdA* and *tcdB*, respectively, and are located within a five-gene locus known as the PaLoc (6). Both toxins A and B are potent monoglucosyltransferases that have been shown to target small GTP-binding proteins, including Rho, Rac, and Cdc42, which are involved in the regulation and formation of the actin cytoskeleton in eukaryotic cells (18, 19).

In addition to producing toxins A and B, some *C. difficile* strains, including the NAP1/027 epidemic strains, produce a third toxin, known as *C. difficile* binary toxin (CDT) (12, 38, 53). This toxin is an actin-specific ADP-ribosyltransferase that consists of two subunits, namely, CDTa, which is the enzymatic component, and CDTb, the binding component (3, 39). It has a high degree of amino acid sequence similarity to iota toxin from *Clostridium perfringens* type E strains and *Clostridium spiroforme* toxin, the subunits of which are reportedly interchangeable (38). CDTb binds to an as-yet-unidentified cellular receptor, leading to the internalization of CDTa into the cytosol, where it catalyzes the ADP-ribosylation of monomeric actin and the resultant disruption of the actin cytoskeleton (1, 47). Since most human *C. difficile* strains do not produce functional CDT, its role in the pathogenesis of CDAD is yet to be determined. Recent studies have shown that CDT production is much more prevalent in strains isolated from animals, with the CDT structural genes being found in approximately 23% to 100% of these isolates (43). However, the significance of this finding for *C. difficile*-mediated disease in animals is also unknown.

CDT is encoded by two genes, *cdtA* and *cdtB*, which are transcriptionally linked and are located on the *C. difficile* chromosome at a locus separate from the PaLoc (38). In contrast to the situation with toxin A and toxin B, very little research has been carried out to examine how the expression of this potentially important virulence factor is regulated. Here we report

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics and/or origin ^a	Source or reference
Strains		
<i>C. difficile</i>		
630	Clinical isolate, Tc ^r Em ^r , Switzerland	13
CD196	Binary toxin-producing reference strain, France	39
VPI 10463	Toxinotype 0 reference strain, United States	56
L253	Toxigenic isolate	14
CD37	Nontoxigenic isolate, United States	49
JIR8094	630 Em ^r ; has a spontaneous deletion of an <i>erm</i> (B) gene from Tn5398	35
KZ1623	Nontoxigenic isolate, Japan	32
662	Nontoxigenic isolate, Switzerland	60
M7404	NAP1/027 Canadian isolate, 2005	J. Pepin
661	Toxigenic isolate, France, 1992	M. Sebald
660/2	Toxigenic isolate, France, 1992	M. Sebald
685	Toxigenic isolate, France, 1992	M. Sebald
AM421	Nontoxigenic isolate; Fairfield Hospital, Australia; 1980	R. Wilkinson
AM662	Nontoxigenic isolate; St. Vincents Hospital, Australia; 1982	R. Wilkinson
AM1180	Toxigenic isolate; Latrobe Valley Regional Hospital, Australia; 1985	R. Wilkinson
M334581	Toxigenic isolate; Austin Hospital, Australia; 2002	L. Grayson
M336632	Toxigenic isolate; Austin Hospital, Australia; 2002	L. Grayson
M336948	Nontoxigenic isolate; Austin Hospital, Australia; 2002	L. Grayson
M345206	Nontoxigenic isolate; Austin Hospital, Australia; 2002	L. Grayson
M345300	Toxigenic isolate; Austin Hospital, Australia; 2002	L. Grayson
M361441	Nontoxigenic isolate; Austin Hospital, Australia; 2002	L. Grayson
WA7	Toxigenic isolate; Rockingham Kwinana District Hospital, Australia; 2006	T. Riley
WA9	Toxigenic isolate; Katanning Hospital, Australia; 2006	T. Riley
MMC CD1	Toxigenic isolate; Monash Medical Centre, Australia; 2006	G. Jenkin
MMC CD2	Toxigenic isolate; Monash Medical Centre, Australia; 2006	G. Jenkin
MMC CD8	Toxigenic isolate; Monash Medical Centre, Australia; 2006	G. Jenkin
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80 <i>dlacZ</i> M15 (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA supE44 thi-1 gyrA96 relA1</i> λ ⁻	Life Technologies
Top10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 deoR recA1 araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (Str ^r) <i>endA1 nupG</i>	Invitrogen
HB101	<i>thi-1 hsdS20</i> (r _B ⁻ m _B ⁻) <i>supE44 recAB ara-14 leuB5 proA2 lacY1 galK rpsL20</i> (Sm ^r) <i>xyl-5 mtl-1</i>	5
Plasmids		
pVS520	Tra ⁺ Mob ⁺ RP4 derivative, Tc ^r	37
pMTL9301	<i>E. coli</i> - <i>C. difficile</i> mobilizable shuttle vector, carries pCD6 replication region, Em ^r	40
pMTL500E	<i>E. coli</i> - <i>Clostridium</i> shuttle vector (pAM β 1 replicon), Em ^r	36
pMTL9361Cm	<i>E. coli</i> - <i>C. difficile</i> mobilizable shuttle vector, carries pCD6 replication region, Tm ^r	G. Carter, N. Minton (unpublished)
pCR2.1-TOPO	<i>E. coli</i> PCR cloning vector, ColE1, Ap ^r Km ^r	Invitrogen
pT7Blue3	<i>E. coli</i> PCR cloning vector, ColE1, Ap ^r	Novagen
pJIR3107	pMTL9361Cm Ω CD196 4.5-kb <i>cdtAB</i> fragment (SacI/SphI), CDT binary toxin expression plasmid, Tm ^r	Recombinant
pJIR3394	pMTL9301 (FspI) Ω JIR8094 <i>cdtR</i> 1.13 kb (EcoRV/5maI), CdtR expression plasmid, Em ^r	Recombinant
pJIR3395	pMTL3394 Δ <i>cdtR</i> (BamHI/PstI), internal deletion in <i>cdtR</i> , Em ^r	Recombinant

^a Tm^r, thiamphenicol (chloramphenicol) resistance; Em^r, erythromycin resistance; Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Sm^r, streptomycin resistance.

the identification of a functional LytTR family response regulator, which we have named CdtR, and we show that it is able to activate CDT production in *C. difficile*. Furthermore, our results reveal that the *cdtR* and the *cdtAB* genes are genetically linked as part of the CDT locus (CdtLoc). Finally, in CDT-negative isolates we have identified a novel 68-bp nucleotide sequence that is absent in isolates carrying the CdtLoc.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The characteristics and origins of all recombinant strains and plasmids are shown in Table 1. All bacteriological culture media were obtained from Oxoid. For mating procedures and extraction

of plasmid and genomic DNAs, *C. difficile* strains were cultured in BHIS broth (49) supplemented with 0.1% L-cysteine HCl and 0.5% glucose or on BHIS agar with the additional supplement of 0.09% FeSO₄. Cycloserine (250 μ g \cdot ml⁻¹), cefoxitin (8 μ g \cdot ml⁻¹), thiamphenicol (10 μ g \cdot ml⁻¹), or erythromycin (10 μ g \cdot ml⁻¹) (all from Sigma-Aldrich) were used for selection of recombinant strains, as required. *C. difficile* cultures were grown in an atmosphere of 10% H₂, 10% CO₂, and 80% N₂ at 37°C in a Coy anaerobic chamber. *Escherichia coli* strains were derivatives of either DH5 α , Top10, or HB101(pVS520) and were cultured aerobically at 37°C in 2 \times YT agar or broth (30). Recombinant *E. coli* cultures were supplemented with chloramphenicol (25 μ g \cdot ml⁻¹), erythromycin (400 μ g \cdot ml⁻¹), or tetracycline (10 μ g \cdot ml⁻¹), as required.

Isolation and manipulation of nucleic acids. Plasmid DNA was isolated from *E. coli* strains grown overnight in 5 ml of 2 \times YT broth or from *C. difficile* strains grown in 5 ml of BHIS broth, with appropriate antibiotic selection, using the

TABLE 2. Oligonucleotide primers

Primer	Sequence (5'→3')	Use ^a
JRP1744	GGGAAAGAAAAGAAGCAGAAAG	Amplify internal fragment of <i>cdtA</i> (+)
JRP1745	TGTATTTTCATTGTTTCTCCTC	Amplify internal fragment of <i>cdtA</i> (-)
JRP2342	CCGGAATTCGCTCTATTGGACTAGACCGTTG	Amplify internal fragment of <i>tcdA</i> (+)
JRP2343	CCGGAATTCCTAAAGTTTTTCTATAGTATT	Amplify internal fragment of <i>tcdA</i> (-)
JRP2344	CCGGAATTCGTTCCAGTAACAGTGGATTTTTG	Amplify internal fragment of <i>tcdB</i> (+)
JRP2345	CCGGAATTCCTCAAATGGATATTCATACTGCC	Amplify internal fragment of <i>tcdB</i> (-)
JRP2423	CCGGAATTCGGCCATATATGTCGTATATCTA	Amplify <i>C. difficile</i> CD196 <i>cdtA</i> and <i>cdtB</i> genes and upstream, region introduces EcoRI site (+)
JRP2424	CCGGAATTCCTGTGGGGACAAATTTAAATCA	Amplify <i>C. difficile</i> CD196 <i>cdtA</i> and <i>cdtB</i> genes and upstream region, introduces EcoRI site (-)
JRP2523	AGTTCTAGAGGAATGAAACTAAAAGAAAAAAT	Amplify internal fragment of <i>cdtR</i> (+)
JRP2524	AGTTGCGCAGCATCATCTAAATCTGGTAAAG	Amplify internal fragment of <i>cdtR</i> (-)
JRP3631	GTTGCGGGGATCCTCTTACACCG	Amplify Δ <i>cdtR</i> by SOE-PCR (+)
JRP3632	CTCATTTTAATATTTCTTACAACATCATTATCAAAACCTTC	Amplify Δ <i>cdtR</i> by SOE-PCR (-)
JRP3633	TAAGAAATATTAATAAGTAAATGGCCATATATG	Amplify Δ <i>cdtR</i> by SOE-PCR (+)
JRP3634	GGTCAGGCTGCATCTGCAGAATTCG	Amplify Δ <i>cdtR</i> by SOE-PCR (-)
JRP3720	CTTCTATAAATATGTTAAATAATTCTTC	Amplify <i>cdtR</i> gene and upstream region (+)
JRP3721	GAGACATCTCCTTTTTCTATTTATTATG	Amplify <i>cdtR</i> gene and upstream region (-)
JRP3722	GTTGATAAAATTTATTATAACTCTCTAG	Amplify internal fragment of <i>CD2602</i> (+)
JRP3723	GTAACACTAGAATAATATCTATAG	Amplify internal fragment of <i>CD2602</i> (-)
JRP3724	CATGTTTACAATCTCTTCTACACTC	Amplify internal fragment of <i>trpS</i> (+)
JRP3725	CAATCACATGTCAAAGAGCATGTTGAAC	Amplify internal fragment of <i>trpS</i> (-)
JRP3726	CTACTATTAGATATTATAACAATGTTCC	Amplify putative <i>cdtA</i> promoter region (+)
JRP3727	CCCTACAAATATTAGTTTGTAAAC	Amplify putative <i>cdtA</i> promoter region (-)
JRP3728	GCCACCTGATACTATAGATATTATTC	Amplify/sequence <i>CD2602/trpS</i> intervening region (+)
JRP3729	AAGTTGGATTTATAGAGAGAAG	Amplify/sequence <i>CD2602/trpS</i> intervening region (-)
JRP3845	TGCAATACTACTTACAAGGCTCCTATAGA	Amplify internal <i>cdtA</i> fragment in QRT-PCR (+)
JRP3846	TCTTTCCATTCTTTAGCCTTTTC	Amplify internal <i>cdtA</i> fragment in QRT-PCR (-)

^a +, leading-strand-specific oligonucleotide primer; -, lagging-strand-specific oligonucleotide primer.

QIAPrep spin miniprep kit (QIAGEN) according to the manufacturer's instructions. For *C. difficile* plasmid preparations, cell pellets were incubated in buffer P1 (supplied with the kit) supplemented with 30 μ g \cdot ml⁻¹ lysozyme (Sigma-Aldrich) for 30 min at 37°C, before extraction as per the manufacturer's instructions. *C. difficile* genomic DNA was prepared using the DNeasy tissue kit (QIAGEN) according to the manufacturer's instructions. DNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies) and stored at -20°C. Standard methods for the digestion, modification, ligation, and analysis of plasmid and genomic DNA and PCR products were used (44). Nucleotide sequence analysis was carried out using a PRISM BigDye Terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Sequence detection was carried out on an Applied Biosystems 3730S genetic analyzer and was performed by Micromon at Monash University. Sequences were analyzed using ContigExpress (Invitrogen).

PCR. Oligonucleotide primer sequences are listed in Table 2. Unless otherwise stated, all PCR experiments were carried out with *Taq* DNA polymerase and the supplied 10 \times PCR buffer with magnesium according to the manufacturer's instructions (Roche). The following parameters were utilized for all PCR experiments: initial denaturation was performed at 94°C for 4 min, followed by 30 cycles consisting of a denaturation step at 94°C for 30 s, an oligonucleotide primer annealing step at 50°C for 30 s, and an extension step at 72°C. The extension time was 1 min per 1 kb of amplified DNA. A final extension step was performed at 72°C for 12 min.

Construction of recombinant plasmids. For the construction of the CDT expression plasmid, PCR was performed using primers JRP2423 and JRP2424 (Table 2), chromosomal DNA from strain CD196, and the Triple Mastermix PCR system (Roche). The resultant 4.5-kb fragment containing the CDT region was cloned into the EcoRV site of pT7Blue3 using the Perfectly Blunt cloning kit according to the manufacturer's instructions (Novagen). After sequencing, the 4.5-kb fragment carrying both the *cdtA* and *cdtB* genes was excised from the pT7Blue3 derivatives with *Sph*I and *Sac*I before cloning into the equivalent sites of the *E. coli*-*C. difficile* shuttle plasmid pMTL9361Cm, resulting in pJIR3107.

For construction of the *cdtR*-plasmid pJIR3394, PCR was performed using primers JRP3720 and JRP3721 (Table 2). Chromosomal DNA from strain JIR8094 was used in the Failsafe PCR system with buffer E (Epicenter Biotechnologies) to amplify the *cdtR* region. The PCR product was then TA cloned into pCR2.1-TOPO, according to the manufacturer's instructions (Invitrogen). The

1.13-kb *cdtR* fragment was excised from this plasmid with *Xho*I and *Bam*HI and subcloned into the equivalent sites of pMTL500E. The insert was subsequently excised from this intermediate vector using *Eco*RV and *Sma*I and inserted into the *Fsp*I site of pMTL9301, resulting in pJIR3394.

The deletant *cdtR* allele was generated using splice overlap extension PCR (SOE-PCR) (15, 16). PCR was used to amplify the left and right sides of the *cdtR* region using primers JRP3631 with JRP3632 and JRP3633 with JRP3634, respectively (Table 2). This reaction used pJIR3394 as the template together with the Failsafe PCR system enzyme mix and buffer E. Each fragment was purified by extraction from a 1% agarose gel using the Qiaquick gel extraction kit (QIAGEN). An aliquot of each of the purified PCR products was mixed in an equimolar ratio and used as the DNA template in a second PCR with primers JRP3631 and JRP3634, which resulted in a DNA fragment with a 561-bp deletion within *cdtR*, which was then TA cloned into pCR2.1-TOPO (Invitrogen). The fragment was excised from pCR2.1-TOPO using *Bam*HI and *Pst*I, and cloned into the equivalent sites of pJIR3394, resulting in the final construct, pJIR3395.

Transfer of plasmid DNA into *C. difficile* by conjugation. The conjugation procedure was carried out as previously described (40), except that HB101(pVS520) (37) was used as the *E. coli* donor. Bacterial pellets collected from 1-ml aliquots of an overnight *E. coli* donor culture were resuspended in 200- μ l aliquots of the *C. difficile* recipient strain, which had been collected from 20-ml overnight cultures incubated at 37°C in an anaerobic chamber. This mating mixture was then spread onto a thick BHIS agar plate and incubated for 6 h or overnight at 37°C under anaerobic conditions. Bacterial growth was harvested by flooding the agar three times with 500 μ l of sterile phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.4 mM KH₂PO₄, 4.3 mM Na₂HPO₄). Aliquots (100 μ l) of the combined cell suspension were then spread onto BHIS agar supplemented with cycloserine and cefoxitin to prevent growth of the *E. coli* donor and with either thiamphenicol or erythromycin to select for *C. difficile* transconjugants carrying the appropriate plasmids. The plates were incubated under anaerobic conditions for 24 to 72 h.

Determination of the integration site of the CdtLoc. The region flanked by *CD2602* and *trpS* from *C. difficile* strain CD37 was amplified by PCR using primers JRP3722 and JRP3725 (Table 2) and the Failsafe PCR system buffer G. The sequence of the amplified product was then determined using primers JRP3722, JRP3725, JRP3728, and JRP3729 (Table 2). The CDT integration sites of all other nontoxic *C. difficile* strains were determined by PCR amplifica-

tion of this region using primers JRP3728 and JRP3729 and Failsafe buffer E, followed by sequencing with primers JRP3728 and JRP3729.

Detection of CDTa and CDTb proteins by Western blotting. Isolation of CDTa and CDTb and Western blotting were carried out as previously described (38). Proteins were precipitated at a concentration of 70% ammonium sulfate from cell-free supernatants of *C. difficile* cultures grown overnight in 100 ml of BHIS broth. The precipitate was collected, dissolved in 2 ml of distilled water, and dialyzed against 10 mM Tris-HCl buffer, pH 7.0, for 24 h with three changes of buffer. Proteins were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (23) and transferred by electrophoresis (44) to nitrocellulose membranes (Whatman). The membranes were incubated for 1 h in phosphate-buffered saline containing 5% dry milk powder and then incubated overnight with immunopurified antibodies specific for *C. perfringens* iota toxin components A (Ia) and B (Ib), which are cross-reactive with the *C. difficile* CDTa and CDTb proteins, respectively (38). CDTa- and CDTb-bound antibodies were then detected with peroxidase-conjugated anti-rabbit sheep antibodies (Chemicon) and the Western lightning chemiluminescence reagent kit (Perkin Elmer), according to the manufacturer's instructions.

ADP-ribosyltransferase assays. ADP-ribosyltransferase assays were carried out essentially as before (55). In brief, precipitated supernatant protein was incubated for 1 h in a reaction solution containing 50 mM triethanolamine-HCl buffer (pH 7.5), 5 mM MgCl₂, 10 mM dithiothreitol, 10 mM thymidine, and 10 μg rabbit skeletal muscle actin (Sigma-Aldrich), together with [³²P]NAD (3 μCi). The entire reaction volume was then applied to a glass fiber filter (Millipore) and allowed to dry. Protein was then precipitated onto the filter by washing with ice-cold 10% (wt/vol) trichloroacetic acid, followed by three washes in ice-cold 5% (wt/vol) trichloroacetic acid and a final wash using 95% ethanol. The filters were allowed to dry before being placed in Optiphase Hisafe 3 scintillation fluid (Perkin-Elmer), and radioactivity was counted using a Tri-Carb 2100TR liquid scintillation analyzer (Packard Biosciences).

Quantitative real-time PCR (QRT-PCR) analysis of *cdtA* expression. *C. difficile* cultures (10 ml) were grown to a turbidity of approximately 1.0, and RNA was extracted using the Ribopure Bacteria kit (Ambion Inc.) according to the manufacturer's instructions. To remove contaminating genomic DNA, the purified RNA was treated with the DNA-free kit (Ambion Inc.), according to the manufacturer's instructions. Reverse transcription reactions were then performed using 2 μg of purified RNA, 0.5 μM primer, and the Omniscript reverse transcription kit (QIAGEN) according to the manufacturer's instructions. The reaction mixtures were diluted twofold, and real-time PCR analysis was performed in a final volume of 25 μl with SYBR green PCR master mix (Applied Biosystems), 2 μl of diluted reverse transcription reaction mixture, and 120 nM primers using an Eppendorf Realplex 4 Mastercycler. Triplicate reactions were performed in multiple experiments using RNAs from three biological replicates. The data were normalized to *C. difficile rpoA* mRNA levels.

Nucleotide sequence accession number. The GenBank accession number of the CD196-derived sequence determined in this study is EF581852.

RESULTS

More binary toxin is produced in a strain 630 background than in a strain CD37 background. To facilitate the functional analysis of CDT in *C. difficile*, the *E. coli*-*C. difficile* shuttle plasmid pJIR3107 (Fig. 1A) was constructed. This plasmid encoded the complete binary toxin region of *C. difficile* strain CD196. The nucleotide sequence of the cloned 4.5-kb fragment was determined to see if any PCR-derived mutations had been introduced. When this sequence was compared to the CD196-derived binary toxin region in the GenBank database (accession number L76081), several differences were observed. Sequencing of two independent CDT-specific PCR products revealed that these changes were not the result of PCR-derived errors.

The most significant difference occurred within *cdtA*, after nucleotide position 267, where the residues ACCAGAAGA were present in the sequence determined here but not in the database sequence. The addition of these residues would lead to the insertion of three amino acids, Thr Arg Arg. Within the sequenced region, there were two other changes that would

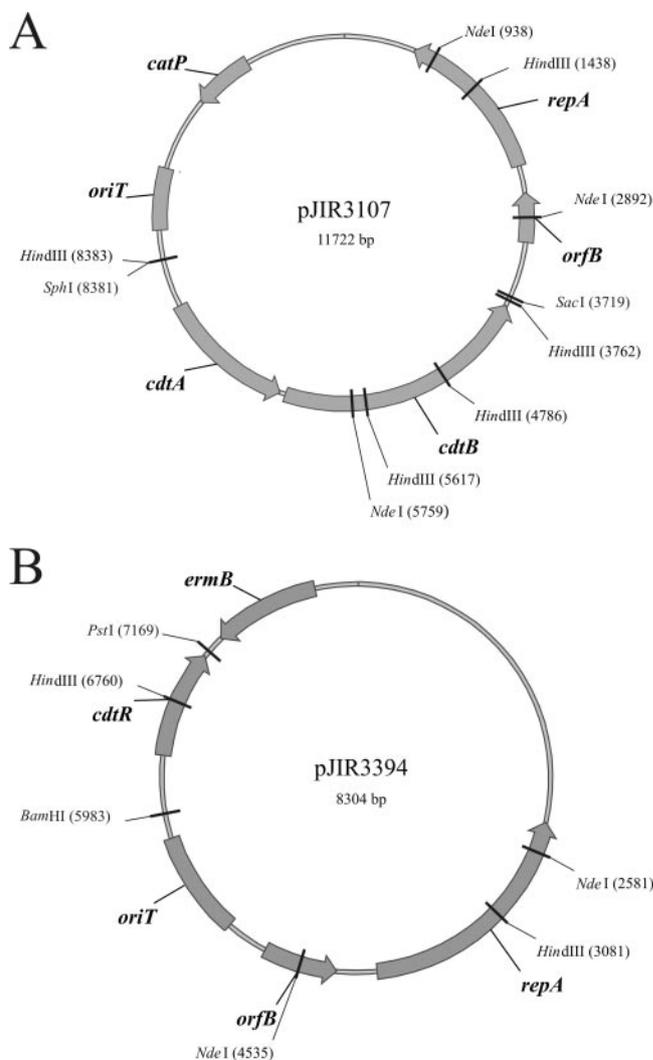


FIG. 1. Relevant shuttle plasmids. Schematic representations of the CDT binary toxin expression plasmid pJIR3107 (A) and the *cdtR* complementation plasmid pJIR3394 (B) are shown. The position and orientation of the pCD6 plasmid replication genes (*repA* and *orfB*) are shown, as is the transfer origin (*oriT*), the selectable markers (*ermB* or *catP*), the CDT binary toxin genes (*cdtA* and *cdtB*), and the *cdtR* gene.

lead to a change to the resultant protein sequence. Within *cdtA* there was a G at position 871, not an A, resulting in a change from Asn to Ser. Within *cdtB*, the T at position 1720 was an A, changing the encoded amino acid from Ser to Thr. It is not known if errors in the previous CD196-derived sequence or strain variation is the reason for the differences that were observed. However, comparison of the *cdtAB* sequence that we obtained from CD196 with those of the sequenced NAP1/027 strain QCD-32g58 (<http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=120673853&view=gbwithparts>) and of an additional *cdtAB* positive isolate, CCUG 20309 (accession number AF271719) (7), revealed that the differences observed here were present in both of these strains. This analysis showed that the CDT region that had been cloned into pJIR3107 was identical to that from the chromosome of strain CD196, as well as

strains QCD-32g58 and CCUG 20309, validating the use of this plasmid in subsequent studies.

The CDT⁺ plasmid pJIR3107 was introduced by conjugation into JIR8094, an erythromycin-susceptible derivative of the *tcdA*⁺ *tcdB*⁺ strain 630, which does not have intact *cdt* genes and therefore produces toxins A and B but not binary toxin. The plasmid was also introduced into the nontoxicogenic strain CD37. The integrity of the plasmids from several independently derived transconjugants was confirmed by introducing plasmid DNA into *E. coli* DH5 α and carrying out restriction analysis.

To determine if the *C. difficile* transconjugants produced the CDTa and CDTb proteins, Western blotting was performed. Neither JIR8094, CD37, nor derivatives of these strains carrying the vector plasmid pMTL9361Cm produced any detectable CDTa or CDTb protein (Fig. 2A and B). As expected, both CDT components could be detected in the supernatant of the reference binary toxin-producing strain CD196. The JIR8094 derivative that carried pJIR3107 produced substantial quantities of CDTa and CDTb, much more than was produced by CD196. Unexpectedly, the CD37 derivative carrying the same plasmid produced very low levels of the CDT proteins (Fig. 2A and B).

To quantitatively measure CDT production and to confirm that functional CDT binary toxin was being produced, ADP-ribosyltransferase assays were performed using ammonium sulfate-precipitated culture supernatants prepared from JIR8094 and CD37 derivatives carrying pJIR3107, together with the appropriate controls (Fig. 2C). In agreement with the Western blots, the level of ADP-ribosyltransferase activity associated with JIR8094(pJIR3107) was more than 15-fold higher than that associated with CD37(pJIR3107) and more than 4-fold higher than that associated with the CD196 reference strain (Fig. 2C). No significant ADP-ribosyltransferase activity was detected in extracts from strain JIR8094(pMTL9361Cm), as expected (Fig. 2C).

A LytTR family response regulator gene is adjacent to the CDT-encoding genes. To determine if a regulatory gene was responsible for the increased levels of CDT production by JIR8094(pJIR3107), bioinformatic analysis of the CdtLoc of the strain 630 genome sequence (45) and of strain QCD-32g58 was undertaken. Note that although strain 630 does not produce CDT or have an intact *cdtAB* operon, it does have *cdtAB* pseudogenes (Fig. 3), which have accumulated several frameshift mutations and premature stop codons (45).

Our analysis revealed the presence of a gene (CD2603) encoding a putative LytTR family response regulator, which was located immediately upstream of the predicted *cdtA* pseudogene (Fig. 3). Due to its close proximity to the *cdtAB* genes, and in view of the results reported in this paper, we have named CD2603 the “*Clostridium difficile* binary toxin regulatory gene,” or *cdtR*. The *cdtR* gene was 747 bp in length and encoded a putative 30-kDa CdtR protein that had approximately 28% amino acid sequence identity to the accessory gene regulator A (AgrA) protein from *Staphylococcus aureus* and that had similarity to several other AgrA orthologs (Fig. 4). Unlike AgrA from *S. aureus*, *cdtR* was an orphan response regulator gene, since it did not appear to have a cognate sensor histidine kinase gene associated with it.

The similar genetic organizations of the *cdt* locus in strains

630 and QCD-32g58 (Fig. 3) suggested that *cdtR* might encode a regulator of binary toxin production. The low level of CDT production by CD37(pJIR3107) might be due to the absence of *cdtR* or to a mutation within this gene in CD37. To investigate this hypothesis, primers JRP3720 and JRP3721 (Table 2) were used in an attempt to PCR amplify the *cdtR* gene region from strains JIR8094 and CD37. While a PCR product specific to *cdtR* was amplified using chromosomal DNA from JIR8094, no such product could be generated when CD37-derived DNA was used (data not shown). This result indicated either that *cdtR* was absent from CD37 or that the nucleotide sequence of the gene was significantly different from that of the gene in strain 630. PCR analysis using other primers that anneal within the *cdtR* coding region yielded similar results (data not shown), indicating that strain CD37 does not carry the *cdtR* gene.

The *cdt* locus was found only in toxigenic *C. difficile* isolates.

The *cdt* loci from 26 *C. difficile* strains, isolated over a period of at least 27 years, were investigated in more detail. Genomic DNA from each strain was subjected to PCR amplification using primers specific for the *cdtR* gene, the predicted promoter region of *cdtA*, and CD2602 and *trpS*, which are located immediately upstream and downstream, respectively (Fig. 3). The *cdtA* promoter region was analyzed in preference to an internal *cdtA* fragment since there are a number of isolates that have large deletions within the coding regions of *cdtAB* (53). Any such strains would have appeared as false negatives in *cdtA*-specific analysis. In addition, the major toxin genes, *tcdA* and *tcdB*, were also PCR amplified. Of these 26 strains, eight isolates did not yield amplification products for either *tcdA* or *tcdB* and therefore were classed as nontoxicogenic isolates. Both the *cdtR* gene and the predicted *cdtA* promoter region were found exclusively in the remaining 18 isolates, which had both *tcdA* and *tcdB*, although all 26 strains carried both CD2602 and *trpS*.

To define the *cdt* locus, the region located between the CD2602 and *trpS* genes of strain CD37 was PCR amplified. A product of only 1.8 kb in size was generated (data not shown), indicating that a substantial portion of the equivalent 4.2-kb and 6.2-kb regions present in strains 630 and QCD-32g58, respectively, was absent from strain CD37. The nucleotide sequence of this 1.8-kb fragment was determined, allowing the boundaries of the *cdt* locus to be defined. The *cdtR*, *cdtA*, and *cdtB* genes, as well as some flanking sequences, were all absent in strain CD37. Instead, a unique 68-bp nucleotide sequence was found at this location (Fig. 3). This region did not have any similarity with sequences within the *cdt* locus of strain 630 or QCD-32g58 or with any other sequences found in the database.

To determine whether the same 68-bp region was present in the remaining seven nontoxicogenic strains, PCR was used to amplify a smaller internal fragment located between the CD2602 and *trpS* genes. In every strain, a product of approximately 700 bp was generated, which was identical in size to that from CD37 (data not shown). Subsequent sequence analysis of each PCR product revealed the presence of the same 68-bp sequence in every nontoxicogenic isolate examined (Fig. 3).

CdtR positively regulates binary toxin production in *C. difficile*. To determine if CdtR regulates the expression of the *cdtAB* operon, the *cdtR* gene and a 300-bp upstream region

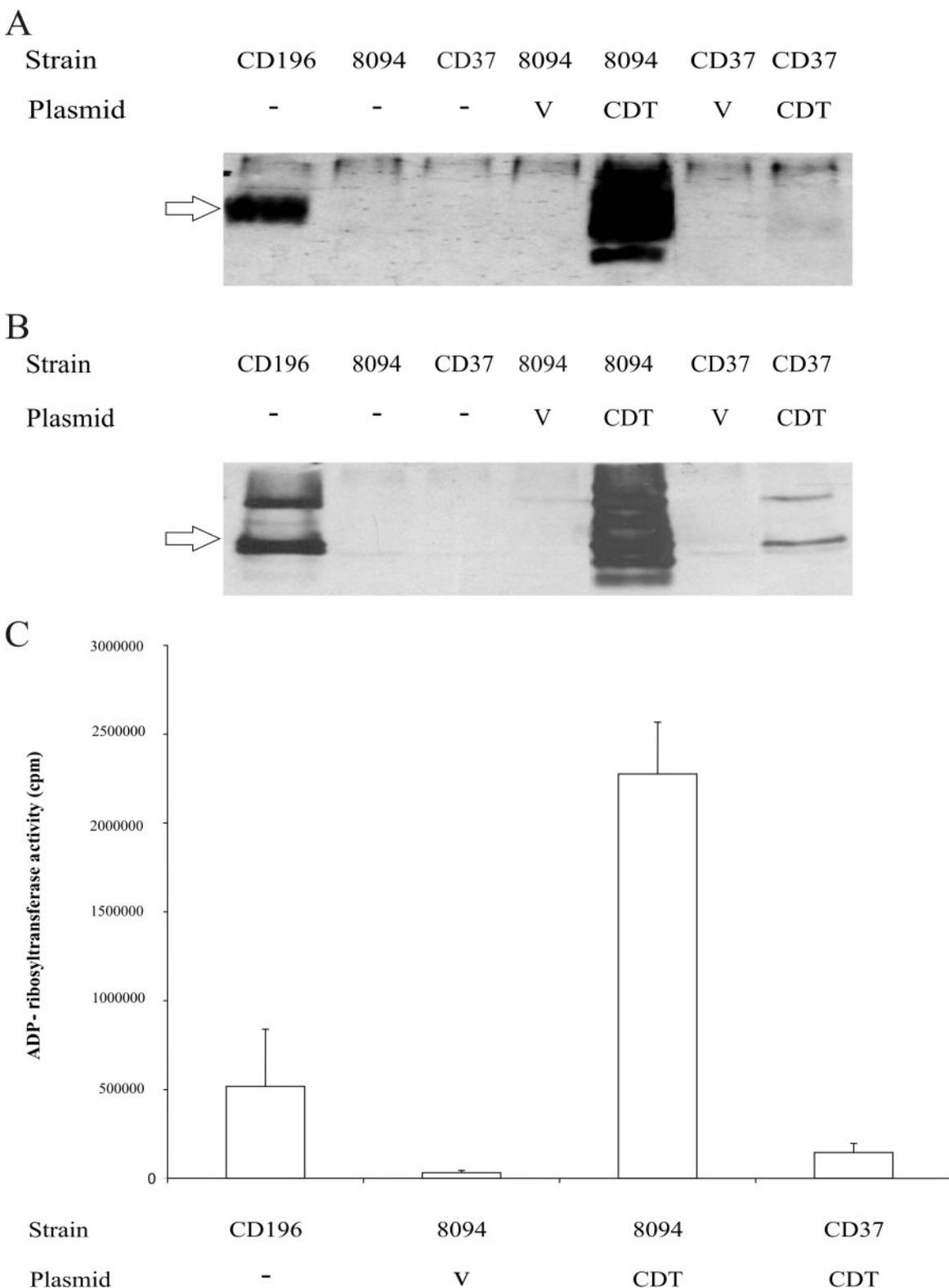


FIG. 2. Analysis of binary toxin production by complemented strains. (A) CDTa Western immunoblot using cross-reactive Ia-specific antibodies and precipitated supernatant proteins from the strains indicated. (B) CDTb Western immunoblot using cross-reactive Ib-specific antibodies and precipitated supernatant proteins. (C) ADP-ribosyltransferase activity assay using purified rabbit skeletal muscle actin, [³²P]NAD and precipitated supernatant proteins. The data are the mean values and standard deviations from three independent experiments. CD196, binary toxin-positive reference strain; JIR8094, binary toxin-negative strain; CD37, nontoxicogenic strain; V, pMTL9361Cm vector plasmid; CDT, pJIR3107, which carries the wild-type *cdtAB* genes. The arrows indicate the 48-kDa CDTa protein and 75-kDa CDTb protein. The higher-molecular-mass products are most likely higher-order aggregates and the smaller protein bands breakdown products.

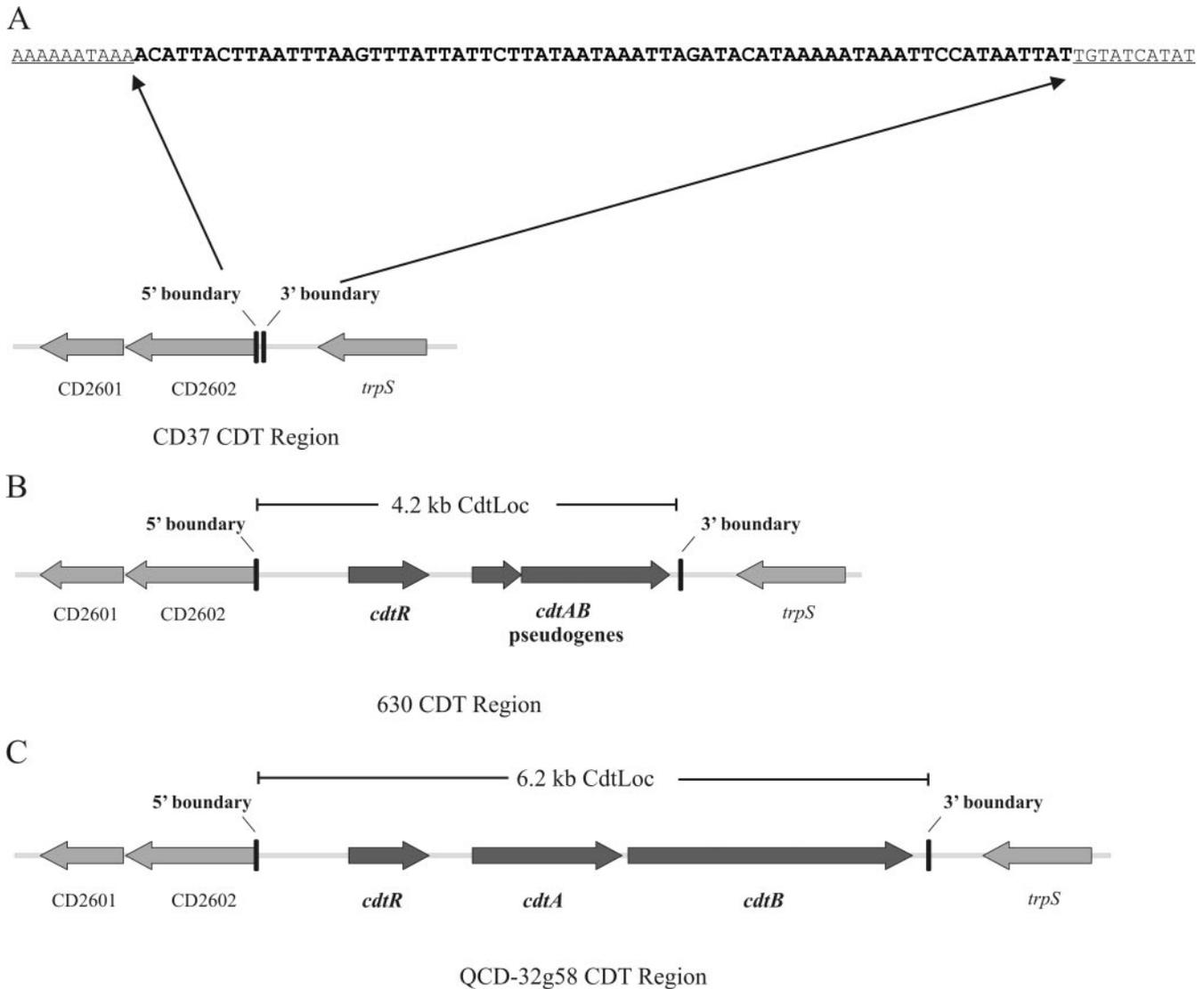


FIG. 3. Schematic representation of the CDT region and flanking genes. The regions from the nontoxic isolate CD37 (A), the binary toxin-negative isolate strain 630 (B), and the binary toxin-positive isolate QCD-32g58 (C) are shown. The positions of the 5' flanking genes CD2601 and CD2602, the 3' flanking gene *trpS*, the response regulator gene *cdtR*, and the CDT binary toxin-encoding genes *cdtAB*, or their pseudogenes, are shown. For each variant of the CDT region the positions of the 5' and 3' conserved boundaries are shown, and the size of the entire CdtLoc is indicated. The unique 68-bp sequence that is present in CD37 and other nontoxic isolates in place of the CdtLoc is shown in bold, and the nucleotide boundaries of the CDT region that are conserved in all three variants are underlined.

were PCR amplified from strain JIR8094 and cloned into the shuttle plasmid pMTL9301, generating pJIR3394 (Fig. 1B), which encodes erythromycin resistance. This plasmid was transferred by conjugation into the CD37 derivative that carries the *cdtA*⁺*B*⁺ plasmid, pJIR3107. The transconjugants were isolated and maintained on medium supplemented with both thiamphenicol and erythromycin to ensure the continued presence of both plasmids, each of which was derived from a pCD6 replicon. PCR analysis of several transconjugants confirmed the presence of both *cdtA* and *cdtR* (data not shown), verifying the presence of both pJIR3107 and pJIR3394 in the resultant strain.

To provide the appropriate control for this experiment, pJIR3395 was constructed. This plasmid was a derivative of pJIR3394 that contained a large in-frame internal deletion of

cdtR, spanning codons 36 to 222, designated Δ *cdtR*. This plasmid was transferred by conjugation into strain CD37 (pJIR3107), and thiamphenicol and erythromycin resistant transconjugants were selected, maintained, and analyzed as before.

Western blot analysis was then performed on the isogenic CD37 derivatives carrying pJIR3107 (*cdtA*⁺*B*⁺) alone or pJIR3107 together with pJIR3394 (*cdtR*⁺) or pJIR3395 (Δ *cdtR*), as well as CD37 carrying the shuttle vector pMTL9361Cm and pJIR3394. Other controls included the CD196 binary toxin reference strain and a CDT⁺ NAP1/027 strain, M7404. The results showed that the presence of pJIR3394 in CD37(pJIR3107) led to a dramatic increase in the production of both CDTa and CDTb (Fig. 5A and B). By contrast, pJIR3395 had no effect on CDTa or CDTb produc-

<i>S. aureus</i>	M-KIFICEDDPKQRENMTI	IKNYIMIEEKPMETALATDNPEYEVLEQAKN	49				
<i>S. epidermidis</i>	M-KIFVCEDDQKQREHMVSI	IKNYIMIEEKPMELALATNDPEYEVLEQSKE	49				
<i>C. acetobutylicum</i>	MLEVFVCEDNKEQKFNFRKI	IDNFIIMENLDMKISVVTENPDDIINYVIK	50				
<i>C. difficile</i>	M-DILIFDNDVCFGMKLEKEI	INNILLKEGFDNDVVRLYHNANLLKELTE	49				
<i>S. aureus</i>	MNDIGCYFLDIQLSTDI	----GIKLGSEIRKHD	PVGNII	FVTS	HSELT	94	
<i>S. epidermidis</i>	LNDIGCYFLDIQLEADMN	----GKLA	EIRKHD	PVGNII	FVTS	HSELT	94
<i>C. acetobutylicum</i>	NSVSGLYFLDIDLNASIN	----GTQLAAEIRKYD	PRGFIV	FVTT	HAEMS	95	
<i>C. difficile</i>	KNKVRIYFIVVDAKYKISNELCD	GLWIAQKIRESDYISPI	IFELTN	HTEMI		99	
<i>S. aureus</i>	YLTFVYKVAAMDFIFKDDPAELRTR	IIDCLETAHTRLQLLSKDNSVETIE	144				
<i>S. epidermidis</i>	YLTFVYKVAAMDFIFKDDPSELKMR	IIDCLETAHTRLKLLSKESNVDTIE	144				
<i>C. acetobutylicum</i>	YLTFELYKVEAMDYI	IKDNYKNIGDRIYQCIVDAQRKYSAKTTD-LQKIFT	144				
<i>C. difficile</i>	LGIFDYRLEVMDFILKHMEIAESKIKACIKIAHKRY-IKEKNYRSNFFT		148				
<i>S. aureus</i>	LKRGNSVYVQYDDIMFES	SSTKS	HRLIAHL	DNRQIEFYGNLKELS-QLD	193		
<i>S. epidermidis</i>	LKRGNSVYVQYDDIMFES	SSTKS	HRLIAHL	DNRQIEFYGNLKELA-QLD	193		
<i>C. acetobutylicum</i>	IKADDRIINIEFQKILFFETS	SFTIEHKVVLHSVNRQIEFYAKMKDIEGELD			194		
<i>C. difficile</i>	IYSDFSLWKISFDEVIYFET	SSTIPEKIKLVTTSRIEFYKSLKSLP-DLD			197		
<i>S. aureus</i>	DRFFRCHNSFVVRHNI	ESIDSKERIVYFKNKEHCYAS---	VRNVKKI---	238			
<i>S. epidermidis</i>	ERFFRCHNSFVINRHNI	ESIDSKERIVYFKNGENCFAS---	VRNVKKI---	238			
<i>C. acetobutylicum</i>	DSFYRCHKSYIVNKKNI	KEININKRRIYMINGEECLIS---	TRMLKGLIK-	241			
<i>C. difficile</i>	ACFTRVHKSFVVRNKHV	IVSLDLKKNIKMSNGHICRISNTYRNILKNI	IKT	248			

FIG. 4. Alignment of CdtR orthologs. The predicted CdtR protein from *C. difficile* strain 630 and predicted AgrA orthologs of *S. aureus* (ABB17535), *Staphylococcus epidermidis* (AAO25552) and *Clostridium acetobutylicum* (AAK78066) were aligned using CLUSTALW (58). Identical amino acid residues are shaded with black, and strongly similar amino acid residues are shaded with gray.

tion. As expected, a CD37 derivative carrying pMTL9361Cm and pJIR3394 did not produce any detectable CDTa or CDTb, since it did not contain the *cdtA* or *cdtB* gene. Note that CDTa and CDTb production by CD37(pJIR3107) carrying the *cdtR*⁺ plasmid appeared to be greater than that by either of the binary toxin-producing positive controls.

To confirm the data obtained by Western blot analysis, ADP-ribosyltransferase assays were performed on the same strains (Fig. 5C). The results showed that the functional *cdtR* gene results in a 17-fold increase in CDT activity. The CD37 (pJIR3107)(pJIR3394) strain had approximately fivefold more activity than the positive control strains CD196 and M7404. The introduction of the internal deletion into *cdtR* led to an approximately ninefold reduction in ADP-ribosyltransferase activity, to a level that was not significantly different from that of the isogenic negative controls. These results provide good experimental evidence that the *cdtR* gene encodes a positive activator of binary toxin production in *C. difficile*.

To verify that CdtR regulates the expression of the binary toxin genes at the transcriptional level, *cdtA*-specific QRT-PCR analysis was performed on RNAs extracted from strains CD37 (pJIR3107)(pJIR3394) and CD37(pJIR3107)(pJIR3395). The results showed that there was a 90-fold \pm 10.5-fold increase in the levels of *cdtA*-specific mRNA in the strain carrying the intact *cdtR* gene compared to the strain carrying the Δ *cdtR* plasmid ($P < 0.0002$). These data confirm that *cdtR* is a transcriptional regulator of the binary toxin genes.

DISCUSSION

The results presented in this paper clearly show that binary toxin production in *C. difficile* is positively regulated by the LytTR family response regulator, CdtR. The evidence for this conclusion comes from the observation that strain 630-derived recombinant strains carrying the binary toxin structural genes *cdtA* and *cdtB* produced large amounts of binary toxin, but the equivalent CD37 derivatives, which do not have a *cdtR* gene, produced approximately 15-fold less binary toxin, despite these strains carrying the same CDT-encoding plasmid. In addition, when a recombinant CD37 derivative carrying *cdtA* and *cdtB* was complemented with a functional *cdtR* gene, binary toxin production was activated and greater than 17-fold more binary toxin was produced.

To rule out the possibility that another plasmid-encoded factor was responsible for the increase in binary toxin production seen in the complemented strains, the intact *cdtR* gene was replaced with a mutant *cdtR* allele. This Δ *cdtR* gene contained an in-frame deletion that spans codons 36 to 222, resulting in the removal of most of the *cdtR* sequence. When complemented with Δ *cdtR*, CDT production was not statistically different from that of the noncomplemented parental strain, indicating that CdtR is indeed responsible for the observed up-regulation of binary toxin production.

Compared to the NAP1/027 strain M7404 and the reference CDT⁺ strain CD196, which both produced similar levels of

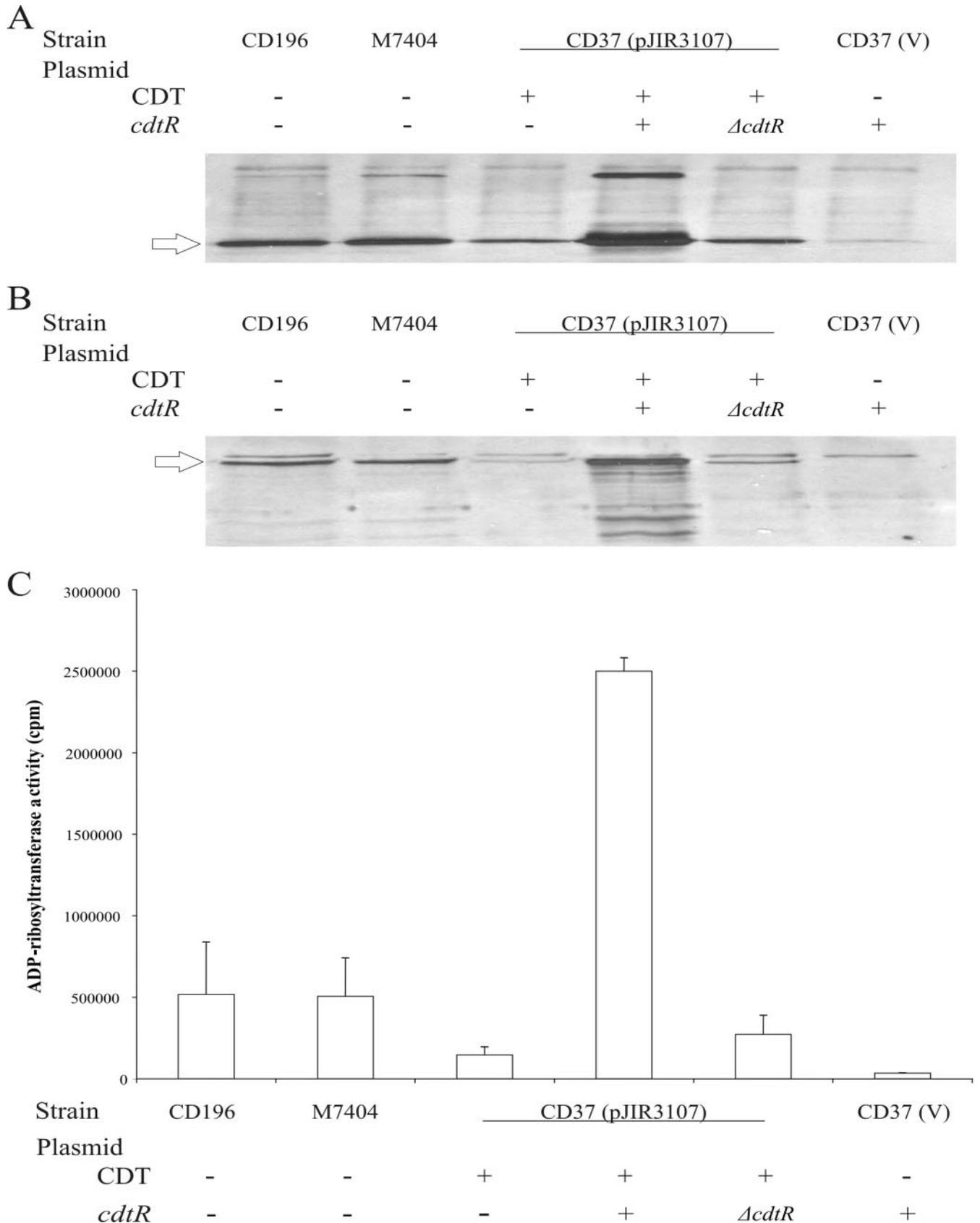


FIG. 5. Analysis of binary toxin production in derivatives of CD37. (A) CDTa Western immunoblot using cross-reactive Ia specific antibodies and precipitated supernatant proteins from the strains indicated. (B) CDTb Western immunoblot using cross-reactive Ib specific antibodies and

binary toxin, the *cdtAB*-complemented strain 630 derivative and the *cdtAB*- and *cdtR*-complemented CD37 derivative had significantly more ADP-ribosyltransferase activity. This result most likely reflects the higher copy number of the plasmid-carried *cdtAB* genes in the recombinant strains.

The comparative genomic studies showed that there was an absolute correlation between the presence of the *cdtR* gene and the upstream *cdtA* region. Strains that did not have *cdtA* or a *cdtA* pseudogene did not carry *cdtR*. The term PaLoc is widely used to describe the cytotoxin gene locus of *C. difficile*, a locus that includes both toxin structural genes and regulatory genes. Based on the findings presented here, and in keeping with the PaLoc nomenclature, it is proposed that the term CdtLoc be used to describe the *cdt* locus, which is hereby defined as the region containing *cdtR*, *cdtA*, and *cdtB* (as shown in Fig. 3), irrespective of whether in a particular isolate *cdtA* and *cdtB* are functional genes or pseudogenes.

Of the strains examined here, only those isolates that had the *tcdA* and *tcdB* genes carried *cdtR* and therefore had the CdtLoc. Recently, other workers performed genomic microarray analysis that compared diverse *C. difficile* isolates (52), very few of which were included in our study. Of the 75 strains analyzed, 68 provided definitive data with regard to the presence or absence of *cdtR*. Our analysis of the microarray data appears to reinforce the findings reported here, since approximately 96% of *cdtR*-positive isolates also carried the *cdtAB* genes or, at the very least, fragments of these genes, indicating that *cdtR* and the *cdtAB* genes are genetically linked. Interestingly, we found that approximately 82% of the *cdtR*-negative isolates were clustered within the A⁻B⁺ clade. The remaining 18% were located in the HA1 clade and were not from disease outbreaks. Upon closer inspection of the entire A⁻B⁺ clade, we discovered that only four of these 22 isolates carried the CdtLoc. Remarkably, each of these four strains was actually genotypically classified as A⁺B⁺ and not A⁻B⁺, and they were all of animal origin (52). Analysis of the seven A⁻B⁻ isolates included in the microarray study revealed that only two contained the CdtLoc. These strains were located within the HA1 and HA2 clades, respectively. The remaining five genotypically A⁻B⁻ isolates were *cdtR* negative and were clustered within the A⁻B⁺ clade.

Other studies have identified CDT-positive but toxin A- and toxin B-negative strains, providing evidence that these loci are not always linked (10, 11). However, the observations described above, together with the results presented in this study, suggest that, with the exception of strains found within the A⁻B⁺ clade, there is a correlation between the presence of the PaLoc and the CdtLoc. More than 98% of CdtLoc-positive strains also have the PaLoc. Furthermore, it is apparent, again with the exception of A⁻B⁺ strains, that the CdtLoc is widely

disseminated, with greater than 90% of the non-A⁻B⁺ clade isolates analyzed with the microarrays containing this region.

Further analysis of the site of insertion/deletion of the CdtLoc in CD37 and seven other PaLoc-negative isolates showed that the entire CdtLoc was absent in these isolates and had been replaced by a novel 68-bp sequence of unknown function. This finding is reminiscent of the analysis of the PaLoc, where nontoxic strains were found to harbor a unique and highly conserved 115-bp sequence instead of the PaLoc (6). As with the PaLoc, there is no evidence of transposon-, plasmid-, or bacteriophage-related genes in close proximity to the CdtLoc. Since both of these toxin loci share some important characteristics, namely, the loss of an apparently innocuous nucleotide sequence upon their acquisition and the absence of any obvious genes or nucleotide sequences related to mobile genetic elements, it is postulated that the integration of the PaLoc and the CdtLoc may have occurred through similar, but as yet unknown, mechanisms.

It is estimated that between 1.6% and 6% of all *C. difficile* isolates produce a functional binary toxin, including the highly virulent toxinotype III NAP1/027 epidemic strains (53). However, the role of this toxin in the pathogenesis of *C. difficile* infections remains to be determined. Several studies have suggested an association between binary toxin-producing strains and more severe disease and community-acquired infections (2, 29, 57). Furthermore, CDT is known to be cytotoxic to Vero cells (38) and causes fluid accumulation in the rabbit ileal loop model (10). Indirect evidence therefore does suggest that CDT plays a role in *C. difficile*-mediated disease. However, a recent study showed that toxin A- and toxin B-negative, CDT-positive isolates were unable to cause disease in the hamster model (10).

Many bacteria tightly control their ability to produce toxins. *C. difficile* regulates the expression of its major toxins, toxin A and toxin B, in response to several environmental cues, including nutritional signals, cell density, and temperature (8, 17, 20, 27). This process involves the *tcdR* and *tcdC* genes, which are encoded within the PaLoc. However, the PaLoc does not carry genes encoding a two-component signal transduction system.

Members of the LytTR response regulator family, named after the LytT and LytR response regulators from *Bacillus subtilis* and *S. aureus*, respectively, are characterized by the presence of a novel DNA-binding domain, which shows little similarity to the helix-turn-helix or winged-helix domains that are more typical of other response regulators (33). They are widely distributed in low-G+C gram-positive bacteria, such as the clostridia, and many members have a well-documented association with bacterial virulence. Some play an integral role in quorum sensing, such as the AgrA response regulator, which is the master regulator of the quorum-sensing-controlled vir-

precipitated supernatant proteins. (C) ADP-ribosyltransferase activity assayed using purified rabbit skeletal muscle actin, [³²P]NAD and precipitated supernatant proteins. The data are the mean values and standard deviations from three independent experiments CD196, binary toxin-positive reference strain; M7404, binary toxin-positive NAP1/027 strain; CD37, nontoxic strain; pJIR3107, encodes the wild-type *cdtAB* genes (shown as + in the CDT rows); V, pMTL9361Cm vector plasmid (shown as - in the CDT rows); pJIR3394, encodes the wild-type *cdtR* gene (shown as + in the *cdtR* rows); pJIR3395, encodes the Δ *cdtR* gene (shown as Δ *cdtR* in the *cdtR* rows). The arrows indicate the 48-kDa CDTa protein and 75-kDa CDTb protein.

ulence response of *S. aureus* (31). Others are important in the regulation of toxin production, as demonstrated by the VirR response regulator of *C. perfringens*, which controls the synthesis of several toxins, including perfringolysin O (26, 42, 46), while others are involved in the production of extracellular polysaccharides, such as alginate in *Pseudomonas aeruginosa*, which is regulated by the AlgR response regulator (24). CdtR is now another virulence-related member of this family, controlling the production of CDT in *C. difficile*. CdtR may activate *cdtAB* gene expression either directly or by a secondary regulator.

LytTR response regulators are activated by the transfer of a phosphoryl group to a conserved aspartate residue located in their receiver domain, through the action of a cognate sensor histidine kinase (54). The genes encoding these proteins often comprise an operon, as observed for both *virR* and *agrA*, which are adjacent to *virS* (26) and *agrC* (34), respectively. By contrast, the *cdtR* gene is an orphan response regulator; there is no sensor kinase gene located in close proximity, and the histidine kinase that presumably is responsible for the activation of CdtR is unknown. However, the integration site of the CdtLoc positions *cdtR* only 11 bp from the predicted start site of a sensor histidine kinase gene (CD2602), although this gene is transcribed on the opposite DNA strand (Fig. 3). CD2602 appears to be associated with another downstream LytTR response regulator (CD2601). It is possible that the acquisition of the CdtLoc might result in the differential regulation of CD2602. Current studies in this laboratory are aimed at determining whether CD2601 and CD2602 are involved in the regulation of binary toxin expression.

In conclusion, we have identified a LytTR family response regulator that activates binary toxin production in *C. difficile* and together with the *cdtAB* operon comprises a newly defined locus, the CdtLoc. The genetic boundaries of this region were defined by the comparison of different CDT⁺ and CDT⁻ isolates. Key questions that remain to be resolved but which are the subject of current studies in this laboratory involve the determination of the environmental signals and signal transduction pathways that lead to the activation of CdtR and binary toxin production and the determination of the role of binary toxin production in diseases caused by this increasingly important human pathogen.

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