The function and regulation of the Clostridium difficile binary toxin (CDT) is currently poorly understood and the role of this toxin in pathogenesis of infection has yet to be defined. The objective of this study was to compare full binary toxin loci sequences from different C. difficile ribotypes and toxinotypes in an effort to further the understanding of the regulation of the binary toxin and its putative upstream regulator (cdtR). We used pyrosequencing to sequence isolates for a truncating single nucleotide cdtR mutation and we also sought to perform sequence and phylogenetic analysis to determine discordance between ribotype and binary toxin sequence relatedness.

### Materials and Methods

Isolate Selection and Culture Conditions. All C. difficile isolates were obtained from a collection of clinical isolates from human patients in Ontario, Canada, collected between 2004-2006 (1) except one porcine isolate collected in 2002. Ten isolates were chosen representing 6 different binary toxin positive ribotypes belonging to 9 different toxinotypes. All C. difficile strains were cultured on Columbia Blood Agar plates (Oxoid), and incubated anaerobically for 24 hours at 37°C. Genomic DNA was extracted using a commercial kit following the manufacturer’s instructions (Instagene Matrix, Biorad, Richmond, CA). Twenty-four primers were used to sequence the binary toxin loci including the cdtR promoter, cdtR, cdtAB promoter, cdtA, and cdtB genes.

### Binary Toxin Locus Analysis

Binary toxin locus sequencing and assembly was performed using the Geneious bioinformatics software (Biomatters, Auckland, NZ). Signal sequences and their respective cleavage sites were predicted using the online SignalP 3.0 Server (2). Multiple sequence alignments were performed using ClustalW2 tool (3) and phylogenetic trees were created using ClustalX version 2.0. Secondary structure prediction was performed using the Advanced Protein Secondary Structure Prediction (APSSP) Server (4).

### qPCR

Quantitative real-time PCR was used to assess expression of cdtA and cdtR in some C. difficile isolates. C. difficile isolates were grown to early stationary phase (OD$_{600}$=1.0) and RNA was isolated using a Maxwell®16®, a robotic, magnetic bead-based system (Promega). Samples were treated with the Turbo DNA-free DNase system (Ambion) and subjected to a reverse transcription reaction using the Omniscript Reverse Transcription Kit (Qiagen) and gene specific primers (as described by Carter et al. (5) for cdtA, and for cdtR, cdtR-F 5’ ccgaaaaatataagataaagtt 3’ and cdtR-R 5’gggatatttttttcttt 3’) following the manufacturer’s instructions. Expression was normalized using the rpoA gene.

### Pyrosequencing

Pyrosequencing. A pyrosequencing protocol was developed to identify a single nucleotide polymorphism (SNP) in the cdtR gene. Forward, sequencing, and biotinylated reverse primers were designed using the PyroMark Assay Design Software 2.0 (Qiagen). A 100 bp region covering the SNP was PCR amplified in 54 ribotype 078 or toxintype V strains, 22 ribotype 027 and 6 strains belonging to other ribotypes. Pyrosequencing was performed using Pyro Gold reagents on a PyroMark ID instrument following the manufacturer’s instructions (Qiagen).

### Results

- Analysis of the full binary toxin loci of 10 C. difficile isolates yielded information on putative promoter elements of the cdtR and cdtAB genes not previously identified (Figure 1).

- The SNP was only found in ribotype 078 or non-078 cdtA genes not previously sequenced.

- The significance of the SNP was more common in recent isolates suggesting this mutation has recently emerged.

- Pyrosequencing identified the cDR SNP in 39/82 C. difficile isolates from animal, human, and other sources including food and the environment (Table 1).

- The SNP was significantly more common in C. difficile strains isolated in or after 2008 compared to before 2008 (P=0.0001).

- Phylogenetic analysis of the binary toxin loci revealed an overall conserved relationship between isolates of the same ribotype or toxinotype with one exception. ADE667 is a toxinotype III isolate and has a binary toxin locus sequence more closely resembling toxinotype IX isolates.

- The toxinotype IV isolate appears to be more closely related to toxinotype V isolates than any other type.

### Discussion

- Analysis of the promoter sequences of the cdtR and cdtA gene indicates consensus sequences for several promoter elements are present in some C. difficile promoters.

- Despite no expression of cdtA in a 078 strain and the presence of a mutation that would truncate CdtR even if it was expressed, cdtA expression was still detected by qPCR. This suggests CdtR may not be the only positive regulator of cdtA and other regulatory mechanisms are likely involved in regulating expression of the binary toxin.

- The significance of the cdtR SNP only being found in toxinotype V isolates remains unknown. In general, the SNP was more common in recent isolates suggesting this mutation has recently emerged.

- Bovet and Popoff (6) found the same mutation in the cdtR gene and also reported the SNP to be restricted to ribotype 078 or toxintype V strains.

- Phylogenetic analysis of the whole binary toxin loci tended to reflect the relationship indicated by pyrosequencing and toxintyping.

### References