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Time-resolved transcriptome analysis of *Clostridium difficile* R20291 response to cysteine

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ABSTRACT

The incidence of *Clostridium difficile* infection has been steadily rising over the past decade. The increase in the rate of incidence is associated with the specific NAP1/BI/027 strains which are "hypervirulent" and have led to several large outbreaks since their emergence. However, the relation between these outbreaks and virulence regulation mechanisms remains unclear. It has been reported that the major virulence factor TcdA and TcdB in *C. difficile* could be repressed by cysteine. Here, we investigated the functional and virulence-associated regulation of *C. difficile* R20291 response to cysteine by using a time-resolved genome-wide transcriptome analysis. Dramatic changes of gene expression in *C. difficile* revealed functional processes related to transport, metabolism, and regulators in the presence of cysteine during different phases of growth. Flagellar and ribosomal genes were significantly down-regulated in long-term response to cysteine. Many NAP1/BI/027- specific genes were also modulated by cysteine. In addition, *cdsB* inactivation in *C. difficile* R20291 could remove the repression of toxin synthesis but could not remove the repression of butyrate production in the presence of cysteine. This suggests that toxin synthesis and butyrate production might have different regulatory controls in response to cysteine. Altogether, our research provides important insights into the regulatory mechanisms of *C. difficile* response to cysteine.

1. Introduction

Clostridium difficile is a Gram-positive, anaerobic bacterium which is widely recognized as the most frequent cause of antibiotic-associated colitis and health care-acquired diarrhea worldwide (Burke and Lamont, 2014). In the last decade, the incidence rate of *Clostridium difficile* infection (CDI) has been on the increase (Butler et al., 2016; Dávila et al., 2017; Mabardy et al., 2017). Based on statistics from the Centers of Disease Control and Prevention, over 250,000 people need hospital care and at least 14,000 people die from CDI each year in the United States (International, 2013).

Different *C. difficile* strains can be distinguished by PCR ribotyping (Knetsch et al., 2013). Since 2004, the incidence of CDI cases dramatically increased in North America, the UK, and mainland Europe. Most of these cases were associated with a specific PCR-ribotype 027 subtype, also known as North American pulsed field type 1 (NAP1) or restriction endonuclease group BI strain (NAP1/BI/027) (McDonald et al., 2005; Pépin et al., 2005; Warny et al., 2005). Since then, CDI cases have been reported worldwide. In Asia, the awareness and surveillance of CDI remained poor and the prevalence of CDI remained unclear (Collins et al., 2013). But some cases of NAP1/BI/027 infection have already been reported (Cheng et al., 2009, 2016; Wang et al., 2014). More work still need to be done to investigate CDI epidemic state and improve the diagnostic capacity, as well as to explore the association between NAP1/BI/027 subtype and virulence.

The toxins TcdA and TcdB are the main virulence factors of CDI (Rupnik et al., 2009). The genes encoding these toxins are located in a pathogenicity locus (PaLoc), which also contains three genes encoding a negative regulator TcdC, a sigma factor TcdR, and a holin like protein TcdE respectively (Dupuy and Sonenshein, 1998). The NAP1/BI/027 strains carry characteristic mutations, that's Δ117 frameshift mutation and 18 bp deletions in the *tcdC* gene, which have been thought to be associated with increased toxins (Carter et al., 2011; Curry et al., 2007a,b; Matamouros et al., 2007). The regulation of toxin synthesis is very complex and modulated in response to various environmental signals, including nutrients, antibiotics, biotins, temperature and oxidation-reduction potential (Antunes et al., 2012; Gerber et al., 2008; Karlsson et al., 1999, 2003). Previous studies showed that cysteine

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significantly repressed toxin synthesis in both the *C. difficile* VPI 10463 and 630Δ*erm* (Dubois et al., 2016; Karlsson et al., 2000, 2008). The cysteine-dependent differentially expressed genes of strain 630Δ*erm* were analyzed by microarray technologies and the results showed that cysteine modulates the expression of 201 genes involved in multiple pathways, such as amino acid biosynthesis, carbon metabolism, and stress response (Dubois et al., 2016).

The epidemic *C. difficile* NAP1/BI/027 strain is characterized by elevated early toxin production, increased sporulation, efficient germination, and resistance to fluoroquinolones, which was thought to be correlated to the increased severity and transmission (Carlson et al., 2013; Merrigan et al., 2010; Vohra and Poxton, 2011). Compared to the non-epidemic 630Δ*erm* strain, the NAP1/BI/027 strain R20291 has five unique genetic regions including 234 additional genes, which may contribute to the differences in toxicity, motility, and antibiotic resistance (Stabler et al., 2009). The NAP1/BI/027 strains have binary toxin (CDT) encoded by *cdtA* and *cdtB* genes in a particular genomic locus (CdtLoc), which thought to be associated with increased severity, high fatality rate and high recurrence rate of CDI (Bacci et al., 2011; Hensgens and Kuijper, 2013; Stewart et al., 2012). In addition, toxin expression in *C. difficile* was also related to the growth phase of the bacterium (Dupuy and Sonenshein, 1998). In this study, we evaluated the physiological alterations of *C. difficile* R20291 response to cysteine during different growth phases. Gene expression patterns of *C. difficile* cultured in the presence and absence of cysteine were compared by genome-wide transcriptome analysis at different time points. The transcriptomic patterns and regulation obtained from the RNA-sequencing analysis were then verified by additional experimental evidence. These results provide insight into system-wide metabolism and virulence regulation in *C. difficile* R20291 response to cysteine availability.

2. Materials and methods

2.1. Bacterial strains and growth conditions

C. difficile strains were cultured in BHI, TY or TYC (TY with 5 mM cysteine) broth under an anaerobic chamber (10% H_2, 5% CO_2, and 85% N_2), supplemented with appropriate antibiotics. *E. coli* strains were cultured in LB medium at 37 °C supplemented with the appropriate antibiotics (chloramphenicol, 25 μg/mL). Bacterial strains and plasmids used in this study are showed in S1 Table.

2.2. cDNA library preparation and sequencing for RNA-sequencing

For RNA isolation, 5 mL of *C. difficile* grown in TY or TYC broth for 4 h, 8 h and 12 h were harvested by centrifugation at 4000 *g* for 10 min at 4 °C. Total RNA was extracted using the RNAprep Pure Cell/Bacteria Kit (TIANGEN) following the manufacturer's instructions and checked for the RNA integrity (RIN) by an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, US). All 12 samples had RIN values > 9. Qualified total RNA was further purified by RNeasy micro kit (QIAGEN) and RNase-Free DNase Set (QIAGEN).

For sequencing of *C. difficile* mRNA, rRNA was removed from total RNA using the Ribo-Zero rRNA Removal kit (Gram-Postive Bacteria), and then each mRNA sample was fragmented into short sequences with divalent cations and heat. Using these short fragments as templates, first-strand cDNA was synthesized using random hexamer primers, and second-strand cDNA was synthesized using a dNTP mixture containing dUTP with DNA polymerase I and RNase H. After the adenylation of the 3' ends of DNA fragments, illumina PE adaptor oligonucleotides were ligated to the cDNA fragments. To select preferentially cDNA fragments, the library fragments were purified with the AMPure XP system (Beckman Coulter). DNA fragments with ligated adaptor molecules on both ends were selectively enriched using Illumina PCR Primer Cocktail in a 10 cycle PCR. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using the TruSeq PE Cluster Kit v3-HS (Illumina, CA, USA). After clustering, the libraries were sequenced on an Illumina Hiseq 2500 platform and 125-bp paired-end reads were generated.

2.3. Raw sequence analysis and validation of mRNA-Seq by qRT-PCR

Clean data were obtained from raw data by removing reads containing adapter sequences, unknown or low-quality sequences (reads with unknown bases 'N', reads less than 20 bp, or reads in which Q20 bases represented < 50%), then were used for the downstream analyses. Qualified sequences were mapped to the *C. difficile* R20291 genome using FANSe2. Differential expression analysis for RNA-sequencing data was performed using the edgeR package. To test for differential expression, we used Bayesian adjusted t statistics and performed the multiple testing correction of Benjamini and Hochberg based on the false discovery rate (FDR). A gene was considered to be differentially expressed when the p value was ≤ 0.01 and fold change (FC) was ≥ 2 or ≤ -2. Differentially expressed genes were selected to conduct functional enrichment analysis according to their GO terms and KEGG pathways.

Relative expression levels of target transcripts were determined using SYBR *Premix Ex Taq* (TaKaRa) following the manufacturer's protocol. Primers used for real-time PCR (RT-qPCR) in this study were presented in S2 Table. The data were analyzed by using the ΔC_T method as previously described (Schmittgen and Livak, 2008). Expression levels of the genes were normalized using the amplification efficiencies and the expression level of the reference gene 16SrRNA. At least three biological replicates were assayed. Statistical analysis was performed using two-way analysis of variance tests and a p value ≤ 0.05 was considered significant.

2.4. Construction of the cdsB mutant in C. difficile R20291

The *cdsB* gene was inactivated in *C. difficile* R20291 by using the ClosTron system as described previously (Heap et al., 2010). The intron DNA fragments of the *cdsB* gene were amplified by overlap PCR using specific retargeted primers in S2 Table and then cloned into the*Hin*dIII and *Bsr*GI restriction sites of pMTL007 using *E. coli* TOP10 as the host. The recombinant pMTL007-$cdsB_{291}$ was transferred into *C. difficile* R20291 by conjugation. The transconjugants were further verified by PCR using the primers shown in S2 Table and southern blotting using a DIG-High Prime Labelling and Detection Kit (Roche). For complementation studies, the *cdsB*gene together with its native constitutive promoter and ribosome binding site was cloned into pMTL84151, generating pMTL-$cdsB_{291}$. The plasmid pMTL-$cdsB_{291}$ was transferred into *C. difficile* R20291 *cdsB*::*ermB* strain to give the complemented strain. To exclude the effects of the heterologous plasmid on the strains, the pMTL84151 vector was transferred into *C. difficile* R20291 strain and *C. difficile* R20291 *cdsB*::*ermB* strain respectively to give the parental strain and the *cdsB* mutant which were used in this study.

2.5. Cell cytotoxicity assays and dot blotting analysis

The supernatants of *C. difficile* cultures from different time points were harvested and filter sterilized. Vero cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (Gibco) at 37 °C in a humidified atmosphere containing 5% CO_2. For cytotoxicity assays, Vero cells were seeded into 96-well plates at densities of approximately 5×10^4 cells/mL and incubated for 20–24 h. The filter sterilized supernatants were diluted in four-fold series and added into monolayers of Vero cells. The cytotoxicity was recorded after 24 h. Negative controls were treated with fresh medium. The end-point titer was defined as the first dilution in the series in which the morphology of the Vero cells was the same as the negative controls. Each experiment was performed in duplicate three times.