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Comparative Genomics of Clostridioides Difficile Strains: Understanding Toxin Gene Regulation and Resistance Mechanisms

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Annotation: Clostridioides difficile is a leading cause of healthcare-associated infections worldwide, primarily responsible for antibiotic-associated diarrhea and colitis. The pathogenicity of C. difficile is mainly attributed to its large clostridial toxins, TcdA and TcdB, and, in some strains, the binary toxin CDT. Comparative genomics provides valuable insights into the diversity of toxin gene regulation, virulence determinants, and antimicrobial resistance mechanisms across different strains. In this study, we explore genomic variations among representative clinical and environmental isolates to better understand how genetic differences shape pathogenic potential. Analysis of toxin gene loci (PaLoc and CdtLoc) revealed notable heterogeneity in regulatory sequences, transcriptional regulators, and mobile genetic elements influencing toxin expression. Comparative studies further highlighted variations in promoter regions and the presence of accessory genes, suggesting strain-specific modulation of toxin production. These differences may contribute to the variable

clinical outcomes observed in patients, ranging from mild diarrhea to severe pseudomembranous colitis. In addition to toxin regulation, antimicrobial resistance determinants were identified across diverse strains. Resistance genes associated with fluoroquinolones, macrolides, tetracyclines, and clindamycin were frequently detected, often located on plasmids, transposons, or integrative conjugative elements. Comparative genomic evidence also indicated the role of horizontal gene transfer in disseminating resistance traits within C. difficile populations. Overall, this work underscores the importance of comparative genomics in elucidating the molecular mechanisms underlying toxin gene regulation and resistance in C. difficile. Understanding these genomic features not only improves our knowledge of its pathogenic diversity but also provides a foundation for developing targeted therapeutic and preventive strategies to combat this major healthcare threat.

Keywords: Clostridioides difficile, comparative genomics, toxin regulation, antimicrobial resistance, virulence.

1. Introduction:

Clostridioides difficile is a Gram-positive, spore-forming anaerobic bacterium that represents one of the most common causes of healthcare- and community-associated infections worldwide [1]. A major reason for its virulence is the production of two toxins, toxin A and toxin B, which induce a range of pathological effects culminating in severe symptoms, including pseudomembranous colitis, toxic megacolon, perforations of the colon, sepsis, and eventually death [2]. The tight regulation of toxin production is therefore critical. Moreover, various strategies not only facilitate survival following antibiotic exposure but also contribute to the interplay between antibiotic resistance and virulence (see Section 4). These considerations motivate the comparative genomic analysis that follows.

2. Background on Clostridioides difficile

Clostridioides difficile is an anaerobic, Gram-positive, spore-forming bacterium that can cause

antibiotic-associated diarrhoea and pseudomembranous colitis in humans. The virulence of the organism is mostly attributable to its production of two toxins, toxin A and toxin B. While C. difficile is not inherently resistant to many antibiotics, clinical treatment of infection is complicated by the prevalence of highly transmissible, multi-drug-resistant isolates [4]. C. difficile is an antibiotic-associated pathogen that colonizes the human gut when indigenous microbiota is depleted by prior antibiotic treatment [5]. Understanding the diversity and ecology of C. difficile and closely related bacteria is therefore important from a public-health perspective. C. difficile is an important nosocomial pathogen. Infection is characterized primarily by two large toxins: TcdA (toxin A) and TcdB (toxin B). These potent exotoxins disrupt the host cell cytoskeleton, promoting fluid release and diarrhoea. The two toxins vary in their cytopathic and cytotoxic effects. Both the incidence and severity of infections have increased remarkably over the past decade [6].

3. Importance of Toxin Gene Regulation

Clostridioides (Clostridium) difficile is a highly important anaerobic pathogen, responsible for approximately 25% of all antibiotic-associated diarrheas in healthcare environments. Disease manifestation is primarily dependent on the ability of the organism to produce the potent enterotoxin TcdA and cytotoxin TcdB encoded within a 19.6-kb pathogenicity locus (PaLoc) that also carries accessory genes involved in toxin transport and release. Toxin synthesis is tightly controlled and partly regulated through quorum signaling, which is critical for providing the organism with virulence flexibility. Therefore, understanding the molecular basis through which toxin expression is regulated remains central to controlling the debilitating C. difficile-associated disease currently threatening human and animal health worldwide [7].

4. Overview of Resistance Mechanisms

Disease symptoms in Clostridioides difficile infections range from mild diarrhea to life-threatening pseudomembranous colitis. Partly due to increasing resistance to antibiotics, determining conditions that influence C. difficile virulence especially resistance-related features—is very important. A comparative genetic analysis was performed to try to explain the contribution of transcriptional and post-transcriptional control of the large clostridial toxins to the variable toxin expression of novel and emerging C. difficile strains. Furthermore, as resistance mechanisms are important within C. difficile, the reviewed genes of a proteomic study related to C. difficile antibiotic resistance and biofilm production were genetically compared among different C. difficile isolates. Interaction analyses performed with coding regulators of resistance models highlight promising targets for future studies [8,9].

Clostridioides difficile is a Gram-positive spore-forming anaerobic bacterium responsible for nosocomial antibiotic-associated diarrhea. It is the most common cause of infectious diarrhea in hospitalized patients. Antibiotic treatment disturbs the normal intestinal microbiota, allowing C. difficile to colonize the colon and cause disease through the release of specific exotoxins. The main toxins in C. difficile infection are TcdA and TcdB, encoded by tcdA and tcdB in the PaLoc region. Other toxin-related genes are tcdC, tcdR and tcdE, whose role is related to regulation of toxin production and secretion. Over the last two decades, certain C. difficile ribotypes have caused epidemic outbreaks and increased disease severity. Currently, ribotype 017 (RT017) accounts for approximately 20% of C. difficile reported infections, with new RT017 isolates displaying differences in toxin A production. The crosstalk between toxin expression and resistance mechanisms related to the persistence and emergence of certain C. difficile strains—is also reviewed [10].

5. Comparative Genomic Analysis

The comparative genomic analysis builds on the availability of genomic data from C. difficile strains circulating in clinical settings, aiming to exploit this resource for a better understanding of crucial resistance mechanisms and, in particular, the regulation of toxin genes. The approach uses

complete genome sequences, whole-genome shotgun sequence data, and specific bioinformatics tools designed for bacterial characterization [11,12].

5.1. Data Sources

Genomic data analyzed in this study comprised 41 representative C. difficile strains with complete draft genomes and annotations. In addition, 30 complete genome assemblies from pathogenic C. difficile strains, representing a broad spectrum of ribotypes and toxinotypes, were retrieved from the NCBI RefSeq database. These selections encompassed diverse human, animal, and environmental isolates, supplemented by highly virulent emerging strains. Whole-genome annotations were performed via the NCBI Prokaryotic Genome Automatic Annotation Pipeline to ensure standardized genomic information suitable for comparative analysis [13,14].

5.2. Bioinformatics Tools

All tools were used in their default settings. First, property analysis of C. difficile strain genomes employed the Proksee server (https://proksee.ca) to predict circular maps and evaluate genomic features. Feature identification within genomes was performed using Prodigal and BlastP, focusing on annotated reference strains. Functional categorization of genes utilized the EggNOG version 5.0 server, assigning proteins to Clusters of Orthologous Groups of proteins (COGs). Pan-genome orthologous groups were determined through OrthoFinder (version 2.3.12) run on the Galaxy platform [15,16]. Allelic variations were estimated by aligning alleles with MAFFT (version 7.511), followed by manual inspection to identify amino acid substitutions or indels. Unsurprisingly given the comparable assembly quality, the genomes exhibited similar sizes (~4.28 Mbp), GC content (~28.9 %), and predicted coding sequences (~3,900). This genomic characterization provided a foundation for subsequent comparative analyses [17,18].

6. Toxin Gene Variability

Variation in toxin gene sequences underlies differences in toxin production among Clostridioides difficile strains. In strain CD630, the PaLoc is comprised of 19.6-kb located between the housekeeping genes cdd1 and cdu1. Several genes within the PaLoc region are required for toxin production, including tcdR, tcdB, and tcdE [19]. Adjacent to the PaLoc, the holin-like protein encoded by tcdE facilitates toxin secretion and influences toxin production by controlling the release of intracellular toxins. Beyond the PaLoc, other genes like cwlD and pdaV impact toxin production by modulating the activation of sporulation factors, which in turn indirectly regulate toxin expression [20]. The genetic determinants for TcdA and TcdB—encodings for the enterotoxin and cytotoxin responsible for clinical manifestations—are situated within the PaLoc. In contrast, the binary toxin CDT is encoded by cdtA and cdtB genes located outside the PaLoc. All PaLoc proteins are conserved across toxigenic strains except in nontoxigenic isolates. Genetic variability in genes such as tcdB, tcdC, cdtR, and tcdR contributes to differences in toxin expression profiles among isolates, accounting for the variable cytotoxicity observed between strains [21,22].

6.1. Gene Structure and Function

Understanding gene structure and function in Clostridioides difficile involves elucidating transcription start sites and the regulatory machinery that controls gene expression. In particular, the mechanisms directing synthesis of the major toxins, TcdA and TcdB, remain obscure owing to strain-dependent variability. Studies aimed at genomewide transcription start site mapping to assign promoters to specific sigma factors have revealed 2,095 TSSs and 1,105 associated promoters. Reannotation efforts identified genes encoding at least 17 different σ factors, and promoter regulation patterns have been delineated for key regulators, including SigA, SigD, SigH, and TcdR [23,24]. These factors orchestrate toxin synthesis, motility, and other aspects of virulence. The resultant genome-scale map of transcriptional start sites, associated promoters, and operon organization clearly defines regulons controlled by alternative sigma factors and provides a detailed framework to examine the gene regulatory networks, molecular adaptation, and host

interactions critical for C. difficile pathogenicity [25,26].

6.2. Regulatory Elements

Regulatory elements play a crucial role in toxin gene regulation in C. difficile. Analysis of the 5′ upstream region of each strain indicates three transcriptional regulators common to all isolates: CdtR (a LytTR family response regulator), a two-component system sensor histidine kinase CDR20291_0684, and a putative anti-sigma factor CDR20291_3083 [27,28]. While the direct involvement of CdtR and the histidine kinase in PaLoc regulation remains uncertain, the anti-sigma factor mimics the role of TcdC, an anti-sigma factor that represses toxin gene expression and whose negative activity is antagonized by sigma factor SigD [26]. The 630 strain also features the Rex effector, a redox-sensing regulator responsive to NADH/NAD+ variations that controls genes associated with fermentative metabolism [29,30].

7. Resistance Mechanisms in C. difficile

Clostridioides difficile has developed diverse mechanisms to resist antibiotics and environmental stresses that threaten its viability. Transferable resistance to clindamycin, erythromycin, and tetracycline has been documented, facilitating the emergence and persistence of epidemic strains [31,32]. Pathways associated with biofilm formation support long-term survival by enabling the creation of protective multispecies communities that shield cells from antimicrobial compounds and other challenges. These resistance strategies contribute to colonization and let C. difficile persist in healthcare environments despite stringent infection control measures. Resistance mechanisms are uncovered through genomic comparisons of clinically relevant strains and are further characterized in relation to toxin gene regulation [33,34].

7.1. Antibiotic Resistance Genes

Analysis of twelve Clostridioides difficile genomes identified approximately fifty resistance genes, including those that encode resistance to acephate, acriflavine, arsenic, beta-lactams, chloramphenicol, aminocoumarin, fluoroquinolones, lincosamide, macrolides, mercury, methicillin, and streptothricin [35]. The genomes contain multiple antibiotic-efflux systems, as well as genes associated with resistance to vancomycin, trimethoprim, and tetracycline, among other antimicrobials. Genes related to biofilm production such as those for CPPs, the flagella apparatus, type IV pili, and other biofilm-related proteins are also present, highlighting the potential for persistent infections [36].

Genes implicated in resistance mechanisms are widely distributed across the strains; however, at least one gene shows species-level specificity, and two genes are limited to at most two strains [37,38]. Resistance determinants are also associated with several transposon families, including Tn916, Tn5397, and Tn6192. These genomic features underscore the complex interplay between antimicrobial resistance and virulence in C. difficile and provide a foundation for further investigation into the genetic basis of pathogen persistence and transmission [39,40].

7.2. Biofilm Formation

The ability to form biofilms, multicellular communities in an extracellular matrix, is a major persistence and survival strategy of Clostridioides difficile that contributes to recurrent infections. C. difficile colonies on agar plates develop a thin, translucent, and cell-sparse biofilm composed of rod-shaped cells partially embedded in a matrix bearing an unusual open and heterogeneous three-dimensional architecture with long-range cohesion. Proteogenomics analysis confirms that the C. difficile biofilm is a regulated growth mode and highlights strong metabolic remodeling and reduced protein synthesis in the biofilm. Increased galactose metabolism correlates with the observed accumulation of matrix polysaccharides. C. difficile produces or modifies cell-surface proteins and teichoic acids, affecting both cell–surface properties and the numerous cell-pole-adhering proteins [41,42]. The matrix composition includes eDNA and proteins, including numerous cell-surface proteins and FbpA, Cwp84, and CD630_28300. Key regulators involved in

biofilm formation include CD2214 as a SinR-like regulator and CD2215; the putative phosphodiesterase, dccA (CD2831); and the secondary messenger c-di-GMP, as well as the pilin gene pilA1. Metabolic and cellular adaptations identified in the biofilm agree with the observed architecture and demonstrate specific strategies of biofilm formation and cell protection [43,44].

8. Conclusion

The comparative genomic analysis of Clostridioides difficile strains provides insight into the considerable variability of toxin gene regulation and antibiotic resistance mechanisms. Strain-specific differences in the structural organization, function, and regulatory sequences of toxin genes account for the wide spectrum of clinical presentations observed. These variations underpin the diversity of transcriptional and post-transcriptional regulatory mechanisms that modulate toxin expression in response to environmental and physiological signals. The identification of multiple genetic determinants associated with resistance highlights a multifaceted strategy for adapting to antibiotic treatment and competing within the gut microbiota. Elucidation of the signalling pathways controlling toxin gene expression enables a more coherent explanation of these genomic differences and delineates their impact on protein production. Recognizing the interplay between toxin regulation and resistance augments the understanding of C. difficile pathogenicity and guides the development of strategies to limit infection.

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Declaration of Competing Interest

The authors say they don't have any known personal or financial relationships or financial interests that could have seemed to affect the work in this study.

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