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Diversity and Evolution in the Genome of *Clostridium difficile*

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SUMMARY

Clostridium difficile infection (CDI) is the leading cause of antimicrobial and health care-associated diarrhea in humans, presenting a significant burden to global health care systems. In the last 2 decades, PCR- and sequence-based techniques, particularly whole-genome sequencing (WGS), have significantly furthered our knowledge of the genetic diversity, evolution, epidemiology, and pathogenicity of this once enigmatic pathogen. *C. difficile* is taxonomically distinct from many other well-known clostridia, with a diverse population structure comprising hundreds of strain types spread across at least 6 phylogenetic clades. The *C. difficile* species is defined by a large diverse pangenome with extreme levels of evolutionary plasticity that has been shaped over long time periods by gene flux and recombination, often between divergent lineages. These evolutionary events are in response to environmental and anthropogenic activities and have led to the rapid emergence and worldwide dissemination of virulent clonal lineages. Moreover, genome analysis of large clinically relevant data sets has improved our understanding of CDI outbreaks, transmission, and recurrence. The epidemiology of CDI has changed dramatically over the last 15 years, and CDI may have a foodborne or zoonotic etiology. The WGS era promises to continue to redefine our view of this significant pathogen.

INTRODUCTION

Clostridium difficile Infection

Clostridium difficile is a spore-forming, Gram-positive, anaerobic bacillus found ubiquitously in the environment and the gastrointestinal tracts of humans and animals. *C. difficile* is a formidable pathogen and currently the leading cause of antimicrobial and health care-associated infectious diarrhea in humans (1). The incidence and severity of *C. difficile* infection (CDI) present a significant burden to global health care systems due to increasing costs associated with treatment, infection control, disease recurrence, patient length of hospital stay, and mortality, especially among the elderly (2). A recent report from the Centers for Disease Control and Prevention (CDC) ranks *C. difficile* as the most important antimicrobial-resistant threat to public health in the United States, with 250,000 infections and 14,000 deaths per year and annual excess medical costs (attributable to the cost of extra bed days and associated treatment) totaling $1 billion (3). Another North American study reports that in 2011 alone, the clinical burden of CDI accounted for almost 500,000 infections and 29,000 associated deaths (4).

Originally named *Bacillus difficilis* due to difficulties in cultivation *in vitro*, the bacterium was first described in 1935 as a component of the healthy neonatal intestinal microflora (5). Later, investigators verified its toxigenic potential through toxin studies in guinea pigs (6), and in the 1970s, work by John Bartlett et al.

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identified *C. difficile* as the cause of antibiotic-associated pseudomembranous colitis (PMC) (7). CDI is a toxin-mediated disease of the colon, with three or more watery, nonbloody stools per 24-h period being the hallmark of symptomatic illness (8). Clinical characteristics of CDI include abdominal pain, cramps, and fever (9), and extraintestinal manifestations are rare (10). CDI is also associated with leukocytosis, hypoalbuminemia, and high serum creatinine levels (8). Disease severity can vary from mild or self-limiting to severe and, in some instances, fatal sequelae, including PMC, toxic megacolon, bowel perforation, and sepsis (7–9). Asymptomatic carriage of *C. difficile* is also common in health care settings and may play a role in CDI transmission (11).

There are many risk factors for the development of CDI, including comorbidities, surgical and nonsurgical gastrointestinal procedures, duration of hospital stay, admission to an intensive care unit (ICU), immunocompromised status (particularly oncology and hematology patients), and advanced age (>65 years of age) (12, 13). Antimicrobial exposure is the single most important risk factor for the acquisition of CDI due to the disruption and dysbiosis of endogenous colonic microbiota (colonization resistance), allowing *C. difficile* to colonize and proliferate (12). Almost all antimicrobials have been implicated, especially those with high gut concentrations and activity against bowel flora to which *C. difficile* is resistant, including clindamycin, penicillin, ampicillin, amoxicillin, cephalosporins, and, for some strains, fluoroquinolones (14, 15).

C. difficile forms spores that are resistant to desiccation, extremes of temperature, and many chemicals and disinfectants (16, 17). Spores are highly transmissible and responsible for contamination of health care environments, often persisting for long periods of time and contributing to the burden of disease (2, 17, 18).

Current treatment options for CDI include antimicrobial therapy (vancomycin, metronidazole, or fidaxomicin) and restoration of colonic microbiota through fecal microbiota transplantation (FMT) (19–21). Phage therapy and treatment with monoclonal antibodies are also active areas of interest (22, 23). In up to 20% of fulminant colitis cases, surgical intervention (subtotal colectomy, resection, and/or ileostomy) is required (24).

CDI is mediated by the production of two large clostridial toxins (LCTs), TcdA and TcdB, which, following expression, inactivate host cell GTP-binding proteins, resulting in actin disassembly, enterocyte apoptosis, and severe inflammation (25–27). In some strains, a third unrelated binary toxin (cytolethal distending toxin [CDT]) is produced. The exact role of CDT in pathogenesis remains unclear; however, it is thought to be involved in epithelial adhesion (25, 27, 28). Additionally, variations in flagella, sporulation factors, and adhesins are thought to play a role in virulence (27, 29, 30).

An optimal diagnostic strategy for laboratory detection of CDI remains controversial (31). Current guidelines recommend PCR-based methods to detect the toxin-encoding genes *tcdA* and *tcdB*, either alone or in conjunction with a toxin detection enzyme immunoassay (EIA) (20, 24, 32). Culture of the bacterium from feces does not differentiate toxigenic from nontoxigenic bacteria or asymptomatic carriers from those with CDI; however, it provides an isolate for epidemiological typing.

Methods for Determining Strain Relatedness

Several typing methods have been used to investigate the epidemiology, genetic diversity, and evolution of *C. difficile*. Some methods are based on macroanalysis of genome architecture (restriction endonuclease analysis [REA] and pulsed-field gel electrophoresis [PFGE]), while some focus on analysis of single regions within the genome (PCR ribotyping and toxinotyping). Sequence-based methods provide differentiation of strains on a single-nucleotide level and can target multiple loci (multilocus sequence typing [MLST] and multilocus variable-number tandem-repeat analysis [MLVA]) or even the entire length of the bacterial genome (whole-genome sequencing [WGS]) (33, 34). Strain nomenclature is often based on one or more of these schemes, as shown by the designation for epidemic strain *C. difficile* 027/BI/NAP1, where 027 refers to the PCR ribotype (RT), BI refers to the restriction endonuclease group, and NAP1 refers to the North American pulsotype. The applications, limitations, and future perspectives of these techniques have been extensively described elsewhere (33–38), including an excellent 2013 review by Knetsch and colleagues (33).

For *C. difficile*, PFGE and PCR ribotyping have been most widely adopted methods in North America and the rest of the world, respectively. In PFGE, chromosomal DNA is digested by restriction endonucleases such as SmaI, and DNA fragments are separated in an agarose medium under a pulsed electric current, producing a strain-specific fingerprint or pulsotype (33). PFGE provides a highly discriminatory method for surveillance of CDI outbreaks and tracking of patient transmission events (33, 34). PCR ribotyping exploits the variations in the intergenic spacer region (ISR) located between the 16S and 23S rRNA genes, resulting in a RT-specific set of amplicons after PCR amplification (39). It is worth noting, however, that the use of the word "intergenic" in this context may be misleading, as for some isolates, numerous tRNA genes have been identified in this region (40). The collation of existing and the assignment of new RTs were the responsibilities of the Public Health Laboratory Service Anaerobe Reference Unit in Cardiff, United Kingdom; however, these responsibilities have now moved to the Health Protection Agency-funded *C. difficile* Ribotype Network (CDRN) based in Leeds, United Kingdom. Currently, there are >600 RTs in the CDRN database (W. Fawley, personal communication). RT nomenclature is under constant review, and recently, there has been a concerted effort to reconcile conventional (agarose-based) and newer (capillary-based) library data.

Toxinotyping is a restriction fragment length polymorphism (RFLP)-based PCR method for differentiating *C. difficile* strains on the basis of variability in restriction sites in the 19.6-kb pathogenicity locus (PaLoc) (with respect to a reference strain, VPI 10463, toxinotype 0). Currently, 31 variant toxinotypes have been described (types I to XXXI) (41, 42).

MLST is a robust and accurate typing method for identifying clonal relationships among strains of bacteria. For *C. difficile*, MLST has discriminatory power comparable to that of PCR ribotyping and provides unambiguous data that are easily shared between laboratories (33). Based on an earlier scheme described by Lemee et al. (43), the scheme developed by Griffiths et al. (44), in which sequence types (STs) are assigned based on allelic variants of seven highly conserved housekeeping genes (*adk*, *atpA*, *dxr*, *glyA*, *recA*, *sodA*, and *tpi*), has been widely adopted for studying CDI epidemiology (44–47). Furthermore, a large, well-curated database (PubMLST [http://pubmlst.org/]) provides simple and rapid ST assignment and allows submission of novel alleles.

MLVA and *in silico* typing based on WGS (single nucleotide

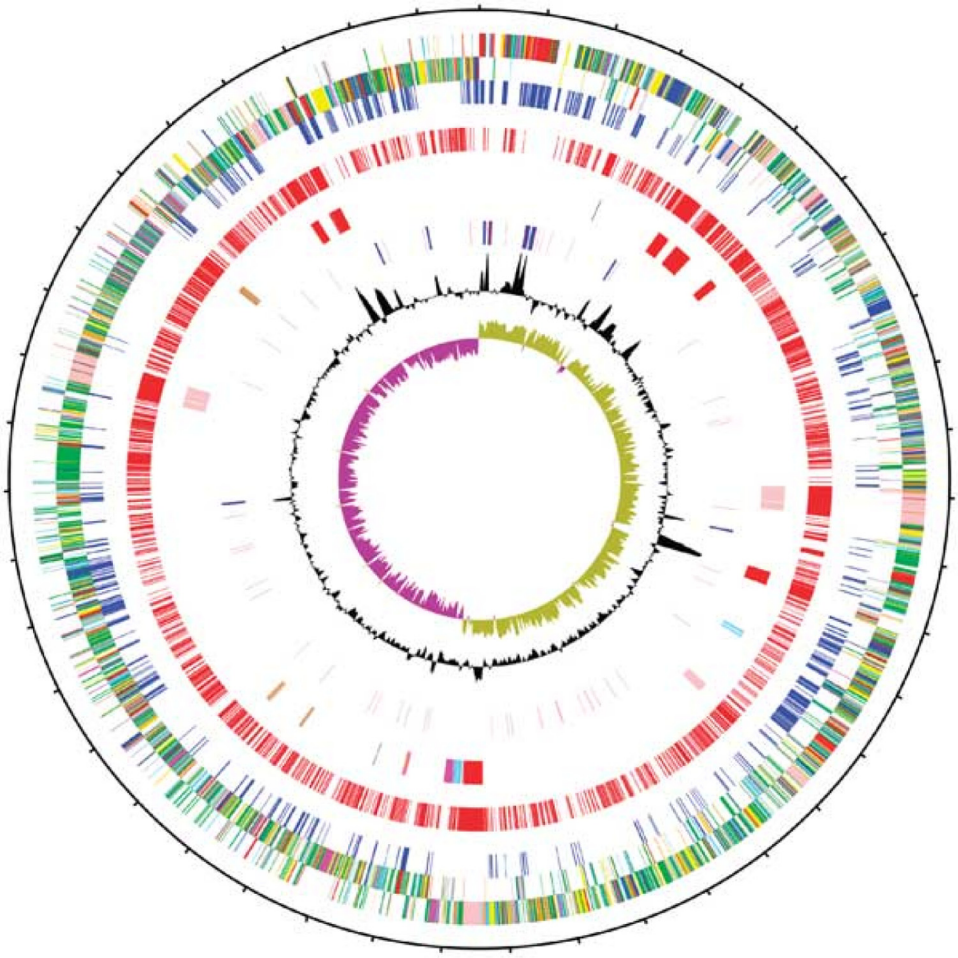

FIG 1 Circular illustration of the 4.3-Mb chromosome of *C. difficile* strain 630. The concentric circles are as follows (from the outside in): circles 1 and 2, 3,776 putative CDSs (transcribed clockwise and counterclockwise); circle 3, CDSs shared with other sequenced *Clostridia* (blue); circle 4, CDSs unique to *C. difficile* (red); circle 5, mobile elements (red/pale red, transposons; pink, prophages; brown, partial prophages/transposons; blue, skin element; magenta, genomic island); circle 6, RNA genes (blue, rRNAs; red, tRNAs; purple, stable RNAs); circles 7 and 8, G+C content/GC deviation (plotted using a 10-kb window). (Reproduced from reference 52 by permission from Macmillan Publishers Ltd.)

polymorphism [SNP] typing, MLVA, and MLST) currently offer the highest level of bacterial strain discrimination and are powerful tools for studying transmission events in *C. difficile* (35) and other important global pathogens, including *Staphylococcus aureus* (48), *Mycobacterium tuberculosis* (49), and *Escherichia coli* (50). In the last decade, with the advent of high-throughput or "next-generation" sequencing (NGS) methods such as the Roche 454 and Illumina methods, the number of sequenced *C. difficile* genomes has risen sharply. To date, several fully "closed" high-quality genomes have been reported (see the section on the *C. difficile* genome, below); however, of the thousands of individual genomes currently archived in online depositories such as GenBank and the Sequence Read Archive (SRA), the vast majority remain incomplete or "draft" genomes. While adequate for most studies, these draft genomes present some challenges in determining "complete" genomic content, and whole-genome phylogenetic inferences should be made with this limitation in mind (51).

For *C. difficile*, the NGS era has contributed to significant advances in a number of key areas, many of which are discussed below. WGS has helped to define the architecture, diversity, conservation, and plasticity of the *C. difficile* genome; describe the mechanisms and forces influencing the evolution of the *C. difficile* core and pangenome; and provide a robust global phylogeny, particularly of virulent and epidemic lineages. Moreover, WGS has built upon knowledge from MLST studies; improved our understanding of CDI outbreaks, transmission, and recurrence; and further highlighted the potential for zoonotic transmission of *C. difficile*.

C. DIFFICILE PHYLOGENOMICS AND STRAIN DIVERSITY

The *C. difficile* Genome

In 2006, Sebaihia and colleagues described the first fully sequenced and annotated closed genome of *C. difficile* (strain 630; RT012) (52). This virulent, highly transmissible, and multidrug-resistant strain of *C. difficile* was originally isolated in 1982 from a patient with PMC in Zurich, Switzerland. Sequencing and annotation of strain 630 revealed a large circular chromosome of 4,290,252 bp (4.3 Mb), 3,776 putative protein-coding sequences (CDSs), and a GC content of 29.06% (Fig. 1) (52). A circular

plasmid (pCD630) of 7,881 bp containing 11 CDSs was also identified (52). Five years later, Monot and colleagues (53) reannotated the genome of strain 630 by using a combined transcriptomic and proteomic approach to update the putative functions of >500 previously putative or unknown genes. Since this time, several other genomes ranging in size from 4.1 to 4.3 Mbp have been fully sequenced and annotated: CD37 (RT009; isolated in the United States in 1980), M68 (RT017; isolated in Ireland in 2006), CF5 (RT017; isolated in Belgium in 1995), M120 (RT078; isolated in the United Kingdom in 2007), G46 (RT027; isolated in the United Kingdom in 2006) (54), R20291 (RT027; isolated in the United Kingdom in 2006), 196 (RT027; isolated in France in 1985), 2007855 (RT027; isolated in the United States in 2007), and BI1 (RT027; isolated in the United States in 1988) (55–57). Reference genomes such as those of strains 630 and M120 play an important role in the NGS data analysis pipeline. They comprise an unambiguous and contiguous sequence of known nucleotides spanning the entire chromosome and plasmids (if present), therefore providing an extremely high-quality reference for mapping of draft genomes.

As was the case with strain 630, WGS of these *C. difficile* strains revealed much about the architecture of the *C. difficile* genome. *C. difficile* has a highly dynamic and mosaic genome comprising a high proportion (~11% in strain 630) of mobile genetic elements. These include bacteriophages, group I introns, insertion sequences (IS), *sigK* intervening (skin) elements, clustered regularly interspersed short palindromic repeat (CRISPR)-*cas* elements, genomic islands, and transposable and conjugative elements, accompanied by an extensive range of accessory genes (52, 53, 55, 58, 59).

Many of the CDSs identified in the genome of *C. difficile* are associated with adaptation and proliferation in the gastrointestinal tract (germination, adhesion, and growth) and survival in challenging suboptimal environments (endospore formation) (52, 53). These findings support the view that *C. difficile* lives within a highly dynamic niche and is able to spend a long time coexisting with its host (52). This is in contrast to the genome of *Clostridium botulinum*, where many of the genes unique to this species encode proteins associated with rapid killing (cytolysins and neurotoxin) and saprophytic feeding (extracellular proteases and chitinases). Moreover, the genome of *C. botulinum* is much more stable than that of *C. difficile*, reflecting the short-lived host association compared to that of *C. difficile* (60).

Large genomes are typically indicative of a bacterium that is able to adapt to and thrive in multiple, often adverse, environments, as seen with *Pseudomonas aeruginosa* (genome size, 6.3 Mb) (61). This is also true for *C. difficile*, with a genome up to 42% larger than those of other closely related clostridial species such as *C. bifermentans* and *C. sticklandii* and larger than those of most other *Firmicutes* (62, 63). This large, complex genome reflects the ability of the bacterium to survive, often for long periods of time, within a diverse range of human, animal, and abiotic environments.

The PaLoc

Encompassing a 19.6-kb region of the chromosome, the PaLoc has received significant attention, as it contains the genes encoding the major virulence factors toxin A and toxin B and thus plays an essential role in the pathogenesis of CDI. The PaLoc is present in all toxigenic strains but absent in nontoxigenic strains, where it is replaced by a 115-bp noncoding and highly conserved region known as the integration site (26, 64, 65). In addition to *tcdA* and *tcdB*, which encode toxins A and B, respectively, the PaLoc contains three other genes, *tcdR*, *tcdE*, and *tcdC*, as well as CD630_06620, a putative *N*-acetylmuramoyl-L-alanine amidase identified during the reannotation of the *C. difficile* 630 genome (53). While *tcdR* encodes an RNA polymerase sigma factor, a positive regulator of toxin expression (66), *tcdE* encodes a protein structurally and functionally similar to holin proteins found in bacteriophages (67), and *tcdC* is considered to be a negative regulator of toxin expression (68–70). For several years, strains with an aberrant *tcdC* gene (deletions and premature stop codons) have been linked to hypervirulence, although this has been disputed (71, 72). In addition to *tcdC* mutations, variations in *tcdB* (specifically the receptor-binding domain [RBD]) have also been associated with a hypervirulence phenotype (45, 73). These hypervirulence-promoting locus variants are common in certain RTs and have a strikingly congruent association with specific clades (45).

Interestingly, there has been very recent direct evidence that toxin synthesis in *C. difficile* is modulated by an accessory gene regulator (*agr*) quorum-signaling system distinct from the PaLoc (74). In *C. difficile*, the *agr* locus comprises genes encoding a quorum signal generation pathway (*agrB* and *agrD*) and genes encoding a quorum response pathway (*agrA* and *agrC*) (74). *agr*-mediated quorum signaling presents an advantageous mechanism for *C. difficile* toxin production, as there is a fitness advantage through coordinating information about cell density and synchronizing gene expression on a population level rather than on a single-cell level (74). Notably, significant differences in *agr* locus content between hypervirulent strains (RT027 and RT017 [*agrBDAC*]) and nonhypervirulent strains (RT012 [*agrBD*]) have been described, which may suggest a role for quorum sensing in the evolution of virulent lineages (74, 75).

Ultralow Level of Genome Conservation

C. difficile can be defined by its pangenome, the genetic repertoire of the species or "gene pool." The pangenome is comprised of a core genome (those genes present in all isolates) and an accessory or adaptive genome (genes absent from one or more strains or unique to a particular strain) (76). Scaria et al. (77), using microarray and WGS data derived from a small but diverse collection of clinical and animal strains, estimated that the *C. difficile* pangenome is comprised of 9,640 CDSs. This figure is comparable to that for *Salmonella enterica* ($n = 9{,}966$) and higher than those for *Staphylococcus aureus* ($n = 4{,}221$) and *Streptococcus pneumoniae* ($n = 3{,}934$) (78). However, this figure is likely an underestimate based on the narrow geographic area (United States and United Kingdom) from which most of the strains originated. Furthermore, as discussed below, *C. difficile* possesses an "open" genome with extreme levels of plasticity, with access to and frequent exchange with multiple host environments and bacterial gene pools. Consequently, as more *C. difficile* strains from divergent lineages and diverse animal and environmental sources are sequenced, this estimate will likely increase.

Estimates of the size of the core genome of *C. difficile* are many orders of magnitude lower (~600 to 3,000 CDSs) (57, 77, 79). Stabler et al. (73) and Janvilisri et al. (80) showed that much of the core CDSs in *C. difficile* encode proteins involved in essential cellular processes such as metabolism, biosynthesis, DNA replication, transport, and cell division as well as processes associated with pathogenicity (colonization, adhesion, motility, and antibi-

otic resistance), indicating their essential role. In addition, many of the core genes show divergent sequences that may indicate host adaption and specificity (80). As with the pangenome, the size of the core genome is influenced by the strains analyzed. Thus, it is probable that as strains belonging to divergent phylogenetic lineages are sequenced, the size of the core genome will likely decrease further.

The contrasting estimates of the sizes of the pan- and core genomes of *C. difficile* highlight the ultralow levels of genome conservation in this species. To date, three studies have used comparative genomic hybridization (CGH) to measure the size of the genome in *C. difficile*, with estimates that the amount of shared core genome of *C. difficile* might be as low as 16%, lower than that of any bacterial species described to date (52, 73, 80). An ultralow level of conservation is rare in bacteria, even in species considered to have high levels of genetic variability, e.g., *Campylobacter jejuni* (59.2%), *Helicobacter pylori* (58.5%), *Streptococcus pneumoniae* (46.5%), and *E. coli* (~40.0%) (81–83), and is more typical for phylogenetic distances between genera within a family rather than strains within a species. Such large phylogenetic distances between *C. difficile* lineages threaten the very definition of *C. difficile* as a species and support recently suggested taxonomic revisions (84–87).

Taxonomy

The *Clostridium* group represents an ancient prokaryotic lineage, estimated to have diverged from the bacterial domain 2.34 Ga (billion years) ago, earlier than the *Escherichia*, *Campylobacter*, and *Helicobacter* groups (ca. 1.37 to 1.89 Ga) and around the time when concentrations of molecular oxygen in the atmosphere began to increase (88). Described under the phylum *Firmicutes*, the class *Clostridia* incorporates a group of obligately anaerobic, endospore-forming (and thus resistant to desiccation), Gram-positive organisms. Classification of *Clostridia* was initially made based on these phenotypic characteristics; however, 16S rRNA sequencing showed that the *Clostridia* were phylogenetically incoherent and required significant taxonomic revision (86). According to the scheme of Collins et al. (86), *C. difficile* belongs to cluster XI, which represents a taxonomically heterogeneous group more closely related to the non-spore-forming species *Peptostreptococcus anaerobius* and *Eubacterium tenue* than the type species of the *Clostridia*, *Clostridium butyricum*. Notably, by this scheme, *C. difficile* does not cluster with many other familiar clostridial species, such as *C. botulinum*, *C. tetani*, and *C. perfringens*, all of which can be pathogenic for humans and animals via toxin-mediated virulence mechanisms.

Bergey's Manual of Systematic Bacteriology (87) now places *C. difficile* in the *Peptostreptococcaceae* along with a number of other *Clostridium* species, including *C. bifermentans*, *C. glycolicum*, *C. bartlettii*, *C. sordellii*, and *C. sticklandii*, as well as members of the genera *Eubacterium*, *Peptostreptococcus*, *Sporacetigenium*, and *Filifactor*. In 2013, further taxonomic revision of the *Clostridia* was called for, with a proposed name change from *Clostridium* to *Peptoclostridium* (85) for *C. difficile*. Despite NCBI taxonomy adopting this name change, it appears unlikely that the *C. difficile* community will follow.

Phylogenetics and Molecular Epidemiology

C. difficile has a clonal population structure. In 2004, Lemee et al. (43) conducted the first analysis of *C. difficile* isolates using MLST. Those authors identified three distinct phylogenetic lineages but noted that geographical affiliation, host species, or a particular phenotype (e.g., strains causing severe disease) was not associated with any particular lineage. In 2006, Stabler and colleagues (73), using DNA microarrays, a Bayesian evolutionary model, and a more diverse *C. difficile* population, identified four phylogenetic lineages or clades. The majority of STs clustered into a single heterogeneous lineage, but the remaining three represented emergent virulent lineages: RT017 (ST-37), RT027 (ST-1), and a more distantly related group, RT078 (ST-11) (73). Later, the same authors undertook a phylogenetic analysis of *C. difficile* WGS data and confirmed the lineage topology known at that time (four clades) and, in addition, provided a more in-depth and robust phylogeny (57). Through evaluation of WGS data from six strains representing these four clades, calculations of the evolutionary distance between the clades were made. Based on these and other data, the last common ancestor was estimated to have emerged somewhere between 1.1 and 85 Ma (million years) ago (57). However, it is worth noting that methods for dating bacteria are imperfect and based on models and assumptions about evolutionary rates (in this case divergence of orthologous genes between *C. difficile* and *C. tetani*), which may not be entirely accurate.

Using a different MLST scheme, Griffiths and colleagues (44) identified an additional lineage containing toxigenic RT023 (ST22), bringing the total number of clades to five. This population structure has since been confirmed by other studies, including some using WGS (45, 46, 89), and has been summarized in a recent review by Janezic and Rupnik (90). MLST clade 1 represents a highly heterogeneous cluster of toxigenic and nontoxigenic STs (numbering over 100) and RTs, including many of clinical significance, such as RT014 (*tcdA* positive [A^+], *tcdB* positive [B^+], and *cdtA* and *cdtB* negative [CDT^-]; STs 2, 14, and 49), RT002 (A^+ B^+ CDT^-; ST8), and RT018 (A^+ B^+ CDT^-; ST17), all of which are RTs consistently among the most frequently recovered from patients with CDI (91–94). Clade 2 contains hypervirulent RT027 (A^+ B^+ CDT^+; ST1) and several other RTs of clinical importance, including RT244 (A^+ B^+ CDT^+; ST41) and RT176 (A^+ B^+ CDT^+; ST1) (95, 96). To date, clade 3 has received little interest in comparison, but this clade contains RT023 (A^+ B^+ CDT^+; ST5 and ST22), which has been isolated from humans in Europe (92). Clade 4 contains RT017 (ST37), which has a variant toxin profile (A^- B^+ CDT^-) and is often clindamycin and fluoroquinolone resistant. Despite the absence of toxin A and binary toxin expression, RT017 causes widespread disease; has been associated with outbreaks in Europe (97, 98), North America (99), and Argentina (100); and is responsible for much of the CDI burden in Asia (94). Clade 5, containing RT078 (ST11), has been the focus of much interest because of its significant divergence from the other clades and its association with animals, particularly livestock (101). However, recent MLST and WGS studies have shown that clade 5 is more heterogeneous than first thought, including not only RT078 but also numerous RTs (RT033, RT045, RT066, RT126, RT127, RT237, RT280, RT281, and RT288) from a diverse collection of clinical, animal, and food sources worldwide (46, 102, 103).

Some clade 5 strains show an atypical arrangement of the PaLoc, specifically the genes for LCTs A and B. RT237, which has been recovered from pigs and humans in Australia, is positive for toxin B but negative for toxin A (A^- B^+) while also possessing binary toxin (CDT^+) (104). In RT033 and RT288, CDT is present;

however, the entire *tcdB* gene and the majority of *tcdA* are absent (41, 105). RT033 was recently isolated from a cluster of six epidemiologically unrelated cases of CDI in France, suggesting that despite the absence of LCTs, there remains a pathogenic potential (105). Additionally, the prevalence and clinical burden of these two LCT-negative, CDT-positive RTs are likely to be underestimated. A recent study by Androga et al. (106) found that current molecular diagnostic assays (which rely on amplification of the toxin A and B genes) fail to detect *C. difficile* RT033.

More recently, there have been reports of two novel lineages. One lineage, designated clade 6, contained a single ST (ST122; RT131) and appeared as a sister lineage to clades 1 and 2 (47). Recently, its status has been questioned, as in another study, ST-122 was not an outlier but rather part of heterogeneous clade 1 or possibly a hybrid of clades 1 and 2 (107). The other novel lineage, designated C-I, as it was reminiscent of the cryptic clades of *E. coli* (107), was highly divergent, entirely nontoxigenic, and potentially a new species or subspecies of *C. difficile* (107). Figure 2 shows the currently described *C. difficile* population structure of six clades (107).

MLST demonstrates the high level of genetic diversity within the species and shows that RTs are clade specific (45). Recently, however, the concept of clonal *C. difficile* lineages has been extended to include more than just RT affiliations. Kurka et al. (108) examined the genomes of strains belonging to 21 different RTs from different MLST lineages, looking at differences in a number of conserved genes, including *rpoA* and *gyrB*, encoding RNA polymerase A and gyrase B, respectively. Those authors found that strains with the same sequence deviations in these and many other genes clustered into groups which mirrored the RT diversity inferred by MLST; e.g., all strains of RT126, RT127, and RT033 (clade 5) clustered together. This is interesting, as it shows that RT is indicative of not only differences in the amplified 16S-23S rRNA gene ISR but also specific differences in the nucleotide sequences of a number of conserved genes. Similarly, gene variations in hypervirulent RT027 and RT078 are MLST lineage specific (109).

MECHANISMS SHAPING DIVERSITY AND EVOLUTION IN *C. DIFFICILE*

The remarkable genetic diversity in the *C. difficile* genome mirrors the wide variety of phenotypes, ecological adaptations, and physiological versatilities seen in this species. This diversity is a result of the acquisition of foreign DNA coding for novel phenotypes and has been shaped over long and short time periods by mechanisms of lateral gene flux, such as homologous recombination and horizontal gene transfer (HGT) (45, 57, 107, 110).

Transposable Elements

Of the numerous genetic elements found in the genome of *C. difficile*, many are transposable and can change their position within the genome. Some *C. difficile* transposons (Tns) are mobilizable, meaning that they rely on complex host-mediated mechanisms for conjugation and full mobility (111, 112). Some elements are self-transmissible and are known as conjugative transposons (CTns) or integrative and conjugative elements (ICEs). CTns are capable of excision, transfer, and integration into the genome of *C. difficile* and species of other genera through the expression of integrase (*int*), excisionase (*xis*), and, in some elements, a site-specific recombinase (*tndX*) (113). Consequently, there is the potential for various bacteria to acquire new DNA from the highly diverse *C. difficile* pangenome as well as allowing *C. difficile* to acquire genes from the intestinal metagenome (114). Many *C. difficile* mobilizable transposons and CTns have been found throughout different lineages, including those that are separated by large phylogenetic distances, e.g., clades 1 to 4 and clade 5, suggesting insertion of the element prior to clade divergence (45, 57).

The introduction of these elements into the *C. difficile* genome leads to numerous heritable changes not only in the acquisition of new, possibly advantageous genes but also, as in the case of insertion within an open reading frame (ORF), in gene disruption and phenotypic alteration (112, 114). Dissemination of these elements and their accessory genes by lateral transfer has significantly contributed to the genetic diversity seen in *C. difficile* and possibly contributed to the success of *C. difficile* as an opportunistic pathogen (52, 73, 112, 114, 115).

Exposure to antimicrobials has a significant role in the pathogenesis of CDI, and resistance should be considered a virulence factor, as it is in other nosocomial infections such as those caused by extended-spectrum-β-lactamase- and carbapenemase-producing *Enterobacteriaceae* (12, 116). Unlike these and other related pathogens, resistance to antimicrobials in *C. difficile* is unusual in that it is mediated predominantly by Tns as opposed to plasmids (52, 112, 114). Through traditional PCR- and, more recently, WGS-based studies, numerous Tns associated with antimicrobial resistance in *C. difficile* have been described, including Tn*916* {tetracycline resistance [*tet*(M)]} (117), Tn*4453*a/b (chloramphenicol resistance) (118), Tn*5397* [*tet*(M)] (119), Tn*B1230* [*tet*(W)] (120), and Tn*5398*. Tn*5398*, first described in 1995, contains two copies of *erm*(B), which encodes a 23S rRNA methylase conferring the MLS (macrolide-lincosamide-streptogramin B) phenotype, the most common resistance type in *C. difficile* (112, 121). Tn*6215* also carrying *erm*(B) was reported to be transferred between *C. difficile* strains by bacteriophage-mediated transduction (115). Tn*6218* is a stably integrated transposon with a number of described variants occupying different PaLoc-independent chromosome locations in different RTs (107). In addition to *erm*(B), some variants of Tn*6218* also possess the *cfr* gene. *cfr* encodes a methyltransferase that alters binding sites within the bacterial ribosome, resulting in resistance to clindamycin, florfenicol, and chloramphenicol (122). The *cfr* gene has been found previously in many Gram-positive and -negative bacteria (122), particularly in plasmids of *Staphylococcus* spp. (123), but this was the first description of it in *C. difficile* (107). Recently, Marin et al. (124) reported reduced susceptibility to linezolid (MIC, 6 to 16 mg/liter) in 9/891 clinical isolates of *C. difficile*. These 9 strains belonged to RT001, RT017, and RT078, and they all possessed the *cfr* gene, which showed 100% sequence identity with a fragment of Tn*6218* (124).

Several other putative transposons have also been described. Analysis of the genome of *C. difficile* strain 630 identified six putative CTns (CTn*1*, CTn*2*, CTn*4*, CTn*5*, CTn*6*, and Ctn*7*) associated with genes encoding efflux pumps and ATP-binding cassette (ABC) transporters, which confer resistance to tetracycline, chloramphenicol, and erythromycin (52, 53). One of these putative CTns, Tn*6164*, contains aminoglycoside and tetracycline resistance genes. In reference strain M120, Tn*6164* is located in a novel 106-kb genetic island that is made up largely of mobile elements from nonclostridial species such as *Streptococcus pneumoniae*, *Enterococcus faecalis*, and *Thermoanaerobacter* sp. (125). The pres-

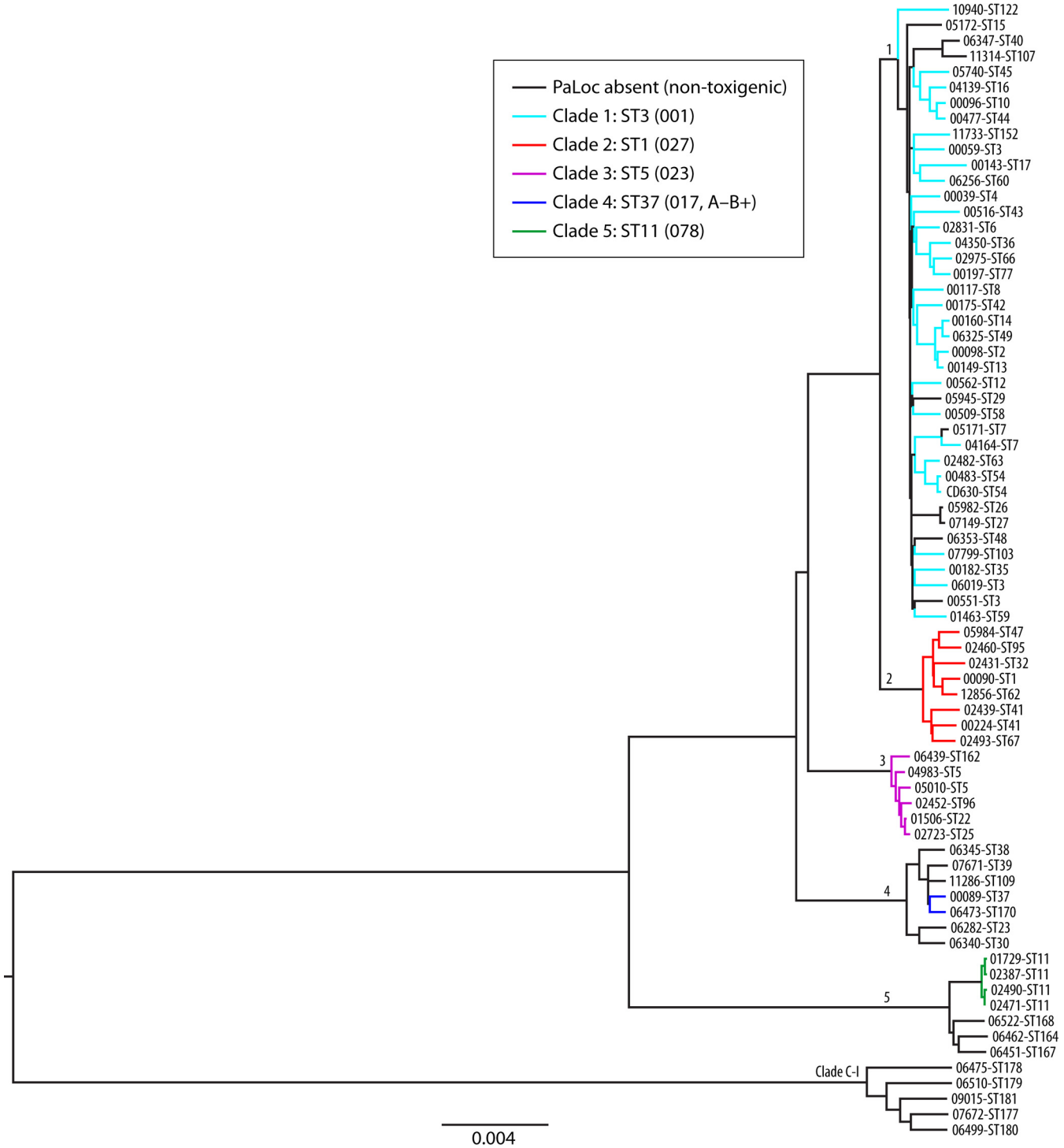

FIG 2 Phylogenetic tree showing representatives of six currently described *C. difficile* clades and the relationship between toxigenic and nontoxigenic isolates. A maximum likelihood tree was generated from the alignment of 1,426 core genes of 73 *C. difficile* isolates. Isolates represented extremes of clinical severity, geographic diversity, and toxigenic status. Clades are indicated by their designated number. Nontoxigenic isolates are indicated by black branches. Toxigenic isolates are indicated by branches colored according to clade. The ST and RT (in parentheses) of a well-characterized representative of each clade are indicated. (Reproduced from reference 107 by permission of the Society for Molecular Biology and Evolution.)

ence of this element was found to vary in porcine and human strains of RT078, and this element was absent from other RTs. Notably, although patient numbers were small, patients infected with strains containing Tn*6164* had higher rates of mortality (29% versus 3%) (125). Another CTn of interest is Tn*6194*, the most common *erm*(B)-containing element in clinical isolates in European hospitals (126). Tn*6194* has been associated with epidemic RT027, particularly in strains belonging to a lineage isolated in the

United States and Asia (57). In addition to *erm*(B), Tn*6194* carries genes proposed to function as recombinases and integrases, suggesting the potential to travel laterally between *C. difficile* strains (intraspecies transfer) (57, 127). This potential has been confirmed by the successful excision and transfer of Tn*6194* in strain 630 (114). More recently, Tn*6194* has been shown to be capable of inter- and intraspecies transfer. Wasels and colleagues (128) transferred a variant of Tn*6194* (identified in the genome of a RT001 strain) into the genomes of strains with two different *C. difficile* RTs as well as a strain of *E. faecalis* (128).

630Δ*erm* is a macrolide-susceptible *C. difficile* mutant derived from strain 630, which has often been used as a tractable strain for genetic analysis of *C. difficile*. In a recent study by van Eijk et al. (129), WGS revealed that CTn*5* is one of several genetic features that might explain the underlying phenotypic differences between 630 and its erythromycin-sensitive derivative. Specifically, it was shown that in 630, CTn*5* resides within an adhesin (CD1844); however, in 630Δ*erm*, the same transposon can be found interrupting the methyltransferase gene *rumA* (129).

Bacteriophages

Another important component of the *C. difficile* mobilome is bacteriophages (phages). Phages have coevolved with *C. difficile* over very long periods of time, and phage infection is an inherent part of the natural history and biology of *C. difficile* (22, 130). Phages are capable of mediating HGT via a process known as transduction, whereby host DNA is packaged into the head particle of a phage and subsequently inserted into the genome of a recipient cell. Consequently, the acquisition of phages by and their loss from the *C. difficile* genome are significant genetic events that have impacted host evolution (22, 130).

C. difficile carries a diverse collection of phages, including numerous members of the *Siphoviridae* and *Myoviridae* families, such as ϕC2, ϕMMP04, ϕCD119, ϕCDHM1, ϕCD38-2, and ϕCD27, ranging in size from 31 to 56 kbp with a GC content not dissimilar to that of the *C. difficile* genome (28 to 30%) (52, 57, 73, 113, 131–133). Despite the absence of proven virulence factors in *C. difficile* phage genomes, there is increasing evidence that phages may play a role in *C. difficile* pathogenesis. All sequenced phages identified in the genome of *C. difficile* have contained putative integrase genes, suggesting that they have access to the lysogenic life-style (113). Recently, phage ϕC2, which is common to clinical strains of *C. difficile*, has been shown to mediate the transfer of Tn*6215* containing *erm*(B) between two laboratory strains of *C. difficile* (115). Viral DNA identical to that of phages (ϕMMP02 and ϕMMP04) found within *C. difficile* has been recovered from stool samples obtained from patients with CDI, indicating that these phages are induced during infection. Furthermore, the *in vitro* induction of these phages was increased significantly in the presence of fluoroquinolone antimicrobials, demonstrating how the established CDI risk factor of antimicrobial exposure may influence phage biology and may ultimately promote phage-mediated HGT (113). Notably, Hargreaves et al. (134) describe the presence of *agr* homologues in the genome of phage ϕCDHM1. In *C. difficile*, the *agr* locus is responsible for modulating the expression of 75 genes associated with various cellular functions, such as flagellum assembly and toxin synthesis, particularly during late exponential growth (113). It is hypothesized that the expression of these *agr*-like genes during phage lysogeny may influence gene expression in the host bacterium through a quorum-signaling mechanism (113, 134). Moreover, phages ϕCD119, ϕCD38-2, and ϕCD27 have been shown to modulate toxin production in *C. difficile*; however, the genetic basis of these interactions is not yet understood (113, 134).

Few studies to date have investigated the host range of *C. difficile* phages. Some phages have been shown to infect strains of multiple RTs, and phages have been recovered from human, animal, and environmental populations of *C. difficile* (131, 132). As a result of the coevolution of phages and their hosts, many species of bacteria, including *C. difficile*, have developed ways to resist infection. In *C. difficile*, it was recently shown that strain specificity for phages and the host's ability to resist infection are likely defined by the number, distribution, and diversity of CRISPRs (135). Often associated with *cas* proteins, the CRISPR-*cas* system utilizes a series of phage-specific spacers (CRISPR array) to identify and degrade spacer homologues found in phage DNA, a mechanism reminiscent of RNA interference (RNAi) in eukaryotes and hypothesized to be a putative bacterial adaptive immunity system (135, 136). Hargreaves and colleagues (135) recently showed that some phages can evade CRISPRs through polymorphisms in spacer regions. Remarkably, those authors also showed that some *C. difficile* prophages possess CRISPR arrays of their own, which, if fully functional, present a mechanism that can influence infection by other phages. Given the role of phages in mediating HGT, the interaction between *C. difficile* CRISPR arrays and phages has undoubtedly impacted the evolution of *C. difficile*, particularly the extent of HGT.

Homologous Recombination

Homologous recombination is a powerful driver for shaping genetic diversity in a wide variety of bacteria and archaea, ranging from commensal opportunistic pathogens to free-living terrestrial and marine extremophiles (110). The ratio of the nucleotide substitution rate as a result of recombination to that as a result of mutation (*r*/*m*) is a measure of the rate of homologous recombination in sequence diversification in bacteria, which varies considerably among species (110). For *C. difficile*, this rate has been estimated to be 0.2 (110) or slightly higher (0.63 to 1.13) (57). These rates are low compared to those of other gut pathogens such as *Vibrio parahaemolyticus* (39.8), *S. enterica* (30.2), and *H. pylori* (13.6) but comparable to those of other *Firmicutes* such as *E. faecalis* (0.6) and *S. aureus* (0.1) (110). For *C. difficile*, the *r*/*m* is perhaps an underestimation resulting from the geographical segregation of global *C. difficile* populations, and as many studies have now illustrated, homologous recombination has played a very significant role in shaping the evolution of genes associated with gastrointestinal adaptation and virulence potential in *C. difficile*.

Many *C. difficile* genes and associated operons show a mosaic structure that could have arisen only by homologous recombination. In recent years, much attention has been paid to investigating the evolution and phylogeny of these areas of the genome, particularly the PaLoc. Recently, Brouwer et al. (137) demonstrated that the PaLoc is capable of being transferred between *C. difficile* strains by a conjugation-like mechanism. Those authors were able to demonstrate the transfer of the PaLoc from a toxigenic *C. difficile* strain (630Δ*erm*) to three nontoxigenic strains (RT009 [CD37], RT138, and RT140). Analysis by a cytotoxic assay revealed that the resulting transconjugants produced toxin B at levels similar to that of the donor strain (137). Analysis of the

transconjugants showed that the PaLoc was transferred on variably sized DNA fragments (range, 66 to 272 kbp) and was not contained within an obvious mobile element. Those authors noted that in 630Δ*erm* strains, the regions immediately upstream and downstream of the PaLoc were homologous to regions of chromosomal DNA in nontoxigenic strain CD37, thus facilitating recombination and integration of the PaLoc within this region (137). Such chromosomal transfer of the PaLoc is reminiscent of the high-frequency recombination (Hfr) of bacterial chromosomes mediated by CTns and followed by homologous recombination, as seen in bacteria such as *Vibrio cholerae* (138) and *Bacteroides* sp. (139). CTn*1*, CTn*2*, and Tn*5397* are possible candidates for Hfr-mediated transfer, as they are in close proximity to the PaLoc and are transfer proficient (137). Evidence of PaLoc transfer between strains by this mechanism is thought to be occurring in wild populations and is a key driver of evolution in the *C. difficile* genome (45, 57). Furthermore, there are elements analogous to the *C. difficile* PaLoc in other closely related species, notably the *tcdA*- and *tcdB*-related toxin genes in *C. novyi*, *C. perfringens*, and *C. sordellii* (140), which suggests that the PaLoc in *C. difficile* may have arisen by interspecies recombination. When present, the PaLoc is always found at the same chromosomal location (45, 64); however, the presence and composition of the PaLoc vary between isolates and in some instances even among isolates of the same ST (107), again reflecting the genetic diversity within the species.

Recently, the evolutionary history of the PaLoc has been reconstructed (107). Dingle and colleagues compared the core genomes and PaLoc phylogenies of >1,600 toxigenic and nontoxigenic isolates from the United Kingdom and Australia. By using a subset of these isolates with mixed toxigenic statuses representing all previously described clades, the distribution of the PaLoc among the *C. difficile* population was assessed. Interestingly, the resulting phylogeny (which was based on a maximum likelihood alignment of 1,426 concatenated "core" genes) showed a highly divergent lineage comprised only of nontoxigenic strains (C-I), while the remaining nontoxigenic strains were distributed alongside toxigenic isolates in several clades (107) (Fig. 2).

Remarkably, further analysis of this data set identified 26 independent events of PaLoc acquisition, exchange, and loss, the most recent being ~30 years ago in clade 1 (107). The overall PaLoc phylogeny showed numerous clade-specific acquisitions, many occurring after clades had diverged. For the most recent instances of PaLoc exchange, several clade-specific homologous recombination events involved very long chromosomal fragments (up to 232 kb). Such large-scale recombination in the *C. difficile* genome has been reported previously (57). In that instance, several large regions of SNPs were observed throughout the core genome, suggesting recent exchange between RTs, some of which were separated by large phylogenetic distances. Such findings are significant since they indicate that homologous recombination is a key driver of *C. difficile* (and PaLoc) evolution and, thus, the virulence potential of *C. difficile* (107).

The 16S-23S ISRs of *C. difficile* are highly variable, and differences in ISRs define the PCR ribotype (39, 141). Recently, it was proposed that both inter- and intrastrain recombination events have influenced the evolution of, and account for, the heterogeneity and mosaicism seen in the ISR (40). Similar recombination events have been described for rRNA operons in other bacterial species, including *E. coli*, *Haemophilus parainfluenzae*, and *Vibrio cholerae* (40, 142).

Another example of homologous recombination driving genetic diversity can be seen in the *C. difficile* S layer, a paracrystalline immunodominant cell surface antigen that is the basis of S-layer typing and a component of serological typing in *C. difficile* (143). The S layer forms an important interface between the bacterium and its host. It is thought that the S layer has a central role in adaptation to life within the gastrointestinal tract and evolves in response host immunological selection (144). The S layer is made up of a number of S-layer proteins (SLPs), principally SlpA. SlpA is encoded by the *slpA* gene located within a 36.6-kb cell wall protein (*cwp*) gene cluster. In addition to *slpA*, the genes *secA2* (encoding a secretory protein) and *cwp66* (encoding an adhesin) comprise a genetically variable 10-kb cassette (89). Recently, through an analysis of genome sequence data, the extent of *cwp* cluster diversity has been determined, at the same time providing clues about the evolution of these important loci (89). Analysis of the nucleotide sequences of the 10-kb *slpA-cwp66-secA2* cassettes from 58 genetically diverse *C. difficile* strains revealed 12 distinct stable variants spread across five phylogenetic clades. These findings suggest that frequent and independent horizontal transfer of the *cwp* cluster has taken place throughout the *C. difficile* population, a process referred to by the authors of that study as S-layer switching (89). Adding to the diversity in this locus is the finding of a novel cassette in three of the five clades that appeared to encode components of a putative S-layer-glycosylating cluster, the first such finding for a *Clostridium* species (89).

Forces of Selection

Clonal diversification is influenced by the process of natural selection, whereby synonymous nucleotide changes in CDSs evolve under a neutral model of selection, while mutations that provide a reproductive advantage or "fitness" are fixed in a bacterial lineage by positive or Darwinian selection, and deleterious mutations in the genome are subject to purifying or negative selection (145). To date, a single study by He and colleagues (57) investigated the selective forces acting on the *C. difficile* genome. To do so, those authors used a robust codon-based substitution model of molecular evolution to analyze CDSs from the nonrepetitive core genomes of 9 *C. difficile* isolates representing divergent clonal lineages (MLST clades 1 to 5). The calculation of the relative ratio ($\omega = dN/dS$) of nonsynonymous substitutions (dN) and synonymous substitutions (dS) in these core CDSs allowed for inference of signatures of selection. Between highly divergent lineages such as clade 5 (strain M120) and clades 1 to 4 (including strains BI, 630, CD196, CF5, and M68), there was evidence of strong purifying selection (mean $\omega = 0.08$). This paucity of dN in the core genome of divergent strain M120 suggests a long divergence time and further supports its status as an ancient lineage. In contrast, between recently diverged clones (strains representing clades 1 to 4), the value of ω was close to 1, indicating enrichment of dN in the core genome of these lineages. While this may suggest that these core CDSs were under neutral selection, it is probable that purifying selection is somewhat delayed in these recently divergent lineages due to insufficient evolutionary time passing for purging of nonsynonymous substitutions (57). Those authors also noted that selection was not homogenous in the core genome, with 12 CDSs under positive selection. These CDSs encode proteins associated with the bacterial cell surface, membrane, and response

regulators and likely reflect the influence of host immune selection on the *C. difficile* genome (57). It is worth pointing out that this analysis was restricted to the core nonrepetitive genome of a select group of *C. difficile* clones. It is highly likely that the accessory genome and recombinant/repetitive parts of the core genome that are heavily enriched with SNPs (40, 45, 57, 89, 107, 146, 147) are under various degrees of purifying and Darwinian selection.

THE COMPLEX AND DYNAMIC EPIDEMIOLOGY OF CDI

Evolutionary History of Epidemic Lineage RT027

In the last 2 decades, RT027 (toxinotype III; ST-1 [MLST clade 2]; BI/NAP1; A^+ B^+ CDT^+) has emerged as a major pathogen of humans that has been associated with large, highly publicized outbreaks of CDI, initially in North America and later in Europe (148–150). One notable example was an outbreak of CDI at the Stoke Mandeville hospital in the United Kingdom. In a 3-year period spanning April 2003 to March 2006, 498 patients were diagnosed with CDI while admitted to the hospital, 127 of whom died (151). RT027 possesses a number of attributes that result in a hypervirulent phenotype, including increased production of LCTs (152), the presence of binary toxin (28), higher sporulation rates (153, 154), aberrant forms of *tcdC* (69), production of toxin B variants with an enhanced spectrum of cytotoxicity (155), and mutations in the DNA gyrase resulting in fluoroquinolone resistance (FQR) (156). Consequently, patients with RT027 infection showed a poor response to treatment and a marked increase in morbidity and mortality (148, 149).

The emergence and dissemination of RT027 brought about a massive change in the global molecular epidemiology of *C. difficile*. This was likely a result of the culmination of a number of events related to epidemiological, host, and pathogen factors, in particular the selective pressure applied by the extensive use of fluoroquinolone antimicrobials in health care settings as well as human travel (157, 158). WGS has played a significant role in the underlying genetic reasons behind this change. In 2009, Stabler and colleagues (56) undertook a three-way genomic comparison of a nonepidemic "historic" (1985) RT027 *C. difficile* strain (CD196), a recent epidemic and hypervirulent RT027 strain (R20291), and an RT012 strain (630), the genome of which had been reported previously. Those authors aimed to relate genetic differences in the genomes of these strains to phenotypic differences in antibiotic resistance, toxicity, survival, and motility. The study identified five large genetic regions present within the recent RT027 strain but absent from the preepidemic CD196 counterpart, including transcriptional regulators, a unique phage island, and a two-component regulatory system (56). Those authors looked for the presence of these genetic markers in a larger set of *C. difficile* RT027 genomes and found that many were acquired very recently, potentially explaining the genetic basis for the emergence of RT027 and its successful dissemination (56). In addition to the acquisition of new genes, numerous point mutations and nucleotide inversions have been identified within or upstream of putative coding regions in epidemic strains of RT027, which likely result in changes in gene functionality and phenotype (59).

In 2013, He et al. (127) sequenced the genomes of 151 *C. difficile* RT027 strains collected from 1985 to 2010 with the aim of accurately inferring the population structure. Those authors demonstrated that RT027 acquired fluoroquinolone resistance independently on two separate occasions, resulting in two distinct epidemic lineages (FQR1 and FQR2) with different patterns of global spread (Fig. 3). The FQR1 lineage was thought to have originated in Pittsburgh, PA (earliest isolate dated 2001) and contained epidemic strains associated with severe outbreaks throughout the United States and later sporadic cases in South Korea and Switzerland. The majority of epidemic strains belonged to the FQR2 lineage. FQR2 showed a notable star-like topology in North America, suggestive of a rapid population expansion, likely from a single progenitor clone. Transcontinental dissemination epidemic of FQR2 occurred on no fewer than four occasions in Europe and once in Australia (Fig. 3). The separate acquisitions of FQR and a novel conjugative transposon (CTn*5*-like, Tn*6194*) common to both lineages were thought to be the key genetic changes responsible for the rapid emergence and subsequent successful worldwide dissemination of this lineage (127).

The RT027 lineage is more variable than first thought. Previously presumed to be RT027-REA type BI, three novel and clinically relevant RTs (RT176 [BI-6], RT198 [BI-11], and RT244 [BI-14]), first isolated in the United States between 2001 and 2004, appear to have emerged from the RT027 lineage (96). Of these emergent RTs, RT244 has generated much recent interest, specifically in its pathogenic potential and community acquisition. RT244 infection is associated with a higher mortality rate, and patients with RT244 infection are more likely to develop severe disease and hypoalbuminemia and to have renal impairment (95, 159). WGS of a RT244 strain isolated from a patient who died of severe CDI in Australia revealed a variant toxin B resulting in an enhanced cytopathic effect *in vitro* (95). In another recent study by Eyre et al. (160), 15 outbreak isolates of RT244 from across Australia were sequenced. All strains were genetically highly related (within 16 single-nucleotide variants [SNVs] of each other), and isolates from a cluster of seven cases from three states differed by just 4 SNVs. However, despite this high degree of genetic similarity, no geographic clustering could be identified, suggesting a single source, possibly in the food chain. Furthermore, these outbreak strains were found to be highly related to a strain isolated from a patient with CDI in the United Kingdom who had recently returned from Australia (160). These findings highlight both the pathological and dissemination potential of RT244 and emphasize the need for ongoing surveillance of strains of this lineage and other newly emergent RTs. The study by Eyre et al. (160) also provided a novel insight into the evolution of this RT. Initially thought to be a relatively recent evolutionary event (96), RT244 and RT027 lineages actually share a relatively ancient common ancestor with current outbreak strains CD196 (RT027) and MDU-064e (RT244), separated by 12,026 SNVs and many hundreds/thousands of years of evolution (160).

Microevolution and Transmission in the Hospital Environment

CDI has traditionally been considered hospital acquired (161, 162), and it was a widely held assumption that much of the *C. difficile* transmission in hospitals occurs horizontally between symptomatic patients (162, 163). In the past, examination of the molecular epidemiology of CDI outbreaks by MLVA, MLST, and PCR ribotyping has supported this view. However, while MLVA is useful in outbreak investigations, MLST and PCR ribotyping are not sufficiently discriminatory to distinguish between strains or investigate patterns on an ultrafine scale (35, 36, 164, 165). WGS and estimates of the *C. difficile* molecular clock (within-host mu-

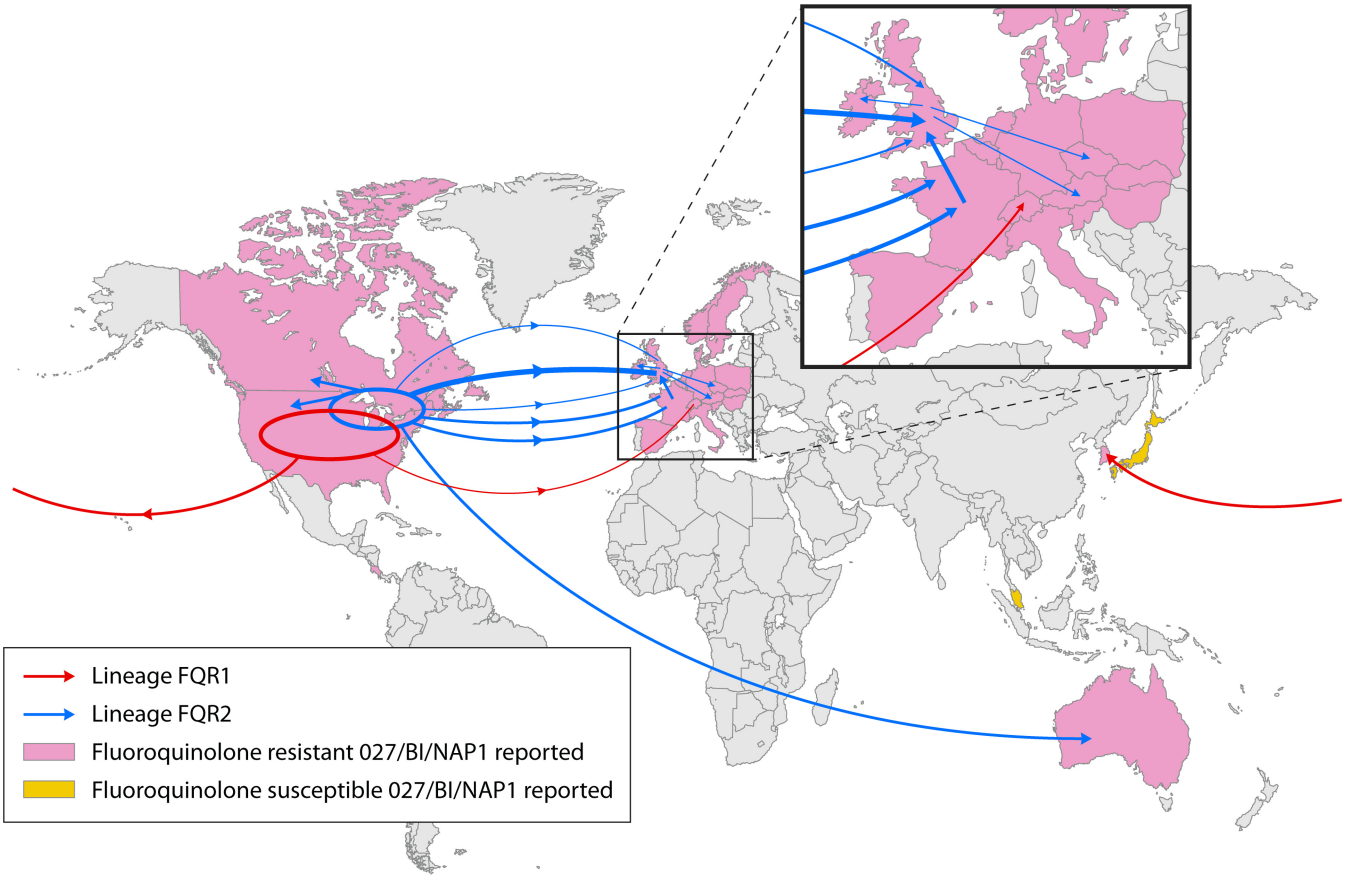

FIG 3 Transcontinental dissemination of epidemic RT027. Shown is the global spread (arrows) of lineages FQR1 and FQR2 inferred from phylogeographic analysis. The width of the arrow is approximately proportional to the number of descendants from each sublineage. The inset shows an enlarged view of transmission in Europe. (Reproduced from reference 127 by permission from Macmillan Publishers Ltd.)

tation rate) have begun to reshape *C. difficile* surveillance and outbreak investigations (36). Didelot et al. (166) estimated the *C. difficile* evolutionary rate to be 3.2×10^{-7} mutations per nucleotide per year (95% confidence interval [CI], 1.3×10^{-7} to 5.3×10^{-7} mutations per nucleotide per year), equating to ~1.4 mutations per genome per year. This estimate of the within-host mutation rate is based on the application of a complex evolutionary model based on coalescent theory to the genomes of serially isolated strains from 91 cases of CDI. Those authors analyzed a total of 486 *C. difficile* genomes obtained from CDI cases arising in Oxfordshire, United Kingdom, during 2006 to 2010. By combining this estimate with meaningful epidemiological data (hospital admission and patient ward movement), those authors were able to generate a genealogical timeline for pairs of genomes with similar STs and identify plausible transmission events. As expected, these events were all highly associated with pairs of patients sharing the same space and time in hospital. Surprisingly, there was a large proportion of genome pairs, isolated within 30 days of each other and matched by traditional typing, e.g., MLST or PCR ribotyping, that were too distantly related to be direct transmissions. This was an important finding and suggested that transmission between symptomatic patients in hospitals contributed far less to the overall rate of infection than first thought (166).

In light of these results, the question remained as to the extent that alternative sources, such as patients with asymptomatic colonization, or community and environmental sources of *C. difficile* contribute to the overall burden of disease. In a 2013 landmark study by Eyre and colleagues (167), >1,200 isolates of *C. difficile* obtained from symptomatic patients in Oxford University Hospitals throughout 2007 to 2011 were sequenced, with the aim of identifying the genetic relationship between strains. Those authors began by estimating the evolution rate and mean within-host diversity by evaluating the genomes of isolates from the first and last samples of 145 patients. By applying a coalescent model of evolution and taking into account the interval between the collection times of individual samples, those authors estimated an evolutionary rate of 0.74 SNVs (95% CI, 0.22 to 1.40 SNVs) per genome per year and a mean within-host diversity of 0.30 SNVs (95% CI, 0.13 to 0.42 SNVs). By using these prediction intervals, pairs of isolates separated by <124 days from each other and differing by 0 to 2 SNVs were considered to be a result of direct transmission. A comparison of 957 isolates collected between 2008 and 2011 revealed that just 35% of isolates showed evidence of direct transmission from an earlier case (2007 to 2011), a figure significantly lower than anticipated. Moreover, in one-third of these patients, no plausible epidemiological link could be made (e.g., no contact with another patient in the hospital or in the community) (Fig. 4). Remarkably, 45% of isolates had >10 SNVs, indicating that they were genetically distinct from all other cases (Fig. 4) and likely from a source other than the hospital environ-

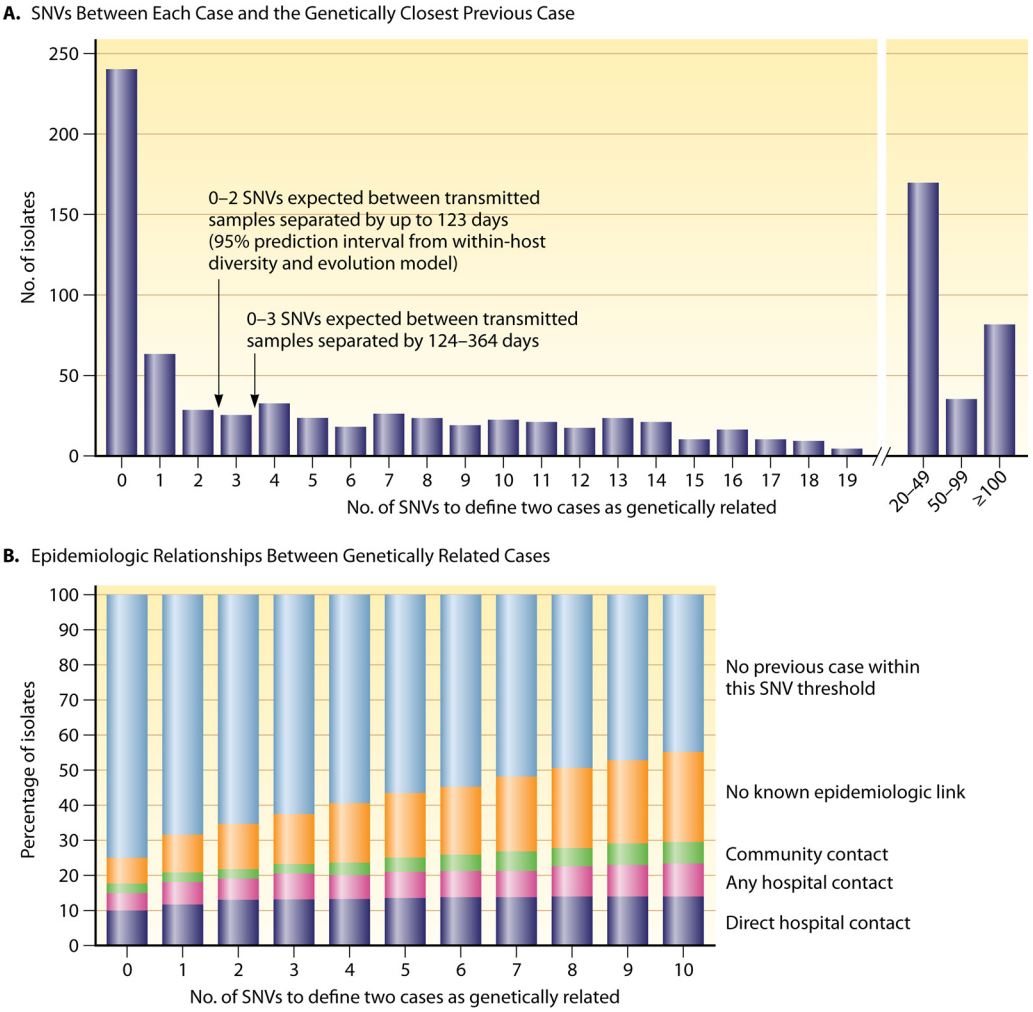

FIG 4 Diverse sources of *C. difficile* in the hospital environment. Shown are the genetic variation and epidemiological relationships among 957 isolates obtained from patients with CDI. (A) Numbers of single-nucleotide variants (SNVs) between each sample obtained during the period from 1 April 2008 through 31 March 2011 and the most closely related previous sample obtained after 1 September 2007. (B) Percentages of isolates that were classified as genetically related, according to the different SNV thresholds, along with the epidemiological links between related isolates. (Reproduced from reference 167 with permission from the Massachusetts Medical Society.)

ment. Importantly, these data demonstrated that genetically diverse sources of *C. difficile* play a more substantial role in *C. difficile* transmission than first thought. Although asymptomatic carriage may be an important source of CDI and could account for many unexplained cases (11), these recent findings (11, 166, 167) represent a significant milestone in *C. difficile* transmission research, as they challenge the prevailing view that horizontal transmission from symptomatic patients is the source of most cases of CDI in health care settings, a concept that is currently the basis for infection control and prevention guidelines (163, 168).

Historically, recurrent or refractory CDI occurs in ~20 to 25% of CDI patients after treatment of primary infection with metronidazole or vancomycin (169). In the past decade, however, coinciding with increases in the frequency and severity of CDI caused primarily by the emergence of the epidemic RT027 strains, the rates of recurrent CDI have also increased, presenting clinicians with a difficult challenge (169, 170). To better understand the epidemiology of CDI recurrences, Eyre and colleagues applied WGS to 93 paired isolates of *C. difficile* from patients with recurrent CDI (65 treated with vancomycin and 28 treated with fidaxomicin), with the aim of resolving the nature of the recurrence (171). By using a methodology similar to the one used in their previous study (167), an evolutionary rate of 0.74 SNVs/called genome/year was used to define relapse (≤2 paired SNVs) and reinfection (≥10 SNVs) (171). For 79.6% of participants, there were <2 SNVs between paired isolates, which is indicative of infection with the same strain. Interestingly, for cases of recurrent CDI attributable to RT027, fidaxomicin showed levels of protection comparable to those with vancomycin; however, for non-RT027 strains, fidaxomicin was superior to vancomycin in preventing both reinfection with a new strain and relapse (171).

Animal Reservoirs and Zoonotic Potential

The incidence of community-acquired CDI (CA-CDI) has been increasing globally, in some regions accounting for up to a quarter of all cases (172). Individuals acquiring disease in the community setting do not have the classic risk factors for CDI acquisition and are generally young and healthy, without contact with hospital-

ized patients and often without prior antimicrobial exposure (173–175). Notably, the genotypes of *C. difficile* strains acquired in the community differ from those of predominant hospital strains. In particular, RT078 (toxinotype V; ST-11 [MLST clade 5]; NAP7/8; A^+ B^+ CDT^+) has emerged as a significant pathogen associated with the majority of CA-CDI cases in the Northern Hemisphere (1, 37). It is currently among the top three most frequently encountered RTs in European hospitals (92, 93, 176). Furthermore, RT078 shares similar genetic features (binary toxin and *tcdC* mutations) and disease phenotypes (increased mortality and morbidity) with RT027 (28, 109, 176, 177).

In an attempt to understand the changing epidemiology of CDI in humans, particularly in the community setting, numerous studies have sought to determine if CDI has a foodborne etiology by investigating the prevalence and genotype of *C. difficile* in animals and food. *C. difficile* is widely recognized as a commensal and enteric pathogen in a wide range of host species (178–180). To date, *C. difficile* been recovered from numerous animal sources, including livestock (pigs, piglets, cows, calves, sheep, lambs, goats, and chickens), domestic animals (cats and dogs), equines (horses and foals), wildlife (rabbits, wild birds, shrews, raccoons, feral swine, ostriches, Kodiak bears, zebras, kangaroos, elephants, ibex, tamarin monkeys, and chimpanzees), and marine organisms (bivalve molluscs) (103, 178, 181–196). Many of these studies described differences in prevalence (particularly a decline with age), toxigenic status, antibiotic resistance, clonal lineage, and host susceptibility to disease, as well as differences in veterinary and agricultural practices (178, 180). Furthermore, these studies highlight the ability of *C. difficile* to adapt to a wide range of host immune systems and gastrointestinal environments, again reflecting the diversity seen in the pangenome.

The predominant strain of *C. difficile* identified in many of these studies was an RT078 strain. In particular, RT078 is well established in food-producing animals, comprising 75 to 100% of porcine and >90% of bovine isolates (187, 197–199). Detection of *C. difficile* in livestock has raised concerns that animals are a potential source of CDI in humans and that spores could be transmitted through either direct contact, shedding, or contamination of meat products with fecal material during slaughter (200). RT078 and RT027 strains have been isolated from pork, beef, and chickens in the Northern Hemisphere, with recovery rates varying from 3% in Europe to 42% in North America (201, 202). These data are alarming and provide support for the theory that *C. difficile* has the potential for zoonotic transmission (178, 201, 203).

Interestingly, RT078 has not been isolated from livestock in Australia (103, 191, 204). However, several other RTs belonging to MLST clade 5 have been found in 7-day-old calves (RT126 [A^+ B^+ CDT^+] and RT127 [A^+ B^+ CDT^+]) and in neonatal pigs (RT033 [A^- B^- CDT^+] and RT237 [A^- B^+ CDT^+]) (103, 204). Albeit in low numbers, these non-RT078 clade 5 RTs have all been isolated from humans with CDI in Australia in recent years and are of emerging One Health importance (205, 206). In the absence of RT078 in Australian livestock, other RTs have become established. In a recent Australian study of *C. difficile* RTs in neonatal pigs, the predominating RT was found to be RT014 (A^+ B^+ CDT^-) (204). This is noteworthy because RT014 belongs to MLST clade 1, is binary toxin negative, and for many years has been the most common RT isolated from humans with CDI in most geographic regions (91–93, 204).

Characterization of the genetic overlap of *C. difficile* strains isolated from different reservoirs facilitates a better understanding of possible transmission routes. Several studies have attempted to determine the extent of genetic relatedness between *C. difficile* isolates of human, animal, and food origins. Initially, isolates of *C. difficile* sharing the same RT or PFGE pattern (e.g., RT078 or NAP7/8, respectively) were typed by MLVA or MLST, providing greater discriminatory power than non-sequence-based methods. Bakker et al. (207) found that 85% of RT078 isolates of human and porcine origins were genetically related by MLVA. Stabler et al. (46) used MLST to analyze a large collection of isolates ($n = 385$) from different geographical locations (Europe, North America, and Australia) and sources (humans, food, and animals). Isolates from diverse sources belonged to the same lineage, and many isolates from humans, food, and animals were indistinguishable (46). Most recently, Dutch researchers used whole-genome SNP typing to compare the genomes of 65 *C. difficile* RT078 isolates of human and porcine origins (208). Analysis of the core genomes of these isolates revealed a total of 401 phylogenetic SNPs, which were used for phylogenetic tree building. The RT078 population-specific mutation rate was estimated to be 2.72×10^{-7} substitutions per site per year (95% CI, 1.43×10^{-7} to 3.99×10^{-7} substitutions per site per year), which is equivalent to 1.1 SNPs per genome per year, a figure comparable with previous estimates (166, 167). Maximum likelihood phylogeny showed isolates of human and porcine origins clustering together. Furthermore, the genomes of pigs and humans harbored identical antimicrobial resistance genes (tetracycline and streptomycin). Notably, these analyses showed a pair of human and pig isolates from the same pig farm in The Netherlands to be indistinguishable (zero SNP differences), suggesting that interspecies transmission had occurred. While this certainly contributes to the theory that CDI is a zoonosis, a common source cannot be ruled out. Moreover, it is possible that zooanthropomorphic (human-to-animal) transmission may have occurred.

CONCLUSIONS AND FUTURE PERSPECTIVES

C. difficile remains a formidable pathogen, and the financial and clinical burden of CDI continues to challenge health care systems the world over. In this review, we have described how sequencing of the genomes of strain 630 and other clinically important reference strains has been a key milestone in unraveling the complexities of this once enigmatic species. We have also described how the application of genomic techniques such as MLST and WGS to robust clinically relevant data sets has significantly advanced our knowledge of both the epidemiology of CDI and the genetics of *C. difficile*.

The genome of *C. difficile* is large and genetically diverse, showing remarkable levels of plasticity and ultralow levels of conservation among strains. Complex mechanisms of HGT and recombination between close and distantly clonal lineages, and also between different genera, have had a profound effect on the evolution of many clinically important loci, such as the PaLoc, ISR, S layer, CRISPR-*cas*, and antimicrobial resistance genes. Many of these mechanisms are mediated by an extensive and diverse collection of transposons and phages that have coevolved with *C. difficile* over long time periods. These evolutionary events have occurred numerous times and collectively led to the species diversifying into hundreds of strains types spread over at least 6 phylogenetic lineages. WGS and estimates of the in-host mutation rate (molecular clock) have provided novel insights into important

aspects of CDI, including ward-based transmission, outbreaks, refractory disease, and the diverse nature of *C. difficile* in the hospital environment. Moreover, recent studies have used WGS to elucidate the genetic basis of the microevolution and transcontinental dissemination of epidemic RT027 strains and have shown for the first time the possibility of interspecies transmission of *C. difficile* between pigs and humans.

Notwithstanding these advances, numerous areas of study of *C. difficile* biology are still in their infancy, and there remains much to learn. Further studies are needed to elucidate the complexities of CRISPR-*cas* elements and the *agr* locus in *C. difficile*. With mounting evidence that livestock are a potentially significant reservoir of CDI, further work is needed to investigate the extent and direction of *C. difficile* transmission between animals and humans as well as genetic exchange between animal and human isolates. In particular, there is a need for a genealogical timeline for the emergence of livestock-associated clones with clinical relevance, such as RT126, RT127, RT237, and RT033, and a greater understanding of their relationship to RT078. There is a need to better understand the overall phylogeny of clade 2, particularly the global epidemiology of RTs other than RT027, and determine their contribution to CA-CDI. By occupying niches within multiple host species, *C. difficile* is able to access and exchange DNA with an enormously diverse metagenome. Further analysis of the vast array of mobile elements such as phages and CTns present in *C. difficile* from diverse sources will advance our understanding of *C. difficile* pathogenesis and diversification of lineages and at the same time expand our knowledge of the *C. difficile* pangenome. In the coming years, WGS will continue to provide insights into this important pathogen, providing researchers and clinicians with information that can be used to reduce the overall burden of disease caused by *C. difficile* in humans and animals.

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