The replication machinery of Clostridium difficile:a potential target for novel antimicrobials

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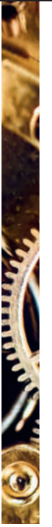

Chapter 1

General Introduction and outline thesis

Erika van Eijk

General Introduction and outline thesis

Clostridium difficile infection

Clostridium difficile (*Clostridioides difficile*) is a Gram-positive, spore-forming obligate anaerobic bacterium that can asymptomatically colonize the intestine of humans, other mammals, reptiles, birds and insects [1,2]. The bacterium is ubiquitous in the environment and particularly present in soil. Ingestion of spores and the subsequent germination into vegetative toxin-producing cells or prior colonization by *Clostridium difficile* within healthy or immunocompromised individuals, and/or elderly patients may induce *Clostridium difficile* infection (CDI) [3]. The spectrum of disease ranges from mild, self-limited diarrhoea to life-threatening pseudomembranous colitis [4,5]. Transmission of the bacterial spores occurs via the faecal-oral route and further spread is promoted by contact of healthcare workers with contaminated surfaces and infected patients. Eradication of spores in healthcare settings is extremely difficult due to their metabolically dormant state and resistance against a variety of environmental stresses such as desiccation, high temperatures, aerobic conditions and many hospital disinfectants and -cleaning agents [4,6-10]. *C. difficile* spores may survive for up to 5 months on environmental surfaces [11,12]. Moreover, spores are not only shed by symptomatic patients, but also by asymptomatic patients which hampers infection control in respect to identifying the source and implementing appropriate preventive measures.

Clostridium difficile infection (CDI) can occur when the normal protective intestinal microbiota is disrupted. Under these conditions, *C. difficile* can proliferate in the gut, causing an inflammatory response induced by the clostridial toxins, toxin A (TcdA) and toxin B (TcdB) [13]. Although the specific roles of these exotoxins are not entirely uncovered, direct effects on the integrity of the tight junctions of the colonic epithelium and apoptotic properties targeted against this tissue has been well described [14-17] and are associated with the main clinical manifestations of CDI: diarrhoea and colitis. Disruption of the colonic epithelium allows the toxins to interact with other cells such as immune cells and neurons and may stimulate, indirectly, the production of chemokines, pro-inflammatory cytokines, neuropeptides and other neuro-immune signals resulting in a systemic inflammation response [18].

Originally identified as part of the intestinal microbiota of healthy infants (by Hall and O'Toole in 1935 [19]), it took more than forty years to identify the causal relationship between *Clostridium difficile* and life-threatening pseudomembranous colitis [20,21]. Fatality of CDI infection has been dramatically demonstrated in the Stoke Mandeville Hospital outbreaks in the United Kingdom in 2004 and 2005 [22]. After recognition of European hospital outbreaks of *Clostridium difficile* infections (CDIs) associated with the emergence of PCR ribotype 027, surveillance at national level was encouraged by the European Centre for Disease Prevention and Control (ECDC) [23]. In the United States, the Center for Disease Control and Prevention (CDC) estimated on basis of a large population- and laboratory-based surveillance that the overall incidence of *C. difficile* infection in 2011 was 453.000 and 29.000 deaths were attributable to this infection [24]. Within the health care-associated population of CDI, the rate of first recurrence of infection was estimated at 20.9 percent (61.400 cases) [24], which is consistent with other reports (18-25 percent) [25-29]. In the US, the CDI incidence rose from 4.5 to 8.6 cases per 1.000 hospital discharges between 2001 and 2008. Furthermore, the overall mortality of CDI patients in the USA increased significantly from 6.5 to 7.2 percent in 2001-2010, with a total of 154.184 deaths (7.1 percent) during this time-period [30]. In a pilot study, conducted by the European *C. difficile* Infection Surveillance Network (ECDIS-NET) involving 37 European acute care hospitals, it was determined that the incidence rate of hospital-acquired CDI ranged from 0.6 to 18.5 per 10.000 patient days (median 3.7) [31]. It should be noted that the incidence rate in this study was based on aggregated hospital data. In the Netherlands, sentinel surveillance overseen by the National Reference Laboratory for *C. difficile* in 23 hospitals showed that the incidence rate per 10.000 patient days was 3.1 for the period of May 2015 to May 2016 [32]. Aside from the clinical implications, CDI also represents a substantial economic burden with estimated annual costs ranging from $ 5.4 billion [33] to $ 6.3 billion [34] in the US. The bulk of the economic burden of CDI consists of the costs of hospital-ization and recurrence of infection [29,35]. In the USA, the national annual cost of recurrent CDI is estimated at $ 1.5 billion [33]. The costs of CDI case management in Europe are difficult to determine due to heterogeneous methods [35,36]. In 2006, Kuijper *et al.* roughly estimated that the potential cost of CDI in Europe was €3 billion, a result which was extrapolated from the (estimated) annual cost of management of CDI in the United Kingdom [37]. Recently, in a multicentre study that was conducted in the UK it was estimated that the median total management cost for a first episode of CDI and recurrent CDI was approximately €7.100 and

€ 8.500, respectively[38]. Data is scarce on the costs of CDI case management in the Netherlands specifically. However, a retrospective cost analysis that was conducted to gain insight on the financial burden of an outbreak of *C. difficile* in a tertiary hospital in the Netherlands provided some much-needed information [39]. It was estimated that the costs attributed to this outbreak, involving 72 patients in a time-period of one year, was € 1.222.376 [39].

Recent years have seen an increase in the incidence and severity of *C. difficile* infections (CDI) in both the United States and Europe, due to the emergence of certain PCR ribotypes (RT) [13,40]. The increased incidence and severity of the disease are associated with outbreaks of 'hyper-virulent' *C. difficile* strains, particularly PCR ribotype 027 (also known as NAP1/027/BI) and PCR-ribotype 078 [37,41-44]. The epidemic PCR ribotype 027 was first recognized in Western Europe and North America [2] and emergence of this strain has been linked to fluoroquinolone resistance [45]. *C. difficile* strains of both these PCR-ribotypes cause severe CDI with high mortality rates, though infections with *C. difficile* RT 078, contrary to RT 027, are often located outside the hospital environment and affect younger patients [46-49].

Antibiotic use is a well-established risk factor for CDI, but age of the patient and/or underlying comorbidities play an important role in both aetiology and severity of the disease [2,13,50]. The antibiotic use increases the risk for CDI during therapy and in the period of 3 months after cessation of antibiotic therapy. The highest risk in contracting CDI after antibiotic therapy was found to be in the first month following cessation [51]. Antibiotics associated with CDI risk, such as clindamycin, broad spectrum penicillins, cephalosporins and fluoroquinolones significantly deplete the Gram-negative microflora and augment colonization of *C. difficile* [4,52,53]. Patients who are treated with these antibiotics for another infection are prone to infection with this opportunistic pathogen [4]. In most cases, patients develop antibiotic-associated diarrhoea (AAD). Although *C. difficile* is not the sole microorganism implicated in AAD, it is the most common causative agent of infectious antibiotic-associated diarrhoea, responsible for 10-25 percent of the cases [54,55] and the leading cause of nosocomial infectious diarrhoea in adults [56]. Other pathogens associated with infectious AAD are *Clostridium perfringens*, *Klebsiella oxytoca*, *Staphylococcus aureus*, and *Candida albicans* (approximately 40 percent of AAD, *C. difficile* included), though in a substantial number of cases, the causative agent remains unknown [4,57].

Antimicrobial treatment and resistance

Paradoxically, CDI is generally treated using antibiotics that simultaneously prohibit regeneration of the protective gut microflora, so infection may persist and relapses may be promoted. At present three antibiotics, metronidazole, vancomycin and fidaxomicin, are commonly used to treat CDI [58,59]. The drug of choice is dependent on the severity of CDI and the risk to develop a recurrent episode [53]. Metronidazole is often administered when the infection ranges from mild to moderate, as this antibiotic has a low cost and does not increase the risk for the development of vancomycin resistance in *C. difficile* or other microorganisms (*Enterococcus spp.*) as overuse of vancomycin might [2,4,53,60]. Oral vancomycin is often indicated when patients suffer from a severe or complicated CDI and is superior to treatment with metronidazole under these circumstances [2,60]. Recently, treatment with vancomycin has been increased due to decreasing costs, lower side effects and evidence that vancomycin is more efficacious in mild to moderate infections compared to metronidazole [59,61–64]. In case of recurrent disease and patients with high relapse risk, treatment with fidaxomicin is preferred over vancomycin [2,4,60]. Another advantage of fidaxomicin over the first-line therapies metronidazole and vancomycin is that the former antibiotic agent has a minimal impact on the microbiota of the host as it targets specific anaerobic Gram-positive bacteria [4,60]. Despite the superiority over vancomycin in preventing relapses of CDI, fidaxomicin is not yet prescribed on a large scale due to the high cost of this treatment [2,4,60,65,66].

Significant resistance to the standard antimicrobial therapy has not yet occurred in the clinic, although an increase of treatment failure associated with metronidazole has been observed [63,64,67,68] and sporadic resistance has been reported [69–72]. However, in light of the development of resistance to clindamycin and fluoroquinolones through their extensive use in the past, it is most probable that resistance to the standard therapy will arise over time [4]. Indeed, selective pressure induced by increased prescription of even a narrow-spectrum antibiotic as fidaxomicin may increase the risk of development of resistant *C. difficile* strains as has been shown *in vitro* [53,73]. It should be noted that the broad-spectrum antibiotics clindamycin and fluoroquinolones are not used to treat CDI but are commonly administered to resolve other bacterial infections [53]. Nonetheless, their impact on the integrity of the microbiota is a predisposing factor in the occurrence and recurrence of CDI [13,53].

Resistance to clindamycin and fluoroquinolones are exemplary for the extensive arsenal of antimicrobial resistance of *C. difficile* [53,74]. Multidrug resistant (MDR) strains of *C. difficile* are common [3,74,75] and resistance patterns among MDR strains are very diverse, as are the mechanisms that confer resistance. For instance, in a recent study conducted in the US, it has been shown that out of 139 clinical isolates from patients diagnosed with CDI almost 60 percent of strains were resistant to three types of antibiotics or more [75]. Fluoroquinolones are inhibitors of type II topoisomerases, which include gyrase and topoisomerase IV, enzymes essential for DNA replication. *C. difficile* lacks genes encoding topoisomerase IV but does contain *gyrA* and *gyrB* genes that encode subunits of the gyrase [76]. Resistance to fluoroquinolones can occur through point mutations in the quinolone-resistance determining region (QRDR) of the DNA gyrase subunits, GyrA and/or GyrB, that decrease the affinity for fluoroquinolones [77-79]. The most frequent amino acid substitution found in *C. difficile* is a threonine to isoleucine mutation in the GyrA subunit (Thr82Ile) [79]. Interestingly, this particular GyrA amino acid substitution is found in epidemic PCR ribotype 027 strains that have emerged in the beginning of this century but are not found in historical isolates of the same ribotype [74,76,80]. In contrast, changes in the pathogenicity locus (PaLoc) of *C. difficile* RT 027 previously hypothesized to account for the hyper-virulence and transmissibility of this particular ribotype were present in both pre- and post-epidemic isolated of RT 027. Thus, the acquisition of fluoroquinolone resistance marked a pivotal point in the evolution of RT 027 [45,81]. Despite its clear link to epidemicity in PCR ribotype 027 strains, fluoroquinolone resistance is not an exclusive trait of this ribotype: it is also common in other ribotypes [74,80]. Therefore, the emergence of the epidemic RT 027 strains is most likely multifactorial. In support of this view, in a recent study it was proposed that the use of the disaccharide trehalose in the food industry significantly contributed to the emergence of RT 027 strains [82]. Sensitivity to low concentrations of trehalose was attributed to a single point mutation in the trehalose repressor (*treR*), thereby creating a fitness advantage over several other ribotypes lacking this mutation [82]. Interestingly, another epidemic strain, RT 078, also showed enhanced growth in presence of low concentrations of trehalose, although the molecular basis for the increased sensitivity to this specific carbon source differed from RT 027 [82].

In contrast to fluoroquinolone resistance, which is mediated by a chromosomal resistance determinant and non-transferable, clindamycin resistance is acquired by horizontal gene transfer of the mobilizable non-conjugative transposon Tn*5398*,

which contains two copies of erythromycin ribosomal methylase (*erm*) genes of class B [83]. In *Clostridium difficile*, ribosomal methylation of bacterial 23S rRNA caused by the products of these genes, is the most common mechanism of resistance [74], resulting in prevention of antibiotic binding, and thus, antimicrobial activity [81]. Like clindamycin resistance, tetracycline resistance in *C. difficile* is acquired through horizontal gene transfer. Transposons linked with tetracycline resistance are Tn*5397*, Tn*916*, Tn*916*-like, and Tn*6164* [84-91]. Although these elements are capable of transferring a number of the *tet* class of genes (*tetM*, *tet44* and *tetW*), *tetM* is the predominant class identified in *C. difficile* [53]. The product of the *tetM* gene, the TetM ribosome protection protein, confers resistance through binding and displacing tetracycline from its binding site on the ribosome [92]. It is noteworthy that 11 percent of the genome of the *C. difficile* reference strain 630 is comprised of mobile genetic (i.e. horizontally acquired) elements [89].

The mechanisms described above, that are specific to a particular class of antimicrobials, are not the only ones capable of conferring resistance to fluoroquinolones and tetracyclines. In many bacteria, general mechanisms exist that confer resistance to multiple classes of antimicrobials [93]. For instance, the multidrug resistant chloramphenicol-florfenical resistance (*cfr*) gene may confer resistance to different classes of clinically relevant antibiotics [53,74,94,95]. A *cfr*-like gene was also identified in *C. difficile* [96] and it was established that the product of this gene can function as a legitimate Cfr protein [94]. Similarly, active drug efflux by ATP-binding cassette (ABC) transporters can contribute to resistance to different classes of antimicrobials [97,98] and a recent study demonstrated that the ABC transporter CD2068 of *C. difficile* could potentially function as multidrug efflux transporter [99]. Moreover, the highly resistant endospores of *C. difficile* are intrinsically resistant to many antimicrobials, due to metabolic dormancy [4,100,101]. Clearly, *C. difficile* has developed multiple mechanisms to avoid the activity of many classes of antimicrobials [53,99].

The extensive arsenal of resistance mechanisms alone already underscores the need for development of new therapeutic options. But there are other reasons for pushing the development of novel antimicrobials against CDI as well. First, metronidazole and vancomycin are both broad-spectrum antibiotics targeting not only *Clostridium difficile*, but also certain bacteria that are part of the protective microbiota. Dysbiosis of the microbiota caused by these antibiotics may perpetuate CDI [13].

This may at least partly explain why the average cure rate achieved by the standard therapy recommended for CDI does not exceed 80-90 percent [102]. Other underlying causes that may lead to suboptimal results in terms of clinical cure and prevention of relapse in relation to the standard therapies are the spore-forming ability of *C. difficile* and the altered immunity of CDI patients [103]. An additional concern is that vancomycin-use leads to increased prevalence of vancomycin-resistant enterococci (VRE), other important nosocomial pathogens [104], and/or result in VRE overgrowth. Using narrow-spectrum antimicrobials limits these off-target effects and thus contributes to both the integrity of the microbiota and reduces the development and spread of antimicrobial resistance in other bacteria. Second, prescribing fidaxomicin instead of the first-line antibiotics is still considered as not favourable, in terms of cost-effectiveness [13,105]. Third, it has been reported that although administering fidaxomicin leads to a reduction of the relapse rate, sustained cure may be limited to 75 percent among treated patients who have experienced multiple CDI recurrences [103].

Novel drugs for CDI targeting DNA replication

Evidently, there is an urgent demand for more efficient therapies and tools to combat CDI [103]. In recent years, tremendous efforts have been made by pharmaceutical industry and academia to develop new treatments for CDI, which are directed at the various stages of infection [105]. The latter is reflected in diversity the strategies that have been employed such as, microbiologic approaches for treatment of CDI (faecal microbiotica transplantation (FMT), microbiota supplements), non-microbiologic approaches for CDI treatment and prevention (passive immunization and vaccination) and antibiotic inactivation for CDI prevention and alternative antibiotics (new antimicrobials, off-label and derivatives) [60,62,65,103,105-107]. However, drug discovery and development of new antibiotics against *C. difficile* is challenging in terms of the characteristics that have to be met [108]. Ideally, the new antimicrobial agents should possess specific physiochemical properties, such as low solubility, high molecular weight, high polarity and low permeability and absorption, coupled with the ability to withstand excretion by efflux pumps [108]. Furthermore, the spectrum of activity should be narrow to safeguard the integrity of the normal gut flora. As the 'one drug-one target' model has proven its limited viability in regard to emergence of resistance and recurrence, novel targets and mechanisms of action

should be explored [108,109]. Additionally, new agents should be superior or at least non-inferior to the standard therapy, in terms of efficacy and tolerability, and should display decreased permeability at the site of action [108]. Finally, the drug acquisition cost of the new agent should be reasonable, so its use will not be limited due to high pricing. Promising candidates (such as ramoplanin, cadazolid, ridinazole, and tigecycline) are in different stages of development and reviewed extensively [59,60,65,103,105,106]. Hereafter, a few interesting compounds with direct relevance for this thesis are highlighted.

Cadazolid (Actelion Pharmaceuticals) has dual mode of action directed at the 50S ribosome unit and DNA gyrase due to its chimeric structure with quinolonyl and oxazolidinone moieties [65,103,105]. This antibiotic has received a fast track status by the FDA to accelerate its development but has shown no superiority over vancomycin [110].

Another FQ-hybrid antibiotic under investigation is MBX-500 (Microbiotix) [111], which consists of a FQ-pharmacophore covalently linked to anilinouracil (AU) component [112]. Mechanistically, MBX-500 functions as a dual gyrase/topoisomerase inhibitor and DNA polymerase inhibitor [103,108]. Although *in vivo* efficacy has been demonstrated in several animal studies [111,113], no data is available for efficacy in humans [103].

The compound 362E exerts it action on DNA polymerase in a similar fashion as the AU component of MBX-500, though belongs to another class of PolC inhibitors: both inhibitors bind via specific domains to the DNA strand and DNA polymerase simultaneously leading to the formation of an inert ternary complex [114,115]. The antimicrobial activities of 362E were similar to those of the standard therapy, when tested in a small panel of *C. difficile* strains [116]. Furthermore, the specificity of 362E regarding *C. difficile* towards purified *C. difficile* PolC [115] was supported by the results of agar dilution data, where it demonstrated lower activity against the majority of a modest collection of Gram-positive anaerobes [116]. Currently, this compound is still in the phase of preclinical development.

The common factor between the agents discussed above is that they are – at least partially – directed against DNA replication proteins (**Figure 1**). DNA replication is the process in which a mother cell duplicates its DNA semi-conservatively, to ensure that upon division both daughter cells contain the same genetic information.

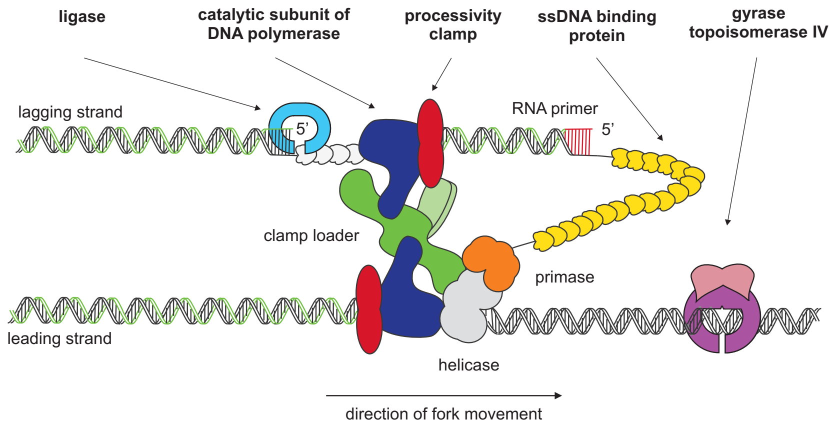

Figure 1. Schematic representation of the bacterial replisome and accessory replication proteins

Indicated is the core of the replisome and the other accessory replication proteins. For simplicity, replication initiation proteins and regulators have been omitted from this figure.

By and large, the mechanisms of DNA replication are conserved across all domains of life. Faithfull replication of the bacterial DNA is an essential requirement for viability of bacterial cells, and stringent coordination of proteins involved in this process is needed to accomplish this. In contrast to *C. difficile*, DNA replication has been extensively studied in the non-pathogenic Gram-positive model bacterium *Bacillus subtilis* (*B. subtilis*), which is a high A+T content Firmicute, like *C. difficile* [117-119]. The process of DNA replication can be divided in an initiation and an elongation phase. In this section, the description of the function of proteins involved in these particular phases is based on extensive work done in *B. subtilis*.

In most organisms, DNA replication starts by binding of a replication initiator protein at a specific chromosomal location. In bacteria with a single chromosome, such as *B. subtilis*, this protein is the highly conserved DnaA [120], and the chromosomal location is the origin of replication, *oriC*. DnaA binds to specific DnaA boxes in the *oriC* where it melts and unwinds the double-stranded (ds) DNA helix and recruits the replicative DNA helicase and other proteins [120-123]. Subsequently, the replicative helicase DnaC is loaded onto ssDNA by three proteins: DnaI, DnaB and

DnaD, which are all proven to be essential for DNA replication [124]. DnaI is the loader ATPase and is responsible for the assembly of the helicase hexamer on single-stranded (ss) DNA [124]. DnaI, DnaB and DnaD proteins are conserved in many, but not all, low G+C Gram-positive bacteria [118]. The replicative DNA helicase, encoded by the *dnaC* gene in *B. subtilis*, is responsible for unwinding of the DNA helix at the replication fork and is conserved in all prokaryotes [118,125,126]. As *B. subtilis* contains one chromosome, two replication forks are assembled at the singular origin of replication which move bi-directionally [127]. For lagging strand DNA synthesis, short RNA primers have to be synthesized by primase (DnaG) [128] that are extended by DNA polymerase into Okazaki fragments. *B. subtilis* primase interacts with the replicative DNA helicase and is thought to form a complex that modulates primase activity [129,130]. Loading of the helicase marks the start of the elongation phase, in which the leading and lagging DNA templates are replicated. This highly progressive process is carried out by the DNA polymerase III holoenzyme. The main subunits in the *B. subtilis* PolIII complex are two α subunits; PolC and DnaE. Both polymerases are essential for DNA replication in *B. subtilis*, and also for cell viability [131]. PolC possesses a proofreading exonuclease domain and is responsible for rapid elongation of both the leading strand and the lagging strand [124,131]. In contrast, *subtilis* DnaE is an inefficient and error prone polymerase, which is indispensable for synthesis of the lagging strand but not for leading strand synthesis [124,132]. The combination of PolC and DnaE is not found in Gram-negative bacteria, as they do not possess PolC [133]. Other subunits of the *B. subtilis* polymerase III holoenzyme are the β-clamp, τ (and γ) subunit, δ subunit and the δ' subunit encoded by *dnaN*, *dnaX*, *holA* and *holB* respectively. The β-clamp encircles primed DNA strands and is able to slide across the strands. It intimately links the DNA polymerases to the DNA template, thereby ensuring the high processivity of DNA replication [132,134]. Loading of the β-clamp on DNA depends on initiation of Okazaki fragment synthesis and the actual loading is performed by the clamp-loader complex, which in *B. subtilis* consists of a τ trimer, a δ and δ' monomer [129,130,135]. The β-clamp and the τ/γ subunits are conserved in most bacteria, while the δ and δ' subunits are more variable [118]. The whole complex responsible for DNA synthesis (that includes one or more DNA polymerases, proofreading enzymes and factors ensuring processivity such as the beta-clamp) is commonly termed replisome (**Figure 1**).

Other accessory proteins are also important for DNA replication, including DNA ligase, single-stranded DNA-binding protein (SSB) and DNA gyrase. The primary

function of DNA ligase is to catalyse the joining of breaks in duplex DNA that are left as a result of the discontinuous lagging strand synthesis, repair or recombination [136]. Disruption of the gene encoding ligase is detrimental for cell growth in *B. subtilis* and therefore considered to be essential [136]. SSB maintains and protects the ssDNA strands produced by the replicative helicase [137]. The unwinding and reannealing of double stranded DNA can result in topological problems that are resolved by topoisomerases. DNA gyrase is a topoisomerase type II and its main function is the introduction of negative supercoils that relax the DNA helix ahead of the replication fork [138]. Therefore, it is essential for the progression of the replication machinery, and thus for process of DNA replication as a whole.

Though this overall mechanism of DNA replication is conserved, details differ substantially between organisms, with respect to the proteins performing the various functions [117], the molecular mechanisms [139] and the way their activity is regulated [129,140-147].

Due to their essential nature, proteins involved in DNA replication are attractive targets for the development of antimicrobials. However, to date, none of the antimicrobials used in the clinical setting, target the main components of the DNA replication machinery [148] and topoisomerase II is the only replication-associated protein that is targeted by commonly used therapeutics. The development of new therapeutics is hindered by a limited characterization of the replication machinery of bacterial pathogens, such as *C. difficile*.

Outline of this thesis

Chapter 1 is this introduction.

Chapter 2 provides an overview of DNA replication proteins that potentially may serve as targets for antimicrobials in drug-resistant pathogens and includes the *in silico* identification of the replication proteins of *C. difficile*. Additionally, the mode of action and the current developmental status of the compounds directed at the core replication machinery and accessory replication proteins are discussed.

In **chapter 3** we determined the complete genome sequence of our reference laboratory strain, *C. difficile* 630Δ*erm*, and compared this sequence to the sequence of *C. difficile* 630. This revealed an unexpected transposition of the mobile genetic element CTn*5*. The genome sequence now offers an appropriate reference for all molecular work on this strain.

Apart from the identification and characterization of gyrase and DNA polymerase in previous studies, no information on DNA replication proteins or the mechanism of replication was available. To address this hiatus, we sought out to identify core replication proteins and to experimentally validate the functional role of these proteins. The findings of this experimental work**,** with a focus on helicase and primase, are presented in **chapter 4** and **chapter 5**, respectively.

In **chapter 6**, we tested the antimicrobial activity of the DNA polymerase inhibitor 362E against a large and diverse panel of clinical *C. difficile* isolates. Furthermore, we have determined the transcriptional response of *C. difficile* to replication inhibition by 362E.

Finally, in **chapter 7**, we place our findings in a broader perspective and provide an outlook for the field.

References

1. Hensgens, M. P. *et al.* Clostridium difficile infection in the community: a zoonotic disease? *Clin Microbiol Infect* **18**, 635-645, doi:10.1111/j.1469-0691.2012.03853.x (2012).
2. Leffler, D. A. & Lamont, J. T. Clostridium difficile Infection. *N Engl J Med* **373**, 287-288, doi:10.1056/NEJMc1506004 (2015).
3. Spigaglia, P., Barbanti, F., Morandi, M., Moro, M. L. & Mastrantonio, P. Diagnostic testing for Clostridium difficile in Italian microbiological laboratories. *Anaerobe* **37**, 29-33, doi:10.1016/j.anaerobe.2015.11.002 (2016).
4. Johanesen, P. A. *et al.* Disruption of the Gut Microbiome: Clostridium difficile Infection and the Threat of Antibiotic Resistance. *Genes (Basel)* **6**, 1347-1360, doi:10.3390/genes6041347 (2015).
5. Katzianer, D. S., Yano, T., Rubin, H. & Zhu, J. A high-throughput small-molecule screen to identify a novel chemical inhibitor of Clostridium difficile. *Int J Antimicrob Agents* **44**, 69-73, doi:10.1016/j.ijantimicag.2014.03.007 (2014).
6. Gerding, D. N., Muto, C. A. & Owens, R. C., Jr. Measures to control and prevent Clostridium difficile infection. *Clin Infect Dis* **46 Suppl 1**, S43-49, doi:10.1086/521861 (2008).
7. Lawley, T. D. *et al.* Use of purified Clostridium difficile spores to facilitate evaluation of health care disinfection regimens. *Appl Environ Microbiol* **76**, 6895-6900, doi:10.1128/AEM.00718-10 (2010).
8. Weber, D. J., Rutala, W. A., Miller, M. B., Huslage, K. & Sickbert-Bennett, E. Role of hospital surfaces in the transmission of emerging health care-associated pathogens: norovirus, Clostridium difficile, and Acinetobacter species. *Am J Infect Control* **38**, S25-33, doi:10.1016/j.ajic.2010.04.196 (2010).
9. Maillard, J. Y. Innate resistance to sporicides and potential failure to decontaminate. *Journal of Hospital Infection* **77**, 204-209, doi:10.1016/j.jhin.2010.06.028 (2011).
10. Rutala, W. A., Gergen, M. F. & Weber, D. J. Efficacy of different cleaning and disinfection methods against Clostridium difficile spores: importance of physical removal versus sporicidal inactivation. *Infect Control Hosp Epidemiol* **33**, 1255-1258, doi:10.1086/668434 (2012).
11. Fekety, R. *et al.* Epidemiology of antibiotic-associated colitis; isolation of Clostridium difficile from the hospital environment. *Am J Med* **70**, 906-908 (1981).
12. Claro, T., Daniels, S. & Humphreys, H. Detecting Clostridium difficile spores from inanimate surfaces of the hospital environment: which method is best? *J Clin Microbiol* **52**, 3426-3428, doi:10.1128/JCM.01011-14 (2014).
13. Smits, W. K., Lyras, D., Lacy, D. B., Wilcox, M. H. & Kuijper, E. J. Clostridium difficile infection. *Nat Rev Dis Primers* **2**, 16020, doi:10.1038/nrdp.2016.20 (2016).
14. Hecht, G., Koutsouris, A., Pothoulakis, C., LaMont, J. T. & Madara, J. L. Clostridium difficile toxin B disrupts the barrier function of T84 monolayers. *Gastroenterology* **102**, 416-423 (1992).
15. Hecht, G., Pothoulakis, C., LaMont, J. T. & Madara, J. L. Clostridium difficile toxin A perturbs cytoskeletal structure and tight junction permeability of cultured human intestinal epithelial monolayers. *J Clin Invest* **82**, 1516-1524, doi:10.1172/JCI113760 (1988).
16. Aktories, K. & Just, I. Monoglucosylation of low-molecular-mass GTP-binding Rho proteins by clostridial cytotoxins. *Trends Cell Biol* **5**, 441-443 (1995).
17. Feltis, B. A. *et al.* Clostridium difficile toxins A and B can alter epithelial permeability and promote bacterial paracellular migration through HT-29 enterocytes. *Shock* **14**, 629-634 (2000).
18. Sun, X., Savidge, T. & Feng, H. The enterotoxicity of Clostridium difficile toxins. *Toxins (Basel)* **2**, 1848-1880, doi:10.3390/toxins2071848 (2010).

1

19 Hall, I. C. & O'Toole, E. Intestinal flora in new-born infants: With a description of a new pathogenic anaerobe, bacillus difficilis. *American Journal of Diseases of Children* **49**, 390-402, doi:10.1001/archpedi.1935.01970020105010 (1935).

20 George, R. H. *et al.* Identification of Clostridium difficile as a cause of pseudomembranous colitis. *Br Med J* **1**, 695 (1978).

21 George, W. L., Sutter, V. L., Goldstein, E. J., Ludwig, S. L. & Finegold, S. M. Aetiology of antimicrobial-agent-associated colitis. *Lancet* **1**, 802-803 (1978).

22 Inspection, C. f. H. A. a. Investigation into outbreaks of Clostridium difficile at Stoke Mandeville Hospital, Buckinghamshire Hospitals NHS Trust. (2006).

23 Kuijper, E. J. *et al.* Update of Clostridium difficile-associated disease due to PCR ribotype 027 in Europe. *Euro Surveill* **12**, E1-2 (2007).

24 Lessa, F. C. *et al.* Burden of Clostridium difficile infection in the United States. *N Engl J Med* **372**, 825-834, doi:10.1056/NEJMoa1408913 (2015).

25 Deshpande, A. *et al.* Risk factors for recurrent Clostridium difficile infection: a systematic review and meta-analysis. *Infect Control Hosp Epidemiol* **36**, 452-460, doi:10.1017/ice.2014.88 (2015).

26 Louie, T. J. *et al.* Fidaxomicin versus vancomycin for Clostridium difficile infection. *N Engl J Med* **364**, 422-431, doi:10.1056/NEJMoa0910812 (2011).

27 Lowy, I. *et al.* Treatment with monoclonal antibodies against Clostridium difficile toxins. *N Engl J Med* **362**, 197-205, doi:10.1056/NEJMoa0907635 (2010).

28 Rodriguez-Pardo, D. *et al.* Epidemiology of Clostridium difficile infection and risk factors for unfavorable clinical outcomes: results of a hospital-based study in Barcelona, Spain. *J Clin Microbiol* **51**, 1465-1473, doi:10.1128/JCM.03352-12 (2013).

29 Zhang, D., Prabhu, V. S. & Marcella, S. W. Attributable Healthcare Resource Utilization and Costs for Patients With Primary and Recurrent Clostridium difficile Infection in the United States. *Clin Infect Dis* **66**, 1326-1332, doi:10.1093/cid/cix1021 (2018).

30 Reveles, K. R., Lee, G. C., Boyd, N. K. & Frei, C. R. The rise in Clostridium difficile infection incidence among hospitalized adults in the United States: 2001-2010. *Am J Infect Control* **42**, 1028-1032, doi:10.1016/j.ajic.2014.06.011 (2014).

31 van Dorp, S. M. *et al.* Standardised surveillance of Clostridium difficile infection in European acute care hospitals: a pilot study, 2013. *Euro Surveill* **21**, doi:10.2807/1560-7917.ES.2016.21.29.30293 (2016).

32 Tenth Annual Report of the National Reference Laboratory for Clostridium difficile and results of the sentinel surveillance, May 2015- May 2016 <https://www.rivm.nl/Documenten_en_publicaties/Algemeen_Actueel/Uitgaven/Infectieziekten/CDiffNL/Tenth_Annual_Report_of_the_National_Reference_Laboratory_for_Clostridium_difficile_and_results_of_the_sentinel_surveillance> (2016).

33 Desai, K. *et al.* Epidemiological and economic burden of Clostridium difficile in the United States: estimates from a modeling approach. *BMC Infect Dis* **16**, 303, doi:10.1186/s12879-016-1610-3 (2016).

34 Zhang, S. *et al.* Cost of hospital management of Clostridium difficile infection in United States-a meta-analysis and modelling study. *BMC Infect Dis* **16**, 447, doi:10.1186/s12879-016-1786-6 (2016).

35 Heimann, S. M., Cruz Aguilar, M. R., Mellinghof, S. & Vehreschild, M. Economic burden and cost-effective management of Clostridium difficile infections. *Med Mal Infect* **48**, 23–29, doi:10.1016/j.medmal.2017.10.010 (2018).

36 Nanwa, N. *et al.* The economic impact of Clostridium difficile infection: a systematic review. *Am J Gastroenterol* **110**, 511–519, doi:10.1038/ajg.2015.48 (2015).

37 Kuijper, E. J. *et al.* Emergence of Clostridium difficile-associated disease in North America and Europe. *Clin Microbiol Infect* **12 Suppl 6**, 2–18, doi:10.1111/j.1469-0691.2006.01580.x (2006).

38 Wilcox, M. H. *et al.* Impact of recurrent Clostridium difficile infection: hospitalization and patient quality of life. *J Antimicrob Chemother* **72**, 2647–2656, doi:10.1093/jac/dkx174 (2017).

39 van Beurden, Y. H. *et al.* Cost analysis of an outbreak of Clostridium difficile infection ribotype 027 in a Dutch tertiary care centre. *J Hosp Infect* **95**, 421–425, doi:10.1016/j.jhin.2016.12.019 (2017).

40 Bauer, M. P. *et al.* Clostridium difficile infection in Europe: a hospital-based survey. *Lancet* **377**, 63–73, doi:10.1016/S0140-6736(10)61266-4 (2011).

41 Clements, A. C., Magalhaes, R. J., Tatem, A. J., Paterson, D. L. & Riley, T. V. Clostridium difficile PCR ribotype 027: assessing the risks of further worldwide spread. *Lancet Infect Dis* **10**, 395–404, doi:10.1016/S1473-3099(10)70080-3 (2010).

42 Goorhuis, A. *et al.* Spread and epidemiology of Clostridium difficile polymerase chain reaction ribotype 027/toxinotype III in The Netherlands. *Clin Infect Dis* **45**, 695–703, doi:10.1086/520984 (2007).

43 Kuijper, E. J. *et al.* Update of Clostridium difficile infection due to PCR ribotype 027 in Europe, 2008. *Euro Surveill* **13** (2008).

44 Pepin, J. *et al.* Clostridium difficile-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. *CMAJ* **171**, 466–472, doi:10.1503/cmaj.1041104 (2004).

45 He, M. *et al.* Emergence and global spread of epidemic healthcare-associated Clostridium difficile. *Nat Genet* **45**, 109–113, doi:10.1038/ng.2478 (2013).

46 Goorhuis, A. *et al.* Clostridium difficile PCR Ribotype 078: an Emerging Strain in Humans and in Pigs? *J Clin Microbiol* **46**, 1157–1158, doi:10.1128/jcm.01536-07 (2008).

47 McDonald, L. C. Clostridium difficile: responding to a new threat from an old enemy. *Infect Control Hosp Epidemiol* **26**, 672–675, doi:10.1086/502600 (2005).

48 Patterson, L. *et al.* Morbidity and mortality associated with Clostridium difficile ribotype 078: a case-case study. *J Hosp Infect* **82**, 125–128, doi:10.1016/j.jhin.2012.07.011 (2012).

49 Pepin, J., Valiquette, L. & Cossette, B. Mortality attributable to nosocomial Clostridium difficile-associated disease during an epidemic caused by a hypervirulent strain in Quebec. *CMAJ* **173**, 1037–1042, doi:10.1503/cmaj.050978 (2005).

50 Abt, M. C., McKenney, P. T. & Pamer, E. G. Clostridium difficile colitis: pathogenesis and host defence. *Nat Rev Microbiol* **14**, 609–620, doi:10.1038/nrmicro.2016.108 (2016).

51 Hensgens, M. P., Goorhuis, A., Dekkers, O. M. & Kuijper, E. J. Time interval of increased risk for Clostridium difficile infection after exposure to antibiotics. *J Antimicrob Chemother* **67**, 742–748, doi:10.1093/jac/dkr508 (2012).

52 Davies, J. & Davies, D. Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev* **74**, 417–433, doi:10.1128/MMBR.00016-10 (2010).

1

53 Peng, Z. *et al.* Update on Antimicrobial Resistance in Clostridium difficile: Resistance Mechanisms and Antimicrobial Susceptibility Testing. *J Clin Microbiol* **55**, 1998-2008, doi:10.1128/JCM.02250-16 (2017).

54 Bartlett, J. G. Clostridium difficile: history of its role as an enteric pathogen and the current state of knowledge about the organism. *Clin Infect Dis* **18 Suppl 4**, S265-272 (1994).

55 Bartlett, J. G. & Gerding, D. N. Clinical recognition and diagnosis of Clostridium difficile infection. *Clin Infect Dis* **46 Suppl 1**, S12-18, doi:10.1086/521863 (2008).

56 Kelly, C. P. & LaMont, J. T. Clostridium difficile—more difficult than ever. *N Engl J Med* **359**, 1932-1940, doi:10.1056/NEJMra0707500 (2008).

57 McFarland, L. V. Update on the changing epidemiology of Clostridium difficile-associated disease. *Nat Clin Pract Gastroenterol Hepatol* **5**, 40-48, doi:10.1038/ncpgasthep1029 (2008).

58 Johnson, A. P. & Wilcox, M. H. Fidaxomicin: a new option for the treatment of Clostridium difficile infection. *J Antimicrob Chemother* **67**, 2788-2792, doi:10.1093/jac/dks302 (2012).

59 Ooijevaar, R. E. *et al.* Update of treatment algorithms for Clostridium difficile infection. *Clin Microbiol Infect* **24**, 452-462, doi:10.1016/j.cmi.2017.12.022 (2018).

60 Martin, J. & Wilcox, M. New and emerging therapies for Clostridium difficile infection. *Curr Opin Infect Dis* **29**, 546-554, doi:10.1097/QCO.0000000000000320 (2016).

61 Cohen, S. H. *et al.* Clinical practice guidelines for Clostridium difficile infection in adults: 2010 update by the society for healthcare epidemiology of America (SHEA) and the infectious diseases society of America (IDSA). *Infect Control Hosp Epidemiol* **31**, 431-455, doi:10.1086/651706 (2010).

62 Debast, S. B., Bauer, M. P., Kuijper, E. J., European Society of Clinical, M. & Infectious, D. European Society of Clinical Microbiology and Infectious Diseases: update of the treatment guidance document for Clostridium difficile infection. *Clin Microbiol Infect* **20 Suppl 2**, 1-26, doi:10.1111/1469-0691.12418 (2014).

63 Johnson, S. *et al.* Vancomycin, metronidazole, or tolevamer for Clostridium difficile infection: results from two multinational, randomized, controlled trials. *Clin Infect Dis* **59**, 345-354, doi:10.1093/cid/ciu313 (2014).

64 Pepin, J., Valiquette, L., Gagnon, S., Routhier, S. & Brazeau, I. Outcomes of Clostridium difficile-associated disease treated with metronidazole or vancomycin before and after the emergence of NAP1/027. *Am J Gastroenterol* **102**, 2781-2788, doi:10.1111/j.1572-0241.2007.01539.x (2007).

65 Roshan, N., Hammer, K. A. & Riley, T. V. Non-conventional antimicrobial and alternative therapies for the treatment of Clostridium difficile infection. *Anaerobe* **49**, 103-111, doi:10.1016/j.anaerobe.2018.01.003 (2018).

66 Surawicz, C. M. *et al.* Guidelines for diagnosis, treatment, and prevention of Clostridium difficile infections. *Am J Gastroenterol* **108**, 478-498; quiz 499, doi:10.1038/ajg.2013.4 (2013).

67 Musher, D. M. *et al.* Clostridium difficile colitis that fails conventional metronidazole therapy: response to nitazoxanide. *J Antimicrob Chemother* **59**, 705-710, doi:10.1093/jac/dkl553 (2007).

68 Musher, D. M., Nuila, F. & Logan, N. The long-term outcome of treatment of Clostridium difficile colitis. *Clin Infect Dis* **45**, 523-524, doi:10.1086/520008 (2007).

69 Barbut, F. *et al.* Antimicrobial susceptibilities and serogroups of clinical strains of Clostridium difficile isolated in France in 1991 and 1997. *Antimicrob Agents Chemother* **43**, 2607-2611 (1999).

70 Brazier, J. S., Fawley, W., Freeman, J. & Wilcox, M. H. Reduced susceptibility of Clostridium difficile to metronidazole. *J Antimicrob Chemother* **48**, 741-742 (2001).

71 Indra, A. *et al.* First isolation of Clostridium difficile PCR ribotype 027 in Austria. *Euro Surveill* **11**, E060914 060913 (2006).

72 Pelaez, T. *et al.* Metronidazole resistance in Clostridium difficile is heterogeneous. *J Clin Microbiol* **46**, 3028-3032, doi:10.1128/JCM.00524-08 (2008).

73 Leeds, J. A., Sachdeva, M., Mullin, S., Barnes, S. W. & Ruzin, A. In vitro selection, via serial passage, of Clostridium difficile mutants with reduced susceptibility to fidaxomicin or vancomycin. *J Antimicrob Chemother* **69**, 41-44, doi:10.1093/jac/dkt302 (2014).

74 Spigaglia, P. Recent advances in the understanding of antibiotic resistance in Clostridium difficile infection. *Therapeutic Advances in Infectious Disease* **3**, 23-42, doi:10.1177/2049936115622891 (2016).

75 Peng, Z., Addisu, A., Alrabaa, S. & Sun, X. Antibiotic Resistance and Toxin Production of Clostridium difficile Isolates from the Hospitalized Patients in a Large Hospital in Florida. *Front Microbiol* **8**, 2584, doi:10.3389/fmicb.2017.02584 (2017).

76 Dridi, L., Tankovic, J., Burghoffer, B., Barbut, F. & Petit, J. C. gyrA and gyrB mutations are implicated in cross-resistance to Ciprofloxacin and moxifloxacin in Clostridium difficile. *Antimicrob Agents Chemother* **46**, 3418-3421 (2002).

77 Ackermann, G. *et al.* Resistance to moxifloxacin in toxigenic Clostridium difficile isolates is associated with mutations in gyrA. *Antimicrob Agents Chemother* **45**, 2348-2353, doi:10.1128/AAC.45.8.2348-2353.2001 (2001).

78 Drudy, D., Kyne, L., O'Mahony, R. & Fanning, S. gyrA mutations in fluoroquinolone-resistant Clostridium difficile PCR-027. *Emerg Infect Dis* **13**, 504-505, doi:10.3201/eid1303.060771 (2007).

79 Spigaglia, P., Barbanti, F., Louie, T., Barbut, F. & Mastrantonio, P. Molecular analysis of the gyrA and gyrB quinolone resistance-determining regions of fluoroquinolone-resistant Clostridium difficile mutants selected in vitro. *Antimicrob Agents Chemother* **53**, 2463-2468, doi:10.1128/AAC.01252-08 (2009).

80 Spigaglia, P. *et al.* Fluoroquinolone resistance in Clostridium difficile isolates from a prospective study of C. difficile infections in Europe. *J Med Microbiol* **57**, 784-789, doi:10.1099/jmm.0.47738-0 (2008).

81 Baines, S. D. & Wilcox, M. H. Antimicrobial Resistance and Reduced Susceptibility in Clostridium difficile: Potential Consequences for Induction, Treatment, and Recurrence of C. difficile Infection. *Antibiotics (Basel)* **4**, 267-298, doi:10.3390/antibiotics4030267 (2015).

82 Collins, J. *et al.* Dietary trehalose enhances virulence of epidemic Clostridium difficile. *Nature* **553**, 291-294, doi:10.1038/nature25178 (2018).

83 Farrow, K. A., Lyras, D. & Rood, J. I. Genomic analysis of the erythromycin resistance element Tn5398 from Clostridium difficile. *Microbiology* **147**, 2717-2728, doi:10.1099/00221287-147-10-2717 (2001).

84 Brouwer, M. S., Roberts, A. P., Mullany, P. & Allan, E. In silico analysis of sequenced strains of Clostridium difficile reveals a related set of conjugative transposons carrying a variety of accessory genes. *Mob Genet Elements* **2**, 8-12, doi:10.4161/mge.19297 (2012).

85 Brouwer, M. S., Warburton, P. J., Roberts, A. P., Mullany, P. & Allan, E. Genetic organisation, mobility and predicted functions of genes on integrated, mobile genetic elements in sequenced strains of Clostridium difficile. *PLoS One* **6**, e23014, doi:10.1371/journal.pone.0023014 (2011).

1

86 Corver, J. *et al.* Analysis of a Clostridium difficile PCR ribotype 078 100 kilobase island reveals the presence of a novel transposon, Tn6164. *BMC Microbiol* **12**, 130, doi:10.1186/1471-2180-12-130 (2012).

87 Roberts, A. P., Johanesen, P. A., Lyras, D., Mullany, P. & Rood, J. I. Comparison of Tn5397 from Clostridium difficile, Tn916 from Enterococcus faecalis and the CW459tet(M) element from Clostridium perfringens shows that they have similar conjugation regions but different insertion and excision modules. *Microbiology* **147**, 1243-1251, doi:10.1099/00221287-147-5-1243 (2001).

88 Roberts, A. P. & Mullany, P. Tn916-like genetic elements: a diverse group of modular mobile elements conferring antibiotic resistance. *FEMS Microbiol Rev* **35**, 856-871, doi:10.1111/j.1574-6976.2011.00283.x (2011).

89 Sebaihia, M. *et al.* The multidrug-resistant human pathogen Clostridium difficile has a highly mobile, mosaic genome. *Nat Genet* **38**, 779-786, doi:10.1038/ng1830 (2006).

90 Spigaglia, P., Barbanti, F. & Mastrantonio, P. New variants of the tet(M) gene in Clostridium difficile clinical isolates harbouring Tn916-like elements. *J Antimicrob Chemother* **57**, 1205-1209, doi:10.1093/jac/dkl105 (2006).

91 Spigaglia, P., Carucci, V., Barbanti, F. & Mastrantonio, P. ErmB determinants and Tn916-Like elements in clinical isolates of Clostridium difficile. *Antimicrob Agents Chemother* **49**, 2550-2553, doi:10.1128/AAC.49.6.2550-2553.2005 (2005).

92 Arenz, S., Nguyen, F., Beckmann, R. & Wilson, D. N. Cryo-EM structure of the tetracycline resistance protein TetM in complex with a translating ribosome at 3.9-A resolution. *Proc Natl Acad Sci U S A* **112**, 5401-5406, doi:10.1073/pnas.1501775112 (2015).

93 Blair, J. M., Webber, M. A., Baylay, A. J., Ogbolu, D. O. & Piddock, L. J. Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol* **13**, 42-51, doi:10.1038/nrmicro3380 (2015).

94 Hansen, L. H. & Vester, B. A cfr-like gene from Clostridium difficile confers multiple antibiotic resistance by the same mechanism as the cfr gene. *Antimicrob Agents Chemother* **59**, 5841-5843, doi:10.1128/AAC.01274-15 (2015).

95 Long, K. S., Poehlsgaard, J., Kehrenberg, C., Schwarz, S. & Vester, B. The Cfr rRNA methyltransferase confers resistance to Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin A antibiotics. *Antimicrob Agents Chemother* **50**, 2500-2505, doi:10.1128/AAC.00131-06 (2006).

96 Schwarz, S. & Wang, Y. Nomenclature and functionality of the so-called cfr gene from Clostridium difficile. *Antimicrob Agents Chemother* **59**, 2476-2477, doi:10.1128/AAC.04893-14 (2015).

97 Lubelski, J., Konings, W. N. & Driessen, A. J. Distribution and physiology of ABC-type transporters contributing to multidrug resistance in bacteria. *Microbiol Mol Biol Rev* **71**, 463-476, doi:10.1128/MMBR.00001-07 (2007).

98 Putman, M., Van Veen, H. W., Degener, J. E. & Konings, W. N. Antibiotic resistance: era of the multidrug pump. *Mol Microbiol* **36**, 772-773 (2000).

99 Ngernsombat, C., Sreesai, S., Harnvoravongchai, P., Chankhamhaengdecha, S. & Janvilisri, T. CD2068 potentially mediates multidrug efflux in Clostridium difficile. *Sci Rep* **7**, 9982, doi:10.1038/s41598-017-10155-x (2017).

100 Awad, M. M., Johanesen, P. A., Carter, G. P., Rose, E. & Lyras, D. Clostridium difficile virulence factors: Insights into an anaerobic spore-forming pathogen. *Gut Microbes* **5**, 579-593, doi:10.4161/19490976.2014.969632 (2014).

101 Smits, W. K. Hype or hypervirulence: a reflection on problematic C. difficile strains. *Virulence* **4**, 592–596, doi:10.4161/viru.26297 (2013).

102 Cornely, O. A. *et al.* Clinical efficacy of fidaxomicin compared with vancomycin and metronidazole in Clostridium difficile infections: a meta-analysis and indirect treatment comparison. *J Antimicrob Chemother* **69**, 2892–2900, doi:10.1093/jac/dku261 (2014).

103 Feher, C., Soriano, A. & Mensa, J. A Review of Experimental and Off-Label Therapies for Clostridium difficile Infection. *Infect Dis Ther* **6**, 1–35, doi:10.1007/s40121-016-0140-z (2017).

104 Al-Nassir, W. N. *et al.* Both oral metronidazole and oral vancomycin promote persistent overgrowth of vancomycin-resistant enterococci during treatment of Clostridium difficile-associated disease. *Antimicrob Agents Chemother* **52**, 2403–2406, doi:10.1128/AAC.00090-08 (2008).

105 Ivarsson, M. E., Leroux, J. C. & Castagner, B. Investigational new treatments for Clostridium difficile infection. *Drug Discov Today* **20**, 602–608, doi:10.1016/j.drudis.2014.12.003 (2015).

106 Basseres, E., Endres, B. T., Dotson, K. M., Alam, M. J. & Garey, K. W. Novel antibiotics in development to treat Clostridium difficile infection. *Curr Opin Gastroenterol* **33**, 1–7, doi:10.1097/MOG.0000000000000332 (2017).

107 McDonald, L. C. *et al.* Clinical Practice Guidelines for Clostridium difficile Infection in Adults and Children: 2017 Update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). *Clin Infect Dis* **66**, 987–994, doi:10.1093/cid/ciy149 (2018).

108 Tsutsumi, L. S., Owusu, Y. B., Hurdle, J. G. & Sun, D. Progress in the discovery of treatments for C. difficile infection: A clinical and medicinal chemistry review. *Curr Top Med Chem* **14**, 152–175 (2014).

109 Worthington, R. J. & Melander, C. Combination approaches to combat multidrug-resistant bacteria. *Trends Biotechnol* **31**, 177–184, doi:10.1016/j.tibtech.2012.12.006 (2013).

110 Wilcox, M. H. *et al.* in *European Congress of Clinical Microbiology and Infectious Diseases (ECCMID).*

111 Butler, M. M. *et al.* MBX-500, a hybrid antibiotic with in vitro and in vivo efficacy against toxigenic Clostridium difficile. *Antimicrob Agents Chemother* **56**, 4786–4792, doi:10.1128/AAC.00508-12 (2012).

112 Butler, M. M. *et al.* Antibacterial activity and mechanism of action of a novel anilinouracil-fluoroquinolone hybrid compound. *Antimicrob Agents Chemother* **51**, 119–127, doi:10.1128/AAC.01311-05 (2007).

113 Steele, J. *et al.* MBX-500 is effective for treatment of Clostridium difficile infection in gnotobiotic piglets. *Antimicrob Agents Chemother* **57**, 4039–4041, doi:10.1128/AAC.00304-13 (2013).

114 Clements, J. E., D'Ambrosio, J. & Brown, N. C. Inhibition of Bacillus subtilis deoxyribonucleic acid polymerase III by phenylhydrazinopyrimidines. Demonstration of a drug-induced deoxyribonucleic acid-enzyme complex. *J Biol Chem* **250**, 522–526 (1975).

115 Torti, A. *et al.* Clostridium difficile DNA polymerase IIIC: basis for activity of antibacterial compounds. *Curr Enzym Inhib* **7**, 147–153 (2011).

116 Dvoskin, S. *et al.* A novel agent effective against Clostridium difficile infection. *Antimicrob Agents Chemother* **56**, 1624–1626, doi:10.1128/AAC.06097-11 (2012).

117 Briggs, G. S., Smits, W. K. & Soultanas, P. Chromosomal replication initiation machinery of low-G+C-content Firmicutes. *J Bacteriol* **194**, 5162–5170, doi:10.1128/JB.00865-12 (2012).

1

118 Lemon, K., Moriya, S., Ogasawara, N. & Grossman, A. in *Bacillus subtilis and its closest relatives* (eds AL Sonenshein, JA Hock , & R Losick) 73–86 (ASM Press, 2002).

119 Wolf, M., Muller, T., Dandekar, T. & Pollack, J. D. Phylogeny of Firmicutes with special reference to Mycoplasma (Mollicutes) as inferred from phosphoglycerate kinase amino acid sequence data. *International journal of systematic and evolutionary microbiology* **54**, 871–875, doi:10.1099/ijs.0.02868-0 (2004).

120 Messer, W. The bacterial replication initiator DnaA. DnaA and oriC, the bacterial mode to initiate DNA replication. *FEMS Microbiol Rev* **26**, 355–374 (2002).

121 Richardson, T. T., Harran, O. & Murray, H. The bacterial DnaA-trio replication origin element specifies single-stranded DNA initiator binding. *Nature* **534**, 412–416, doi:10.1038/nature17962 (2016).

122 Smits, W. K., Goranov, A. I. & Grossman, A. D. Ordered association of helicase loader proteins with the Bacillus subtilis origin of replication in vivo. *Mol Microbiol* **75**, 452–461, doi:10.1111/j.1365-2958.2009.06999.x (2010).

123 Soultanas, P. Loading mechanisms of ring helicases at replication origins. *Mol Microbiol* **84**, 6–16, doi:10.1111/j.1365-2958.2012.08012.x (2012).

124 Sanders, G. M., Dallmann, H. G. & McHenry, C. S. Reconstitution of the B.subtilis replisome with 13 proteins including two distinct replicases. *Molecular cell* **37**, 273–281, doi:10.1016/j.molcel.2009.12.025 (2010).

125 Sakamoto, Y., Nakai, S., Moriya, S., Yoshikawa, H. & Ogasawara, N. The Bacillus subtilis dnaC gene encodes a protein homologous to the DnaB helicase of Escherichia coli. *Microbiology* **141 (Pt 3)**, 641–644, doi:10.1099/13500872-141-3-641 (1995).

126 Velten, M. *et al.* A two-protein strategy for the functional loading of a cellular replicative DNA helicase. *Molecular cell* **11**, 1009–1020 (2003).

127 O'Donnell, M., Langston, L. & Stillman, B. Principles and concepts of DNA replication in bacteria, archaea, and eukarya. *Cold Spring Harbor perspectives in biology* **5**, doi:10.1101/cshperspect.a010108 (2013).

128 Frick, D. N. & Richardson, C. C. DNA primases. *Annual review of biochemistry* **70**, 39–80, doi:10.1146/annurev.biochem.70.1.39 (2001).

129 Noirot-Gros, M. F. *et al.* An expanded view of bacterial DNA replication. *Proc Natl Acad Sci U S A* **99**, 8342–8347, doi:10.1073/pnas.122040799 (2002).

130 Rannou, O. *et al.* Functional interplay of DnaE polymerase, DnaG primase and DnaC helicase within a ternary complex, and primase to polymerase hand-off during lagging strand DNA replication in Bacillus subtilis. *Nucleic acids research* **41**, 5303–5320, doi:10.1093/nar/gkt207 (2013).

131 Dervyn, E. *et al.* Two essential DNA polymerases at the bacterial replication fork. *Science (New York, N.Y.)* **294**, 1716–1719, doi:10.1126/science.1066351 (2001).

132 Bruck, I. & O'Donnell, M. The DNA replication machine of a gram-positive organism. *J Biol Chem* **275**, 28971–28983, doi:10.1074/jbc.M003565200 (2000).

133 Timinskas, K., Balvociute, M., Timinskas, A. & Venclovas, C. Comprehensive analysis of DNA polymerase III alpha subunits and their homologs in bacterial genomes. *Nucleic acids research* **42**, 1393–1413, doi:10.1093/nar/gkt900 (2014).

134 Su'etsugu, M. & Errington, J. The replicase sliding clamp dynamically accumulates behind progressing replication forks in Bacillus subtilis cells. *Molecular cell* **41**, 720–732, doi:10.1016/j.molcel.2011.02.024 (2011).

135 Afonso, J. P. *et al.* Insights into the structure and assembly of the Bacillus subtilis clamp-loader complex and its interaction with the replicative helicase. *Nucleic acids research* **41**, 5115-5126, doi:10.1093/nar/gkt173 (2013).

136 Petit, M. A. & Ehrlich, S. D. The NAD-dependent ligase encoded by yerG is an essential gene of Bacillus subtilis. *Nucleic acids research* **28**, 4642-4648 (2000).

137 Lohman, T. M. & Ferrari, M. E. Escherichia coli single-stranded DNA-binding protein: multiple DNA-binding modes and cooperativities. *Annual review of biochemistry* **63**, 527-570, doi:10.1146/annurev.bi.63.070194.002523 (1994).

138 Sissi, C. & Palumbo, M. In front of and behind the replication fork: bacterial type IIA topoisomerases. *Cellular and molecular life sciences : CMLS* **67**, 2001-2024, doi:10.1007/s00018-010-0299-5 (2010).

139 Davey, M. J. & O'Donnell, M. Replicative helicase loaders: ring breakers and ring makers. *Current Biology* **13**, R594-R596, doi:10.1016/s0960-9822(03)00523-2 (2003).

140 Goranov, A. I., Breier, A. M., Merrikh, H. & Grossman, A. D. YabA of Bacillus subtilis controls DnaA-mediated replication initiation but not the transcriptional response to replication stress. *Mol Microbiol* **74**, 454-466, doi:10.1111/j.1365-2958.2009.06876.x (2009).

141 Hayashi, M., Ogura, Y., Harry, E. J., Ogasawara, N. & Moriya, S. Bacillus subtilis YabA is involved in determining the timing and synchrony of replication initiation. *FEMS microbiology letters* **247**, 73-79, doi:10.1016/j.femsle.2005.04.028 (2005).

142 Kaguni, J. M. DnaA: controlling the initiation of bacterial DNA replication and more. *Annual review of microbiology* **60**, 351-375, doi:10.1146/annurev.micro.60.080805.142111 (2006).

143 Katayama, T., Ozaki, S., Keyamura, K. & Fujimitsu, K. Regulation of the replication cycle: conserved and diverse regulatory systems for DnaA and oriC. *Nat Rev Microbiol* **8**, 163-170, doi:10.1038/nrmicro2314 (2010).

144 Leonard, A. C. & Grimwade, J. E. Initiation of DNA Replication. *EcoSal Plus* **4**, doi:10.1128/ecosalplus.4.4.1 (2010).

145 Leonard, A. C. & Grimwade, J. E. Regulating DnaA complex assembly: it is time to fill the gaps. *Current opinion in microbiology* **13**, 766-772, doi:10.1016/j.mib.2010.10.001 (2010).

146 Merrikh, H. & Grossman, A. D. Control of the replication initiator DnaA by an anti-cooperativity factor. *Mol Microbiol* **82**, 434-446, doi:10.1111/j.1365-2958.2011.07821.x (2011).

147 Mott, M. L. & Berger, J. M. DNA replication initiation: mechanisms and regulation in bacteria. *Nat Rev Microbiol* **5**, 343-354, doi:10.1038/nrmicro1640 (2007).

148 Fossum, S. *et al.* A robust screen for novel antibiotics: specific knockout of the initiator of bacterial DNA replication. *FEMS microbiology letters* **281**, 210-214, doi:10.1111/j.1574-6968.2008.01103.x (2008).