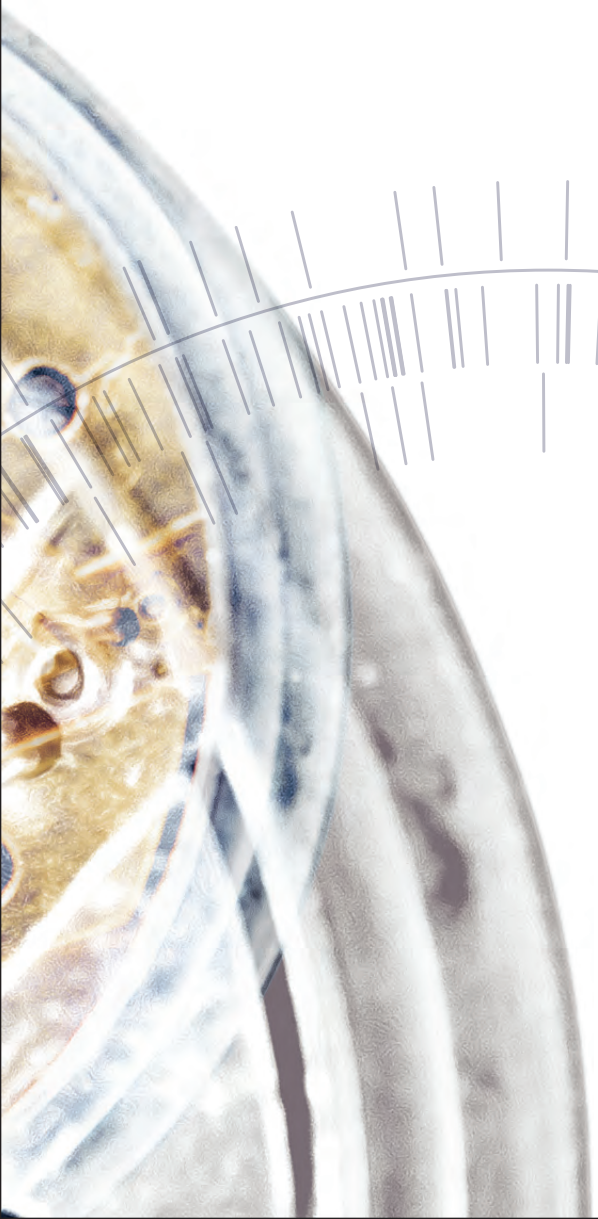
The replication machinery of Clostridium difficile:a potential target for novel antimicrobials

Eijk, H.W. van

Chapter 7

General Discussion

Erika van Eijk



General discussion

Phenotypic consequences of genetic diversity

In 2006, the complete genome sequence of a multiple drug resistant clinical isolate of *C. difficile* was published [1], and this particular strain (strain 630) became the reference strain for laboratories interested in the characterization of the organism itself and/or studying the genetic basis of the pathogenicity of this bacterium. A relatively large proportion of the genome of this historical PCR ribotype 012 isolate consists of mobile genetic elements (11 percent) [1]. Most of these are present in the chromosome as conjugative transposons, non-conjugative transposons, or integrated bacteriophages [1-3], thereby increasing genetic diversity in this species. The availability of the genome sequence spurred the development of tools for genetic manipulation of *C. difficile*, which is essential for gaining insight in pathogenesis of *C. difficile* infection [4]. Whereas the historical 630 strain demonstrated transferable erythromycin resistance [1], this resistance was lost due to serial culturing on non-selective media, to allow for the use of the *erm*B gene as antibiotic-selective marker in genetic studies in *C. difficile* [5]. The generation of these erythromycin sensitive strains, commonly referred to as 630Δ*erm* and 630E/JIR8094, occurred independently in laboratories based in Europe and Australia, respectively [4-7]. Both erythromycin-sensitive strains were distributed for research-purposes to other laboratories around the world. Additionally, the 630 and 630Δ*erm* strains were deposited and can be obtained from culture collections based in the US (ATCC), UK (NCTC), and Germany (DSMZ) [5], further contributing to the global distribution of these strains for laboratory-based studies [5,8]. However, when subjecting these different erythromycin-sensitive strains to similar experiments, differences in phenotypes - and thus primary outcomes - were observed, for instance in studies investigating the role of toxins in *C. difficile* infection [9,10]. Indeed, side-by-side comparison of 630E and 630Δ*erm* strains used for exploring the relative role of toxin A and toxin B confirmed their phenotypic- and genotypic distinction [6]. Similarly, significant phenotypic differences with respect to sporulation were observed within a collection of epidemic strains of PCR ribotype 027, refuting the assumption that the rate of sporulation was PCR ribotype-specific [11-13]. Taken together, the phenotypic differences demonstrated between the 630E and 630Δ*erm* strain as well as the parental

strain 630 underline the importance of documenting provenance, and hint at an underappreciate source of inter-laboratory experimental variation [5,6,8]. Moreover, the diversification of genetically distinct sub-strains caused by different passaging conditions in the different laboratories has been demonstrated for the attenuated strain of *Mycobacterium bovis*, Bacillus Calmette-Guérin (BCG), which is used as a prophylactic measure against tuberculosis [14]. Thus far, the molecular factors that impact the properties of the of BCG strains used for the vaccines are not known, but studies delineating these strains into phenotypic and genetic categories may provide insight into vaccine efficiency [14]. Such an approach may also be feasible for the laboratory strains of *C. difficile* that are currently used and determination of the complete genome sequence of our reference strain *C. difficile* 630Δ*erm* and comparison to its parental strain may be a starting point (**Chapter 3**).

In hindsight, the world-wide distribution of the *C. difficile* reference strain 630 and its erythromycin-sensitive derivatives and their propagation within numerous laboratories can be viewed as a long-term evolution experiment, akin to the ongoing long-term evolution experiment of *E. coli* (LTEE). This project started in the late eighties in order to study genetic changes as a result of repeated subculturing of initially identical populations of *Escherichia coli*, in a controlled laboratory environment [8,15,16]. In contrast to the LTEE, the *C. difficile* strains, however, encountered many different conditions in the individual laboratories, for instance in culturing and storage conditions [8]. Adaptation to these different conditions may have altered the genetic background these strains, most probably causing phenotypic variations [8]. For *C. difficile*, it is conceivable that growth conditions in a laboratory environment requires adaptation to nutrients that are readily available in the culture medium, but absent in the host environment of the bacteria. This may be reflected in an altered metabolic pathway or other adaptations. Such an adaptation to altered growth conditions has been observed in the LTEE, in which *E. coli* acquired a novel trait, aerobic citrate utilization, after being propagated for thousands of generations in a glucose-limited medium that also contains citrate [15,17]. This carbon source is normally not utilized under oxic conditions [15,17]. This observation in the long-term evolutionary experiment was a starting point to decipher the molecular basis of this specific adaptation [17]. The similarity between LTEE and the dissemination of *C. difficile* laboratory strains is that evolution of the species can be studied over time. However, in *C. difficile* strains, different selective pressures due to variability in laboratory conditions may have may accelerated the

accumulation of single nucleotide polymorphisms (SNPs) as opposed to a controlled environment such as the LTEE. Overall, though, in both species adaptation occurred over a vast period of time. Studying the (experimental) evolution of these bacteria may provide insight into the molecular basis of adaptation [18].

Even a single SNP can be responsible for phenotypic differences between genetically closely related strains. For instance, a single mutation in the gyrase gene is the underlying genetic mechanism for fluoroquinolone resistance in *C. difficile* [2,19–21]. When comparing the reference genome sequence of strain 630 to the sequence of our laboratory strain 630Δ*erm* (LUMC), we uncovered numerous insertions, deletions, single nucleotide polymorphisms (SNPs), and large scale chromosomal rearrangements such as the transposition of a mobile genetic element and the acquisition of an additional ~5 kb rRNA/tRNA cluster (**Chapter 3, Table 1, Figure 4**). It is not inconceivable that these genetic changes influence the phenotype of 630-derived strains. Moreover, when such phenotypic differences are already evident for genetically closely related strains, differences may be even more pronounced in genetically distant strains. For instance, studies in *Streptococcus pneumoniae* have shown that genomic content between strains can vary to such an extent, that an important part of experiencing and processing antibiotic stresses of a bacteria may be strain-dependent [22]. Indirectly, an antimicrobial can trigger a complex, multi-factorial process that resonates through the bacterium, involving numerous and diverse pathways such as regulation, metabolism and/or energy generation [22–26]. Due to the genetic diversity, or more specific, the absence and presence of genes between strains, the genome has to display remarkable plasticity to maintain function [22]. This implies that newly acquired genetic elements have to be integrated into regulatory networks, leading to changes within these networks (i.e. establishing new connections and altering existing connections). As a result, genomes may function differently, which may cause phenotypic variation ranging from adaptation of antimicrobials to virulence [22]. This may also apply to *C. difficile*, considering that the a large portion (11 percent) of the genome of *C. difficile* 630 consists of horizontally acquired elements [1], the average proportion of core genome (genes shared between strains) may be as little as 40 percent [27] and strains may or may not contain plasmids [28]. We observed already considerable differences between closely related strains belonging to PCR ribotype 012 (**Figure 1**), described in **Chapter 3** of this thesis.

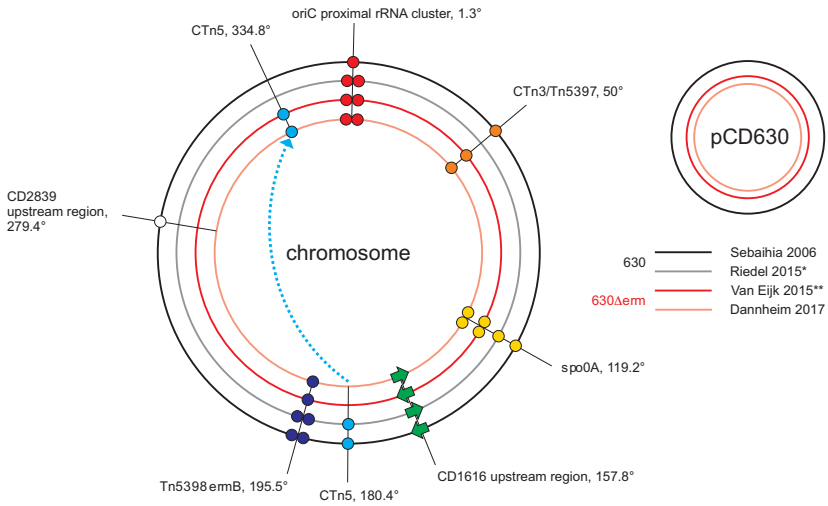


Figure 1. Whole genome comparison of *C. difficile* strain 630 and derivatives

Genome sequences (black/grey) and 630 *erm* sequences (red/orange) are represented as concentric circles. Structural variants are highlighted using symbols. Dots are used to indicate presence (dot) /absence (no dot) or duplications (two dots) of a sequence. Arrows indicate inversions. The dashed arrow between positions 180.4° and 334.8° indicates transposition of the CTn5 element. Note that this global overview does not allow discrimination of single nucleotide polymorphisms, but highlights presence/absence and location of certain larger structural variants (Figure reproduced from Roberts *et al.* (2018)[8] with permission from the publisher)

This PCR ribotype, however, is not the most prevalent circulating ribotype based on surveys conducted in Europe and the US [29,30] and large differences exist between ribotypes [1,3,31,32]. For RT 078, it even has been suggested that it forms a separate sub-species [33]. This indicates that there is a large variable component between genomes (accessory genome), that may be relevant for coping with stress, for instance exposure to antimicrobials [27,34]. The genetic diversity discussed above was one of the reasons to test a large collection of clinical isolates of *C. difficile* against the antimicrobial properties of new drugs such as 362E (**Chapter 6**). By testing a diverse collection, it can be established if a new antimicrobial is potent against all isolates despite the potential differences in genetic background, or only a subset. Our results show that there was no significant difference in 362E susceptibility

between clades (**Chapter 6, Table 1**) and that the different PCR ribotypes demonstrated a similar distribution in MIC values (range MIC: 0.5-4.0 µg/ml; MIC_{50}: 2 µg/ml; MIC_{90}: 4 µg/ml) (**Chapter 6, Figure 2**).

Genome organisation as regulator

When subjecting *C. difficile* vegetative cells to a sub-MIC level of the DNA polymerase inhibitor 362E, we observed an increase in the *oriC*:*terC* ratio, indicating the presence of an increased number of replication forks (**Chapter 6, Figure 6**). Although the replisome was (presumably) slowed or stalled by the inhibitor, initiation of replication seemed to continue or was possibly even stimulated [35]. Similarly, a coupling between replication inhibition and replication initiation was reported for *B. subtilis* where inhibition of gyrase by novobiocin leads to increased over-initiation [36]. As a direct consequence of this multi-fork replication, the copy-number of genes which are located in close proximity to the origin increase compared to genes located distally from the origin. A correlation between gene copy number and gene expression implies that the transcriptome of a bacterial cell is influenced by gene dosage [37-40] (**Chapter 6, Figure 5**). It should be noted that fluctuation in gene copy number due to genomic location is also affected by growth rate. In our experiment, cells were grown in nutrient-rich liquid medium which promotes fast growth and leads to multi-fork replication (i.e. more than one round of DNA replication occurs at the same time). In fast growing *E. coli*, which has a circular chromosome comparable in size to *C. difficile*, 8 copies of the origin of replication can be observed (doubling time ~ 20 minutes). As a result, the copy number of origin-proximal genes in comparison to genes located near the terminus may increase by 4-fold [41]. However, in our study, the *oriC*:*terC* ratio of the bacterial cells treated with 362E was compared to untreated cells and results of the Marker Frequency Analysis showed a relative increase up to 16-fold (**Chapter 6, Figure 6**). This result is independent of the increase of *oriC*:*terC* ratio caused by growth rate. The effect of 362E is concordant with experiments performed in *S. pneumoniae*, *B. cereus* and *S. aureus*, in which other antimicrobials affecting DNA replication significantly increased the *oriC*:*terC* ratio [42]. When cells of these organism were subjected to DNA-polymerase inhibitor HPUra, the *oriC*:*terC* ratio increased three- to tenfold [42]. Ciprofloxacin-treated cells (*S. pneumoniae*, *B. cereus*) showed a 6-fold increase in *oriC*:*terC* ratio compared to untreated cells [42]. In several organisms, such as *S.*

pneumoniae and *Vibrio cholerae*, it was shown that genes which are directly or indirectly important for dealing with (replication) stress and cell differentiation are located close to the origin of replication and that the functional genome organization is important for these bacteria to respond adequately to specific conditions [42-44]. Moreover, in *E. coli*, in which the origin of replication was relocated to an ectopic chromosome location, a significant loss of fitness was demonstrated when exposed to sub-inhibitory concentrations of ciprofloxacin [45]. Like 362E, ciprofloxacin generates replication fork stalling, resulting in increased number of copies of genes near the origin in wild-type *E. coli*. Cells with an ectopic origin display a different gene dosage effect, which affects replication fork repair and causes chromosome instability [45]. This experiment shows that the gene distribution in the origin region is pivotal for dealing with specific stresses, such as ciprofloxacin [45]. Nevertheless, the importance of chromosomal gene location has not received much attention yet compared to transcriptional regulation [39].

When analysing the sequence of our laboratory strain 630Δ*erm*, we found that it had acquired an additional copy of an origin-proximal rRNA/tRNA cluster, resulting into a total of twelve ribosomal RNA (*rrn)* operons [1]. Higher copy numbers of *rrn* operons seem to occur more often in fast growing organisms and those with a relatively large genome size [46,47]. It is plausible that strain 630Δ*erm* adapted to fast growth in the laboratory to cope with the increased demand for protein imposed by growth in nutrient-rich media [48]. Our assumption regarding adaptation to fast growth is further supported by the specific location of this extra copy of *rrn* operon, near the origin, which effectively amplifies rRNA gene dosage during rapid growth [49]. Comparative analysis of genomes of more than a thousand bacterial species showed that *rrn* copy number predicts traits associated with resource availability [50]. From an ecological perspective, a nutrient-rich environment enables the bacterium to acquire an additional *rrn* operon, as the substantial energetic cost of ribosome synthesis may be compensated by the excess of nutrients [47], whereas in nutrient-poor conditions an excess of ribosomes may result in suboptimal growth [51,52]. In *E. coli*, it was experimentally demonstrated that the optimal number of *rrn* operons is determined by the advantage of both rapid adjustment to influx of nutrients and the capacity for fast growth [47]. In conditions with fluctuating nutrient levels isogenic *E. coli* strains with 7 (wild-type copy number) and 8 operons were the most effective competitors, whereas in stable, relatively nutrient-poor conditions it was suggested that the number of operons would be lower

over time [47]. In our laboratory, our laboratory strain is subjected to fluctuating conditions when performing growth curves in which overnight starter cultures are diluted to a certain optic density into a fresh medium. To our knowledge, it is unknown if, or to what extent, nutrient levels fluctuate in the gut. The conditions, in terms of temperature and nutrients, between the laboratory and the environment to which a bacterium is subjected, vary considerably. For instance, growth (and doubling-time) of a soil-dwelling organism such as *B. subtilis* will be much slower in its natural habitat where (ambient) temperature will be far below 37 degrees Celsius. Moreover, growth at extreme temperatures causes stress for *B. subtilis* (laboratory conditions) and may even lead to activation of the alternative sigma B factor, and thereby the general stress response [53-56]. Perhaps, growth under laboratory conditions should be considered a stress for *C. difficile* as well.

Interestingly, the gene encoding the alternative sigma factor B homologue (*sigB*) is directly adjacent to a rRNA/tRNA cluster, that includes the additional copy found in our laboratory strain 630Δ*erm*. The linkage between the protein synthesis machinery and the protein σ^B, and the genomic location close to the origin was also noticed by others, and it was suggested that as a result, *sigB* might transiently be expressed (at an early stage of DNA replication) [1]. In most Firmicutes, σ^B governs the so-called general stress response that is important to cope with environmental stresses and starvation [53]. However, the conservation of genes that are σ^B-dependent in one organism cannot be taken as an indication that their σ^B-dependency is equally conserved, as genes regulated by σ^B can differ per species [57]. In *Bacillus subtilis*, many of the factors involved in regulating σ^B activity are encoded in the *sigB* operon (Rsb proteins) [58-60]. When analysing the gene-clusters which involve the genes responsible for regulating *sigB* from other bacteria, it is clear that large differences in operon-structure between species exist; *Bacillus subtilis* and *Listeria monocytogenes* both possess an 8-gene cluster, in contrast to the 4-gene cluster found in *Bacillus cereus* and *Bacillus anthracis* [58]. *S. aureus* has also a 4-gene *sigB* operon, that encodes three homologs of the *sigB* regulatory genes identified in *B. subtilis* (*rsbU*, *rsbV* and *rsbW*). *C. difficile* only has 2 homologues of the *sigB* regulatory genes (*rsbV*, *rsbW*) in common with *B. subtilis* in its 3-gene cluster [61]. The additional factors that influence the regulatory proteins encoded by the operon in *B. subtilis* (*rsbR*, *rsbS*, *rsbT*, *rsbX*) are absent from *S. aureus* [60] and *C. difficile* [1,61], strongly suggesting that *sigB* activation in these organisms is different.

In *C. difficile*, σ^B seems to control a wide array of genes involved in sporulation, metabolism, cell surface biogenesis and the management of stresses [61]. Inactivation of *sigB* reduced survival in the stationary growth phase and increased sensitivity to for instance, acidification, cationic antimicrobial peptides, nitric oxide and reactive oxygen species [61]. Mutants of *sigB* of other bacterial species demonstrated increased susceptibility to a diverse range of environmental and physiological stresses, including ethanol, acidic or alkaline pH, high osmolarity, bile acids, certain antibiotics, temperature variations or oxidative stress [62-66].

Despite that differential expression of *sigB* in response to 362E inhibition was not demonstrated in our experiment, a substantial number of genes that are presumed to be under the control of σ^B were differentially expressed (**Chapter 6, Table 3**). Transient upregulation of *sigB* may have occurred before harvesting the cells in our experiment; such kinetics are known from other organisms and believed to minimize the fitness costs associated with *sigB* expression [53,61,67,68]. Our hypothesis is that the origin-proximal position of *sigB* and the inhibitor induced gene dosage increases transcription of *sigB* and σ^B-dependent genes in order to cope with this particular stress. To test this, *sigB* could be translocated from the origin-proximal location to an ectopic (origin-distal) location on the chromosome and repeat our experiment and transcriptome analysis (**Chapter 6**). The sensitivity to 362E of such a strain in comparison with a wild-type and a *sigB*-mutant strain will reveal the importance of the origin proximal location of *sigB* to cope with inhibition by 362E. Of note, Kint and co-workers reported an increased sensitivity of a *sigB* Clostron mutant towards several antibiotics, supporting the notion that σ^B can be important for dealing with growth inhibition [61]. It is most likely that the transcriptional response to 362E inhibition is driven by a combination of the direct effect of gene dosage based on genomic location, and an indirect effect exerted through the induction of genes dependent on an origin-proximal transcriptional regulator, such as *sigB*.

As mentioned earlier, expression and function of the general stress response deviates between species [53]. Within the group of pathogenic *Clostridia*, strikingly, a *sigB* operon was only identified in *C. difficile* [1] and *C. sordellii* [61], suggesting that the other species may have other stress-responsive transcriptional regulators. The fact that in *C. difficile sigB* is origin-proximal and that in other organisms stress-regulators are functionally clustered at the origin of replication [42,44], raises the intriguing possibility that these regulators in other Clostridia are also located near the origin.

When analysing the genome sequences of *Clostridium tetani* (E88 GenBank: AE015927.1), *Clostridium perfringens* (ATCC 13124; GenBank:CP000246) and *Clostridium botulinum* (ATCC 19397; GenBank:CP000726.1), several transcriptional regulators could be identified. An origin-proximal putative transcriptional regulator (CLB_0016; 14910-15431 bp) was found in *C. botulinum*. In *C. perfringens* and *C. tetani*, genes encoding TetR-family transcriptional regulators are located near the origin (CPF_0034; 42133-42702 bp, and CTC00079; 26462-27028, respectively). This family of regulators is a vast and diverse family of one component signal transduction systems. Most known for its role as regulator of antibiotic efflux, they are known to interact with an exceptionally diverse set of small molecules, including metabolites and cell-cell signalling molecules[69] and regulate a variety of important functions, including osmotic stress, catabolic pathways, homeostasis, biosynthesis of antibiotics, efflux pumps, multidrug resistance, and virulence of pathogenic bacteria[70,71]. TetR-family regulators are more abundant and diverse in microbial species exposed to environmental changes, suggesting important regulatory roles in microbial adaptation[70–72]. Although the function of both regulators in *C. perfringens* and *C. tetani* are not described in literature, it is interesting that they are located origin-proximal.

Antimicrobials targeting DNA replication proteins

In our view, compound 362E has the potential to become a new therapeutic against *C. difficile* infections, as it has a unique mechanism of action, with a low potential for resistance and a lack of cross-resistance with existing agents [73–77]. Furthermore, it has low bio-availability which ensures high local concentration at the site of infection in the gut[77]. Though specificity to *C. difficile* was claimed, activity to this compound was only tested against a small panel of intestinal bacteria, including *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bifidobacterium breve*, *Eubacterium lentum*, and *Bacteroides fragilis* [74]. The conclusion may therefore be premature; indeed, we demonstrated in our study that *S. aureus* is highly sensitive to 362E (**Table 1**).

Table 1. MIC values of control organisms used for testing PolC inhibitors with agar dilution method (expressed in µg/ml)

	HPUra		362E	
Strain	**24h**	**48h**	**24h**	**48h**
Bacteroides fragilis ATCC 25285	32	>64	>16	>16
Bacteroides thetaiotaomicron	32	>64	>16	>16
Clostridioides difficile ATCC 43603 #	2	16	2	4
Clostridioides difficile ATCC 700057 $	2	16	1	2
Clostridium glycolicum	2	16	1	2
Clostridium perfringens	4	16	8	8
Staphylococcus aureus ATCC 29213	0,5	1	1	1

This strain is PCR ribotype 085, tcdA– tcdB–, cdtB not amplified by PCR, serogroup X
$ This strain is PCR ribotype 038, tcdA– tcdB–, cdtB not amplified by PCR, non-toxigenic

Admittedly, *S. aureus* does not reside in the gut, but this finding underlines the importance of assessing how a microbial community, such as the gut microbiome, changes upon exposure to 362E. It would be very interesting to compare the effect on the microbiome of 362E with the one of the standard treatment options of CDI, vancomycin. Considering that broad-spectrum antibiotic treatment is one of the most important risk factors for CDI [78,79], it is desirable that a new antimicrobial against *C. difficile* has a minimal impact on the microbiome. Another approach to improve the specificity of an antimicrobial is to combine antimicrobials with different spectra, or antimicrobials and non-antibiotic compounds, that show synergistic activity towards a specific pathogen, such as *C. difficile* [80,81]. Such combinations may also shorten the duration treatment and/or decrease dosage compared to mono-therapy [82], thereby minimizing the time of exposure and facilitating a decrease in antibiotic concentrations. Improving the specificity of treatment in this fashion may also have a profound effect on the prevention of development of resistance. Combination therapy does not have to imply the use of two separate antibiotics but may be engineered into a single molecule. These hybrid antibacterial agents may be the ultimate combination of two antibiotics, and prevention or reduction of *de novo* generation of complete resistance is based on the assumption that the targets of both moieties are inhibited [83]. However, true dual targeting may not be achieved for certain hybrid agents [84]. This includes the anti-*C. difficile* agent cadazolid, an oxazolidinone-fluoroquinolone hybrid. Despite promising results in Phase I and Phase II clinical trials [85,86], Phase III clinical studies demonstrated it was equivalent or inferior

to vancomycin [87]. Mechanistically, cadazolid is a potent inhibitor of bacterial protein synthesis, but barely inhibits the fluoroquinolone target [88]. Nonetheless, the fluoroquinolone pharmacophore contributes to favourable physicochemical properties of the hybrid molecule, which probably increases the activity of the hybrid [84]. For some molecules, such as MBX-500, there is evidence that they do act as dual-target hybrids. MBX-500 consists of a fluoroquinolone moiety covalently linked to a PolC (PolIIIC)- inhibitor pharmacophore (HB-EMAU) (**Chapter 2**). It was developed as an agent to treat antimicrobial-resistant Gram-positive aerobic pathogens, but is also active against certain *Bacillus* species, *C. difficile* and *S. aureus* strains [89,90]. To this date, no clinical studies in humans have been announced for MBX-500 in relation to treatment of *C. difficile* [91]. The evidence for its true dual targeting is based on a demonstration that full resistance to MBX-500 in *S. aureus* required mutations that individually confer resistance to fluoroquinolones as well as PolC-inhibitors [89]. However, also in this study, the hybrid molecule proved to be strong inhibitor compared to the weak antimicrobial activity of an equimolar combination of parent molecules. This suggests that other antibacterial properties than the effect of dual targeting may be involved. It is possible that the fluoroquinolone-moiety of MBX-500 also influences bacterial cell permeation, as suggested for cadazolid. Unfortunately, no *in vitro* biochemical assays with purified *C. difficile* PolC or DNA topoisomerase have been reported. Performing DNA polymerase activity assay for *C. difficile* PolC [74,92] and DNA supercoiling-and decatenation assays, as described by Locher *et al.* 2014 [88] could shed light on the activity of the compound towards the individual targets. To gain further insight into the mode of action of MBX-500 in *C. difficile* it would be interesting to construct a PolC-fluoroquinolone resistant (double) mutant and to assess if resistance will occur, as seen for *S. aureus* [89]. Elucidation of pathways and target identification of MBX-500 or other antimicrobials in *C. difficile* may be further explored using targeted transcriptional repression using CRISPR interference (CRISPRi) [93]. Recently, progress has been made by using CRISPR-based systems in *C. difficile* [93–97].

If the activity of one of the pharmacophores is dominant over the other, an elegant solution to preserve the physico-chemical properties of the combined molecule could be to link both pharmacophores with a peptide bond which can only be cleaved by an enzyme of *C. difficile*, such as endopeptidase PPEP-1 (ZMP1/CD2830) [98], if possible. The advantage of this approach over combination therapy of the individual parent molecules is that the pharmacokinetics of a hybrid

molecule are more predictable. Furthermore, such a cleavable peptide bond-linker may be also be a new approach for designing new hybrid antibiotics which are inactive (prodrug) [83] until they encounter the pathogen of interest, and therefore display high specificity.

Although the spectrum of activity of MBX-500 is reasonably limited, it still targets a fair number of other low-GC Gram-positive organisms and some Gram-negatives [89,90]. Depending on the organism of interest, the niche of that specific micro-organism and the bio-availability of the antimicrobial compound, this may or may not pose a problem. If MBX-500 is aimed at the treatment of *S. aureus*, the PolC-inhibitor pharmacophore could be refined to improve its specificity to this species, as was done for *C. difficile*-specific compound 362E [74]. Surprisingly, we observed that *S. aureus*, which was one of the control organisms in our experiments (**Chapter 6**), displayed a higher sensitivity to 362E than most of the *C. difficile* strains, suggesting this may be a good starting point for designing such hybrids (**Table 1**). A topical formulation of compound 362E could be considered for treatment of various skin infections caused by *S. aureus*, as its bioavailability is low and *S. aureus*' niche is clearly separated from *C. difficile*'s in the human gut. If the therapeutic potential of compound 362E in relation to *S. aureus* would be further explored, a number of experiments should be performed. Apart from the assessment of antimicrobial activity with the agar dilution method (**Chapter 6**), we do not know whether the exquisite sensitivity of *S. aureus* to 362E is due to its PolC-inhibiting activity or if the compound demonstrates an alternative mode of action. A first step in elucidating the mode of action of compound 362E in *S. aureus*, is to perform a marker frequency analysis (MFA) [42]. If *S. aureus* PolC is the primary target of compound 362E, we expect a similar increase in *oriC:terC* ratio as determined for *C. difficile* (**Chapter 6, Figure 6**). If an increase in *oriC:terC* ratio is confirmed, purifying PolC of *S. aureus* [99] would be the next logical step in order to assess the *in vitro* activity of 362E against this essential replication protein. Identification of the target of 362E in *S. aureus in vivo*, can be performed by induced strain sensitivity assays where components of specific pathways are depleted by for instance antisense interference, leading to increased susceptibility towards a particular compound [100-102].

Biochemistry of replication proteins in drug development

Above we mentioned that *in vitro* experiments using purified proteins (gyrase and DNA-polymerase) are important for drug design and target validation. This has been an important consideration for the work discussed in **chapters 4** and **5** of this thesis. Apart from DNA- polymerase and gyrase, no information on replication proteins or the mechanism of replication in *C. difficile* was available at the beginning of our research. In order to facilitate development of antimicrobials against replication proteins, basic research to identify and characterize these proteins, along with the mechanistic details of their activity and interactions is required. The focus of our attention were replication proteins that together form an essential component of the replisome: the primosome [103-105]. In *B. subtilis*, the involvement of the primosomal proteins helicase loader, helicase and primase in the initiation of chromosomal replication has been recognized for a long time and many details about its functioning have been elucidated [106-110]. We hypothesize that helicase and primase form an interesting target for the development of antimicrobials against *C. difficile*. First, the interaction between helicase and primase is essential for bacterial survival and interference with this interaction should therefore lead to cell death [111] (**Chapter 2**). Helicase-primase interactions can be disrupted with peptides [112], but due to unfavourable pharmacological properties these peptides are not antibacterial. It does indicate however, that the interaction may be druggable. Second, the variation in stability of the interaction between species [113-117] and species-specific cross-stimulation by helicase and primase [118,119] suggest that the molecular details of the interaction may be species-specific and this may therefore also apply for the molecule that inhibits the interaction.

In our study, we successfully characterized aspects of the replicative helicase and primase of *C. difficile* (**Chapters 4** and **5**). We established that the helicase and the helicase loader protein interact directly (**Chapter 4**) and provided indirect evidence for an interaction of helicase and primase *in vitro*, through assays measuring helicase and primase activity in the presence and absence of the partner protein (**Chapter 5**). However, attempts to demonstrate a direct interaction between helicase and primase using a bacterial two-hybrid system have been unsuccessful so far. This situation is reminiscent of the transient and/or weak interactions observed for *E. coli* helicase and primase [114-116], which can only be detected by sensitive techniques [117]. One potentially more sensitive technique to demonstrate

an interaction between helicase and primase is a luciferase protein fragment complementation assay, in which both proteins are genetically fused with two inactive fragments of luciferase [120]. Association of these fragments reconstitutes the enzyme activity, allowing for a luminescence read out. The next step to study this interaction is to apply site-directed mutagenesis, to identify the residues important for the interaction as we have performed for the (self-) interaction of helicase and helicase-loader protein (**Chapter 4, Figure 6**). Interaction between helicase and primase may also be demonstrated with cross-linking experiments.

Although the direct evidence of an interaction between helicase and primase was not established, the activation of helicase and/or the helicase-helicase loader complex by primase as determined in the helicase assay (**Chapter 5**) may be sufficient to design a high-throughput small molecule screen and provide new leads for anti-*C. difficile* agents. Potentially, a molecular beacon-based helicase assay (MBHA) can be employed to measure the activity of helicase (**Figure 2**). In such a Förster resonance energy transfer (FRET)-based assay, one strand of a helicase substrate is made of a molecular beacon of which one end is attached to a fluorescent molecule (donor) and the other a quencher (acceptor) [121–123]. The unique feature of a molecular beacon is that is can form a stem-loop structure (hairpin) upon strand separation by the activity of helicase, thereby quenching the fluorescent signal as they are in close proximity [121] (**Figure 2A**).

A major advantage of a molecular beacon-based assay over other FRET-bases assays is that non-specific binding of a compound can be detected by a decrease in fluorescence in absence of helicase (or ATP). Non-specific hits due to DNA-binding compounds are common in helicase assays and their identification is critical for efficient inhibitor development [123]. The MBHA has to be adjusted and optimized in order to meet the unique criteria for inducing *C. difficile* helicase activity and to safeguard that the inhibition of activity observed is a product of disruption of the helicase-primase interaction. This includes the order of addition of the proteins or small molecules, the effect on the activity of non-related (primase-independent) AAA+ helicase (for instance from *B. subtilis* [125,126]), and appropriate negative controls (e.g. Walker-motif mutants of helicase). For instance, we have learned through our experiments that in the presence of ATP and primase, helicase activity is observed (**Chapter 5, Figure 2A**). Therefore, 96-well plates with the small molecule library should be set up under conditions of low helicase activity (in the absence of ATP, and/or

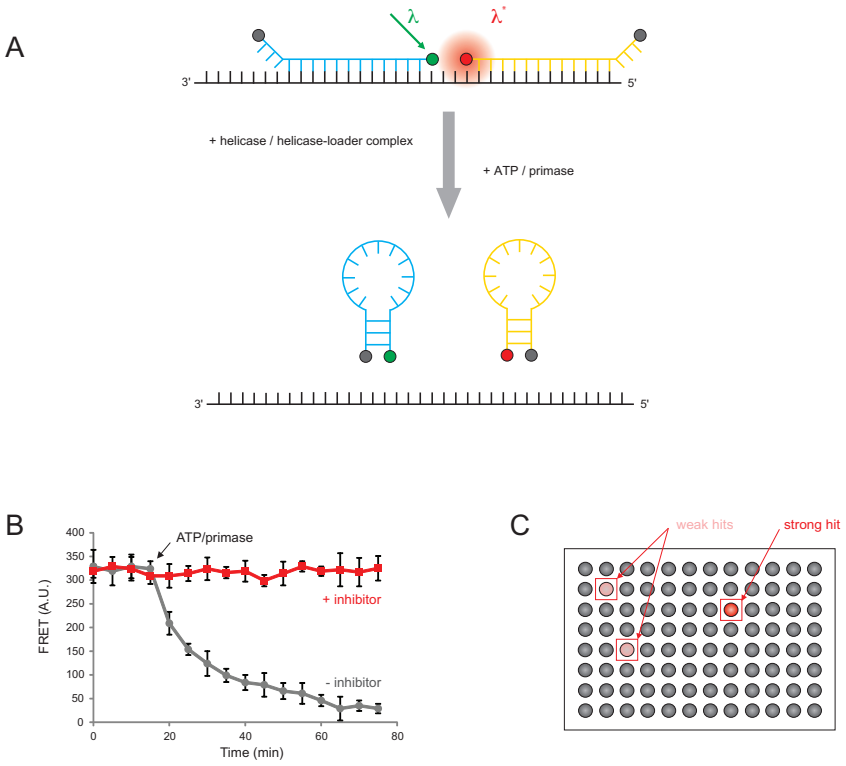


Figure 2. High throughput screen of helicase–primase inhibitors based a molecular beacon-based helicase assay for *C. difficile*.

A. Schematic depiction of the molecular beacon–based assay of helicase activity (based on Belon and Frick (2018)[121], Santangelo (2004)[124]). In the absence of helicase activity, Förster resonance energy transfer between the two fluorescent groups of the molecular beacons (red and green spheres) results in an emission of light (λ*) upon excitation (λ). When the beacons are displaced by helicase, the fluorescent signal is quenched by the proximity of the quencher group (grey sphere). Helicase activity of *C. difficile* helicase requires the presence of ATP and primase.

B. Inhibition of the helicase–primase interaction leads to persistent fluorescence (in arbitrary units, A.U.), whereas helicase activity results in a decrease of fluorescence over time [121]. Data is hypothetical and does not reflect actual experimental data.

C. In a high–throughput screen, inhibitory compounds are readily identified by the persistent fluorescent signal at experimental endpoint. Depicted is a 96–well plate in which to each well a small molecule compound has been added, in addition to helicase, primase and ATP.

primase) and should be monitored for fluorescence upon addition of this compound/protein. Background noise in this assay may be reduced by the addition of the loader protein simultaneously with helicase in the initial plate set-up, as this protein has a negative effect on helicase-activity in a two-hybrid system (**Chapter 5, Figure 2B**).

In conclusion, the work presented in this thesis has provided new insights into the genome of *C. difficile*, and the mechanisms that ensure its faithful replication. We have made important steps in understanding how *C. difficile* responds to antimicrobial compounds targeting DNA replication. Finally, in this discussion we have outlined how our findings can be related to other DNA replication and stress resistance in organisms and how the results of our research can be used in the development of novel antimicrobials.

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