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New tools and insights in physiology and chromosome dynamics of *Clostridioides difficile*

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CHAPTER 1

General Introduction and Thesis Outline

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Clostridioides difficile Infection

Clostridioides difficile, formerly known as *Clostridium difficile*¹ or *Bacillus difficilis*², was originally described in 1935 by Hall and O'Toole² as a member of the intestinal flora of newborns. In the same study, they also showed, however, that *C. difficile* was toxic to guinea pigs and rabbits².

At the time, *B. difficilis* was so-called due to difficulty in isolation and study, as it grew slower than most bacteria at the time and only in an anaerobic environment². In 1978, Bartlett *et al.* isolated *C. difficile* from the faeces of hamsters with clindamycin-induced diarrhoea and colitis, confirming this pathogen as the cause of antibiotic-induced disease in animals³. Later, scientists were able to prove that toxigenic clostridia were the cause of pseudomembranous colitis (PMC)⁴, and that isolation of *C. difficile* was possible from the stools of PMC patients⁵.

Since then, *Clostridioides difficile* Infection (CDI) has been identified as the major cause of hospital-acquired diarrhoea^{6,7}. Several symptoms have been associated with CDI. Mild, and oftentimes self-limiting diarrhoea is the most common, but CDI can also lead to severe inflammation of the colon, pseudomembranous colitis, toxic megacolon, and organ failure, eventually resulting in death^{6,8}. The main virulence factors of *C. difficile* are the large clostridial toxins that induce damage to epithelial cells and lead to an inflammatory response that underlies the symptoms of CDI⁹⁻¹¹.

C. difficile can be found in the intestinal tract, but as an opportunistic pathogen, it relies on the perturbation of the normal gut microbiota to colonize and lead to infection⁶. Healthy individuals have a balanced gut microbiota that prevents the development of CDI¹²⁻¹⁴. The gut microbiota is a complex community of microorganisms that aids in the intake of nutrients, modulates the immune system, and confers protection against colonization by pathogens, thus playing a crucial role in human health¹⁴⁻¹⁷. CDI development is additionally prevented by the commensal gut bacteria through the conversion of primary into secondary bile acids and production of antimicrobial peptides^{18,19}.

For a long time, the development of CDI has been linked to antibiotic use, which greatly reduces the diversity of the intestinal microbiota^{5,8,20}. However, other factors can affect the composition of the microbiota and the resistance of the host to the development of CDI, such

as advanced age of the individuals, the presence of co-morbidities, and the use of other drugs, like proton pump inhibitors or chemotherapy ^{6,21}.

Until recently, CDI was predominantly regarded as a nosocomial infection and was not viewed as a major public health threat. However, the incidence and severity of infections caused by *C. difficile* have increased since the early 2000s ^{8,22}. Outbreaks in healthcare facilities worldwide were reported with higher mortality rates, due to so-called “hypervirulent” or epidemic strains, especially PCR ribotype 027 (RT027) ²³. Several factors may contribute to the higher mortality rates associated with this ribotype, such as the production of a binary toxin by these strains or the increased sporulation ²⁴⁻²⁶. However, other studies report no difference between RT027 strains and other PCR ribotypes ²⁷. Thus, the exact contribution of different factors is still unclear ²⁸.

Nevertheless, spore formation is crucial for *C. difficile* survival and transmission ²⁹. Spores are resistant to a different number of factors, such as elevated temperature, low pH, antimicrobial compounds, and even aerobic conditions. This ensures *C. difficile* viability in many conditions and environments, allowing it to persist for a long period outside the host, thus contributing to transmission ^{30,31}.

Within healthcare facilities, several studies have shown transmission of *C. difficile* from CDI patients to other patients and healthcare workers ³²⁻³⁴. However, only 20-45% of the hospital CDI cases appear to come from direct transmission from other CDI patients ^{33,35,36}. Indeed, an increasing number of community-acquired CDI cases has been noted, also in individuals that did not receive antibiotic therapy and with fewer co-morbidities ^{13,37}. This suggests a potential source of infection outside the healthcare environment. The presence of *C. difficile* spores has also been demonstrated in soil, water, and food, and many animals (e.g. pigs and dogs) were identified as reservoirs ³⁸⁻⁴³. The exact contribution of these different potential sources, however, is unknown ³⁸.

The presence and circulation of different strains in the environment are also underscored by asymptomatic *C. difficile* carriers ⁴⁴. Compared to healthy adults, infants and neonates have a high prevalence of *C. difficile* colonization without clinical symptoms of CDI (up to 17%) ^{13,45-47}. But also in adults, up to 15% can be asymptomatically colonized ^{48,49}. Reasonably, asymptomatic *C. difficile* colonization is higher in adults with underlying diseases or when exposed to a healthcare environment ^{13,49-51}.

C. difficile life cycle and virulence factors

Below, we discuss factors affecting colonization and disease development in the context of the *C. difficile* lifecycle (Fig. 1), and how these factors are regulated.

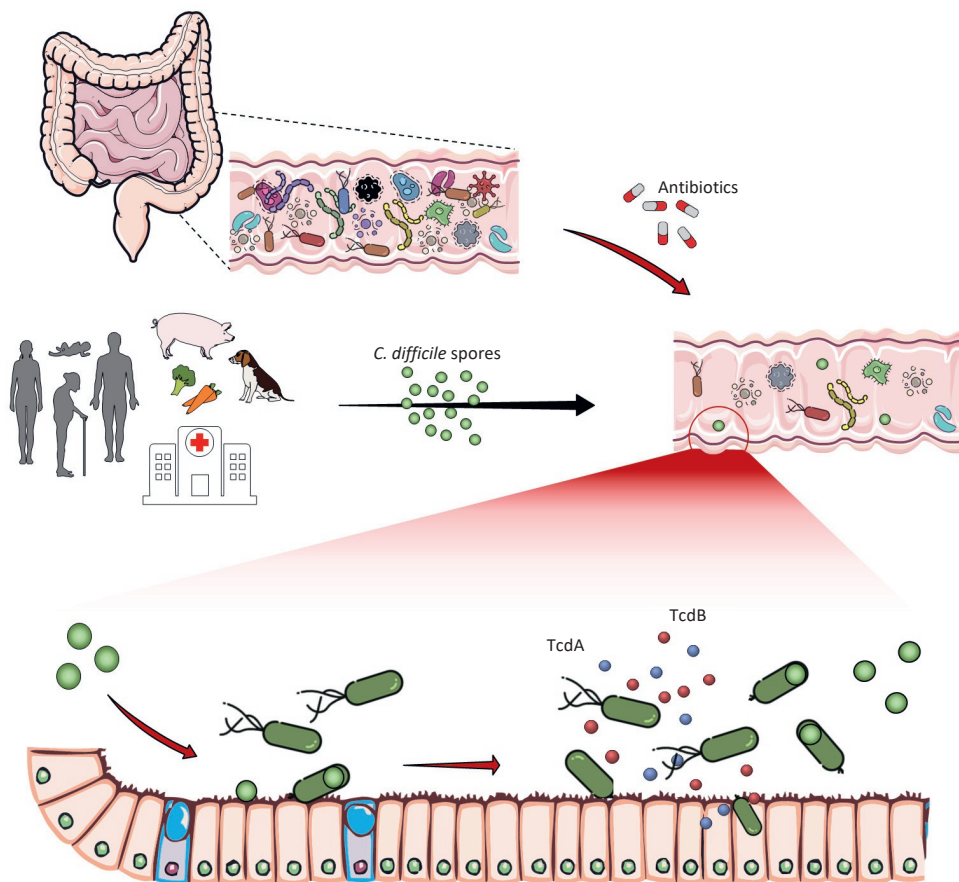


Fig. 1 – Representation of *C. difficile* life cycle in the human gastrointestinal tract. Schematic representation of the healthy microbiota in the gastrointestinal tract. Antibiotic treatment disrupts the healthy gut microbiota, reducing microbial diversity. Acquisition of *C. difficile* spores from different sources is indicated (community, healthcare and environmental). With the disrupted microbiota the spores are able to germinate and grow out to vegetative cells. During the vegetative cycle, *C. difficile* produces toxins, that damage the colonic epithelium, and is able to produce new spores, essential for the transmission. Image was produced with content from Servier Medical Art technical illustrations, under a Creative Commons Attribution 3.0 Unported License (<https://creativecommons.org/licenses/by/3.0/>).

C. difficile is a strict anaerobic organism that is able to form spores. The presence of oxygen is detrimental for the survival of vegetative *C. difficile* cells, and spores are therefore not only important for the survival outside the host environment but also crucial for *C. difficile*

transmission and persistence in the host. In mice, infection with a *C. difficile* strain unable to sporulate prevents *C. difficile* transmission and persistence within the intestinal tract ^{29,52}.

When ingested by the host *C. difficile* spores are able to pass the gastric barrier and reach the intestine. Spore germination and outgrowth are induced by specific cues from the external environment, such as the levels of bile salts present in the intestine. In the absence of balanced gut microbiota, *C. difficile* cells can adhere to epithelial cells of the intestinal tract and establish a population of actively dividing cells.

Once *C. difficile* is able to proliferate, the ability to produce cytotoxins is important to establish CDI. Secretion of the toxins will cause damage to the colonic mucosa and eventually lead to severe diarrhoea. Subsequent shedding of the spores to the environment enables the transmission to new hosts.

Sporulation initiation and spore formation

The sporulation process is tightly controlled. Several regulators of the process have been identified ^{53,54}. The key regulator for entry in sporulation is the Spo0A protein, which influences the transcription of more than 400 genes, including the ones to initiate sporulation ⁵⁵⁻⁵⁷.

Sporulation initiation is generally linked to nutrient depletion. In *B. subtilis*, where sporulation has been extensively studied, the environmental cues are sensed by sensor kinases, triggering a phosphorelay system that leads to activation of Spo0A by phosphorylation ^{58,59}. However, in *C. difficile* no homologs of the known phosphotransfer proteins are present ⁵³. To date, only one kinase has been identified that directly phosphorylates Spo0A, although this protein is still not very well characterized ^{53,59,60}.

Several other proteins involved in *C. difficile* sporulation initiation have been identified. CcpA, a regulator of *C. difficile* carbohydrate metabolism, negatively regulates Spo0A and the sensor histidine kinase expression in the presence of glucose ⁶¹. Disruption of the oligopeptide permeases Opp and App lead to increased sporulation, most likely due to the inability to uptake nutrients ⁶². The regulator RstA has been identified to play a major role in the regulation of sporulation initiation and toxin gene expression. Deletion of *rstA* decreases sporulation frequency and expression of the sporulation specific genes, and positively influences both motility and toxin production through the flagellar-specific sigma factor σ^D ⁶³⁻⁶⁵.

In *C. difficile*, activation of Spo0A leads to the activation of several genes, including the sporulation specific RNA polymerase sigma factors, σ^F in the forespore and σ^E in the mother

cell, as well as genes required for the rearrangement of the chromosome and asymmetric division ^{56,57,66}. Spo0A also affects the expression of several other regulators, resulting in it being a global transcriptional regulator for colonization, virulence, metabolism as well as sporulation ⁵⁷.

Asymmetric division leads to two unequal compartments, the mother cell and the forespore (Fig. 2). Upon asymmetric division, the first sigma factors of sporulation, σ^F and σ^E , are activated and then replaced by σ^G and σ^K , that will direct the final steps of the spore formation and maturation ^{67,68}. The forespore is engulfed by the mother cell membrane and several protein layers are placed around the forespore membrane to ultimately form the mature spore ^{68,69}. The mature spore is then released into the environment by lysis of the mother cell.

Chromosome segregation is a fundamental step for the positioning of the divided chromosomes (Fig. 2). In *B. subtilis*, at the onset of sporulation, Spo0J and Soj (required for chromosome partitioning) are essential for the correct orientation of the chromosome at the pole of the cell, re-organizing the chromosome in a single filament along the long axis of the cell ⁷⁰⁻⁷². The asymmetrically positioned septum divides the cell into two unequal compartments, where approximately 30% of the chromosome is positioned within the forespore compartment. The remaining 70% of the chromosome is then translocated into the forespore by the ATPase SpoIIIE ⁷¹⁻⁷³. In *B. subtilis*, Spo0A is also directly involved in chromosome dynamics, by regulating chromosome copy number by directly binding to the origin of replication. Several Spo0A-boxes were identified in the origin region where the cell replication initiator DnaA protein binds, which suggests that Spo0A plays an important role in the coordination between sporulation and cell replication ^{74,75}. The role of Spo0A in these processes is unexplored in *C. difficile*.

Despite important differences between spore-forming bacteria, the overall structure of the spore is quite similar, with a dehydrated inner core surrounded by several protective layers ⁷⁶. The spore core contains the bacterial chromosome, packed in the presence of the small acid-soluble spore proteins (SASPs) and calcium pyridine-2,6-dicarboxylic acid (Ca-DPA), that protect the DNA and confer resistance to different environmental challenges ^{69,77}. The protective layers consist of the inner spore membrane, the cortex, the coat and the exosporium (Fig. 2) ^{30,78}.

The inner spore membrane has a similar composition as the mother cell membrane, as a result of membrane engulfment during sporulation. The cortex is responsible for maintaining the dehydration of the spore core and is also involved in spore resistance to the environmental challenges. It is formed by modified peptidoglycans which contain low cross-linking, allowing

the deposition of cysteine-rich proteins that will form the spore coat. Several proteins are positioned around the cortex layer forming the inner coat and an electron-dense layer, the outer coat ^{79,80}.

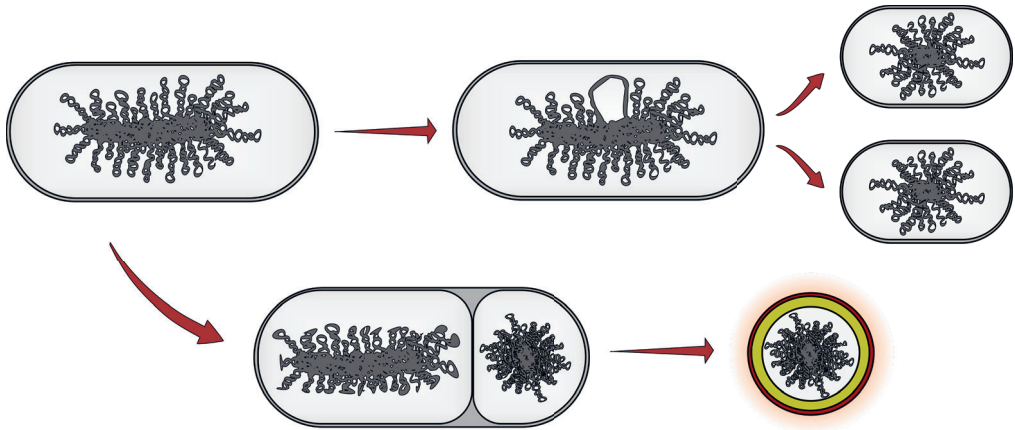


Fig. 2 – Schematic representation of chromosome organization during cell division and differentiation in *C. difficile*. Cell division of the vegetative cell, with initial unwinding at the origin of replication is represented. The chromosome is replicated and segregated to the daughter cells. When in harsh conditions *C. difficile* undergoes asymmetric division, giving two unequal compartments, the mother cell, where the chromosome is re-organized in a single filament, and the forespore. The spore is released to the environment when mature. The inner spore membrane (black line), cortex (yellow), coat (red) and exosporium (orange) surrounding the forespore are depicted.

The coat layer is composed of several proteins and although in *B. subtilis* more than 70 spore coat proteins have been identified, only a few orthologues are found in *C. difficile*, such as SpoIVA, SpoVM and Cote ^{30,81,82}. Nevertheless, several additional coat proteins of *C. difficile* have been characterized, such as SipL (that binds to SpoIVA) and CotL, both important for the assembly of the coat ^{80,83,84}. The intricate layer of proteins confers resistance against several chemical and physical conditions, such as oxidative compounds or UV light, but also to compounds that would affect the peptidoglycan structure, as it shields the cortex and membrane layers. The coat layer also contains receptors essential for the recognition of the compounds that trigger germination (see below).

Several endospore-producing bacteria have been reported to possess an exosporium, which provides an extra layer of protection and can modulate germination ⁸⁵. However, in *C. difficile* this layer appears to be variable between strains, not only in layer stability but also in morphology ^{30,86}. One of the main components of the *C. difficile* exosporium are collagen-like glycoproteins, like BclA, that promote interactions with host cells and are very important to keep the coat integrity ⁸⁷. It has been shown that a *bclA* mutant is unable to colonize mice but

could germinate faster, emphasising the importance of this exosporium protein for adhesion and germination of *C. difficile* spores in the gut ⁸⁷. More recently other cysteine-rich proteins present in the exosporium layer have been identified, like CdeC and CdeM. Mutation of these proteins leads to defects in coat and exosporium assembly and affects the adherence of the spores to the colonic mucosa ⁸⁸.

Germination

The germination process is induced by the recognition of small molecules, so-called germinants, that are sensed through specific germinant receptors ^{30,89}.

In *B. subtilis*, germination is triggered by L- amino acids, recognized by the GerA germinant receptor complex. In *C. difficile*, germination is triggered in the presence of a combination of nutrients and bile salts, specifically cholic acid derivatives, present in the gastrointestinal tract. Taurocholate (TA), a cholic acid derivative, is the most effective germinant *in vitro*. However, for the successful germination to occur co-germinants such as amino acids or divalent cations are required. *C. difficile* germination can respond to a number of amino acids (e.g. L-alanine, L-glycine), which trigger signaling through an unknown receptor ^{90,91}.

In *C. difficile*, there are no homologs of the Ger-type germinant receptors. Instead, bile acids are recognized by the receptor CspC, located at the spore coat. The bile acid recognition by CspC triggers a cascade of events that activates the SleC hydrolase, which is able to degrade the cortex layer and thereby leads to release of Ca-DPA from the spore core ⁹². Ca²⁺ has an important role during germination, probably by being a co-factor required for enzymatic activity involved in *C. difficile* spore germination ⁹³. Several proteins have been identified involved in the spore germination and/or recognition of germinants ^{91,94}. GerS, the muramoyl-L-Alanine amidase, is required for SleC activation and consequently cortex degradation ⁹⁵. More recently, Cwld and the polysaccharide deacetylase PdaA were found to be essential for cortex-specific modifications that are required for SleC-mediated degradation ⁹⁶. Though several germinants exert an effect, a synergy between the different germinants appears to be essential for effective *C. difficile* spore germination, initiation of cell metabolism and a new life cycle.

During germination, major transcriptional events occur that lead to the elongation of the cell, cell division and chromosomal segregation. In *B. subtilis*, cell division proteins, such as the MreBHCD and MinCD complexes, are overexpressed throughout germination ^{97,98}. Interestingly, in *Bacillus megaterium*, the forespore chromosome appears ring shaped and upon germination the nucleoid is compacted, as in vegetative cells ^{99,100}. The conformational changes of the chromosome have been associated with the degradation of the SASP proteins

and possibly involve bacterial chromatin proteins, such as HBSu⁹⁹. However, for *C. difficile* these aspects are still unexplored.

Adhesion and Motility

Upon germination, *C. difficile* interacts with host surfaces and adheres to colonic epithelial cells^{101,102}. This is an important step in the development of the disease. The adherence and mobility of the cells is dependent on proteins present on the bacterial cell surface and secretion of proteins into the environment.

The main components of the flagella, the major flagellar structural subunit (FliC) and the flagellar cap protein (FliD), confer motility to the cells^{103,104}. Disruption of *fliC* or the *fliD* genes results in loss of flagella, and mutant strains adhere better than the wild-type *C. difficile* 630 Δ *erm* to Caco-2 cells¹⁰⁵. In the *fliC* mutant, several genes are differentially expressed, such as genes involved in metabolism, virulence and even sporulation¹⁰⁶. Motility and surface migration regulation, independent of flagella or type IV pilus (TFP), was recently identified through phase-variation of the signal transduction system, CmrRST¹⁰⁷.

The *C. difficile* cell wall proteins (CWPs) are the main components of the outmost surface layer¹⁰⁸. Several members of this protein family have been shown to affect adherence or colonization¹⁰⁸⁻¹¹⁰. The major components of the cell surface of *C. difficile* are the high- and low-molecular-weight surface layer proteins resulting from proteolytic processing of the SlpA protein^{111,112}. Different *slpA* genotypes have been identified across *C. difficile* strains^{113,114}. The processed SlpA proteins bind several components of epithelial cells, such as collagen, and are important for the strain-specific adherence^{109,114-116}.

Other paralogues of SlpA are also present on the cell surface¹⁰⁸. Beyond SlpA, Cwp84 has been the best-characterized of the CWPs. SlpA processing, crucial for the formation of an S-layer, is abolished when the *cwp84* gene is disrupted^{117,118}. Cwp84 also exhibits proteolytic activity against fibronectin and other components of the host tissue and affects biofilm formation¹¹⁹⁻¹²¹. The ability to bind to the host tissue has been observed for several of the CWPs and other proteins present at *C. difficile* surface¹²². Binding to fibronectin has been shown for the fibronectin-binding protein Fbp68. Inhibition of Fbp68 impairs adhesion to Vero cells¹²³. Other surface exposed proteins, such as CbpA and CD2831 have also collagen-binding properties^{124,125}.

Multiple cell-wall associated proteins are regulated through cyclic diguanylate (c-di-GMP) levels, and this molecule is therefore important for the transition from a sessile to a mobile state¹²⁶. C-di-GMP has been implicated in the regulation of flagellum biosynthesis and the

production of type IV pili, and therefore indirectly influences adherence of *C. difficile* to epithelial cells ¹²⁷. Additionally, it regulates the expression of the collagen-binding protein CD2831, as well as the associated metalloprotease PPEP-1. PPEP-1 has been identified amongst the most highly secreted proteins in both the laboratory strain 630 Δ *erm* (RT012) as well as the epidemic strain R20291 (RT027) ¹²⁸. PPEP-1 activity has been suggested to regulate the switch between adhesion and motility phases through the cleavage of Pro-Pro peptide bonds in CD2831 and other proteins ^{125,128,129}. Disruption of the gene encoding PPEP-1 (CD2830) results in higher affinity for collagen type I and attenuated virulence in hamsters ¹²⁵.

The effector molecule c-di-GMP also modulates biofilm formation ¹²⁶. Biofilms may allow *C. difficile* to persist for prolonged periods of time and provide a physical barrier that generates an environment resistant to external factors, such as antibiotics ^{130,131}. Biofilm formation in *C. difficile* is influenced by several different factors, including cell surface components (such as Cwp84) or Spo0A, the master regulator for sporulation ^{132,133}. Recently, the *C. difficile* *sin* locus has been shown to play a crucial role in the regulation of biofilms, as well as multiple other pathways by controlling other global regulators ^{134,135}. The Sin proteins affect mobility of *C. difficile* cells through regulation of σ^D expression, and therefore also regulate the expression of flagellar components, including FlhC ^{134,136}.

Toxin production

Once *C. difficile* has established itself in the colon of the host, it can induce disease. The development of CDI is mainly due to the action of secreted toxins, which compromise the intestinal barrier, for instance by disrupting the actin cytoskeleton of the epithelial cells, leading to morphological alterations and eventually cell death (Fig. 1) ^{6,10}.

C. difficile can contain two large clostridial toxins: TcdA and TcdB. TcdA and TcdB are homologous, high-molecular-weight proteins of 205 and 308 kDa, respectively, and render the Rho-family GTPases, essential for the assembly and organization of the actin cytoskeleton, inactive ^{137,138}. The collapse of epithelial cells and disruption of the tight junctions between the cells enable bacterial cells and toxins to cross the epithelium and induce an inflammatory response ^{10,139}. The mode of action of TcdA and TcdB has been extensively studied ^{9,140-142}. In short, the toxins are internalized by endocytosis and upon acidification of the endosome conformational changes take place, leading to pore formation and translocation of the N-terminal region into the cytosol. This functional domain contains glucosyltransferase activity, and the resulting glycosylation of Rho-family GTPases (e.g. Rho, Rac) prevents the interaction with their substrates ^{10,141}.

Several receptors have been identified for TcdA and TcdB. The glycoprotein gp96 has been identified as a receptor for TcdA, and is present at the membrane of the colonic epithelial cells¹⁴³. Recently, a wide range of glycans present at the host cell membranes were identified to bind TcdA and TcdB¹⁴⁴. Frizzled proteins (FZDs) are a family of transmembrane proteins present at the colonic epithelium and have been identified as TcdB receptors¹⁴⁵. Recently, it has been shown that TcdA binding to the colonic epithelium can also be mediated by sulfated glycosaminoglycans (sGAGs) and the low-density lipoprotein receptor (LDLR)¹⁴⁶.

Both large clostridial toxins are encoded in a 19.6 kb chromosomal region termed pathogenicity locus (PaLoc). The PaLoc generally encodes at least three more proteins^{10,147}. TcdE is a holin-like protein. Overexpression of this protein is lethal to *C. difficile* and it may be required for efficient toxin secretion^{148,149}. TcdR is an alternative sigma factor and when bound to RNA polymerase allows recognition of target gene promoters, activating the expression of *tcdA*, *tcdE* and *tcdB*, as well as its own *tcdR* gene^{11,150}. TcdC is thought to be a negative regulator of toxin transcription¹⁵¹⁻¹⁵³, but the role of TcdC in toxin regulation is still unclear as some studies failed to detect an effect of *tcdC* on toxin gene expression^{25,55,154}.

In addition to the PaLoc-encoded (putative) regulators, *C. difficile* toxin expression is subject to complex regulation by many different factors, in a growth-dependent manner^{60,136,155}. The expression of *tcdA*, *tcdB*, *tcdE* and *tcdR* strongly increases upon entry into stationary growth phase, and low levels are detected during exponential growth^{60,151}. Environmental conditions such as temperature can affect the expression of the toxin genes¹⁵⁶. The expression of *tcdA* and *tcdB* is greatly influenced by the composition of the growth medium, such as certain carbon sources or amino acids that can impair the expression of the toxin genes^{155,157-159}. The carbon catabolite control protein A (CcpA) regulates genes associated with the metabolism of different carbon sources, and inhibits toxin expression in the presence of glucose⁶¹. The nutritional regulator CodY directly regulates the expression of the toxin genes depending on the growth conditions, as the availability of branched chain amino acids (BCAAs)¹⁶⁰⁻¹⁶².

Known general regulators, as the motility-associated sigma factor σ^D and the sporulation regulator Spo0A also affect the expression of the toxin genes. Inhibition of σ^D -activity represses toxin gene expression, as it is a positive regulator of *tcdR* transcription¹³⁶. The effects of Spo0A on toxin expression may be strain dependent, as no effects are observed for strains such as *C. difficile* 630 Δ erm, whereas Spo0A appears to repress toxin expression in some RT027 strains^{60,74,163}. The link between sporulation and toxin expression is reinforced by the discovery of the regulator RstA. RstA regulates σ^D activity, but represses toxin gene expression independently of σ^D -dependent toxin regulation⁶⁴.

Some *C. difficile* strains also encode a third toxin, the binary *Clostridioides difficile* transferase (CDT)²⁶. CDT is encoded by the *cdtA* and *cdtB* genes in the *cdt* locus (CdtLoc), which contains also the positive regulator CdtR¹⁶⁴⁻¹⁶⁶. Uptake of CDT by epithelial cells is mediated by the lipolysis-stimulated lipoprotein receptor (LSR)^{26,167}. Several studies have structurally characterized the binary toxin complex, and elucidated aspects of CDT entry into host cells^{168,169}. Upon entry, CDT is an actin ADP-ribosylating enzyme that inhibits the polymerization of the actin cytoskeleton, thereby inducing cell rounding and increased adherence of *C. difficile* cells, through the formation of microtubule protrusions on host cells¹⁷⁰. CDT has also an important role in the modulation of the immune host response. It suppresses the protective eosinophils present in the colon and blood, although the exact mechanism is still unclear¹⁷¹.

Treatments and new developments

Over the past decades there has been an increase in the incidence and severity of CDI worldwide¹⁷²⁻¹⁷⁴. Although treatments for CDI can be effective in resolving CDI symptoms, recurrent infections are present in 15% to 30% of the cases^{175,176}.

The first step in CDI treatment is the discontinuation of all antibiotic therapy that may have instigated the episode of CDI by destabilizing the microbial community. Further treatment of CDI depends on disease severity and co-morbidities of the patient. Until recently, metronidazole was the first-line treatment for mild to moderate CDI. However, data on inferiority compared to vancomycin and resistance to metronidazole has led to it only being recommended for specific situations^{177,178}. At present, the first-line of treatment is vancomycin or fidaxomicin, for initial CDI episodes¹⁷⁷. Of these, fidaxomicin shows significantly lower recurrence rates, and it is believed this is in part because fidaxomicin associates with the exosporium of *C. difficile* spores, which results in antimicrobial activity upon germination and outgrowth¹⁷⁹⁻¹⁸².

The use of antibiotic therapy for CDI also affects commensal bacteria that can protect against the reinfection and this may in part explain the relatively high recurrence rate observed with antimicrobial therapy. Patients with recurrent CDI have a reduced microbial diversity, not only when compared to healthy subjects but also when compared to CDI patients with a first episode¹⁸³. To address this, faecal microbiota transplantation (FMT) was developed. FMT restores a stable and diverse gut microbiota and is successful in preventing disease recurrence up to 90% of the cases¹⁸⁴⁻¹⁸⁷. The success of FMT in resolving CDI is believed to be due to several factors, such as niche competition and the secretion of *C. difficile*-inhibiting

bacteriocins by other bacterial species¹⁸⁶. The introduced microbial community also leads to a shift in bile acid composition, where the presence of secondary bile acids inhibits germination of spores and outgrowth of vegetative cells^{30,188,189}.

In recent years, several investigational therapies and treatments have been developed. The success of the FMT treatment in curing and prevention of relapse of CDI, has led to the investigation of the microbiota constituents that could replicate the effect of an FMT. In mice, resolution of CDI was possible with a combination of six selected strains¹⁹⁰. However, though important progress has been made with development of several live biotherapeutic products (LBP) and clinical studies are underway^{191,192}, to date no LBPs formulated have been introduced to the market^{191,192}.

As toxins are the primary cause of CDI, they have been widely explored as therapeutic targets. Several vaccines are under development, containing a part of or a full-length recombinant *C. difficile* toxin, or even antibodies against the toxins⁶. The actoxumab and bezlotoxumab (Merck) antibodies against TcdA and TcdB are able to neutralize toxin activity in mice, and bezlotoxumab can reduce recurrence in humans¹⁹³⁻¹⁹⁶. The IC84 vaccine (Valneva) uses the recombinant truncated forms of TcdA and TcdB¹⁹⁷. The potential of targeting the toxins with small molecules has also been demonstrated¹⁹⁸⁻²⁰⁰, but some therapies aimed at toxin neutralization have failed in clinical trials (e.g. Tolevamer)^{201,202}. Nevertheless, toxins remain an attractive target due to their direct involvement in disease and currently several clinical trials are ongoing^{191,203}. Finally, *C. difficile* is able to produce R-type bacteriocins, designated by diffocins, that target specific SlpA genotypes, present in other *C. difficile* strains^{116,204,205}. Diffocins, despite the strain specificity, pose promising candidates against *C. difficile*, as they do not disrupt the healthy microbiota¹¹⁶.

Recent years have seen the preclinical development of several new therapeutics targeting different mechanisms, such as cell surface components or the metabolism²⁰⁶. But due to the success of antimicrobial therapy for CDI in the past, new and novel antimicrobials remain a focus in research on new CDI treatments. However, new CDI treatments are no guarantee for successful clinical development, as demonstrated by the case of surotomycin. Surotomycin is a calcium-dependent cell membrane-depolarizing agent with efficacy against *C. difficile* demonstrated *in vitro*, but it failed to meet the endpoint of superiority over vancomycin in phase III clinical trials and clinical development was discontinued²⁰⁷⁻²¹⁰.

Interestingly, several of the novel compounds have DNA replication as a target: cadazolid (Actelion Pharmaceuticals), that targets DNA gyrase, and ibezapolstat/ACX-362E (Acurx Pharmaceuticals) that targets PolC, for instance^{206,211-213}. Although components of the

replication system are attractive targets for antibiotic development, they are still underexplored ^{211,214}.

Chromosomal DNA replication and genome maintenance

DNA replication is a crucial process for the viability and integrity of the genetic information, and overall is conserved across all organisms ²¹⁵. During replication, double-stranded DNA is unwound and replicated in a semi-conservative manner, where the individual strands serve as templates for synthesis of a complementary strand. Several mechanisms ensure the timing and the fidelity of replication process, allowing a cell to successfully copy its DNA and segregate the two copies of the chromosome into two daughter cells (Fig. 2) ²¹⁶.

DNA replication starts with the action of different cell components at a specific location on the chromosome, the origin of replication. Unwinding of the chromosomal origin region, generally termed *oriC* in bacteria, allows the deposition of a replication complex, the replisome. This complex includes DNA polymerases, that provide the catalytic activity for the synthesis of the new strands. In bacteria, that mostly have a single circular chromosome, two replisomes move in opposite directions, replicating the chromosome bidirectionally ^{216,217}. Eventually, the replication complex reaches the terminus of replication region, where replisome activity is arrested and the complex gets disassembled ^{218,219}.

Many proteins are involved in the formation of the replisome or in the regulation of its activity (for a schematic representations see for instance Fig. 1 in van Eijk *et al* ²¹⁴), ensuring the efficiency of the replication process, coordination with other cellular processes and an appropriate response to environmental conditions ²²⁰⁻²²². Particular features of DNA replication are broadly conserved in bacteria. In nearly all bacteria, the DNA replication initiator protein is DnaA, a protein that binds to specific sequences (DnaA-boxes) within the origin region ²²³. DnaA is a highly conserved AAA+ adenosine triphosphatase (ATPase), essential for the initial duplex unwinding ²¹⁹. The homolog of this protein in *C. difficile* is CD0001 ^{214,219}.

Upon the unwinding of the *oriC*, the replicative helicase and other proteins are recruited to the unwound region in a hierarchical manner ²²². This process demonstrates some remarkable differences between organisms ²²⁴ and the situation in *C. difficile* appears to differ from that of the Gram-positive model organism *Bacillus subtilis* *in vitro*. Though both organisms appear to load their helicase through a ring-maker mechanism, in which the monomers of the helicase are assembled into a hexameric ring around the unwound region, *C. difficile* helicase (CD3657) activity requires the presence of the primase CD1454 in addition to the loader

ATPase (CD3654) *in vitro*, whereas the *B. subtilis* helicase (DnaC) only requires the loader ATPase (DnaI) ²²⁵.

Similarly, differences exist with respect to the primase protein and its activity. Primase synthesizes short DNA primers to allow for the discontinuous synthesis of DNA on the lagging strand by the DNA polymerase. The primase protein in *C. difficile* initiates primer synthesis with a different trinucleotide motif preference than its *B. subtilis* equivalent ²²⁵.

The actual DNA synthesis is performed by the DNA polymerase III holoenzyme. The polymerase is composed of three components: the core with polymerase and proofreading activities (PolC; CD1305 and CD2394); the sliding clamp (DnaN; CD0002), which is required for processivity; and the clamp loader, consisting of the δ (CD2474), δ' (CD3549) and τ/γ (CD0016) subunits. PolC is unique for low-[G+C] Firmicutes and is a primary target for drug development ^{211,213,226,227}. Notably, ibezapolstat/ACX-362E (Acurx Pharmaceuticals) has been developed to specifically inhibit *C. difficile* growth and is believed to have low activity towards other organisms present in the gut, suggesting a potential for species-specific therapeutics within this class of compounds ^{213,227,228}.

Many proteins can influence the efficiency of DNA replication ^{214,215,219}, such as gyrase, single-stranded DNA-binding protein (SSB), and nucleoid associated proteins (NAPs) ^{214,215,219}. DNA gyrase removes positive supercoiling from chromosomal DNA, which is essential for the unwinding of double-stranded DNA at the origin of replication and the progression of the replication complex, and has been explored as a potential therapeutic target in *C. difficile* (cadazolid; Actelion Pharmaceuticals) ^{212,214,229}. The SSB protein binds and stabilizes single-stranded DNA that results from the DNA-unwinding activity of for instance the replicative helicase and DnaA, protecting the exposed DNA from exonucleases ²³⁰. NAPs are crucial for the maintenance and organization of the chromosome in many species. Several of these, like HU and H-NS, modulate DNA topology and thereby affect transcription regulation in different organisms ^{231,232}. In *E. coli*, for example, HU can influence the *oriC* unwinding by stabilizing the DnaA oligomer at the *oriC* region ²³³. Despite the wealth of information from other organisms, these proteins are still unexplored in *C. difficile*.

Many insights in DNA replication are derived from biochemical experiments. For instance, DnaA-dependent unwinding is often explored by analysis of the unwound region *in vitro* and the sequence of the bound DNA region ^{234,235}. Additionally, the use of bacterial two-hybrid complementation assay has allowed researchers to determine the interaction interface of DnaA and DnaD proteins in *B. subtilis* ²³⁶. Fluorescence microscopy is also a valuable tool, enabling the visualization of the DnaA protein and assessing the replisome function ²³⁷⁻²⁴⁰.

However in *C. difficile*, replication is largely unexplored ^{214,225} and despite the development of new methodologies over the years, *C. difficile* manipulation remains a challenge ^{67,241,242}.

Laboratory manipulation: from diagnosis to mutagenesis and physiology

As pointed out above, a great deal of information can be derived from genetic, biochemical and cell biological investigations of cellular pathways. *C. difficile* is no exception to this rule. Increasing knowledge has allowed for the development of different sets of tools, ranging from the diagnosis of CDI in patients to understanding the role of toxins and more fundamental research questions.

To curb the transmission of *C. difficile*, CDI diagnosis needs to be accurate and quick. Over the years diverse methods for diagnosis have been used, although a consensus approach has been the goal worldwide ^{243,244}. Several methods, like the enzyme immunoassays (EIAs), target the toxins or the protein glutamate dehydrogenase (GDH). GDH is an enzyme highly produced by *C. difficile*, which converts L-glutamate into α -ketoglutarate in the presence of the nicotinamide adenine dinucleotide (NAD) co-factor ²⁴⁵. Currently, the use of real-time PCR or nucleic acid amplification test (NAATs) targeting the toxin genes have become common methods for CDI diagnosis. However, the use of only NAATs is not advisable as it could lead to overdiagnosis due to asymptomatic carriage ¹³. Therefore, a two-step algorithm is recommended for quick and reliable CDI diagnosis, combining the use of NAATs with the EIAs ^{177,243}. In spite of these methods, culturing of CDI-associated isolates is required for further characterisation, including typing and antimicrobial susceptibility testing.

In 2006, the first *C. difficile* genome was sequenced, from the strain 630 that was isolated from a CDI patient ²⁴⁶. Whole genome sequencing (WGS) is a powerful tool to compare isolates and identify strain specific differences ^{247,248}. WGS enabled analysis of subtle genetic changes between different *C. difficile* laboratory strains, like *C. difficile* strains 630 and 630 Δ erm ^{249,250}. The analysis of the sequenced genomes is not only essential for analysis of diversity and evolution of bacteria but also instrumental for our understanding of biological pathways and the mechanisms underlying their regulation.

Genetic manipulation can largely be divided into two lines: one aimed at altering chromosomal genes, by mutagenesis or disruption, and another aimed at adding extra genetic information, either by introduction on plasmids or by integration into the chromosome. For instance, these techniques have been crucial in establishing the importance of the toxins for CDI ^{55,142}.

Several methods for the delivery of genetic information to mutate or disrupt a gene of interest in *C. difficile* have been developed. Delivery of plasmids into *C. difficile* is almost exclusively done through conjugation from donor species like *E. coli*, and shuttle vectors have been developed that replicate both in *E. coli* and *C. difficile* ²⁵¹⁻²⁵⁴. O'Connor and co-workers were the first to successfully disrupt genes in *C. difficile*, using a conditional shuttle vector that could generate insertional mutants ²⁵⁵. Subsequently, the TargeTron/ClosTron system (based on a Group II intron) was introduced, which greatly facilitated genetics of *C. difficile* ^{60,256-259}. However, insertional mutagenesis can have polar effects, altering the expression of neighbouring genes ²⁶⁰. At present, sophisticated systems for genetic manipulation of *C. difficile* allow for clean and marker-less mutations, like allele-coupled exchange, including *pyre* ²⁶¹, *codA* ^{261,55}, and CRISPR-Cas based systems ^{242,262-264}.

In parallel with the development of the genetic manipulation toolkit of *C. difficile*, there was an increase in the development and characterization of different reporters to study gene expression and protein localization. Generally, such reporters can be divided in three categories: luminescent, chromogenic, or fluorescent reporters.

Luminescence refers to the spontaneous emission of light in thermal equilibrium. In general, luminescence light production is a chemical process that relies on the interaction of an enzyme with its specific substrate ²⁶⁵. In nature, several organisms are able to emit light (bioluminescence). These are mostly marine organisms, but bioluminescence is also found in insects, such as the firefly, in which light emission is associated with the protection and survival of the species, by attracting a prey or as a defence mechanism ²⁶⁵. To date, several bioluminescent systems have been described, also in bacteria, and have been applied for a wide variety of molecular applications ²⁶⁶.

The use of firefly luciferase requires the addition of the exogenous substrate D-luciferin, that is catalytically oxidized into oxyluciferin in an ATP-dependent manner, resulting in light emission ²⁶⁷. Another, more recently developed and commercially available luciferase enzyme is the NanoLuc luciferase, derived from the deep-sea shrimp *Oplophorus gracilirostris* ²⁶⁸. Emission of light due to Nanoluc activity relies on the substrate furimazine and is of particular interest for biological applications, not only due to the small size (19 kDa) of the Nanoluc enzyme, but also due to the intensity of luminescent signal ²⁶⁸⁻²⁷⁰.

In contrast to luciferases from other organisms, the luciferase system of bacteria does not require the addition of an exogenous substrate. The luciferase activity is encoded by a *lux* operon, that also contains the specific enzymes required for the oxidation of intracellular FMN substrate ²⁷¹. The *lux* operon has been used for example for the detection of *Bacillus anthracis*

spore germination in infected mice ^{272,273}. Although luciferase systems have been used in multiple organisms, no such tools have been developed for *C. difficile* prior to the work described in this thesis.

Similar to most luminescent systems, chromogenic reporters require a chemical conversion. They are based on a substrate that changes its visible colour in the presence of a specific enzyme. The most commonly used chromogenic reporter is the β -galactosidase (*lacZ*), that uses the artificial chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) and produces a blue colour when cleaved by β -galactosidase ^{274,275}. For *C. difficile*, the best known chromogenic reporters are β -glucuronidase (GusA) and alkaline phosphatase (PhoZ). GusA catalyses the hydrolysis of β -D-glucuronides, such as p-nitrophenyl- β -D-glucuronide (PNPG), which can be measured spectrophotometrically. Amongst others, GusA has been used to demonstrate the functionality of the tetracycline-inducible promoter (P_{tet}) in *C. difficile*, but has also been used to investigate the expression of *C. difficile* toxin genes in the heterologous host *Clostridium perfringens* ^{156,253}. PhoZ is widely used with the substrate p-nitrophenyl phosphate (pNP), that results in a blue colour that can also be assessed by measuring the absorbance at a specific wavelength. It has been applied in *C. difficile* for example to characterize the nisin-inducible promoter (P_{cprA}) ²⁷⁶ and to demonstrate the role of RstA as a pleiotropic regulator ⁶⁴. However, the use of both GusA and PhoZ reporter systems requires cell lysis, as these reporters are expressed intracellularly, require the substrate for activity and/or can only efficiently be measured in the absence of interfering cellular debris ^{253,276}. To circumvent some of these issues, chromogenic proteins have been developed that absorb ambient light to display a visible colour ²⁷⁷. The use of these proteins in *C. difficile*, however, is limited, as these proteins require molecular oxygen for colour development. Additionally, chromogenic reporters generally do not allow for single-cell analyses.

Fluorescence, in contrast to luminescence, requires the absorption of high-energy light by specific molecules called fluorophores. This allows the transition of electrons to an excited state. When the electrons return to their ground state the excess of energy is released in the form of fluorescence light, which is characterized by longer wavelengths than the excitation wavelength ²⁷⁸. Fluorescence can result from small molecules, or proteins that are genetically-encoded (such as GFP) and is exploited in flow-cytometry or fluorescent microscopy, that are suitable to analyze fluorescence in single cells. Flow-cytometry has been used to analyze the adherence of *C. difficile* to epithelial cells using the fluorochrome BCECF/AM (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetomethyl ester) ²⁷⁹. In contrast to flow-cytometry, microscopy also enables the analysis of the subcellular localization and the dynamics of proteins inside the cell. As a result, microscopy has become an important tool to

study different cellular components and pathways. Different fluorophores have been used for the labelling of proteins and gene expression in *C. difficile* ^{67,241,280,281}. The localization and function of cell division proteins, such as MldA and MldB, was investigated through the use of a codon-optimized version of the cyan fluorescent protein (CFP^{opt}) ²⁸⁰. Self-labelling tags, like SNAPtag, have also been described, with the advantage of being oxygen-independent. Fusion of SNAPtag to the sporulation sigma factors allowed for the characterization of the sporulation regulatory pathway in *C. difficile* ⁶⁷. Nevertheless, in *C. difficile* two major limitations prevent the successful use of a wide range of fluorescent proteins: the absence of environmental oxygen needed for maturation of the certain fluorophores and the intrinsic green fluorescence ²⁸¹. Thus, there is a continuous search for improved reporters and new methodologies.

Thesis Outline

In this thesis, we aimed to better understand some *C. difficile* physiological processes: genome maintenance, DNA replication, and toxin regulation. We concluded that available tools to investigate *C. difficile* were insufficient, thereby imposing a significant hurdle for research. Thus, during the progress of this work, it became our goal to develop novel methods for further *C. difficile* studies.

We summarized the relevant background on *C. difficile* in **Chapter 1**, the present chapter, of this thesis.

In **Chapter 2** we introduce two novel reporters AmyE^{opt}, encoding for an amylase, that leads to detectable degradation of starch, and sLuc^{opt}, derived from Nanoluc luciferase. We took advantage of the signal sequence of the abundantly secreted protease PPEP-1 to direct the secretion of the two reporters to the environment. We also use the secreted sLuc^{opt} to validate transcriptional regulation of the promoter of the toxin A gene (*tcdA*) in response to glucose.

The role of TcdC in toxin regulation has been highly controversial. In **Chapter 3**, we further characterize this enigmatic protein, that is encoded in the PaLoc, like the toxins. We analyze the topology of the protein in *C. difficile*, with the use of a complementation system (HiBit^{opt}) that allows determination of the subcellular location of the C-terminus of proteins. We found that the TcdC C-terminus is localized extracellularly, which is incompatible with a function as anti-sigma factor in toxin regulation and confirmed these results with additional biochemical experiments.

In **Chapter 4**, we identify the origin of replication (*oriC*) in *C. difficile* and identified several features that are conserved between different clostridial species. We demonstrate DnaA-dependent unwinding at the *oriC2* region in the intergenic region between the *dnaA* and *dnaN* genes *in vitro*, providing important information on the initiation of replication in *C. difficile*.

In **Chapter 5** we gain new insights in genome maintenance in *C. difficile*, through the characterization of the bacterial chromatin protein HupA. We find that overexpression of this protein leads to compaction of the chromosome *in vitro* and *in vivo*. In the characterization of the *in vivo* interaction of the monomers, we successfully adapt a novel two-hybrid system for *in vivo* protein-protein interactions studies (bitLuc^{opt}) and report the use of the HaloTag for fluorescent microscopy in *C. difficile*.

In **Chapter 6**, we describe the use of the fluorophores CFP^{opt}, mCherry^{opt}, phiLOV2.1, HaloTag, and SNAPtag^{Cd}, and explore the possibilities and limitations of *C. difficile* fluorescence microscopy, including an analysis of factors that contribute to *C. difficile* autofluorescence.

Finally, we summarize the results from our studies in **Chapter 7**, with a discussion on some of the implications of our findings and the perspectives for future research on *C. difficile* based on our work.

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