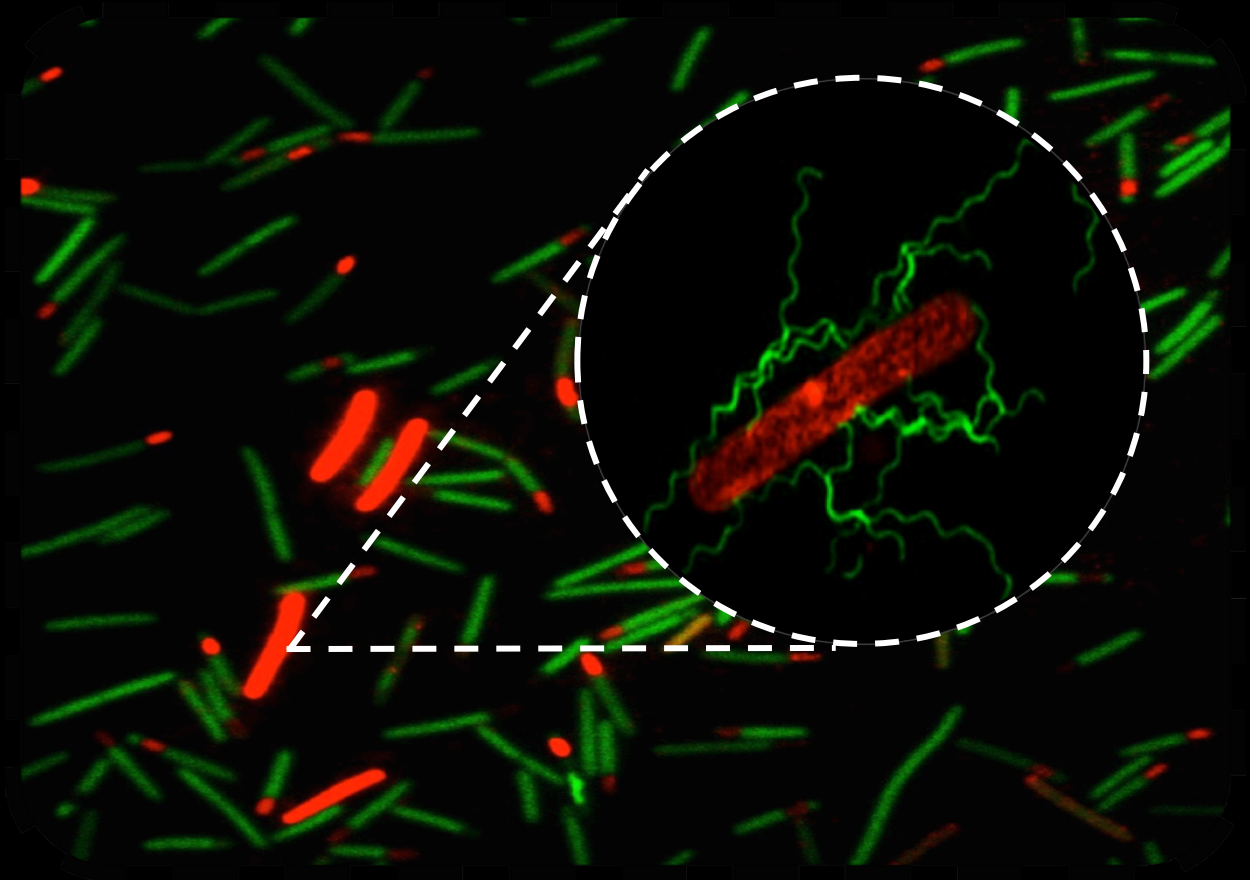


Single cell analysis of toxinogenesis by vegetative and sporulating cells in the enteric pathogen *Clostridioides difficile*

Carolina Piçarra Cassona



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
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Co-supervisor: Dr. Mónica Serrano, Microbial Development Lab., ITQB NOVA

This dissertation is dedicated to my family who always supported me

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LIST OF ABBREVIATIONS

CDI	<i>Clostridium difficile</i> Infection
Cm ^R	Chloramphenicol resistance
BHI	Brain Heart Infusion
BHIS	Brain Heart Infusion Supplemented
bp	Base pair or pairs
c-di-GMP	Cyclic Dimeric Guanosine Monophosphate
CaDPA	Calcium chelate of Dipicolinic Acid
CCR	Carbon Catabolite Repression
CDMM	<i>Clostridium difficile</i> Minimal Medium
DPA	Dipicolinic Acid
DTT	Dithiothreitol
EIA	Enzyme Immunoassay
<i>erm</i>	Erythromycin resistance determinant
FMT	Fecal Microbiota Transplantation
g	gramme
Gal	Galactose
GDH	Glutamate Dehydrogenase
GlcNAc	N-acetylglucosamine
IFN	Interferon
IL	Interleukin
KDa	Kilodalton
L	Liter
LB	Luria-Bertani Medium
lb/in ²	Pound per Square Inch
LCTs	Large Clostridial Toxins
M	Molar

PaLoc	Pathogenicity Locus
PBS	Phosphate Buffered Saline
PC	Phase Contrast
PMC	Pseudomembranous Colitis
PMSF	Phenylmethanesulfonylfluoride
RBS	Ribosome Binding Site
ROS	Reactive Oxygen Species
SCFAs	Short Chain Fatty Acids
TLR	Toll-like Receptor
Tm ^R	Thiamphenicol resistance
TY	Tryptone Yeast Extract Medium
<i>xg</i>	Times Gravity

ABSTRACT

Clostridium difficile, recently re-classified as *Clostridioides difficile*, is a strict anaerobe, with the ability to form spores. *C. difficile* is also a major nosocomial enteric pathogen worldwide and the cause of severe gastrointestinal conditions that can lead to death. The oxygen-resistant spores allow host-host transmission, but also persistence of the organism in the host and the environment, being linked to disease recurrence. The last two decades have witnessed the emergence of epidemic strains that increased the incidence of *C. difficile* infection (CDI) associated with more severe symptoms and high recurrence rates. Together with the dissemination of the organism in the community, the environment and in domestic and stock animals, justifies its labeling as an urgent threat. The disease symptoms are mainly caused by two potent toxins, TcdA and TcdB. Comprehend how spores are formed, their role in infection and the relationship between the two processes is central to our understanding of the infectious cycle and the design of novel strategies to counteract this pathogen. The toxins are encoded in the Pathogenicity Locus, the PaLoc, together with their main regulator, the alternative sigma factor, TcdR, which is under positive auto-regulation.

In this work, we have studied the expression of the PaLoC genes at the single cell level. We have found that expression of the *tcdR*, *tcdA* and *tcdB* genes occurs in a sub-population of cells and is thus bistable (Chapter 2). Bistability most likely arises from the auto-regulation of *tcdR*. Expression of *tcdR* is also positively regulated by the alternative sigma factor σ^D , which is additionally required for a late stage in flagellar assembly; our results support a model in which σ^D primes the TcdR auto-regulatory loop. Thus, the bistable expression

of *tcdR* and of the toxin-encoding genes is further controlled by factors and signals that impinge on the regulation of *sigD* or *tcdR*. These factors include c-di-GMP, proper assembly of the flagellum, the global regulators CodY and CcpA, and the recently described regulator RstA (Chapter 2). Our study reinforces the link between motility and toxinogenesis while the bistable expression of the *tcdA* and *tcdB* genes hints at a division of labour strategy and cooperative virulence.

Additionally, we studied the expression of the PaLoc genes during sporulation. Strikingly, we found that *tcdR*, *tcdA*, *tcdB* and *tcdE* are expressed in sporulating cells. Expression of *tcdR*, *tcdA* and *tcdB* was either confined to the forespore or detected in whole sporangia whereas *tcdE* was only expressed in the forespore (Chapter 3). While expression of the PaLoc genes in the mother cell required σ^D , forespore-specific expression did not. Instead, we show that the forespore-specific regulatory proteins σ^G and SpoVT are required for *tcdR* expression in this cell, which in turn leads to *tcdA*/ *tcdB* expression (Chapter 3). Importantly, we further show that at least TcdA associates with mature spores and thus is present since the onset of infection (Chapter 4). Cytotoxicity assays suggested that both toxins associate with mature spores in an active form, as wild-type spores, but not those of mutants unable to produce the toxins, are cytotoxic to eukaryotic cell lines (Chapter 4). While allowing inter-host dissemination and intra-host persistence, the infectious spores are also carriers of the TcdA and TcdB toxins. Furthermore, lysis of the mother cell upon completion of spore differentiation provides a route for toxin release. We discuss the implications of our findings in the context of infection.

SUMÁRIO

Clostridium difficile, recentemente reclassificado como *Clostridioides difficile* é um anaeróbio estrito com a capacidade de formar esporos. *C. difficile* é um importante patógeno entérico disseminado mundialmente, e que causa patologias gastrointestinais severas que podem levar à morte. Os esporos são resistentes ao oxigénio e permitem a transmissão entre hospedeiros mas também a persistência do organismo no hospedeiro e no ambiente, estando associados à recorrência da doença. As duas últimas décadas têm testemunhado a emergência de estirpes epidémicas que aumentaram a incidência da infecção por *C. difficile* (CDI) associada a sintomas mais severos e a maiores taxas de recorrência. Para além disso a disseminação do organismo na comunidade, no ambiente e em animais domésticos e de alimentação, justificam a sinalização desta infecção como uma ameaça urgente. Os sintomas da doença são essencialmente causados por duas toxinas potentes, TcdA e TcdB. Compreender como os esporos são formados, o seu papel na infecção e a relação entre os dois processos é central para conhecer o ciclo infeccioso e para desenhar novas estratégias para combater este patógeno. As toxinas estão codificadas no *locus* de patogenicidade, o PaLoc, juntamente com o seu principal regulador, o factor sigma alternativo, TcdR, que está sob autorregulação positiva. Neste trabalho, foi estudada a expressão dos genes do PaLoc ao nível da célula individual. Observou-se que a expressão dos genes *tcdR*, *tcdA* e *tcdB* ocorrem numa subpopulação de células e que por isso é biestável (Capítulo 2). A biestabilidade resulta provavelmente da autorregulação do gene *tcdR*. A expressão do gene *tcdR* é também regulada positivamente pelo factor sigma alternativo σ^D que regula as fases tardias de montagem do flagelo e os nossos

resultados apoiam um modelo no qual σ^D inicia o circuito de autorregulação do TcdR. Assim, expressão biestável do gene *tcdR* e dos genes que codificam para as toxinas é adicionalmente controlada por factores e sinais que afectam a regulação dos genes *sigD* e *tcdR*. Estes factores incluem c-di-GMP, a montagem correcta do flagelo, os reguladores globais CodY e CcpA, e o regulador recentemente descrito, RstA (Capítulo 2). O presente estudo fortalece a ligação entre a mobilidade e a toxinogénese, enquanto que a expressão biestável dos genes *tcdA* e *tcdB* apontam para uma estratégia de divisão de funções e virulência cooperativa.

Adicionalmente, foi analisada a expressão dos componentes do PaLoc durante a esporulação. Surpreendentemente, detectámos que os genes *tcdR*, *tcdA*, *tcdB* e *tcdE* são expressos em células a esporular. A expressão dos genes *tcdR*, *tcdA* e *tcdB* foi observada confinada no pré-esporo ou detectada em toda o esporângio, enquanto que o gene *tcdE* apenas foi detectado no pré-esporo (Capítulo 3). A expressão na célula mãe depende do factor σ^D , mas o mesmo não acontece no pré-esporo. Mostrou-se que as proteínas reguladoras específicas do pré-esporo, σ^G e SpoVT são necessárias para a expressão do gene *tcdR* nesta célula, o que por sua vez leva à expressão de *tcdA*/ *tcdB* (Capítulo 3). De notar que se observou também que pelo menos a toxina TcdA está associada com esporos maduros e que por isso está presente desde o início da infecção (Capítulo 4). Ensaaios de citotoxicidade sugerem que ambas as toxinas estão associadas com esporos maduros numa forma activa uma vez que esporos selvagens, mas não esporos mutantes incapazes de produzir toxinas, são citotóxicos em linhas celulares eucarióticas (Capítulo 4). Assim, para além de permitirem a disseminação entre hospedeiros e a persistência dentro do hospedeiro, os esporos também transportam as toxinas TcdA e TcdB. Adicionalmente, a lise da célula

mãe após a diferenciação do esporo pode ser uma nova via de libertação das toxinas. As implicações destas descobertas são discutidas no contexto da infecção.

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Chapter 1

Introduction

1.1. GENERAL ASPECTS OF *CLOSTRIDIUM DIFFICILE*

During the last decade increasing relevance has been given to our microbial co-habitants. The acknowledgement that microbes are an important part of our lives, both as part of our body, as well as in the environment led us to guide our medicine in a different route. Eukaryotes, archaea, bacteria and viruses are part of these microbial communities and bacteria in particular outstand from other microorganisms due to the high number of bacterial cells that cohabitate in our body. In fact, there is a similar number of bacterial cells in our body as human cells (Sender *et al.*, 2016). These bacteria are essential to maintain a healthy organism since they give us important metabolic functions and educate the innate immune system. The gut, skin, the oral cavity and the vagina are examples of niches where a wide microbial diversity can be found.

The gastrointestinal tract is a particularly rich bacterial community. The gut microbiota composition is complex and varies along the tract with a total of 10^{14} bacteria composing this community, which are 70% of the total microbiota (Eckburg *et al.*, 2005). Therefore, not only important interactions are established with the host but also between microbes. Importantly, this interaction network influences physiology and susceptibility to disease (Mandal *et al.*, 2015). Culture-dependent and culture-independent studies allowed defining a core microbiota shared by all healthy adults and a variable microbiota that is highly diversified between populations and through time (Lozupone *et al.*, 2012). In adults, more than 1000 bacterial species can be found in each individual, but species of Bacteroidetes and Firmicutes are predominant (Lozupone *et al.*, 2012). A significant fraction of the Firmicutes belongs to the class Clostridia (Gill *et al.*, 2006, Rodriguez

et al., 2015). Importantly, it is now known that 50-60% of the bacterial genera found in the human gut are spore-formers, suggesting that these organisms are functionally important and that spores are a key mechanism for transmission between different hosts (Browne *et al.*, 2016).

Initially named *Bacillus difficilis* due to the challenging isolation and culture, *Clostridium difficile*, recently renamed *Clostridioides difficile*, was first isolated in 1935 from the stool of healthy neonates (Hall & O'toole, 1935). In 1974 a high rate of pseudomembranous colitis (PMC) was found to be associated with recipients of clindamycin (Tedesco *et al.*, 1974), and in the same year *C. difficile* cytotoxin was first described when found in the stools of guinea pigs treated with penicillin (Green, 1974). However, only in 1977 was established the association between PMC and the *C. difficile* toxin (Barlett JG, 1977). Nowadays, *C. difficile* is a better known Gram-positive spore-forming bacteria that is an obligate anaerobe and an important nosocomial pathogen that causes a spectrum of intestinal diseases, ranging from mild diarrhea to pseudomembranous colitis, toxic megacolon and bowel perforation through the production of two cytotoxins, TcdA and TcdB (Abt *et al.*, 2016, Rupnik *et al.*, 2009, Smits *et al.*, 2016). A third toxin is produced by some isolates, the binary toxin (CDT), for which the importance for infection is not yet fully understood (Goncalves *et al.*, 2004, Popoff *et al.*, 1988).

Besides being a nosocomial pathogen, *C. difficile* is nowadays worldwide spread (Burke & Lamont, 2014) and can be found in the community (Brown *et al.*, 2013, Lessa *et al.*, 2015, van Dorp *et al.*, 2016) as well as in animals (Gould & Limbago, 2010, Hensgens *et al.*,

2012, Rodriguez-Palacios *et al.*, 2013) and in the environment (al Saif & Brazier, 1996, Weese *et al.*, 2010, Zidaric *et al.*, 2010).

1.1.1. Genomic diversity and evolution

The first *C. difficile* genome was sequenced and annotated in 2006 and it belonged to the strain 630 from the ribotype 012 (Sebaihia *et al.*, 2006). This strain presented a circular chromosome with 4.3 Mb, 3776 putative protein-coding sequences and 29.06% of GC content. Furthermore, a circular plasmid, pCD630, with 7881bp and with 11 coding sequences was identified (Sebaihia *et al.*, 2006). Since the first sequenced genome *C. difficile* evidenced having a highly dynamic genome, with 11% of the genome being motile genetic elements (Sebaihia *et al.*, 2006). Besides the plasmid identified, the motile elements also include prophages, transposons, insertion sequences, introns, genomic islands and clustered regularly interspersed short palindromic repeats (CRISPRs) (Elliott *et al.*, 2017, Knight *et al.*, 2015). These mobile elements are important for adaptation to the gut environment and for survival in non-optimal conditions since they encode genes for antimicrobial resistance, virulence, host interaction and surface proteins (Sebaihia *et al.*, 2006). From these elements transposons play an important role (Adams *et al.*, 2002, Roberts *et al.*, 2014). Often transposons encode for important functions involved in antimicrobial resistance and in interactions with the host, like bile salts hydrolases. Furthermore, depending on the location of insertions, the transposons can lead to transcriptional alterations, gene inactivation and can increase recombination rates leading to genome rearrangements (Brouwer *et al.*, 2011).

The high genomic dynamism in *C. difficile* is illustrated by the small core genome, only 16%, comparing with the accessory genome (Janvilisri *et al.*, 2009). Such high variability is not common within the same specie and therefore various taxonomic revisions have been proposed for *C. difficile* (Lawson *et al.*, 2016, Yutin & Galperin, 2013). *C. difficile* belongs to the phylum Firmicutes and from phenotypic characteristics was included into the class Clostridia. Sequencing of the 16S rRNA indicated a close proximity with *Clostridium mangenotii* that belongs to the family Peptostreptococcaceae, and therefore it was included in this family and it was proposed to be renamed *Peptoclostridium difficile* (Yutin & Galperin, 2013). Later, based on phenotypic, chemotaxonomic and phylogenetic analysis a new genus was proposed for *C. difficile*, *Clostridioides*, renaming *Clostridium difficile* to *Clostridioides difficile* (Lawson *et al.*, 2016).

1.1.2. Risk factors of CDI and the importance of the commensal microbiota

In a healthy individual, the commensal microbiota protects the host to susceptibility to infection (Borriello, 1990). *C. difficile* is an opportunistic pathogen, and therefore, *C. difficile* infection (CDI) occurs in case of gut dysbiosis, meaning, when the endogenous microbiota is disrupted (Figure 1.1). The microbial species that habit the human gut produce bacteriocins, compete for nutrients and niches and establish microbe-microbe interactions important for colonization resistance (Rodriguez *et al.*, 2015). The most important risk factor for CDI is antibiotic use, with more than 90% of cases occurring in conjunction with antimicrobial therapy (Rodriguez *et al.*, 2015). Almost all broad-spectrum antibiotics have been shown to cause dysbiosis and

therefore to increase susceptibility to CDI. However, exposure to ampicillin, amoxicillin, cephalosporins, clindamycin and fluoroquinolones are most frequently associated with the disease (Bignardi, 1998, Rodriguez *et al.*, 2015). Antibiotic treatment leads to changes in the microbiota diversity, and studies in animal models showed a decrease in Firmicutes and Bacteroidetes abundance and dominance of Proteobacteria (Antonopoulos *et al.*, 2009, Hill *et al.*, 2010). A reduction in microbial diversity results in a reduction of competition for resources in the gut (Wilson & Perini, 1988) and in disruption of important interactions with the host immune system (Atarashi *et al.*, 2011, Bibbo *et al.*, 2014).

Besides these protective roles, the commensal microbiota is involved in processes such as carbohydrate fermentation, protein digestion, and regulation of lipid and amino acid metabolism (Theriot & Young, 2014). Therefore, changes in the microbiota leads to modifications in the intestinal metabolome, which can facilitate *C. difficile* expansion. For instance, succinate and sialic acids increase because the primary fermenters that break down complex carbohydrates in short chain fatty acids (SCFAs) are no longer present. On the one hand, succinate and sialic acids that can be used as a carbon source by *C. difficile* are increased (Ferreyra *et al.*, 2014); on the other hand, SCFAs that are important as an energy source for the epithelial cells and in maintaining the defense barrier are reduced (Perez-Cobas *et al.*, 2015) (Figure 1.1). Furthermore, it has been shown that antibiotic treatment results in a shift in the bile acid balance, leading to an increase in cholate levels comparing to other primary and secondary bile acids, which promotes *C. difficile* germination *in vivo* (Giel *et al.*, 2010, Sorg & Sonenshein, 2008, Sorg & Sonenshein, 2009,

Sorg & Sonenshein, 2010) (See Section 1.2.4. Germination). This effect is exemplified by the role of *Clostridium scindens* that is associated with resistance to *C. difficile*. *C. scindens* produces dehydroxylating enzymes that are required for the conversion of primary bile acids into secondary bile acids. Without the action of *C. scindens*, the levels of secondary bile acids will reduce and this will facilitate *C. difficile* growth (Buffie *et al.*, 2015, Studer *et al.*, 2016).

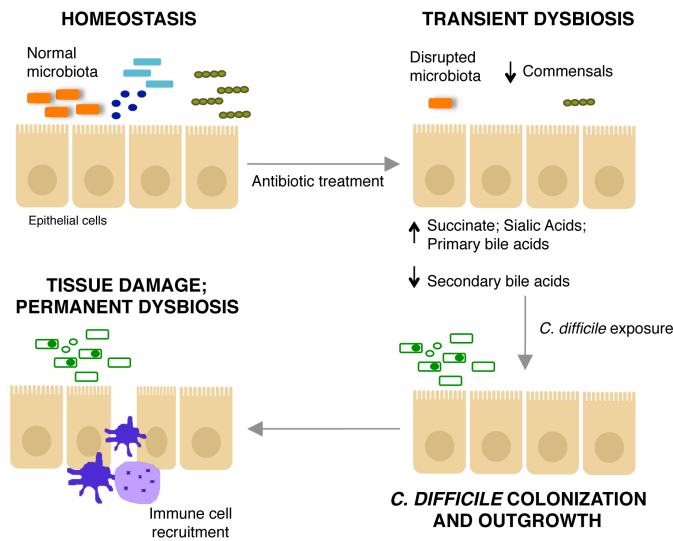


Figure 1.1. Schematic representation of *C. difficile* colonization. In healthy individuals homeostasis is maintained between the microbiota and the host immune system. In case of antibiotic treatment the microbiota is disrupted and the proportion of commensal microorganisms decreases, leading to transient dysbiosis. In this state succinate, sialic acids and primary bile acids increase, while secondary bile acids decrease. In case of *C. difficile* exposure these conditions are optimal for spore germination, leading to *C. difficile* colonization and outgrowth. Due to toxin production, there is tissue damage and is induced a state of permanent dysbiosis, with disruption of the epithelial barrier. The inflammatory response is triggered and immune cells are recruited. Adapted from (Cowardin & Petri, 2014).

1.1.3. Epidemiology of CDI

Increased incidence, severity and mortality have illustrated the changes in the epidemiology of *C. difficile* in the last 10 to 15 years. Although *C. difficile* was initially considered a nosocomial pathogen, it emerged already in the community in healthy individuals (Centers for Disease & Prevention, 2008, Kuntz *et al.*, 2011, Wilcox *et al.*, 2008).

In the United States the incidence remained stable from 1996 to 2000 but increased for almost the double from 2000 to 2003, with an increase in the number of severe cases and in mortality (Redelings *et al.*, 2007, Warny *et al.*, 2005, Zilberberg *et al.*, 2008). In Europe, a study from 2005 performed by the European Study Group of *C. difficile* (ESGCD), indicated a mean incidence of CDI of 2.45 cases per 10 000 patients (Barbut *et al.*, 2007). More recent studies had shown that in general there has been an increased incidence in the last decade in Europe. In Portugal, local studies indicated an increasing incidence since 2007, with the ribotypes 027, 014, 029 and 017 being the most frequent (Santos *et al.*, 2016); among these, ribotype 027 is the most frequent (18.5%) and in general isolated within health-care facilities (Santos *et al.*, 2016).

Outbreaks in the United States, Canada and Europe have been reported since 2003 and were related with the emergence of epidemic strains mainly from the fluoroquinolone-resistant ribotype 027. Strains from this ribotype were first detected in North America in the 2000s but nowadays are worldwide spread (Clements *et al.*, 2010). In Europe, outbreaks have been particularly severe in the United Kingdom, with a six-times increase in mortality from 1999 to 2006. Increased surveillance however, led to a stabilization and decline in incidence

and mortality from 2007 to 2009 (Clements *et al.*, 2010, Jones *et al.*, 2013).

Although mainly a nosocomial infection, CDI has emerged in the community. In fact, in North America and in Europe, the incidence in the community is of 20-30 cases per 100 000 population, being 20-27% of all CDI cases community associated (Lessa *et al.*, 2012). Comparing with hospital acquired infections, community acquired infections tend to be in younger and healthier patients (Jones *et al.*, 2013). The most relevant ribotype in the community is 078, which has been associated with animals, including production animals such as pigs and calves. Nowadays it is in fact the third most common ribotype in Europe suggesting a possible zoonotic transmission (Elliott *et al.*, 2017, Jones *et al.*, 2013).

1.1.4. Diagnosis and new methodologies for *C. difficile* detection

Accurate and rapid diagnosis is important, not only for patient treatment, but also to limit dissemination, especially in hospital environments. The traditional standard diagnostic tests were culture on a selective medium and the cytotoxin neutralization test (Gilligan, 2015). However, these methodologies implicated high costs, required specialized skills, were time consuming and had a slow turnaround time (Burnham & Carroll, 2013). Due to these limitations, the traditional methods were replaced by toxin enzyme immunoassays (EIAs) that detect toxins A and/or B (Gilligan, 2015). Beside the toxin EIAs, the design of enzymatic tests that detect a cell wall-associated antigen, glutamate dehydrogenase (GDH), also allowed detection of the non-toxinogenic strains and therefore it is generally considered a low

specificity tool for CDI diagnosis (Gilligan, 2008). Furthermore, the sensitivity of the enzymatic tests can range from 40% to 100% (Eastwood *et al.*, 2009) and therefore, nowadays, molecular-based methods are being applied. Nucleic acid amplification techniques (NAAT) have a sensitivity of 90%, which led many hospitals to adopt these methodologies (Gould *et al.*, 2013). NAAT usually detect the *tcdA* and *tcdB* genes by PCR amplification. However, these detection methods are costly and the use as the unique diagnosis method leads to false positives, causing overdiagnosis, overtreatment and allocation of unnecessary resources (Polage *et al.*, 2015).

Due to the limitations of the various methodologies available, a multistep diagnosis is more accurate. In Europe the recommended guidelines combine two tests, the first should be a NAAT or glutamate dehydrogenase EIA and, in case of positive result, followed by a sensitive toxin EIA. The first step has high sensitivity but low specificity that is given by the second step. The combination of these two tests allows detecting patients who apparently do not have CDI symptoms but are excreting *C. difficile* to the environment and therefore are possible dissemination points (Jones *et al.*, 2013). In case of outbreak, toxinogenic culture and molecular typing should be the methods applied (Nagy, 2018).

1.1.5. Prevention and treatment of CDI

For prevention it is recommended a good maintenance and cleanness of the hospital surfaces, use of disposable material when possible, an adequate use of antibiotics and a rapid and early detection of CDI. Prevention is mainly made by a control of antibiotic use, mainly clindamycin and cephalosporin (Climo *et al.*, 1998, Khan &

Cheesbrough, 2003). Once the case of CDI is confirmed the patients should be isolated to avoid dissemination (Jones *et al.*, 2013).

For treatment, the more traditional approach has been with antibiotics like metronidazole and vancomycin, but new generation antibiotics, fidaxomicin and tigecyclin have also started to be used. Alternative treatments are also an increasing choice such as fecal transplantation, probiotics, immunotherapy and toxin binding agents. The European Society of Clinical Microbiology and Infectious Diseases (ESCMID) developed a guidance protocol for treatment in Europe (Bauer *et al.*, 2009, Debast *et al.*, 2014). Metronidazole and vancomycin have been the most used antibiotics in cases of mild to moderate CDI. Vancomycin is usually reserved for more severe and recurrent cases since there are increasing concerns about resistance emergence (Nagy, 2018).

Vaccination is a possibility in research. Permpoonpattana *et al.*, showed in the hamster model that *Bacillus* spores displaying toxin A at the surface can be used for immunization (Permpoonpattana *et al.*, 2011a). Further research to develop vaccination using animal models is being developed, and in fact there are already vaccines in phase 2/3 trials (Donald *et al.*, 2013, Karczewski *et al.*, 2014, Knisely *et al.*, 2016, Maynard-Smith *et al.*, 2014, Secore *et al.*, 2017). Probiotics can be used both as prevention as well as a treatment option because it allows the host to re-establish the normal microbiota after antibiotic treatment or other disrupting event. In this way, the gut immune system can recover and an efficient immune response against *C. difficile* can occur (Hickson, 2011, Napolitano & Edmiston, 2017).

The most adopted alternative treatment, especially in cases of recurrent CDI, is the fecal microbiota transplantation (FMT) that

consists in the infusion of faeces from a healthy donor to the patient in order to restore the normal microbiota (Nagy, 2018, Roshan *et al.*, 2018). Donor selection is an important step. The success rate is of 80-95% and the recurrence rate is low making it an appealing method. Some disadvantages, however, are still pointed since the procedure is laborious and the long-term effects are still unknown (Camarota *et al.*, 2017, Nagy, 2018). Efforts are being made to discover which microorganisms are essential in FMT in order to create a formulation that allows oral administration of specific microbiome isolates (Kocielek & Gerding, 2016).

1.1.6. The host immune response to *C. difficile*

During *C. difficile* infection the toxins induce a strong inflammatory response in the epithelial mucosa mediated by the innate immune response. For infection to occur, *C. difficile* needs to colonize the mucosal surfaces at the intestine, where there is a thick layer of mucous important as a first defense barrier for the host and where antimicrobial peptides are present (Usacheva *et al.*, 2016). Cathelicidin and defensins present at the mucosal surface were shown to reduce the inflammation caused by the toxins (Giesemann *et al.*, 2008, Hing *et al.*, 2013). The inflammatory response is the initial response to *C. difficile* cytotoxic effect, and in fact a strong acute response is induced: pro-inflammatory chemokines, like CXCL1, CXCL2, interleukin-8 (IL-8), and cytokines, like IL-1 β , IL-12 and IL23, are secreted by the intestinal epithelial cells and resident innate immune cells; there is neutrophil recruitment from the peripheral blood and the activation of innate lymphoid cells, that produce interferon- γ (IFN- γ) and IL-22; these effector cytokines recruit innate

and adaptive immune cells and induce the expression of antimicrobial peptides, and the production of reactive nitrogen species (RNS) and reactive oxygen species (ROS), which limits dissemination of other bacterial cells and repairs epithelial damage (Abt *et al.*, 2016, Solomon, 2013) (Figure 1.2). Besides the toxins, various cell surface proteins like SLPs, Cwp proteins and flagellin are recognized at this stage by the host cell surface toll-like receptors (TLRs), like TLR4 and TLR5 (Jarchum *et al.*, 2011, Ryan *et al.*, 2011). Important acute clinical symptoms like high white cell counts, fever, profuse diarrhea, hypoalbuminaemia and toxic megacolon result from the inflammatory response. Although the immune response to *C. difficile* infection is protective, it is thought that neutrophils also increase disease severity due to exacerbated inflammation. In fact, a slower recovery rate is associated with higher levels of CXCL5 and IL-8, which are molecules important for neutrophil recruitment (El Feghaly *et al.*, 2013). Furthermore, IL-23 production, also involved in neutrophil recruitment, seems to be induced by TcdB and murine models of infection lacking IL-23 presented significant increased survival rate (Buonomo *et al.*, 2013, El Feghaly *et al.*, 2013). Mice treated with an IL-1R antagonist also presented reduced inflammation and less severe disease (Ishida *et al.*, 2004). Therefore, maintaining a limited inflammatory response is important in containing infection during CDI.

The adaptive immune response has also an important role during CDI. IgA is the immunoglobulin associated with the mucosa and therefore has an important protective role during CDI. Low levels of IgA are associated with prolonged and recurrent CDI. Furthermore,

IgM and IgG responses are also important during CDI (Solomon, 2013).

While the immune response to the surface of vegetative cells has been extensively studied, little is known about the response to the spores. *In vitro*, it was shown that macrophages are able to phagocytize *C. difficile* spores, however spores are able to survive and lead to cell death (Paredes-Sabja *et al.*, 2012).

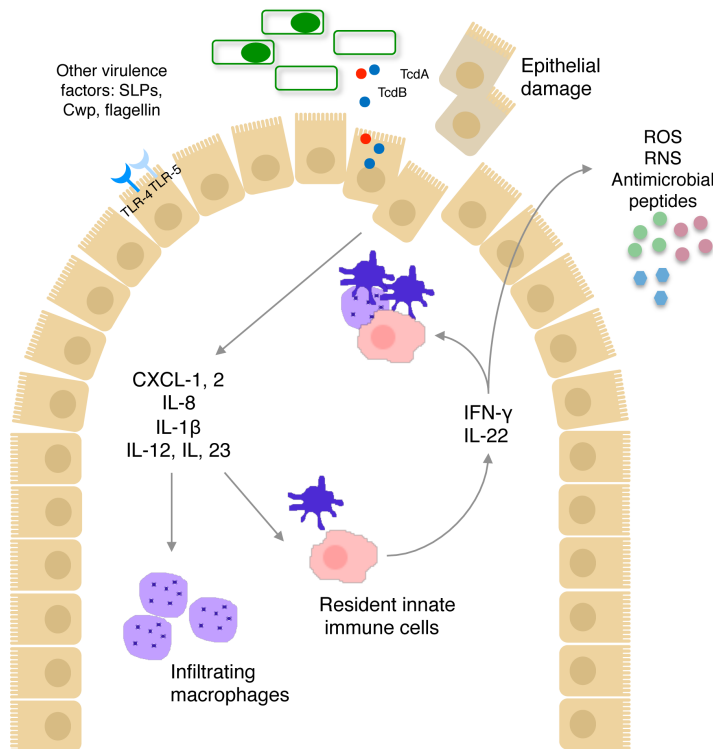


Figure 1.2. The innate immune response to *C. difficile*. The innate immune response to *C. difficile* is mainly triggered by the toxins' effects, but it can also occur in response to other virulence factors. Epithelial cell damage and the consequent translocation of commensal bacteria lead to the secretion of pro-inflammatory chemokines (such as CXCL1, CXCL2, IL-8) and pro-inflammatory cytokines (such as IL-1β, IL-12 and IL-23) by the epithelial cells and innate immune cells. Consequently there is macrophage infiltration and the recruitment of resident innate immune cells that produce IFN-γ and IL-22, which induce the expression of antimicrobial peptides, and the production of reactive nitrogen species (RNS) and reactive oxygen species (ROS). Adapted from (Abt *et al.*, 2016).

1.1.7. Pathogenesis and the main virulence factors

C. difficile infection is an intestinal disease caused through the action of cytotoxins and that rarely occurs outside of the intestine. The disease is presented in different degrees, ranging from mild to severe and fulminant cases. In mild and moderate cases the disease only manifests as a diarrhea, but more severe symptoms can show, such as abdominal pain, fever and leukocytosis. In severe cases, the patients present inflammatory lesions, toxin megacolon, bowel perforation and pseudomembranous colitis, which is characterized by inflammation of the mucosa, with the formation of white and yellow plaques and where there is an accumulation of neutrophils, fibrin, mucin and cellular debris. In fulminant cases, when the treatment is not efficient or rapidly applied, sepsis, shock and death can occur (Poutanen & Simor, 2004, Rupnik *et al.*, 2009).

C. difficile spores are the transmission vehicle and need to be ingested and reach the intestine in order for infection to occur. The spores are acid resistant and therefore can cross the stomach and reach the intestine, where they interact with the host epithelial cells and the bile salts are available to enable germination. This process occurs early in the small intestine, however only when the cecum is reached *C. difficile* finds the conditions for optimal growth and toxin production (Koenigsknecht *et al.*, 2015). The clinical outcomes are mainly caused by the action of the two cytotoxins, TcdA and TcdB, which are glucosyltransferases. The toxins cause disruption of the actin cytoskeleton and of the tight junctions leading to increased vascular permeability and inflammation (Abt *et al.*, 2016) (see also section 1.4. Toxinogenesis in *C. difficile*). Furthermore, it is nowadays known that some strains produce a binary toxin, CDT that is thought

to improve bacterial attachment by leading to the formation of microtubule protrusions in the host cells (Schwan *et al.*, 2009).

The success of the infection also depends on other virulence factors related with escaping the host innate defenses, adherence and motility important for the initial steps of colonization (Janoir, 2016). A mechanism for evasion of the innate immune system is lysozyme resistance. *C. difficile* is naturally resistant to lysozyme due to a high level of N-deacetylation induced by sub-inhibitory concentrations of lysozyme (Ho *et al.*, 2014, Peltier *et al.*, 2011). Surface proteins mediate adherence to the host cells, an important factor for pathogenicity. The Cwp proteins are an important protein family involved in the adherence, characterized by a conserved domain that anchors the protein to the bacterial surface through the polysaccharide II. The “clostridial wall (*cwp*) gene cluster” contains 12 of the Cwp encoding genes, while 17 are distributed throughout the chromosome, and these are highly genetically variable. The genetic variability of protein surfaces is also another strategy for evading the host immune system (Pechine *et al.*, 2005). From the Cwp proteins, the S-layer proteins (SLPs) are important for cell integrity, to contact with environmental molecules and for adhesion to host tissues. SlpA, a protein that is cleaved into two SLP subunits by the cysteine protease Cwp84, has been shown to be a major contributor for host-cell adherence. Adherence is however a multifactorial process and involves various proteins such as the Cwp66 adhesin, the fibronectin-binding protein Fbp68, the collagen-binding protein CbpA, the lipoprotein CD630_08730 and the heat-shock protein GroEL (Janoir, 2016). The flagellum has also been shown to be important for adherence (Tasteyre *et al.*, 2001), however its main role is in motility (See Section 1.3.

Motility in *C. difficile*). The flagellar genes are expressed early during colonization, highlighting its importance at this stage (Janoir, 2016). FliC and FliD, the flagellin and flagellar cap proteins seem to interact with the mucus layer and motility might allow penetrating this layer (Tasteyre *et al.*, 2001). However, the relevance of the flagella for colonization and adherence is discussed due to strain variability (Baban *et al.*, 2013, Barketi-Klai *et al.*, 2014, Dingle *et al.*, 2011b). Furthermore, toxin expression is affected by flagellar components (Aubry *et al.*, 2012, Dingle *et al.*, 2011b, El Meouche *et al.*, 2013) (See Section 1.3.2.1. The role of flagella in pathogenicity).

Sporulation is indisputably important for virulence. Spores are the dissemination vehicles, since they are structures resistant to harsh environmental conditions, and importantly, to oxygen. Therefore spores are resistant to the normal hospital disinfectants and can persist for long periods of time in the hospital environment (Rupnik *et al.*, 2009). In *C. difficile*, as most likely in all sporeformers, the Spo0A response regulator is required for sporulation initiation (Edwards & McBride, 2014) (See Section 1.2.1. Sporulation Initiation). Spo0A is also an important regulator for biofilm formation, together with LuxS, a putative quorum sensing regulator, the protease Cwp84 and cyclic diguanylate (c-di-GMP), a signaling molecule that also regulates motility (Ethapa *et al.*, 2013). The biofilm is composed of multiple layers of bacteria protected by a proteinaceous layer, DNA and polysaccharide making a resistant structure that protects vegetative cells from environmental stresses like oxygen, antibiotics and immune responses from the host. Sporulation can take place within biofilms; both biofilms and spores are therefore important factors for *C. difficile* resistance and persistence (Ethapa *et al.*, 2013, Janoir, 2016).

The increased knowledge on *C. difficile* pathogenicity factors allows to search for new therapeutic targets to prevent dissemination, which is an increase concern with the spreading in the community.

1.2. SPORULATION IN *C. DIFFICILE*

Endosporulation, sporulation for simplicity, is a process of cell differentiation that occurs in various Firmicutes species and that leads to the formation of highly resistant dormant cells known as spores (Henriques & Moran, 2007, Nicholson *et al.*, 2000). Sporulation has been extensively studied in the model organism *Bacillus subtilis*. The main morphological stages of sporulation, as well as the overall architecture of the mature are conserved among spore-formers (Henriques & Moran, 2007). In recent years, sporulation has also been studied in detail in *C. difficile*. As for other gut obligate anaerobes, sporulation is essential for persistence within the host and for transmission in the aerobic environment (Browne *et al.*, 2016).

1.2.1. Sporulation initiation

The first overt sign of sporulation is an asymmetric division of the rod-shaped cell; this division involves placement of the division septum close to one of the poles and partitioning of the chromosome into each of the cell compartments. This originates a smaller compartment, the forespore, and a larger compartment, the mother cell. The forespore and the mother cell will follow different lines of gene expression (Fimlaid & Shen, 2015, Haldenwang, 1995, Higgins & Dworkin, 2012, Pereira *et al.*, 2013, Saujet *et al.*, 2013). In the late stages of sporulation, the mother cell engulfs the forespore, in a process similar to phagocytosis, giving rise to a cell isolated inside

another cell by a double membrane system. The mother cell nurtures development of the spore, but will eventually lyse to release the mature spore in the environment (Henriques & Moran, 2007, Piggot & Hilbert, 2004).

Entry into sporulation involves the activation, by phosphorylation of the master regulatory protein Spo0A, conserved among spore-formers (Abecasis *et al.*, 2013, Galperin *et al.*, 2012). In *C. difficile* Spo0A is essential for sporulation initiation, and additionally regulates other functions important for metabolism and virulence (Deakin *et al.*, 2012, Pettit *et al.*, 2014, Rosenbusch *et al.*, 2012). In *B. subtilis*, a phosphorelay allows cells to integrate and respond to nutritional, metabolic, DNA status, cell density and cell cycle signals that lead to the phosphorylation and activation of Spo0A (Hoch, 1993). However, in *C. difficile*, as in other clostridia, the phosphorelay is absent and Spo0A is directly phosphorylated by orphan histidine kinases that respond to as yet unknown signals (Steiner *et al.*, 2011, Underwood *et al.*, 2009, Worner *et al.*, 2006). The *C. difficile* genome codes for five orphan kinases, three of which, CD1492, CD1579 and CD2492, cluster together in the genome and share a high degree of sequence similarity like the kinases involved in the *B. subtilis* phosphorelay. These were initially proposed to be the kinases responsible for Spo0A phosphorylation. CD1579 was the only kinase to be shown to directly phosphorylate Spo0A and CD2492 positively regulates sporulation (Underwood *et al.*, 2009). In contrast, CD1492 is now known to inhibit sporulation initiation and affects toxin and motility through the regulators RstA and SigD (Childress *et al.*, 2016).

In *C. difficile*, σ^H is also important for sporulation initiation as *spo0A* and CD2492 are transcribed from σ^H -utilized promoters (Saujet

et al., 2011). Additionally, σ^H affects the regulation of genes involved in motility, cell surface proteins, virulence, metabolism and sporulation.

In all, sporulation initiation in *C. difficile*, and in other clostridia, seems to be a simplified version of the phosphorelay that controls sporulation initiation in *B. subtilis*, and that controls the phosphorylation level of the response regulator Spo0A.

1.2.2. Regulation of sporulation

Sporulation is driven by a set of transcriptional factors that are activated and repressed in a hierarchal cascade. In this process, the expression of hundreds of genes is affected and there are central regulators essential for coordinating this complex network (Eichenberger *et al.*, 2004, Wang *et al.*, 2006). Alternative RNA polymerase sigma factors with distinct affinity for unique promoters are an important tool in gene control. For sporulation there are four essential alternative sigma factors, σ^E , σ^F , σ^G and σ^K (Losick & Stragier, 1992). These sigma factors are differentially expressed in the mother cell and in the forespore and at different stages of development: σ^E and σ^F are expressed prior to engulfment completion in the mother cell and in the forespore, respectively, and are substituted at late stages by σ^K and σ^G , respectively (Figure 1.3). The genes regulated by these sigma factors have been extensively studied in *B. subtilis*. Studies in *C. difficile* show that the main morphological stages of sporulation are conserved; moreover, in spite of some important differences between the two organisms (see below), the principal functions in morphogenesis, cell and main periods of activity of σ^E , σ^F , σ^G and σ^K are also conserved (Fimlaid *et al.*, 2013, Pereira *et al.*, 2013, Saujet *et al.*, 2013). In fact, sporulation in *C. difficile* seems to be closer

to the model organism *B. subtilis* when compared with other clostridial species (Pereira *et al.*, 2013, Saujet *et al.*, 2013).

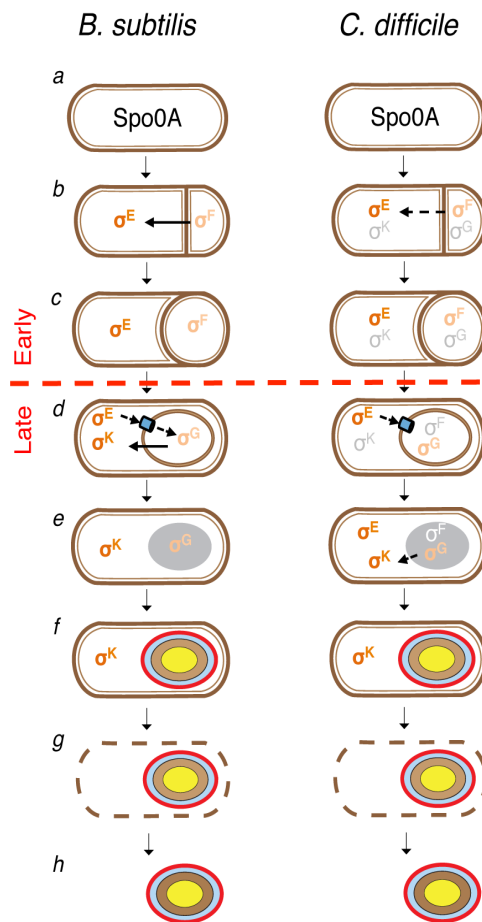


Figure 1.3. *B. subtilis* and *C. difficile* main sporulation stages. Sporulation is initiated by the master regulator Spo0A in the pre-divisional cells (a), followed by asymmetric division (b), engulfment (c), engulfment completion (d), synthesis of the spore layers (e and f) and release of the mature spores due to the mother cell lysis (g and h). σ^E and σ^F are the early stages regulators of the mother cell and forespore lines of gene expression, respectively, while σ^K and σ^G are the corresponding regulators at late stage. The sigma factors are represented in the cell in which they are active and orange indicates their main period of activity. Solid or broken arrows represent dependencies or partial dependencies, respectively. The SpolIQ-SpolIIAH channel is represented in blue. The red horizontal dashed line divides the early stages, prior to engulfment completion, from the late stages of sporulation. Adapted from (Pereira *et al.*, 2013)

In *B. subtilis*, activation of the cell type-specific sigma factors occurs in a “crisscross” between the mother cell and the forespore (Figure 1.3). Phosphorylated Spo0A initiates sporulation by activating transcription of *sigF*, coding for σ^F and *sigE*, coding for σ^E . Transcription of these genes is initiated before asymmetric division, but only after its completion σ^E and σ^F are activated. *sigF* is the third cistron in a three

gene operon under the control of σ^H and Spo0A; the second gene in the *spoIIAA-spoIIAB-sigF* operon, codes for a σ^F anti-sigma factor (SpoIIAB), and the first gene codes for an anti-anti-sigma factor (SpoIIAA) (Figure 1.4) (Duncan *et al.*, 1995, Errington *et al.*, 1996, Feucht *et al.*, 1996). Although not yet studied SpoIIAA and SpoIIAB are also present in *C. difficile*; like in *B. subtilis* *sigF* and *sigE* are expressed in pre-divisional cells (Paredes *et al.*, 2005, Pereira *et al.*, 2013, Saujet *et al.*, 2013).

In *B. subtilis*, σ^F transcribes *spoIIR*, coding for a protein that is secreted across the forespore inner membrane and activates SpoIIGA, a protease required to cleave the pro-peptide that maintains σ^E inactive in the mother cell (Karow *et al.*, 1995, Londono-Vallejo & Stragier, 1995) (Figure 1.4). The pro- σ^E is encoded by *spoIIGB*, the second cistron in the *spoIIG* operon. Transcription of the operon is initiated through a σ^A - and Spo0A-dependent promoter and increases in the mother cell relative to the forespore after asymmetric division (Baldus *et al.*, 1994). Although SpoIIR and SpoIIGA are present in *C. difficile*, σ^E activation is not completely dependent on σ^F , since in a *sigF* mutant some processing of σ^E can be detected. This seems to happen because *spoIIR* expression is, at least in part, independent from σ^F (Fimlaid *et al.*, 2013, Pereira *et al.*, 2013, Saujet *et al.*, 2013).

In *B. subtilis*, once activated, σ^E is required for the activity of the late stage forespore regulator, σ^G . This sigma factor is encoded by *spoIIIG*, which is transcribed by σ^F and later by itself, in a positive auto-regulatory mode (Morlot & Rodrigues, 2018). Several mechanisms delay production of σ^G until engulfment completion, and σ^G replaces σ^F at this stage (Mearls *et al.*, 2018). Late σ^G -dependent gene expression in the forespore, however, depends on the assembly of a transenvelope

secretion complex that is thought to maintain metabolic potential in the forespore (Camp *et al.*, 2011, Doan *et al.*, 2009). The core of this channel is formed by the mother cell protein (σ^E -controlled) SpoIIIAH and the forespore-specific protein (σ^F -controlled) SpoIIQ (Camp & Losick, 2009, Meisner *et al.*, 2008, Serrano *et al.*, 2004) (Figure 1.4). These proteins interact directly in the intermembrane space (Blaylock *et al.*, 2004, Meisner *et al.*, 2008). In *C. difficile*, the SpoIIQ-SpoIIIAH channel also exists and its formation is similarly dependent on σ^E and σ^F (Figure 1.4). In both organisms, the channel is required for the formation of heat-resistant spores (Fimlaid *et al.*, 2015b, Serrano *et al.*, 2016a). However, in *C. difficile*, unlike in *B. subtilis*, the channel also appears to be required for engulfment completion (Fimlaid *et al.*, 2015b, Serrano *et al.*, 2016a). One study proposed that the channel was dispensable for σ^G activity as the activity of this sigma factor is still detected in *spoIIQ* or *spoIIIAH* mutant sporangia (Fimlaid *et al.*, 2015b). σ^F controls *sigG* expression, which is detected in *C. difficile* immediately after asymmetric division (Figure 1.4) (Saujet *et al.*, 2013). Moreover, at least some σ^G activity is detected before engulfment completion in *C. difficile*, contrary to what is observed in *B. subtilis* (Pereira *et al.*, 2013, Saujet *et al.*, 2013). When distinguishing cells at different sporulation stages, *spoIIQ* and *spoIIIAH* mutations did not interfere with the activity of σ^G detected in cells prior to engulfment completion, but appeared to be required for the activity of σ^G following engulfment completion; furthermore, *spoIIQ* and *spoIIIAH* mutations also interfered with the activity of σ^K (Serrano *et al.*, 2016a). This last finding suggests that either the channel transports a metabolite to the forespore that if accumulating in the mother cell inhibits σ^K activity, or that the channel transports a

compound from the forespore to the mother cell necessary to σ^K activity (Serrano *et al.*, 2016a).

The late stage mother cell regulator σ^K is essential for cortex and coat formation. In *B. subtilis* σ^E and SpoIIID activate *spoIVCA* expression, a gene that codes for a recombinase required for excision of a prophage-like element called *skin* that interrupts *sigK* (Haraldsen & Sonenshein, 2003, Kunkel *et al.*, 1989). In *C. difficile* a different *skin* element also interrupts the *sigK* gene; it codes for a recombinase, CD1231 and for a recombination directionality factor, CD1234. While CD1231 is expressed in vegetative cells and during sporulation in both cells, expression of CD1234 is under the control of σ^E and SpoIIID in the mother cell. The products of these two genes are required for *skin* excision to occur, and the requirement of SpoIIID for CD1234 expression is a factor in delaying *skin* excision to a late stage of sporulation (Serrano *et al.*, 2016b) (Figure 1.4). *sigK* has a σ^E -dependent and a σ^K -dependent promoters, one of which further requires SpoIIID; thus, the expression of *sigK* itself is delayed (Pereira *et al.*, 2013, Saujet *et al.*, 2013, Serrano *et al.*, 2016b). While in *B. subtilis* σ^K is produced in an inactive form, pro- σ^K , and requires σ^F and σ^G for activation, in *C. difficile*, lacks a pro-sequence and is produced in an active form (Pishdadian *et al.*, 2015). Thus, σ^G is not required for the activation of σ^K . The timing of *skin* excision, under the control of SpoIIID via *sigK* and CD1234 expression, appears a key factor in the timely appearance of σ^K and proper morphogenesis of the spore surface layers: expression of a *skin*-less *sigK* allele results in misassembly of the coat/exosporium (Serrano *et al.*, 2016b). Further, the main role of SpoIIID in sporulation appears to be the control of σ^K activation, through *skin* excision, because the expression of *skin*-less *sigK* allele

from a SpoIIID-independent promoter bypasses the need for SpoIIID for the production of heat-resistant spores (Pishdadian *et al.*, 2015). SpoIIID is also required at a later stage in sporulation, to enhance expression of genes, such as *cdeC*, under the control of σ^K , required for assembly of the spore surface layers (Pishdadian *et al.*, 2015). Interestingly, while σ^G has no direct role in the activation of σ^K , the forespore exerts a yet uncharacterized role over the activity of σ^K , since σ^F is required for the expression of some σ^K targets (Pereira *et al.*, 2013, Saujet *et al.*, 2013).

Two additional ancillary transcription factors that operate in the mother cell in *B. subtilis*, GerE and GerR (Eichenberger *et al.*, 2004, Zheng *et al.*, 1992), are absent in *C. difficile*. In *B. subtilis*, RsfA and SpoVT further modulate gene expression in the forespore, with the later having an important role in the activation of late, σ^G -controlled genes (Wang *et al.*, 2006) (Figure 1.4). In *C. difficile*, *rsfA* is absent but *spoVT* is transcribed in a σ^F - and σ^G -dependent manner; in contrast to *B. subtilis*, SpoVT also seems to repress genes from the σ^F regulon (Pereira *et al.*, 2013, Saujet *et al.*, 2013).

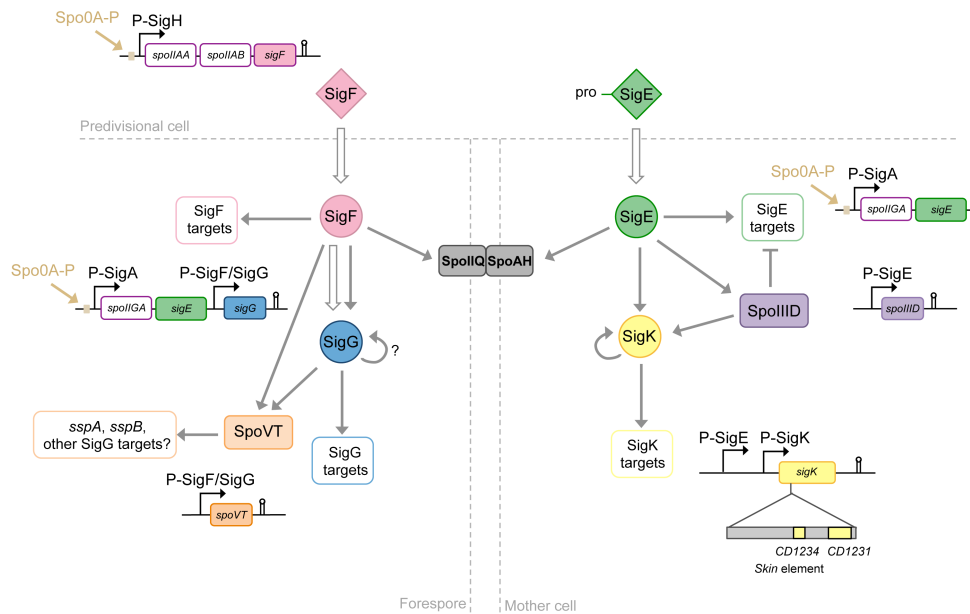


Figure 1.4. The forespore and mother cell regulatory networks in *C. difficile*.

The upper part of the figure represents the predivisional cells. The parallel vertical lines represent the two membranes separating the forespore (left) and the mother cell (right). The active forms of the sigma factors are represented inside circles, while the diamond-shaped boxes represent the inactive forms of σ^F and σ^E . Solid grey arrows indicate activation at the transcriptional level, and crossed arrows correspond to inactivation processes. White arrows indicate activation at the level of protein activity. Question marks represent mechanisms that are not yet fully understood or demonstrated. Adapted from (Saujet *et al.*, 2013).

Because the σ^E regulon is not strictly under σ^F control and because σ^K activity does not depend on σ^G a weaker connection between the mother cell and the forespore lines of gene expression exists in *C. difficile* (Pereira *et al.*, 2013, Saujet *et al.*, 2013). While in *B. subtilis* the tight connection between the two lines of genes expression ensures the fidelity of the differentiation process, it has been hypothesized that the less tight link between the two programs in *C. difficile* may be a strategy for the generation of variation in spore morphology (Pereira *et al.*, 2013, Saujet *et al.*, 2013).

1.2.3. Spore structure and assembly

The bacterial spore is a layered structure composed of three main components: the core, the coat, the cortex and in some species the exosporium. This unique concentric structure confers to the spore its high resistance to external aggressions like heat, chemicals and UV radiation (Henriques & Moran, 2007, Setlow, 2006). The spore core contains the genetic material, however, it is metabolically inactive. Here, small acid soluble proteins (SASP) protect the DNA and there is a low water content and high levels dipicolinic acid (DPA) chelated with calcium (CaDPA); these are important features to confer the spore its characteristic resistance (Setlow, 2014).

An inner membrane involves the core that has a similar lipid composition to the vegetative membranes, however is less permeable due to lipid immobility, an important feature to protect the DNA (Cowan *et al.*, 2004). Over the inner membrane is assembled a thin layer of peptidoglycan, the primordial germ cell wall. This wall will give rise to the vegetative cell wall after spore germination (Henriques & Moran, 2007). A second layer of peptidoglycan, essential to maintain the core dehydration and dormancy state, follows the germ cell wall, the cortex. The cortex is a thicker layer of peptidoglycan and has different features that are important for cortex lytic enzymes to discriminate between the two layers during germination (Setlow, 2014).

An outer membrane involves the cortex and is important for the assembly of a proteinaceous coat (Henriques & Moran, 2007). The characteristic resistance of the spore to chemical and physical harms is mainly due to the coat, composed of multiple layers. In *B. subtilis*, the coat has been shown to be composed of more than 70 proteins that are

orderly assembled. *C. difficile* only has 25% of homologs of the genes coding for these proteins, yet the coat presents a similar laminar structure (McKenney *et al.*, 2013, Permpoonpattana *et al.*, 2013, Permpoonpattana *et al.*, 2011b). The appropriate assembly of the coat proteins to the spore surface is guided by morphogenetic proteins, which are produced by the mother cell. SpoIVA is a morphogenetic ATPase important for the early stages of coat assembly and is conserved in *Bacilli* and *Clostridia* (Henriques & Moran, 2007, Permpoonpattana *et al.*, 2013). SpoIVA assembles into a cable structure in an ATP-dependent manner, which forms a basal layer upon which the rest of the coat is assembled. In *C. difficile*, SpoIVA is essential for coat assembly but contrarily to *B. subtilis*, is dispensable for cortex formation and localizes in a SpoVM-independent manner. These differences suggest that *C. difficile* has divergent proteins with morphogenetic functions (Putnam *et al.*, 2013, Ribis *et al.*, 2017). Another morphogenetic protein important for coat assembly in *B. subtilis* is SpoVID. SpoVID has a LysM domain for peptidoglycan binding and a region that is involved in an interaction with SpoIVA recruiting it to the surface of the developing spore (Wang *et al.*, 2009). SpoVID is essential for a step in coat assembly during which the coat proteins eventually cover the entire surface of the spore; SpoVID controls encasement by all layers of the coat (Wang *et al.*, 2009). *C. difficile* does not have a SpoVID homolog but a protein has been identified with a LysM domain, SipL (SpoIVA interacting protein L), that directly interacts with SpoIVA. In fact a *sipL* mutant has a phenotype similar to a *spoVID* mutant in *B. subtilis*, as it is important for complete coat assembly but dispensable for cortex formation. It is still not known whether SpoIVA and/or SipL directly bind to the

forespore membrane to initiate coat assembly, or whether another unknown protein replaces SpoVM in *C. difficile* or is redundant with it (Putnam *et al.*, 2013).

In some *Bacilli*, such as *B. cereus* and *B. anthracis*, and in some Clostridia, the outermost spore structure is an exosporium. In *C. difficile* an exosporium-like structure closely apposed to the underlying coat is seen; this structure lacks the interspace, which separates the coat from the exosporium basal layer in spores of *B. cereus/B. anthracis*. Furthermore, in *C. difficile*, the exosporium-like layer shows a strain-variable morphology, with some strains like the epidemic R20291 presenting a much thicker exosporium-like layer from which hair-like filaments protrude, and others like the laboratory strain 630 presenting an electrodense compact structure (Pizarro-Guajardo *et al.*, 2016). Some studies suggest that a typical exosporium may be labile, and easily lost (Permpoonpattana *et al.*, 2013, Permpoonpattana *et al.*, 2011b). Nevertheless, *C. difficile* encodes for three homologs of the collagen-like glycoprotein BclA of *B. cereus/B. anthracis*, which forms the hair-like nap of the exosporium; these proteins are coded for by *bclA1*, *bclA2* and *bclA3*, which are expressed during sporulation in the mother cell (Paredes-Sabja *et al.*, 2014, Pizarro-Guajardo *et al.*, 2014). Importantly, a *bclA1* mutant is affected for colonization and infection in the hamster model. Additionally spores for this mutant germinate faster than wild-type spores. These effects suggest that spores are important for the initial stages of colonization and infection since these are affected by an early germination (Phetcharaburanin *et al.*, 2014).

Other proteins have been identified as components of this structure: CdeC and CdeM are considered the exosporium

morphogenetic proteins, expressed during sporulation and that localize at the spore surface, and CdeA and CdeB are other exosporium components exclusively found in *C. difficile*. Furthermore, the previously identified coat proteins, CotA and CotB, were also proposed to be present in the exosporium (Barra-Carrasco *et al.*, 2013, Diaz-Gonzalez *et al.*, 2015). Importantly, as the exosporium is the more external structure in *C. difficile* spores it might have important roles in adherence to the epithelium and therefore might be important during colonization (Paredes-Sabja *et al.*, 2014). However, it is important to note that coat proteins can also be important for colonization (Hong *et al.*, 2017)

1.2.4. Germination

Germination is an irreversible process by which the spore loses its characteristics, resuming metabolic activity and allowing for vegetative growth. Small molecules, designated germinants, are detected by the spore that responds and loses its dormancy.

In *C. difficile* the germination process has been extensively studied due to its relevance during infection. Spores germinate in the hosts gut when the suitable environment is found. The spores respond to specific combinations of amino acids and bile salts, with glycine and cholate derivatives, the most efficient co-germinants (Sorg & Sonenshein, 2008). However, the secondary bile salt deoxycholate prevents vegetative growth and the primary bile salt chenodeoxycholate inhibits germination (Sorg & Sonenshein, 2009, Sorg & Sonenshein, 2010). In this way, the spores find high levels of cholate and glycine in the small intestine where they can germinate, but in normal microbiota conditions the cholate derivatives are rapidly

metabolized in the secondary bile salt deoxycholate and growth is inhibited. However, in case of a disturbed microbiota the metabolization into secondary bile salts does not occur as efficiently, leading to vegetative growth and therefore disease (Sorg & Sonenshein, 2008). Furthermore, only in the large intestine where the anaerobic conditions are found, the levels of cholate are higher than of chenodeoxycholate and therefore that is where outgrowth can occur (Sorg & Sonenshein, 2009, Sorg & Sonenshein, 2010).

CspC is a subtilisin-like pseudoprotease and has been identified as a receptor for cholate derivatives during germination (Figure 1.5) (Francis *et al.*, 2013). Cogermnants were shown to be essential for initiation of germination signaling, however, receptors for this compounds that can be amino acids or divalent cations are still unknown (Shrestha & Sorg, 2018, Sorg & Sonenshein, 2008). Binding of germinants to the receptor CspC activates a proteolytic signaling cascade where the protease CspB (produced bound to the pseudoprotease CspA but cleaved during spore formation by YabG (Kevorkian *et al.*, 2016)), is activated leading to processing of pro-SleC and consequently its activation (Figure 1.5). SleC is a cortex hydrolase that is synthesized in the mother cell during sporulation as a preproprotein. The presequence is cleaved possibly by the action of YabG (Kevorkian *et al.*, 2016). The proprotein is only activated after cleavage by CspB and once activated will cleave the cortex peptidoglycan, leading to germination (Adams *et al.*, 2013, Burns *et al.*, 2010, Francis *et al.*, 2015, Kevorkian *et al.*, 2016).

Contrarily to what occurs in *B. subtilis*, spore cortex hydrolysis occurs before dipicolinic acid (DPA) release. In *B. subtilis* the stage I of germination is characterized by the release of DPA chelated with

calcium (caDPA) from the core in response to small molecule germinants by germinant receptors that exist at the spore inner membrane. This release triggers stage II when occurs cortex degradation by spore cortex lytic enzymes (Setlow, 2014). In *C. difficile* it has been proposed that cortex lysis leads to cell wall expansion, which can be recognized by an unidentified protein that leads to caDPA release or the consequent inner membrane expansion triggers caDPA release directly (Francis *et al.*, 2015).

As in *B. subtilis* cortex hydrolysis also depends on cortex-specific modifications. The spore cortex peptidoglycan is distinguished from the germ cell wall by the presence of muramic- δ -lactam (MAL) residues (Popham & Bernhards, 2015). To generate these residues two enzymes are required: CwlD, an N-acetylmuramoyl-L-alanine amidase and PdaA an N-acetylmuramic acid deacetylase (Fukushima *et al.*, 2005, Fukushima *et al.*, 2002, Gilmore *et al.*, 2004). These enzymes have in *C. difficile* the same functions as in *B. subtilis* (Diaz *et al.*, 2018). Furthermore, GerS is a *Peptostreptococcaceae* family-specific lipoprotein that was shown to be essential for germination (Fimlaid *et al.*, 2015a). GerS interacts directly with the amidase CwlD and it is required for its activity and therefore for cortex modification and SleC hydrolytic activity (Diaz *et al.*, 2018). Additionally GerS requires CwlD for incorporation into the spore. The family specificity of the GerS protein suggests a different pathway for cortex modification when comparing with the model organism *B. subtilis* (Diaz *et al.*, 2018). Efficient germination regulation is essential for a successful infection, making these proteins appealing targets for therapeutics.

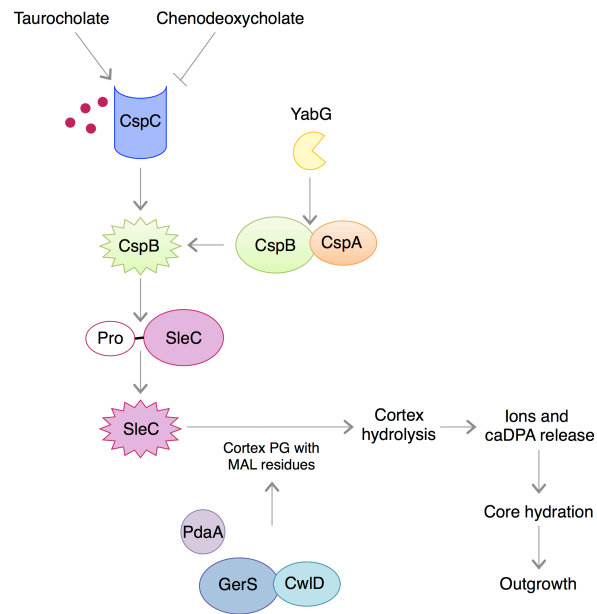


Figure 1.5. Schematic representation of a possible model for *C. difficile* germination pathway. The CspC receptor senses bile salts and will activate the protease CspB. CspB is produced bound to CspA and the protease YabG separates the two proteins. CspB will process pro-SleC activating it. SleC, a cortex lytic enzyme, will recognize the modified peptidoglycan (PG) containing muramic- δ -lactam (MAL) residues. The proteins GerS, CwlD and PdaA are required for this modification specific of the PG cortex. Arrows indicate activation, while lines ending with a bar cross indicate repression. Adapted from (Paredes-Sabja *et al.*, 2014).

1.3. MOTILITY IN *C. DIFFICILE*

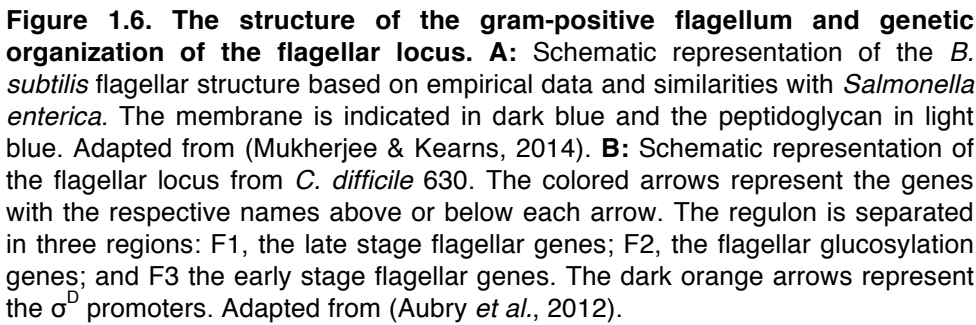
Some bacteria are motile due to the presence at its surface of the flagellum, a semi-rigid rotating filament, important for chemotaxis. The flagellum is a macromolecular organelle, complex in its regulation and assembly. Assembly of this structure implies a high-energy cost and therefore is tightly regulated at the transcriptional and translational levels (Altegoer & Bange, 2015, Diepold & Armitage, 2015, Mukherjee & Kearns, 2014, Terashima *et al.*, 2008). Flagellar motility allows for bacteria to move in fluid environments while sensing environmental cues such as chemicals, oxygen and temperature, due to the presence of chemosensory receptors (Sourjik & Wingreen, 2012). Therefore, the flagellum is a relevant factor in bacterial colonization of biotic and abiotic surfaces, and for pathogens, in establishing the initial interactions with the host and in modulating the host immune system. Other important functions in colonization like adherence, biofilm formation and secretion of virulence factors have been shown for the flagella (Duan *et al.*, 2013). Structurally, the flagellum is a derivative of the type III secretion systems (T3SS), with a similar molecular composition: contains a molecular motor, a hook-basal-body complex and a rotating filament (Evans *et al.*, 2014, Terashima *et al.*, 2008).

Most of the work on flagellar structure and assembly have focused on gram-negative bacteria, with *Salmonella enterica* and *Escherichia coli* being the best studied models (Chevance & Hughes, 2008, Minamino & Imada, 2015). For gram-positive bacteria most studies have been conducted on *B. subtilis* (Mukherjee & Kearns, 2014).

1.3.1. The Gram-positive flagellar structure

Similarly to the gram-negative flagella, the gram-positive flagellum is composed of the basal body, a hook and a filament, with the difference that the basal body does not transverse two membranes but only one (Figure 1.6A). The basal body is the engine of the flagellum, with a rotor and static components embedded in the cell membrane, working as an anchor for the remaining structure and comprising the secretion apparatus required for exporting the external components of the flagellum (Chevance & Hughes, 2008). The export apparatus of the basal body resembles the T3SS, is thought to assemble in the center of a ring, the MS ring, and requires a proton motive force for protein secretion (Kubori *et al.*, 1997) (Chevance & Hughes, 2008, Mukherjee & Kearns, 2014).

The hook is the structure that connects the basal body to the filament, and its subunits are secreted through the basal body structure. The protein FlgD caps the hook and dissociates from the tip when the filament polymerization is initiated (Courtney *et al.*, 2012). The filament is composed of flagellin subunits, which in *B. subtilis* are designated Hag (LaVallie & Stahl, 1989). Hag monomers establish interactions between the N- and C-terminal domains polymerizing to form a hollow cylinder (Figure 1.6A). This structure, although rigid, is able to respond to environmental signals that will control motility (Mukherjee & Kearns, 2014).



Most of *C. difficile* strains are motile due to presence of peritrichous flagella, which has an important role during colonization. Nevertheless, virulent strains have been identified that are non-motile, like the M120 strain from ribotype 078 (Tasteyre *et al.*, 2001, Twine *et al.*, 2009). The genes required for the flagellar apparatus are encoded in three operons, the F1, F2 and F3 regulons and, similarly to other

flagellated bacteria, transcription occurs hierarchically (Figure 1.6B) (Stabler *et al.*, 2009).

The F3 region comprises the early stage genes that are orthologous to the genes at the *fla/che* operon from *B. subtilis*. Similarly to *B. subtilis* it is thought that transcription from this operon occurs through a putative σ^A promoter upstream of *flgB*. Furthermore, it is at this region that *sigD* is encoded. Additionally, the basal body proteins are encoded at this region, such as FliF from the MS ring, FliG and FliM, which are the motor/switch proteins, and FlhB and FliR from the export apparatus (Aubry *et al.*, 2012, El Meouche *et al.*, 2013). The early genes, including *sigD*, are regulated by the nucleotide second messenger c-di-GMP. c-di-GMP binds to the riboswitch Cd1 at the 5' untranslated region of the F3 region and leads to premature transcription termination (McKee *et al.*, 2013, Soutourina *et al.*, 2013, Sudarsan *et al.*, 2008) (Figure 1.6B). Furthermore, in strains from ribotype 027 and 017, between the Cd1 riboswitch and the *flgB* open reading frame there is a flagellar switch that controls phase variable production of flagella, through recombination catalysed by RecV. The flagellar switch comprises a 154 invertible sequence flanked by inverted repeats that are shortened in the 630 laboratory strain, where the switch is locked at the “ON” phase (Anjuwon-Foster & Tamayo, 2017).

The late stage genes are clustered in the F1 region that is importantly regulated by σ^D , with promoters identified upstream of *CD0226*, *flgM* and *fliC* (El Meouche *et al.*, 2013). The F1 region encodes for the putative anti- σ^D factor, FlgM. Similarly to *B. subtilis*, FlgM decreases *sigD* expression and σ^D activity, evidenced by a decrease in expression of *sigD*-dependent genes when *flgM* is

overexpressed, and a consequent loss of motility. Like in gram-negative bacteria and in *B. subtilis*, it has been proposed that temporal regulation of the F1 regulon occurs due to the excretion of the anti- σ^D factor FlgM through the basal body when this has been assembled (Calvo & Kearns, 2015, Chevance & Hughes, 2008, El Meouche *et al.*, 2013). The F1 region also includes the genes *flgK* and *flgL* coding for hook proteins, *fliC* that codes for flagellin and *fliD* that codes for the flagellar cap protein (Aubry *et al.*, 2012, Tasteyre *et al.*, 2001, Tasteyre *et al.*, 2000). Furthermore, the F3 and F1 regions are separated by the F2 region, which encodes flagellar glycan biosynthetic genes (Figure 1.6B). (Twine *et al.*, 2009). Glucosylation is important for flagellar assembly and for interactions with the host (Logan, 2006). In *C. difficile*, disruption of CD0240 that codes for a glucosyltransferase, results in an inability to produce flagella; moreover, flagellin modifications have been shown to be important for colonization (Faulds-Pain *et al.*, 2014, Twine *et al.*, 2009).

Differences have been observed in the flagellar regulons among strains, resulting in phenotypic differences in motility. The F2 region has a variable number of genes: four genes are present in 630 (ribotype 012) and in strains from ribotype 017, while six genes are part of this regulon in R20291 and CD196 (ribotype 027) and in M120 (ribotype 078). This is reflected in strain-specific flagellin modifications with two types of oligosaccharides (Faulds-Pain *et al.*, 2014) and might explain differences observed in motility and in number of flagella, that are however variable between different isolates. Some authors observed peritrichous flagella in 630 and a single polar flagellum in R20291 (Baban *et al.*, 2013), while others also observed multiple peritrichous flagella in R20291 (Anjuwon-Foster & Tamayo, 2017, Martin *et al.*,

2013). Importantly, the factors that might explain the observed differences and that determine the number of flagella are still not identified.

1.3.2.1. The role of flagella in *C. difficile* pathogenicity

Flagella are essential for motility, an important characteristic of various pathogenic bacteria for invasion of the host tissues. Being surface exposed structures, flagella are involved in important interactions with the host, with the concomitant immune response. Furthermore, bacterial flagella have been shown to be important for optimal adherence, particularly for enteropathogenic bacteria (Duan *et al.*, 2013). In *C. difficile*, motility is important for efficient colonization (Tasteyre *et al.*, 2001), but the importance for adherence seems to be strain-specific: in the epidemic strain R20291 flagella are important for adherence, which was not observed for the laboratory strain 630 Δ *erm* (Baban *et al.*, 2013, Dingle *et al.*, 2011b). Highlighting the importance of flagella for pathogenicity is the observation that toxins and flagella work synergistically to induce inflammation during infection, and toxins alone are not enough to lead to epithelial damage (Batah *et al.*, 2017).

Furthermore, in *C. difficile* a stronger link with toxin production exists since *fliC* and *fliD* mutants show higher virulence, most likely due to the production of higher levels of toxins, and the opposite is observed for early stage mutants *fliF*, *fliG*, *fliM*, *flhB*-*fliR* and *sigD* (Aubry *et al.*, 2012, Dingle *et al.*, 2011b, El Meouche *et al.*, 2013). Importantly, the flagellar regulator σ^D is also a positive regulator of toxin production (See section 1.4.4.5. Toxin regulation and motility) (El Meouche *et al.*, 2013, McKee *et al.*, 2013).

1.4. TOXINOGENESIS IN *C. DIFFICILE*

The main virulence factors in *C. difficile* infection are the extensively studied cytotoxins. The two main toxins are TcdA and TcdB that belong to the family of large clostridial glucosylation toxins, together with TcsL and TcsH, the lethal and hemorrhagic toxins from *Clostridium sordellii*, TcnA from *Clostridium novyi* and TpeL from *Clostridium perfringens* (reviewed at (Just & Gerhard, 2004, Schirmer & Aktories, 2004, Voth & Ballard, 2005)). Additionally, some clinical isolates produce *C. difficile* transferase toxin (CDT), a binary ADP-ribosylating toxin that modifies actin (Gerding *et al.*, 2014). The toxins bind to cells receptors, are internalized and their action on the target proteins causes important physiological changes in the cells, which leads to disease (Voth & Ballard, 2005).

1.4.1. The binary toxin CDT

CDT is produced by up to 35% of the *C. difficile* isolates; CDT production was first discovered in the strain CD196 of ribotype 027 (Goncalves *et al.*, 2004, Popoff *et al.*, 1988). CDT is consistently associated with the epidemic ribotype 027 (Stubbs *et al.*, 2000). Furthermore, CDT positive strains were frequently isolated from animals, predominantly of the ribotype 078 (Keel *et al.*, 2007, Rupnik, 2007). CDT belongs to the group of Clostridial iota toxin-like binary toxins and has ADP-ribosyltransferase activity that modifies actin. This toxin is composed of CDTa, the enzymatic component, and CDTb, the binding component. CDTa and CDTb are encoded by *cdtA* and *cdtB*, respectively, and are found at the Cdt locus, or CdtLoc, together with *cdtR* that codes for a regulatory protein (Perelle *et al.*, 1997). The CdtLoc, of 6.2 kb, may be found in a truncated version of 4.2 kb with

cdtAB pseudogenes. When the CdtLoc is absent, a 68 bp sequence is present at its location (Carter *et al.*, 2007, Stare *et al.*, 2007) (Figure 1.7). Production of the binary toxin is regulated by CdtR. CdtR is a response regulator of the LytTR family and is required for CDT-mediated cytotoxicity (Bilverstone *et al.*, 2017, Carter *et al.*, 2007). Interestingly, CdtR also regulates TcdA and TcdB production in strains from ribotype 027, but not from the 078 ribotype or in strain 630 (ribotype 012). Thus, CdtR acts as a global regulator of toxin production in some strains (Lyon *et al.*, 2016).

CDTa is the enzymatic component with ADP-ribosylating activity that modifies actin. Actin is ADP-ribosylated by CDT at arginine-177, which inhibits actin polymerization and induces depolymerization of actin filaments already formed, leading to complete destruction of the action cytoskeleton (Gulke *et al.*, 2001). Another component of the cytoskeleton, the microtubule, is also affected when the actin network is destabilized. In normal conditions the microtubules polymerize into the direction of the cell membrane but the growth is blocked when they reach the actin network. When the actin network is absent due to the action of the CDT, long membrane protrusions filled with microtubules are formed (Schwan *et al.*, 2009). These protrusions were shown to increase *C. difficile* adherence, not only in cell cultures, but also *in vivo* (Schwan *et al.*, 2009).

CDTb is the binding domain that interacts with a receptor at the host cell surface. CDTb is produced with a 42 amino acid signal sequence (Perelle *et al.*, 1997) and before or after cell binding has to be activated by serine-type proteases that cleave the signal sequence and that allow the formation of heptamers. Heptamers will form a prepore

to which CDTa binds and then enters the cell through receptor-mediated endocytosis (Barth, 2004). The cell receptor for CDT is the lipolysis-stimulated lipoprotein receptor (LSR), which is highly expressed in the liver, but also in other tissues including the gut (Hemmasi *et al.*, 2015, Papatheodorou *et al.*, 2011). The low pH of the endosomal compartments leads to conformational changes in the prepore, allowing the formation of a pore, through which the enzymatic unit CDTa is translocated to the cytosol where it will modify the actin. The translocation depends on the cytosolic chaperon system (Barth, 2004, Papatheodorou & Aktories, 2017).

Due to its presence in epidemic strains, CDT has been correlated with severe disease symptoms, with high mortality rates and severity (Bacci *et al.*, 2011, Barbut *et al.*, 2005, Stewart *et al.*, 2013). Interestingly, CDT has been identified in strains from ribotype 033 that are negative for both TcdA and TcdB and are still able to cause disease (Eckert *et al.*, 2015). Furthermore, a *tcdA-tcdB-CDT⁺* mutant made in a ribotype 027 strain was still able to kill three out of nine hamsters (Kuehne *et al.*, 2014). These results raise the question of whether CDT is a virulence factor by itself that should also be tested for diagnosis.

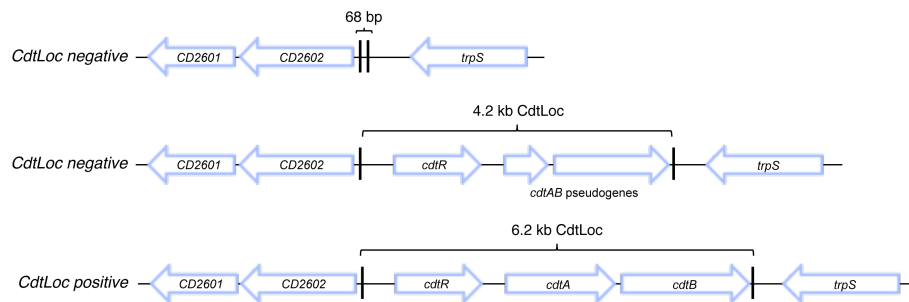


Figure 1.7. Schematic representation of the CDT region and flanking genes. When the CdtLoc is absent a sequence of 68 bp is present (top). A truncated version exist in some strains like *C. difficile* 630 where a CdtLoc of 4.2 kb is

present that contains *cdtAB* pseudogenes (middle). In strains positive for CDT a full CdtLoc is present with 6.2 kb in size containing the gene encoding for the regulator *cdtR* and the genes for the binary toxin CDT, *cdtA* and *cdtB*. Adapted from Gerding *et al.*, 2014.

1.4.2. The Pathogenicity Locus (PaLoc)

The two main toxins, TcdA and TcdB are encoded in a 19.6 kb conserved genomic region termed the pathogenicity locus (PaLoc). Besides *tcdA* and *tcdB*, the PaLoc contains also *tcdR*, *tcdE* and *tcdC* (Figure 1.8) (Braun *et al.*, 1996, Hammond & Johnson, 1995, Hundsberger *et al.*, 1997). *tcdR* codes for an alternative sigma factor that activates toxin expression (Mani & Dupuy, 2001, Moncrief *et al.*, 1997), *tcdE* encodes a holin-like protein possibly involved in toxin release (Govind & Dupuy, 2012, Govind *et al.*, 2015, Tan *et al.*, 2001) and *tcdC* codes for a proposed anti-sigma factor of TcdR proposed that acts as a toxin production repressor, but whose role is still controversial (Bakker *et al.*, 2012, Cartman *et al.*, 2012, Hundsberger *et al.*, 1997, Matamouros *et al.*, 2007) (See Section 1.4.5. Toxin regulation). PaLoc transcription occurs on a polycistronic manner, from a promoter upstream of *tcdR* but mainly as smaller transcripts originating from promoters upstream of *tcdA* and *tcdB* (Dupuy & Sonenshein, 1998, Hammond *et al.*, 1997, Hundsberger *et al.*, 1997). Expression of *tcdR*, *tcdB*, *tcdE* and *tcdA* is repressed during exponential growth but increases at the onset of stationary phase (Dupuy & Sonenshein, 1998).

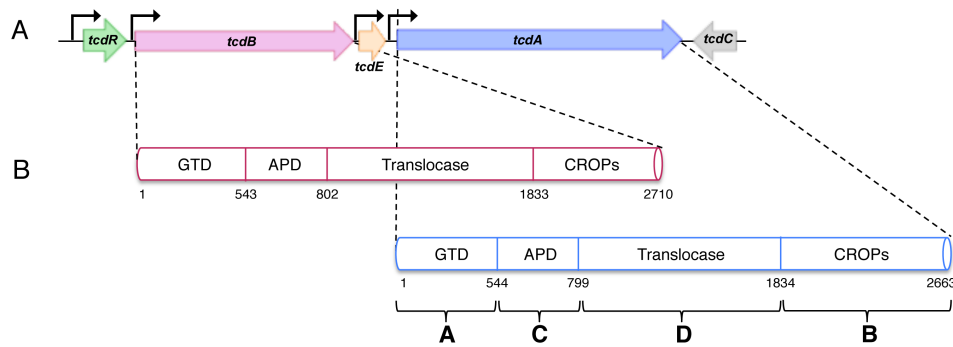


Figure 1.8. Genetic organization of the PaLoc and sequences of the toxins.

A: The PaLoc encodes the regulator TcdR, the two cytotoxins TcdA and TcdB, a holin-like protein TcdE and the possible anti-sigma factor TcdC. The black arrows represent direct regulation by TcdR. **B:** TcdA and TcdB consist of four domains: the glucosyltransferase domain (GTD) (A-Domain), the auto-protease domain (APD) (C-Domain), the delivery and translocase domain (D-Domain) and the combined repetitive oligopeptides (CROPs) (B-Domain). Adapted from (Aktories *et al.*, 2017).

Strains have been identified that lack the PaLoc and are non-toxinogenic; others are toxinogenic producing both toxins, or only producing TcdA or TcdB (for examples see (Goorhuis *et al.*, 2009, Janezic *et al.*, 2015, Kato *et al.*, 1998, Kim *et al.*, 2012, Kuijper *et al.*, 2001, Limaye *et al.*, 2000, Monot *et al.*, 2015, Pituch *et al.*, 2001, Rupnik *et al.*, 2003, Sambol *et al.*, 2000)). Toxinogenic strains additionally present deletions, insertions and single nucleotide polymorphisms (SNPs) in the PaLoc that led to a classification in toxinotypes (Rupnik, 2008). Currently, 31 toxinotypes are distinguished (<http://www.mf.um.si/mf/tox/>).

The genomic location of the PaLoc is largely conserved; in non-toxinogenic strains 75 bp, 115 bp or 7.2 kb sequences of unknown origin, which are absent in toxinogenic strains, are found at the same location (Braun *et al.*, 1996, Dingle *et al.*, 2014, Dingle *et al.*, 2011a, Elliott *et al.*, 2009). Although stably integrated, the presence of the

PaLoc is variable among populations, and even between strains belonging to the same multilocus sequence type (ST) (Dingle *et al.*, 2014, Dingle *et al.*, 2011a). This is usually a characteristic of mobile genetic elements, and in fact toxins are frequently encoded in mobile genetic elements like pathogenicity islands (PAIs). However, the PaLoc does not contain the usual elements found at PAIs such as direct repeats and insertion sequences. Nevertheless, toxin diversity is still a reality mainly due to horizontal gene transfer and recombination events (Dingle *et al.*, 2014, Popoff & Bouvet, 2013). Importantly, the conversion of non-toxinogenic strains into toxinogenic by horizontal gene transfer has already been shown (Brouwer *et al.*, 2013). Recently, whole genome sequencing allowed the identification of the PaLoc at different genome locations (Monot *et al.*, 2015). Furthermore, the same study, based on sequence similarities, proposed a model of evolution of the PaLoc: a mono-toxic PaLoc would have evolved to a binary PaLoc, based on sequence similarities of the intergenic region of the *C. difficile* 630 strain and genes found flanking the region of *tcdA* or *tcdB* in strains producing only one of the two toxins (Monot *et al.*, 2015).

1.4.3. Mechanism of action of TcdA and TcdB

TcdA and TcdB share 63% of identity at the primary sequence level and are both composed of four major functional domains. Initially, the toxins were defined as having a tripartite structure with the N-terminal biologically active domain, the middle hydrophobic region with predicted transmembrane regions and the C-terminal receptor-binding domain (Jank *et al.*, 2007, von Eichel-Streiber *et al.*, 1992). More recently, however, an extra autocatalytic, self-cleaving protease domain required for toxin processing has been proposed

(Figure 1.8). These four modules conform to the ABCD model: the A-domain is the N-terminal active domain with glucosyltransferase activity, the B-domain is the C-terminal receptor binding domain, the C-domain is adjacent to the active domain and is a cysteine protease module involved in processing and cutting of the toxin, and a middle domain (D) contains a hydrophobic region possibly involved in pore formation and delivery of the catalytic domain into the cytosol (Figure 1.8) (Jank & Aktories, 2008, Pruitt *et al.*, 2010).

The toxins act intracellularly in a multistep mode. The initial step is binding to the cell membrane through host cell receptors (see section 1.4.3.1. Cell Receptors) (Figure 1.9). For cell binding the C-terminal domain plays an important role. This domain consists of repeating units designated combined repetitive peptides (CROPs) (Moncrief & Wilkins, 2000, von Eichel-Streiber & Sauerborn, 1990, von Eichel-Streiber *et al.*, 1992) that fold in a solenoid structure of a β -hairpin followed by a loop (Greco *et al.*, 2006, Ho *et al.*, 2005). Structural differences at this domain between TcdA and TcdB justify the use of different cell receptors for the two toxins (Chaves-Olarte *et al.*, 1997). It has been shown, however, that toxin binding to cells can occur independently from the CROPs domain and that the region “upstream” of the CROPS is important in that case (Genisyurek *et al.*, 2011, Gerhard *et al.*, 2013, Olling *et al.*, 2011). Cell binding is followed by cellular uptake, which occurs through endocytosis (Henriques *et al.*, 1987). Endocytosis is mediated by clatrin for TcdB (Papatheodorou *et al.*, 2010), and dynamin- and PACSIN2- (protein kinase C and casein kinase substrate in neurons 2) dependent for TcdA (Chandrasekaran *et al.*, 2016). After cellular uptake, the toxins are translocated into the cytosol through endosomal acidification. Although the translocation

process is not very well characterized, it is known that low pH at the endosomes induces conformational changes that expose the translocation domain, the D domain, where a hydrophobic region is contained that will insert into the endosomal membrane. In this way, a pore can be formed that allows for the N-terminal enzymatic domain to be translocated to the cytosol (Barth *et al.*, 2001, Genisyurek *et al.*, 2011, Qa'Dan *et al.*, 2000). At this point autoproteolytic processing takes place, triggered by inositol hexakisphosphate (InsP₆) from the host, so that only the glucosyltransferase domain is delivered to the cytosol (Reineke *et al.*, 2007). Autocleavage is dependent on a cysteine protease domain (Egerer *et al.*, 2007) and recent studies on TcdA structure showed that a zinc ion at the active site is essential for cleavage (Chumbler *et al.*, 2016). Once in the cytosol, the glucosyltransferase domain can target the Rho proteins (Figure 1.9).

The pathogenic effects of the toxins are mainly due to their action in glucosylation of Rho proteins. TcdA and TcdB transfer a glucose moiety to Rho small GTPases, using UDP-Glucose as a co-substrate. The Rho proteins belong to the Ras superfamily, include around 20 proteins and the toxins mainly target RhoA, RhoB, RhoC, Rac and Cdc42 (Just *et al.*, 1995a, Just *et al.*, 1995b). These small GTPases bind and hydrolyze GTP to GDP, alternating between the GTP-bound and GDP-bound states that correspond to active and inactive forms of the proteins, respectively (Zhou *et al.*, 1998). TcdA and TcdB modify the GDP-bound form of the proteins by transferring a sugar moiety that retains the Rho proteins in its inactive form (Just *et al.*, 1995a, Just *et al.*, 1995b).

The observable effects on cells due to the glucosylation of the Rho proteins are cell rounding and consequent loss of cell-cell contacts;

this in fact is the observation made in the cell cytotoxicity assays with cell cultures that are used for diagnosis (Gilligan, 2015, Torres *et al.*, 1992). Rho protein inactivation causes deregulation of actin polymerization that induces loss of cell-cell contacts and disruption of tight junctions. These alterations lead to increased permeability at the epithelial barrier causing the diarrhea characteristic of the disease (Feltis *et al.*, 2000, Hecht *et al.*, 1992, Hecht *et al.*, 1988, Nusrat *et al.*, 2001). The epithelial barrier is also compromised due to cell apoptosis and cell loss since inactivation of Rho proteins lead to cell cycle arrestment and inhibition of actin-dependent cell cytokinesis (Brito *et al.*, 2002, Fiorentini *et al.*, 1998, Mahida *et al.*, 1996, Nottrott *et al.*, 2007). Rho glucosylation most often leads to inhibition of important regulatory functions. RhoA glucosylation, however, was shown to actually activate the inflammasome. This activation occurs through Pyrin that recognizes glucosylated RhoA and leads to caspase 1 activation, with consequent release of the cytokines IFN- γ and IL-8 (Xu *et al.*, 2014). IL-8 is in fact important to neutrophil recruitment and therefore there is an exacerbated inflammatory response that aids *C. difficile* infection by increasing mucosal damage (El Feghaly *et al.*, 2013) (See also section 1.1.6. The host immune response to *C. difficile*).

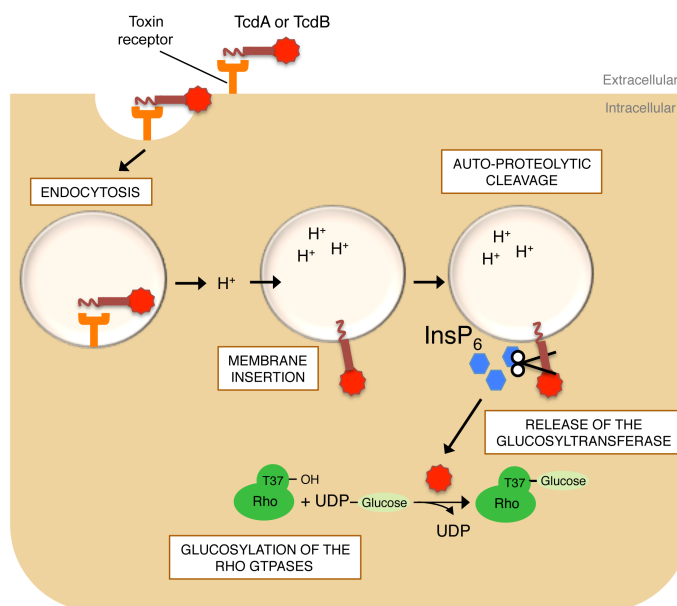


Figure 1.9. Schematic representation of the toxins mode of action. The toxins TcdA and TcdB bind to cell receptors through the C-terminal domain. The toxins are then endocytosed and they will reach the endosomal compartment where acidification allows for conformational changes that enable toxin insertion in the endosomal membrane. Once inserted in the membrane the toxins are autocleaved in the presence of inositol hexakisphosphate (InsP₆). The glucosyltransferase domain is released in the cytosol where it can target the Rho GTPases and glucosylate these proteins at threonine 37 or 35. Adapted from (Jank & Aktories, 2008).

1.4.3.1. Toxin cell receptors

More than one cell receptor has been described for both TcdA and TcdB. TcdA was shown to bind with high affinity to glycans. The glycan structure α -Gal-(1,3)- β -Gal-(1,4)- β -GlcNAc was the first to be described as binding TcdA with high affinity. However, this glycan that is present in the intestine of rodents and other animals is absent in humans (Pothoulakis *et al.*, 1991). Additionally, TcdA was shown to bind the glycan motifs Lewis I, X and Y that are expressed on human intestinal epithelial cells (Smith *et al.*, 1997, Tucker & Wilkins, 1991). More recently, glycan arrays defined that glycan motifs with the core

structure β -Gal-(1,4)- β -GlcNAc, bind TcdA (Gerhard, 2016). It is important to note, however, that this does not necessarily reflect binding in biological conditions. In contrast, for TcdB the entire CROPs region was shown not to be able to bind carbohydrates (Gerhard, 2016).

Various protein receptors have also been described for both toxins. For TcdA, sucrose-isomaltase was the first protein to be described as a possible receptor; this receptor, however, is only found in rabbit intestinal cells (Pothoulakis *et al.*, 1996). Furthermore, gp96, a glycoprotein found in the human colonic mucosa, was also proposed as a receptor for TcdA (Na *et al.*, 2008). It is debatable whether TcdA binds these glycoproteins through protein-protein interactions or through interaction with their glycan motifs. For a long time, no receptors were identified for TcdB; a CRISPR/Cas9-mediated gene knockout screening, however, lead to the identification of chondroitin sulfate proteoglycan 4 (CSPG4) as a possible receptor (Yuan *et al.*, 2015). TcdB binds to this receptor through a region that includes the N-terminal half of the CROPs domain (Orth *et al.*, 2014). Shortly after CSPG4 had been identified as a TcdB receptor, the poliovirus receptor-like 3 (PVRL3) that belongs to the nectin family was also reported as a possible receptor. However, binding to PVRL3 occurs independently from the CROPs domain (LaFrance *et al.*, 2015). The Wnt-receptor frizzled (FZD) proteins are also involved in TcdB binding and cellular uptake. Also for this receptor, TcdB does not bind through the CROPs domain (Tao *et al.*, 2016).

In summary, TcdA and TcdB bind to distinct cell surface receptors, which increase cell tropism. Furthermore, the toxin region involved in binding is now known to not be restricted to the CROPs region as initially pointed.

1.4.4. The possible role of TcdE in toxin release

The toxins are exported and found at the culture supernatant at the stationary phase (Boetzkes *et al.*, 2012, Ketley *et al.*, 1984, Mukherjee *et al.*, 2002). It was initially suggested that the toxins would be released through cell lysis due to their large size and to the absence of a signal peptide; however, the fact that it was found at the supernatant in a fraction superior to other cytoplasmic proteins suggests that a protein secretion mechanism is involved instead of release due to cell lysis (Mukherjee *et al.*, 2002).

TcdE is encoded in the PaLoc between *tcdB* and *tcdA* and was suggested to be involved in toxin release (von Eichel-Streiber *et al.*, 1992). The TcdE amino acid sequence has structural features reminiscent of class I holins that includes the λ phase S protein. In fact, attempts to overexpress *tcdE* in *E. coli* led to bacterial cell death due to membrane disruption (Tan *et al.*, 2001). Holins are small membrane cytolytic proteins produced by lytic bacteriophages that allow to form pores in bacteria, leading to cell lysis and therefore release of the phage progeny (reviewed at (Wang *et al.*, 2000)).

A *tcdE* mutant in the *C. difficile* 630 Δ *erm* did not present differences in the kinetics of toxin release nor in the total amount of toxin secreted (Olling *et al.*, 2011). Like the lambda holin, TcdE has two translation start sites and two putative ribosome binding sites upstream of both start codons (Dove *et al.*, 1990). In the same work, Olling *et al.* have seen that two forms of TcdE with 19kDa and 16kDa were found when *tcdE* was expressed in *E. coli*, while only the 19kDa form was detected in *C. difficile* 630 Δ *erm*. This led to the suggestion that the 16kDa form would be responsible for cell lysis and since it was not produced in *C. difficile* TcdE would not cause cell lysis (Olling *et*

al., 2011). However, in strains JIR8094 and R20291 (ribotypes 012 and 027), *tcdE* mutants presented a defect in toxin release that could be complemented by *tcdE* (Govind & Dupuy, 2012, Govind *et al.*, 2015). Furthermore, in this study TcdE was used to efficiently complement an *E. coli* λ lysogen defective for the λ holin, evidencing that TcdE can function as a holin. Nevertheless, the effect in toxin release was not due to increased cell lysis, since in the *tcdE* mutant no differences were observed in the cell lysis. The authors propose that TcdE forms pores in the cell membrane, where together with the toxins would form channels controlled by the presence of the toxins. Possibly, the toxins could act as plugs to cover the holes and maintain the cell integrity, which was supported by the increased cell lysis in a *tcdA*/*tcdB* double mutant where TcdE is produced (Govind & Dupuy, 2012). The differences between these two studies are possibly due to experimental conditions including the use of different background strains.

More recently, three TcdE isoforms were identified, with 166, 142 and 140 amino acids (TcdE₁₆₆, TcdE₁₄₂ and TcdE₁₄₀), which are translated from start codons at positions 1, 25 and 27, respectively. However, the TcdE₁₆₆ form was not detected when expressed from its own ribosome binding site (RBS), and expression from a modified RBS led to *C. difficile* lysis. Furthermore, the TcdE₁₆₆ and TcdE₁₄₂ isoforms had a much stronger effect on cell lysis and toxin release. The presence of the three isoforms might be required for efficient toxin release while maintaining cell integrity (Govind *et al.*, 2015).

Although with a controversial and strain dependent role, pore formation by TcdE is the only proposed TcdE-dependent mechanism for toxin release.

1.4.5. Toxin regulation

Toxin regulation is a multifactorial and complex process that allows *C. difficile* to adapt its virulence to the external conditions. To date, several nutritional and environmental factors have been identified as influencing toxin production. Furthermore, several regulators affect toxin production in response to these external factors. In order to understand toxin regulation is important to understand how this complex network is established in order to interconnect all the factors involved.

1.4.5.1. *The alternative sigma factor, TcdR*

TcdR is encoded immediately upstream of *tcdB* and is an RNA polymerase sigma factor that positively regulates toxin production (Mani & Dupuy, 2001, Moncrief *et al.*, 1997). TcdR is a small basic protein with 22kDa that interacts with core RNA polymerase. *In vitro* experiments suggested that together with TcdR, RNA polymerase binds the *tcdA* and *tcdB* promoter regions to stimulate transcription (Mani & Dupuy, 2001). Therefore, TcdR is a sigma factor that stimulates toxin production and responds to environmental signals (Mani *et al.*, 2002). Furthermore, it was suggested that TcdR stimulates its own transcription using two potential TcdR-dependent promoters upstream of *tcdR* (Mani *et al.*, 2002).

TcdR belongs to the Group 5 of extracytoplasmic function (ECF) sigma factors from the σ^{70} family, together with BotR from *Clostridium botulinum*, TetR from *Clostridium tetani*, UviA and TpeR from *C. perfringens* and TcsR from *C. sordellii*. These factors respond to extracytoplasmic environmental signals and although similar to members of the Group 4 ECF sigma factors, they were considered

sufficiently different to be assigned a group of their own (Dupuy & Matamouros, 2006, Helmann, 2002). Members of this group present structural similarities that are enough to make them functionally interchangeable, which is perhaps indicative of similar networks regulating toxins and bacteriocins in the *Clostridium* genus (Dupuy *et al.*, 2006). These regulators are encoded in genetic units with similar arrangements, where the sigma factor is encoded upstream and in the same direction of the toxin or bacteriocin that regulates, and is under positive auto-regulation in response to environmental signals (Carter *et al.*, 2014, Dupuy *et al.*, 2005, Mani *et al.*, 2002, Raffestin *et al.*, 2005). Furthermore, the genes regulated by these ECF sigma factors have promoters with a high level of sequence similarity, with the -35 regions being highly conserved and the -10 regions more variable. The consensus sequence has been defined as being TTTACA(16/18bp)CTTTTT (Dupuy & Matamouros, 2006) (Figure 1.10).



Figure 1.10. The TcdR consensus sequence. Sequence alignment of the promoter regions of toxin and bacteriocin genes from *C. difficile*, *C. perfringens*, *C. botulinum*, *C. tetani* and *C. sordellii*. The -35 and -10 regions are based on determination of the transcriptional start sites. Adapted from (Dupuy & Matamouros, 2006).

1.4.5.2. Toxin regulation through *TcdR* or *TcdA/TcdB*

Besides the two potential promoters upstream of *tcdR*, recognized by TcdR itself (Mani *et al.*, 2002), other important regulatory sequences have been identified in this region.

Initially, bioinformatic analysis allowed the identification of a putative σ^D -promoter in the *tcdR* regulatory region and σ^D was in fact showed to directly bind to this promoter together with the core RNA polymerase (El Meouche *et al.*, 2013). Since σ^D stimulates toxin expression through *tcdR*, c-di-GMP levels will also indirectly affect toxin expression. Like in Gram-negative bacteria, in *C. difficile* increased levels of c-di-GMP inhibit motility (Purcell *et al.*, 2012). This inhibitory effect on motility occurs via σ^D that is regulated by c-di-GMP binding to a riboswitch upstream of the F3 region (McKee *et al.*, 2013, Soutourina *et al.*, 2013, Sudarsan *et al.*, 2008). Reduced levels of σ^D result in reduced *tcdR* expression and consequently reduced *tcdA* and *tcdB* transcription (McKee *et al.*, 2013). The genetic switch found upstream of the F3 region and that in strains from the ribotypes 027 and 017 can switch directions, will consequently affect toxin expression across a cellular population (Anjuwon-Foster & Tamayo, 2017). Recently, a new regulator, RstA (Regulator of Sporulation and Toxins) has been identified that upregulates sporulation through an unidentified pathway and represses motility and toxin production through σ^D (Edwards *et al.*, 2016). Thus, any regulator that affects σ^D production will have an impact on toxin production.

CodY, is a global regulator conserved in low G + C Gram-positive bacteria that is involved in adaptation to nutrient deprivation; it was shown to bind directly to the *tcdR* promoter region at three different sites (Dineen *et al.*, 2007). CodY represses a pool of genes that

are unnecessary under conditions of nutrient availability and rapidly de-represses these genes in response to nutrient limitations (Molle *et al.*, 2003, Slack *et al.*, 1995, Sonenshein, 2005). GTP and branched-chain amino acids (BCAA) act as co-repressors by increasing CodY affinity for its targets (Ratnayake-Lecamwasam *et al.*, 2001, Shivers & Sonenshein, 2004, Villapakkam *et al.*, 2009). Nutrient depletion leads to CodY repression relieve since in these conditions the levels of GTP and branched-chain amino acids (BCAA) decrease. CodY has been extensively studied in the model organism *B. subtilis*; in *C. difficile* its relevance in regulating genes involved in amino acid metabolism, nutrient transport, fermentation, membrane components, surface proteins, sporulation, and importantly, toxin production, has been established (Dineen *et al.*, 2010, Dineen *et al.*, 2007). In a *codY* mutant, all the PaLoc genes show increased expression; thus CodY acts as a repressor of the PaLoc expression (Dineen *et al.*, 2007). Furthermore, isoleucine and a seven amino acid mixture that includes BCAAs are known to repress toxin production, which is in agreement with the role of BCAAs and GTP in increasing CodY affinity to its targets including *tcdR* (Dineen *et al.*, 2007, Karlsson *et al.*, 2000). CodY can also affect toxin expression indirectly. For instance, CodY is also a repressor of butyrate synthesis, and the levels of butyrate relate with the levels of toxin expression (Karlsson *et al.*, 2000). Importantly, enzymes involved in c-di-GMP synthesis are also regulated by CodY and GTP levels and therefore nutrient availability affects c-di-GMP signaling. Since c-di-GMP levels are known to repress toxin expression, this indicates that CodY affects toxin production through various mechanisms (Dineen *et al.*, 2010, Purcell *et al.*, 2017).

Besides the regulators acting on the *tcdR* regulatory region, CcpA, a pleiotropic regulator that is a member of the LacI/GalR family of transcriptional regulators that mediates carbon catabolite repression (CCR) in low GC Gram-positive bacteria, binds not only to the *tcdR* regulatory region, but also to the *tcdB* promoter region and to the 5' end of the *tcdA* coding region (Antunes *et al.*, 2011; Antunes *et al.*, 2012). CCR is the mechanism that prioritizes the utilization of glucose as a carbon source in bacteria instead of other secondary carbon sources. Therefore, this mechanism inhibits production of the enzymes involved in the utilization of secondary carbon sources, while activating production of the enzymes involved in glycolysis (reviewed at (Deutscher, 2008, Gorke & Stulke, 2008)). Toxin expression is repressed in response to glucose availability and this repression occurs through CcpA since in a *ccpA* mutant toxin expression is constitutive (Antunes *et al.*, 2011). Furthermore, CcpA affects other important regulators that might on their turn affect toxin production, being CodY on these known regulators.

1.4.5.3. The controversial negative regulator, TcdC

TcdC is a small acidic protein that is localized in the cytoplasmic membrane (Govind *et al.*, 2006). *tcdC* is encoded in the PaLoc but it is divergently transcribed from the other genes and it was early proposed as being a negative regulator of toxin production since *tcdC* expression levels are high at exponential phase of growth when the toxin transcripts are low, and decrease at the stationary phase when the levels of *tcdR*, *tcdA* and *tcdB* transcription increase (Govind *et al.*, 2006, Hundsberger *et al.*, 1997). Supporting this hypothesis was the fact that certain epidemic strains, namely from the ribotype 027, have a deletion or frameshift mutations in *tcdC*, proposed to correlate

with the high levels of toxin being produced by these strains (Carter *et al.*, 2011, Curry *et al.*, 2007, MacCannell *et al.*, 2006, McDonald *et al.*, 2005, Warny *et al.*, 2005).

Matamouros and colleagues showed by *in vitro* assays that TcdC negatively regulates toxin expression by interfering with the initial stages of transcription: TcdC prevented binding of RNA polymerase to the promoter of a *tcdR*-controlled gene by interacting with the sigma factor TcdR or directly with core RNA polymerase (Matamouros *et al.*, 2007). Other studies seem to contradict the proposed role of TcdC as a repressor. Through chromosomal manipulation Cartman *et al.*, showed that there was no association between the *tcdC* genotype and toxin production. In the *C. difficile* R20291 strain (ribotype 027) the $\Delta 117$ frameshift mutation and the 18-nucleotide deletion that occur naturally in the *tcdC* gene were restored and no differences in toxin production were observed. In *C. difficile* 630 *tcdC* was deleted and again no differences in toxin production were observed, indicating that in both strains TcdC is not determinant for the levels of toxin produced (Cartman *et al.*, 2012). In an independent study, a *tcdC* mutant in the background of strain 630 Δ *erm* was constructed using the ClosTron methodology and the results obtained were in agreement with the studies from Cartman and colleagues (Bakker *et al.*, 2012). Furthermore, a strain from ribotype 027 with increased toxin production was found to have a functional TcdC (Murray *et al.*, 2009).

Interestingly, one study has shown, through *in silico* and biochemical analysis, that TcdC has a predicted single-stranded nucleic acid binding fold at the C-terminal domain and that it can actually bind to nucleic acids by forming a dimer. The optimal TcdC

binding site was shown to form a G-quadruplex, which is a guanine rich region that forms a four-stranded structure. However, the relevance of these findings *in vivo* is still unclear, as no G-quadruplex structure exists at the PaLoc (van Leeuwen *et al.*, 2013).

Therefore, further studies are necessary in order to elucidate the role of TcdC in toxin regulation.

1.4.6. Toxins and sporulation

Toxin production increases at the onset of stationary phase, and therefore a connection exists between sporulation initiation and toxin production. SigH, which is an essential sigma factor of transition phase and sporulation initiation might act as a repressor of *tcdA*, *tcdB* and *tcdR* expression (Saujet *et al.*, 2011). Additionally, some studies have shown that Spo0A might stimulate toxin production, since in a *spo0A* mutant TcdA production was reduced (Pettit *et al.*, 2014, Underwood *et al.*, 2009). The role of Spo0A on toxin production, however, is still discussed. In strains of the ribotype 027, Spo0A seems to act as a repressor of toxin gene transcription (Deakin *et al.*, 2012, Mackin *et al.*, 2013), while in a strain of the ribotype 078 Spo0A regulates toxin production in a time-dependent way, repressing toxin production at early stationary phase, but with no effect during late stationary phase (Mackin *et al.*, 2013). In the reference strain 630 Δ *erm*, contradictory results were reported (Deakin *et al.*, 2012, Mackin *et al.*, 2013, Pettit *et al.*, 2014, Rosenbusch *et al.*, 2012, Underwood *et al.*, 2009). Importantly, no SigH or Spo0A binding sequences were found upstream of *tcdR*, *tcdA* or *tcdB* indicating the effects observed should be indirect. In fact, Spo0A is also important for regulating metabolic pathways, inducing processes like glycolysis, glucose uptake and

butyrate production (Pettit *et al.*, 2014), which could affect toxin production. Further work is required in order to elucidate how these regulators of sporulation initiation are related with toxin production.

1.5. GENERAL AIMS OF THIS WORK

Toxin regulation in *C. difficile* has been mainly studied in batches, with results respective to a population of cells. However, nowadays and with recent tools, increasing relevance is being given to genetic and phenotypic heterogeneity (Avery, 2006, Davis & Isberg, 2016, Davis & Isberg, 2018, Weigel & Dersch, 2018). *C. difficile* needs to be able to survive in different environments, surviving to a multitude of stresses and conditions until it finds the optimal conditions to grow and produce the main virulence factors, from which the toxins are the most relevant. Evaluating the regulation of toxin production at the single cell level might elucidate how environmental signals and toxin expression are integrated. Here, we studied toxin expression at the single cell level and how the main regulators affect the expression pattern (Chapter 2).

Sporulation, that allows for dissemination and toxinogenesis are both important processes for *C. difficile* pathogenesis. A clear interconnection between these two processes was never identified. In this work, we identified toxin production by sporulating cells and studied the regulation of this cell type specific expression (Chapter 3). Finally, we identified the toxins at the spore surface and further explored their localization (Chapter 4).

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Chapter 2

Bistable expression of the toxin-encoding genes

Author contributions:

C. P. Cassona performed all experiments except the construction of the initial PaLoc transcriptional fusions that were performed by Mónica Serrano and the construction of R20291 strains that were constructed by Sara Ramalhete.

This chapter contains data from the manuscript in preparation:

Bistable expression of the *Clostridium difficile* toxin-encoding genes.

Note: while the material described in this Chapter was being prepared for submission, Ransom and co-authors published a similar study in which the expression of *tcdA* was examined using FACS analysis (Ransom *et al.*, 2018). The main conclusions of the two studies are in agreement.

2.1. SUMMARY

Clostridioides difficile is a strict anaerobic spore-forming bacterium, a major causative agent of nosocomial diseases associated to antibiotic therapy in adults. Spores produced by *C. difficile* are the main infectious vehicle and the primary cause of persistence and transmission. Ingested spores germinate in the intestine to generate a population of cells that will produce flagella, a key virulence factor, the TcdA and TcdB toxins and spores, which will allow transmission of the organism. The two toxins are the products of genes coded for in a pathogenicity locus, PaLoc, which also carries the regulatory gene *tcdR*. The flagellar alternative sigma factor σ^D transcribes *tcdR*, triggering an auto-regulatory loop involved in the production of TcdR. TcdR, in turn, activates transcription from the *tcdA* and *tcdB* promoters. We show that expression of *tcdR*, *tcdA* and *tcdB* is bistable. Our results support a model in which bistability originates from the *tcdR* positive auto-regulatory loop and highlight the role of σ^D activity in priming this system. By labelling the flagellum with a chemical probe, we further show that most cells in a population are flagellated, and that most of the toxin-producing cells are contained within the flagellated population. We discuss the significance of bistable production of the toxins and the link between toxinogenesis, the activity of σ^D and assembly of the flagellum in the context of infection

2.2. INTRODUCTION

Clostridium difficile (recently reclassified *Clostridioides difficile* (Lawson *et al.*, 2016)), a spore-forming gram-positive anaerobe, is a major nosocomial pathogen, which causes a range of intestinal conditions linked to prolonged antibiotic treatment, as the organism can only colonize its host in the case of dysbiosis (Abt *et al.*, 2016, Lawley *et al.*, 2009, Smits *et al.*, 2016). Importantly, this nosocomial pathogen is now spreading in the community (Lessa *et al.*, 2015, van Dorp *et al.*, 2016). In the last decade *C. difficile* infection (CDI) rates and associated relapse cases have been increasing, due to the global spreading of epidemic strains, many of ribotype 027, which are associated with higher virulence and resistance to treatment (Freeman *et al.*, 2010, He *et al.*, 2013).

C. difficile can be disseminated and maintained in the environment due to its ability to sporulate, since spores are extremely resistant to external aggressions and, importantly, are oxygen resistant (Deakin *et al.*, 2012). Spores are the main infectious vehicles for this organism (Fimlaid *et al.*, 2015b, Pereira *et al.*, 2013, Saujet *et al.*, 2013). Sporulation is initiated during stationary phase under the control of the response regulator Spo0A, conserved amongst spore-forming bacteria (Abecasis *et al.*, 2013, Galperin *et al.*, 2012).

The infectious cycle is initiated by the ingestion of spores; the spores will germinate in response to bile salts and the resulting vegetative forms of the organism produce two potent toxins, TcdA and TcdB, that are the main responsible for the disease symptoms (Aktories *et al.*, 2017, Chandrasekaran & Lacy, 2017, Sorg & Sonenshein, 2009) (Figure 2.1A). TcdA and TcdB have glucosyltransferase activity and belong to the group of Large

Clostridial Toxins (LCTs). These toxins act by inactivating the Ras superfamily of GTPases through glucosylation, leading to interference on essential signalling pathways in the cell and therefore contributing directly to the disease (Aktories *et al.*, 2017). The genes for TcdA and TcdB are encoded in the pathogenicity locus, the PaLoc, together with TcdR, TcdC and TcdE (Hundsberger *et al.*, 1997, Kuehne *et al.*, 2010, Kuehne *et al.*, 2011) (Figure 2.1B). TcdR is encoded immediately upstream of *tcdB* and is an RNA polymerase sigma factor that positively regulates its own expression and toxin production (Mani & Dupuy, 2001, Moncrief *et al.*, 1997). TcdC, a small acidic, membrane protein, has been proposed to be a negative regulator of toxin production (Carter *et al.*, 2011, Matamouros *et al.*, 2007). TcdE is a holin-like protein and is possibly required for toxin release (Govind & Dupuy, 2012, Govind *et al.*, 2015). A link between toxinogenesis and metabolism (Bouillaut *et al.*, 2015) has been reported. Nutritional signals, such as glucose or other carbon sources and the presence of certain amino acids are known to be important in toxin regulation (Bouillaut *et al.*, 2015). Under laboratory conditions, the toxins are mainly produced in stationary phase, upon nutrient limitation (Figure 2.1C) (Dupuy & Sonenshein, 1998, Hundsberger *et al.*, 1997). CcpA is an important regulator in carbon catabolite repression (CCR) and is known to repress toxin production by interacting directly with the promoter regions of the *tcdR*, *tcdA* and *tcdB* genes in the presence of glucose or similar sugars (Figure 2.1C) (Antunes *et al.*, 2012, Antunes *et al.*, 2011, Dupuy & Sonenshein, 1998). Furthermore, proline and/or a mixture of seven amino acids containing glycine and proline also contribute to *C. difficile* toxin repression. PrdR, a σ^{54} -dependent transcriptional activator that responds to proline levels was appointed

as a toxin repressor and Rex, a redox-sensing DNA binding protein, also mediates the proline-dependent repression of toxin expression (Bouillaut *et al.*, 2015, Martin-Verstraete *et al.*, 2016). Finally, CodY is a global regulator present in various gram-positive bacteria and better characterized in the model organism *B. subtilis* that acts mainly as a repressor of dispensable genes in nutrient-rich conditions. In response to nutrient limitation, this set of genes can be rapidly de-repressed allowing for an adaptation to unfavourable environmental conditions (Molle *et al.*, 2003, Sonenshein, 2005). In *C. difficile*, CodY binds to the *tcdR* promoter region at three different locations (Figure 2.1C). Toxin production is repressed in response to an excess of branched-chain amino acids and GTP, since by binding these molecules, the DNA binding affinity of CodY increases (Dineen *et al.*, 2010, Dineen *et al.*, 2007).

As in other gastrointestinal pathogens, motility is important for colonization and virulence in *C. difficile* (Janoir, 2016, Stevenson *et al.*, 2015). The flagellum comprises a molecular motor, a hook-basal-body complex and a rotating filament. The basal body is inserted in the cell envelope and constitutes a complex essential for the export of the external structural proteins (Diepold & Armitage, 2015, Evans *et al.*, 2014). Flagellar motility is important in bacterial adhesion and colonization of biotic and abiotic surfaces, and in establishing interactions with the host, particularly during the early stages of colonization. Flagella are important for biofilm formation, secretion of virulence factors and modulation of the host immune system (Duan *et al.*, 2013). In gastrointestinal pathogens such as *Campylobacter jejuni*, *Vibrio cholerae*, *Helicobacter pylori*, *Escherichia coli* and *Salmonella*

enterica, the regulatory networks for motility and virulence seem to overlap (Rossez *et al.*, 2015).

In the Gram-positive model organism *Bacillus subtilis*, the vegetative sigma factor A (σ^A) regulates the *fla/che* operon where the genes required for the basal body synthesis are encoded. Once the hook is assembled the alternative regulator σ^D , a σ^{28} homolog, is activated due to de-repression by the anti-sigma factor FlgM; σ^D then activates the expression of the late stage genes responsible for filament assembly and rotation (Mukherjee & Kearns, 2014). In *C. difficile* the major flagellar operon is composed of three different regions, designated F1, F2 and F3 (Aubry *et al.*, 2012). The early stage genes are encoded in the F3 region, which includes genes encoding for the MS ring (*fliF*), the C ring motor switch (*fliG* and *fliM*), the export apparatus membrane proteins (*flhB* and *fliR*) and the regulator of the late stage genes, *sigD*. The late stage genes, such as those coding for the flagellar filament protein flagellin (*fliC*) and the flagellar capping protein gene (*fliD*), are encoded in the F1 region. The F2 region encodes additional glycan biosynthetic genes (Aubry *et al.*, 2012).

The flagellar proteins FliC and FliD were shown to be important for adherence to the mucus layer and therefore for colonization (Tasteyre *et al.*, 2001). However, differences have been noted among strains: in R20291, a strain of the 027 ribotype, the flagella is important for adherence, but this was not observed for the commonly used laboratory strain 630 Δ *erm*, of ribotype 012 (Baban *et al.*, 2013, Dingle *et al.*, 2011b). Furthermore, the host immune system generates antibodies against these proteins (Pechine *et al.*, 2005) and mutant strains for *fliC* and *fliD* are more virulent in the hamster model (Dingle *et al.*, 2011b). Importantly, mutations in genes of the

flagellar regulon affect toxin production. Toxin production increases in cells of the late stage mutants *fliC* and *fliD*, and decreases in the early stage mutants *fliF*, *fliG*, *fliM*, *flhB-fliR* and *sigD* (Aubry *et al.*, 2012, Dingle *et al.*, 2011b, El Meouche *et al.*, 2013). The effects of these mutations on toxin production are likely to reflect alterations in the activity of σ^D , which utilizes a promoter upstream of *tcdR*; this promoter is thought to trigger an auto-regulatory loop upon which TcdR directs its own expression from two *tcdR*-dependent promoters (Figure 2.1C) (El Meouche *et al.*, 2013). Thus, expression of *tcdR* is linked to the activity of σ^D and factors that modulate the activity of σ^D also impinge on toxin production. One such factor is the second messenger cyclic di-guanosyl-5'monophosphate (c-di-GMP), which negatively regulates motility by affecting the expression of σ^D , and toxin gene expression (Figure 2.1C) (McKee *et al.*, 2013). The link between assembly of the flagellum and toxin expression may stem from the synergistic effect of the toxins and the flagellum in triggering an efficient mucosal inflammation and epithelial lesions (Batah *et al.*, 2017).

Toxinogenesis and flagellar assembly have not been studied at the single cell level in *C. difficile*. However, it is now recognized that clonal populations exhibit phenotypic heterogeneity, due to the expression of different sets of genes by different or overlapping sub-populations (Davis & Isberg, 2016, Davis & Isberg, 2018, Weigel & Dersch, 2018). This phenotypic heterogeneity is particularly important for pathogens that become in contact with different types of host cells, and pass through various environments, both inside and outside the host (Stewart & Cookson, 2012). Here we studied the expression of *tcdR* and the toxin-encoding genes at the single cell level. We show

direct and indirect factors regulating PaLoc gene expression are indicated (red and blue, negative and positive regulation, respectively; see text for details). The lines below the genetic map show the position and extent of the DNA fragments used to create the indicated transcriptional *SNAP^{Cd}* fusions. Grey rectangles show the position of CcpA binding sites and blue rectangles CodY binding sites. **C:** Expression of *sigD* (top) and *tcdR* (bottom) during growth and stationary phase. *tcdR* is expressed at the end of exponential phase and during stationary phase while *sigD* is expressed during growth and stationary phase.

2.3. MATERIALS AND METHODS

Growth conditions and general methods. Bacterial strains and their relevant properties are listed in Table 2.1. The *Escherichia coli* strain DH5 α (Bethesda Research laboratories) was used for molecular cloning, while the strain HB101 (RP4) was used as the donor in *C. difficile* conjugation experiments (Hussain *et al.*, 2005). Luria-Bertani medium was routinely used for growth and maintenance of *E. coli*. When appropriate, ampicillin (100 μ g/mL) or chloramphenicol (15 μ g/mL) was added to the culture medium. The *C. difficile* strains used in this study are congenic derivatives of strain 630 Δ erm (Hussain *et al.*, 2005) and were routinely grown anaerobically (5% H₂, 15% CO₂, 80% N₂) at 37°C in brain heart infusion (BHI) medium (Oxoid), and when necessary supplemented with L-cysteine (0.1%) and yeast extract (5 mg/mL) (BHIS) (Wilson *et al.*, 1982). For toxin assays, tryptone yeast extract (30g/L tryptone; 20g/L yeast extract) (TY) was used. When necessary, cefoxitin (25 μ g/mL), thiamphenicol (15 μ g/ml) or nisin (1 μ g/mL) were added to *C. difficile* cultures. A defined minimal media (CDMM) (Karasawa *et al.*, 1997) with 1% agar was used as uracil-free medium when performing genetic selections. All the primers used in this study are listed at table 2.2 and all the plasmids used or constructed at this study are listed at table 2.3.

***SNAP^{Cd}* transcriptional fusions.** For the construction of the transcriptional *SNAP^{Cd}* fusions to the PaLoc promoters, plasmid pFT47 previously constructed (Pereira *et al.*, 2013) was used. All the promoters were cloned between the EcoRI/ XhoI sites, upstream of *SNAP^{Cd}*. In order to construct *tcdR*-, *tcdA*-, *tcdB*-, *tcdC*- and *tcdE*-transcriptional *SNAP^{Cd}* fusions, the promoter regions of these genes were PCR-amplified using genomic DNA from *C. difficile* 630 Δ erm with the primer pairs Ptcdr-SNAP-EcoRI-Fw/ Ptcdr-SNAP-XhoI-Rev, Ptcda-SNAP-EcoRI-Fw/ Ptcda-SNAP-XhoI-Rev, Ptcdb-SNAP-EcoRI-Fw/ Ptcdb-SNAP-XhoI-Rev, Ptcdc-SNAP-EcoRI-Fw/ Ptcdc-SNAP-XhoI-Rev and Ptcde-SNAP-EcoRI-Fw/ Ptcde-SNAP-XhoI-Rev, to produce, 815, 520, 530, 430 and 600bp products, respectively. The fragments were cloned between the EcoRI/XhoI sites in pFT47, yielding pMS464, pMS470, pMS471, pMS472 and pMS473. The plasmids were introduced into *E. coli* HB101 (RP4) yielding strains AHEC125, AHEC144, AHEC145, AHEC146 and AHEC147 and transferred to *C. difficile* 630 Δ erm, producing AHCD608, AHCD668, AHCD669, AHCD670 and AHCD671 (Table 2.1). Additionally, pMS470 was transferred to the following *C. difficile* mutants, congenic with 630 Δ erm: *rstA* (AHCD951), *sigD* (AHCD985), *fliC* (AHCD986) and *fliF* (AHCD987), yielding AHCD960, AHCD995, AHCD994 and AHCD996, respectively.

A *tcdA* transcriptional *SNAP^{Cd}* fusion was also constructed in the background of strain R20291 using overlap extension PCR. First, primer pair Ptcda-SNAP-EcoRI-Fw/ tcdASNAP-SOE-Rev amplified the *tcdA* promoter region producing a 546 bp fragment. Secondly, primer pair SNAP-SOE-Fw/ SNAP_C-XhoI-Rev was used to amplify *SNAP^{Cd}* from pFT47 producing a 522 bp fragment. Finally, primer pair Ptcda-

SNAP-EcoRI-Fw/ SNAP_C-XhoI-Rev produced a fragment with 1068 bp. The fragment was cloned between the EcoRI/ XhoI sites in pMTL84121, yielding pSR17. The plasmid was introduced into *E. coli* HB101 (RP4) yielding strain AHEC508 and transferred to *C. difficile* R20291, producing AHCD1001 (Table 2.1).

In order to construct the *tcdA* transcriptional SNAP^{Cd} fusion with the CcpA binding box from 630 Δ *erm* the primer pair P_{tcdA}-SNAP-EcoRI-Fw/ P_{tcdA}-New-XhoI-Rev was used to obtain a 988 bp product, that was cloned between the EcoRI/XhoI sites in pFT47 yielding plasmid pCC79. The plasmid was introduced into *E. coli* HB101 (RP4) yielding AHEC725 and transferred to *C. difficile* 630 Δ *erm*, producing AHCD1196 (Table 2.1).

In order to construct a *fliC* transcriptional SNAP^{Cd} fusion, overlap extension PCR was performed using the primer pair P_{fliC}-EcoRI-Fw/ P_{fliC}-SNAP-SOE-Rev to amplify the promoter region of this gene producing a 361 bp fragment using genomic DNA from *C. difficile* 630 Δ *erm*. The primer pair SNAP-SOE-Fw/ SNAP_C-XhoI-Rev was used to amplify SNAP^{Cd} from pFT47 producing a 522 bp fragment. The final PCR was performed with primer pair P_{fliC}-EcoRI-Fw/ SNAP_C-XhoI-Rev producing a fragment with 883 bp. The fragment was cloned between the EcoRI/ XhoI sites in pMTL84121, yielding pCC34. The plasmid was introduced into *E. coli* HB101 (RP4) yielding AHEC352 and transferred to *C. difficile* 630 Δ *erm*, producing AHCD882. Additionally pCC34 was transferred to *C. difficile* 630 Δ *erm* mutant strains: *sigD* (AHCD985), *fliC* (AHCD986) and *fliF* (AHCD987), yielding strains AHCD984, AHCD1275 and AHCD1276, respectively.

To introduce point mutations in the CodY binding box III upstream of *tcdR*, overlap extension PCR was performed using plasmid

pMS464, initially using the primer pairs PtdR-SNAP-EcoRI-Fw/ PtdR codymut1 Rev and PtdR codymut1 Fw/ SNAP-HindIII-Rev that originated fragments with 768 and 614 bp, and finally with PtdR-SNAP-EcoRI-Fw/ SNAP-HindIII-Rev. The final PCR product was cloned between EcoRI/HindIII sites in pMTL84121 yielding pCC77. The plasmid was transformed into *E. coli* HB101 (RP4) yielding strain AHEC723 and then transferred to *C. difficile* 630 Δ erm by conjugation, producing AHCD1201.

Dual fusions. For the construction of plasmids allowing for inducible DccA, DccA^{mut} or σ^D production while monitoring *tcdA*, plasmid pMS470 (previously constructed) was used. Plasmids pDccA, pDccA^{mut} and pSigD (McKee *et al.*, 2013, Purcell *et al.*, 2012) were digested with PstI, then treated with the Klenow fragment and with EcoRI and inserted between the NdeI/ EcoRI sites of pMS464, yielding pCC62, pCC65 and pCC63, respectively. The plasmids were introduced into *E. coli* HB101 (RP4) yielding strains AHEC475, AHEC478 and AHEC476 and transferred to *C. difficile* 630 Δ erm, producing AHCD958, AHCD966 and AHCD959. In addition, pCC62 was transferred to *C. difficile* 630 Δ erm *rstA* mutant strain (AHCD951), producing strain AHCD972.

FliC^{T195C} construction for flagella visualization. In order to insert a single copy of *fliC* into the genome, coding for a form of flagellin with tyrosine 195 substituted for a cysteine, allelic exchange in the *pyrE* allele was used. The *fliC* allele was obtained by overlap extension PCR using the primer pairs PfliC-BamHI-Fw/ FliC-T195C-Rev and FliC-T195C-Fw/ FliC-XhoI-Rev, which generated fragments with 959 bp

and 386 bp respectively. The final PCR was performed with the primer pair PfliC-BamHI-Fw/ FliC-XhoI-Rev producing a fragment with 1345 bp. The fragment was cloned between the BamHI/ XhoI sites in pMTL-YN1C, yielding pCC70. The plasmid obtained was transformed into *E. coli* HB101 (RP4) yielding strain AHEC523 and subsequently mated with *C. difficile* 630 Δ erm Δ pyrE. The transconjugants obtained were streaked onto CDMM and tested by colony PCR using the primer pair pyrE-vef-Fw/ pyrE-vef-Rev to test for integration in *pyrE*, yielding strain AHCD1015. The strain with a mutated version of *fliC* in *pyrE* was mated with *E. coli* HB101 (RP4) for the introduction of plasmid pMS470, which resulted in strain AHCD1019.

Table 2.1. - Bacterial strains used in this study.

Strain	Relevant properties	Origin/ reference
<i>E. coli</i>		
DH5 α	General cloning host	Invitrogen
HB101 (RP4)	Host for conjugation into <i>C. difficile</i>	Laboratory stock
AHEC076	HB101 (RP4)/ pMTL84121	(Heap <i>et al.</i> , 2009)
AHEC125	HB101 (RP4)/ pMS464	This study
AHEC144	HB101 (RP4)/ pMS470	"
AHEC145	HB101 (RP4)/ pMS471	"
AHEC146	HB101 (RP4)/ pMS472	"
AHEC147	HB101 (RP4)/ pMS473	"
AHEC204	HB101 (RP4)/ pMTL-YN1C	(Ng <i>et al.</i> , 2013)
AHEC352	HB101 (RP4)/ pCC34	This study
AHEC475	HB101 (RP4)/ pCC62	"
AHEC476	HB101 (RP4)/ pCC63	"
AHEC478	HB101 (RP4)/ pCC65	"
AHEC523	HB101 (RP4)/ pCC70	"
AHEC723	HB101 (RP4)/ pCC77	"
AHEC725	HB101 (RP4)/ pCC79	"

C. difficile

AHCD531	<i>C. difficile</i> 630 Δ <i>erm</i>	(Hussain <i>et al.</i> , 2005)
AHCD608	630 Δ <i>erm</i> containing pMS464	This study
AHCD646	630 Δ <i>erm</i> containing pFT47	(Pereira <i>et al.</i> , 2013)
AHCD668	630 Δ <i>erm</i> containing pMS470	This study
AHCD669	630 Δ <i>erm</i> containing pMS471	"
AHCD670	630 Δ <i>erm</i> containing pMS472	"
AHCD671	630 Δ <i>erm</i> containing pMS473	"
AHCD772	630 Δ <i>erm</i> Δ <i>pyrE</i>	(Ng <i>et al.</i> , 2013)
AHCD882	630 Δ <i>erm</i> containing pCC34	This study
AHCD951	630 Δ <i>erm</i> <i>rstA</i> ::intron <i>ermB</i>	(Edwards <i>et al.</i> , 2016)
AHCD958	630 Δ <i>erm</i> containing pCC62	This study
AHCD959	630 Δ <i>erm</i> containing pCC63	"
AHCD966	630 Δ <i>erm</i> containing pCC65	"
AHCD984	AHCD985 containing pCC34	"
AHCD985	630 Δ <i>erm</i> <i>sigD</i> ::intron <i>ermB</i>	(Aubry <i>et al.</i> , 2012)
AHCD986	630 Δ <i>erm</i> <i>fliC</i> ::intron <i>ermB</i>	"
AHCD987	630 Δ <i>erm</i> <i>fliF</i> ::intron <i>ermB</i>	"
AHCD960	AHCD951 containing pMS470	"
AHCD995	AHCD985 containing pMS470	"
AHCD994	AHCD986 containing pMS470	"
AHCD996	AHCD987 containing pMS470	"
AHCD972	AHCD951 containing pCC62	"
AHCD1001	R20291 containing pSR17	"
AHCD1015	630 Δ <i>erm</i> <i>pyrE</i> :: <i>fliC</i> ^{T195C}	"
AHCD1019	AHCD1015 containing pMS470	"
AHCD1180	R20291 Δ <i>pyrE</i> <i>pyrE</i> :: <i>pyrE</i>	"
AHCD1196	630 Δ <i>erm</i> containing pCC79	"
AHCD1201	630 Δ <i>erm</i> containing pCC77	"
AHCD1243	630 Δ <i>erm</i> <i>codY</i> ::intron <i>ermB</i>	(Nawrocki <i>et al.</i> , 2016)
AHCD1256	AHCD1243 containing pMS464	"
AHCD1275	AHCD986 containing pCC34	
AHCD1276	AHCD987 containing pCC34	

Table 2.2. Oligonucleotides used in this work.

Primer	Sequence (5' to 3') ^a
PtcdR-SNAP-EcoRI-Fw	CCCTTAAGCGAATTCTAAAAGTAAACG
PtcdR-SNAP-XhoI-Rev	CATAAAATCCTCGAGTCTTATATTTATAATG
PtcdA-SNAP-EcoRI-Fw	CACAAAGATGAATTCTGGTCAGTTGGT
PtcdA-SNAP-XhoI-Rev	GTATTATTACTCGAGATAATAAATCCAC
PtcdB-SNAP-EcoRI-Fw	TATCAAAGTGAATTCTTTTTGAGGAAG
PtcdB-SNAP-XhoI-Rev	CTATAATACTCGAGCATCTAAATGCTAAAAAC
PtcdC-SNAP-EcoRI-Fw	ACTTCACCTGAATTCTGGTATATTC
PtcdC-SNAP-XhoI-Rev	CATAATACAATCCTCGAGTTATTAGAT
PtcdE-SNAP-EcoRI-Fw	CAATTGGAATTCGATGGATATATGATATG
PtcdE-SNAP-XhoI-Rev	CATTCATCATAGCTCGAGTTTTTTATTG
PfliC-EcoRI-Fw	CGCGCGAATTCGGAGTATAAAAAATATAAGTTTTTC
PfliC-SNAP-SOE-Rev	CATTTTACAATCTTTATCCATTATTTTACCCTCCTTAGTATAG
SNAP-SOE-Fw	ATGGATAAAGATTGTGAAATGAAGAGAACC
SNAP-XhoI-Rev	TTACCCAAGTCCTGGTTTCCCCAAACG
PfliC-BamHI-Fw	CGCGCGGATCCTGGAGTATAAAAAATATAAGTTTTTC
FliC-T195C-Rev	GCATCTGTTCCACAAGTTCCACCTGC
FliC-T195C-Fw	GCAGGTGGAACCTTGTTGGAACAGATGC
FliC-XhoI-Rev	CGCGCTCGAGCCTTTCAAAACTATATTTTC
pyrE-vef-Fw	CAATAATTTTATAACATTAACATGG
pyrE-vef-Rev	GTGTTACTTAAAAAATGTAAAT
PtcdA-XhoI-New-Rev	CGCCTCGAGTTACTGTAATGCTTCAGTGG
tcdASNAP-SOE-Rev	ATTTTACAATCTTTATCCATAAAAAACCTCCTAGTAC
PtcdR codymut1 Rev	GATTTATTTGAAAATTTTcACAACATTGG
PtcdR codymut1 Fw	CCAATGTTGTgAAAATTTTCAAATAAATC
SNAP-HindIII-Rev	CCCAAGCTTTTACCCAAGTCCTGGTTTCCCCAAACG

^a engineered restriction sites are underlined.

Table 2.3. Plasmids used in this study.

Strain	Relevant properties	Origin/ reference
pMTL84121	<i>Clostridium</i> modular plasmid containing <i>catP</i> (Cm ^R /Tm ^R)	Laboratory stock
pMTL-YN1C		(Ng <i>et al.</i> , 2013)
pFT47	pMTL84121-SNAP ^{Cd} (Cm ^R /Tm ^R)	(Pereira <i>et al.</i> , 2013)
pMS464	pFT47 containing the <i>tcdR</i> promoter region	This study
pMS470	pFT47 containing the <i>tcdA</i> promoter region without the CcpA binding box	"
pMS471	pFT47 containing the <i>tcdB</i> promoter region	"
pMS472	pFT47 containing the <i>tcdC</i> promoter region	"
pMS473	pFT47 containing the <i>tcdE</i> promoter region	"
pCC34	pMTL84121 containing the <i>fliC</i> promoter fused to SNAP ^{Cd}	"
pCC62	pMS464 containing P _{opr} - <i>dccA</i>	"
pCC63	pMS464 containing P _{opr} - <i>sigD</i>	"
pCC65	pMS464 containing P _{opr} - <i>dccA</i> ^{mut}	"
pCC70	pMTL-YN1C containing <i>fliC</i> ^{T195C}	"
pCC77	pFT47 containing the <i>tcdR</i> promoter region with point mutations at CodY box III	"
pCC79	pFT47 with <i>tcdA</i> promoter region and the first 462 nucleotides of the gene	"
pSR17	pMTL84121 containing the <i>tcdA</i> promoter region without the CcpA binding box fused to SNAP ^{Cd}	"
pMC-Pcpr	<i>Clostridium</i> modular plasmid containing <i>catP</i> (Cm ^R /Tm ^R)	(Purcell <i>et al.</i> , 2012)
PSigD		(McKee <i>et al.</i> , 2013)
PDccA		(Purcell <i>et al.</i> , 2012)
PDccA ^{mut}		"

Fluorescence microscopy and image analysis. For SNAP labeling, the TMR-Star substrate was added to cells in culture samples inside the anaerobic chamber to a final concentration of 250 nM (New England Biolabs) and the mixture incubated for 30 minutes in the dark. Following labeling, the cells were collected by centrifugation (4000 *xg* for 5 minutes), washed four times with 1 mL of PBS, and finally suspended in 10-20 μ L of PBS. For labeling of the flagella, cells were collected by centrifugation (4000 *xg* for 2 minutes), washed once

with 1 mL of PBS and Alexa Fluor 488 C₅ maleimide (Molecular Probes) was added to a final concentration of 5 µg/mL. The mixture was incubated for 5 min in the dark and the cells were washed twice and finally suspended in 10-20 µL of PBS containing or not the lipophilic styryl membrane dye N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenyl)-hexatrienylpyridinium dibromide (FM4-64; 10 mg/mL). For phase contrast and fluorescence microscopy, cells were mounted on 1.7% agarose coated glass slides and observed on a Leica DM6000B microscope equipped with a phase contrast Uplan F1 100x objective and captured with a CCD Andor Ixon camera (Andor Technologies). Images were acquired and analyzed using the Metamorph software suite version 5.8 (Universal Imaging). Exposure times were adjusted and defined for each SNAP transcriptional fusion analyzed. For quantification of the SNAP signal resulting from transcriptional fusions, 6x6 pixel regions were defined in the desired cell and the average pixel intensity was calculated, and corrected by subtracting the average pixel intensity of the background. The low and high fluorescence signal was defined according to the maximum of intensity per experiment: the maximum was calculated and the cells with intensity below 10% of the maximum were considered with low fluorescence signal and above this value were considered with high fluorescence signal.

Statistical analysis. Distributions obtained from quantifications of the SNAP labeling were statistically analyzed using the non-parametric Kolmogorov-Smirnov test (KS-test). The results with quantification of a fluorescence signal are representative of three

independent experiments. Only the results statistically significant by a KS-test in all three experiments were considered relevant.

Motility assays. BHI motility agar tubes (0.175% agar) were inoculated and grown anaerobically for 24 hours at 37°C, as previously described (Twine *et al.*, 2009).

2.4. RESULTS

2.4.1. Bistable expression of the PaLoc in a population of vegetative cells

To examine the expression of the PaLoc genes at the single cell level, we used the SNAP-tag as a fluorescence reporter under anaerobic conditions (Cassona *et al.*, 2016, Gautier *et al.*, 2008, Pereira *et al.*, 2013, Saujet *et al.*, 2013). We studied the expression of the determinants of toxinogenesis in *C. difficile* encoded in the PaLoc in a population of actively growing cells.

We constructed transcriptional *SNAP^{Cd}* fusions to *tcdA*, *tcdB*, *tcdC*, *tcdE* and *tcdR* promoters (Figure 2.2) and used phase contrast and fluorescence microscopy to analyse how the expression of the PaLoc genes is distributed in the cell population (Figure 2.2). It is to be noted that P_{tcdA} -*SNAP^{Cd}* transcriptional fusion, the CcpA box was not included as no glucose is present in the medium used and no effects are expected from CcpA in the absence of glucose ($P_{tcdA\Delta ccpA}$ -*SNAP^{Cd}*; see also below). In these experiments, a sample from cultures of the wild type strain 630 Δerm bearing a plasmid with each of the transcriptional fusions was collected 12 hours after inoculation into TY, a medium that supports toxin production (Dupuy & Sonenshein, 1998). The cells

were examined by microscopy after SNAP labelling with the red substrate TMR-Star. As shown in Figure 2.2, *tcdA* expression was detected in a sub-population of about 66% of the vegetative cells. Furthermore, the cells can be divided into high (21%) and low (79%) producers (79%) (Figure 2.2, right) (see Materials and methods for the definition of low and high expression levels).

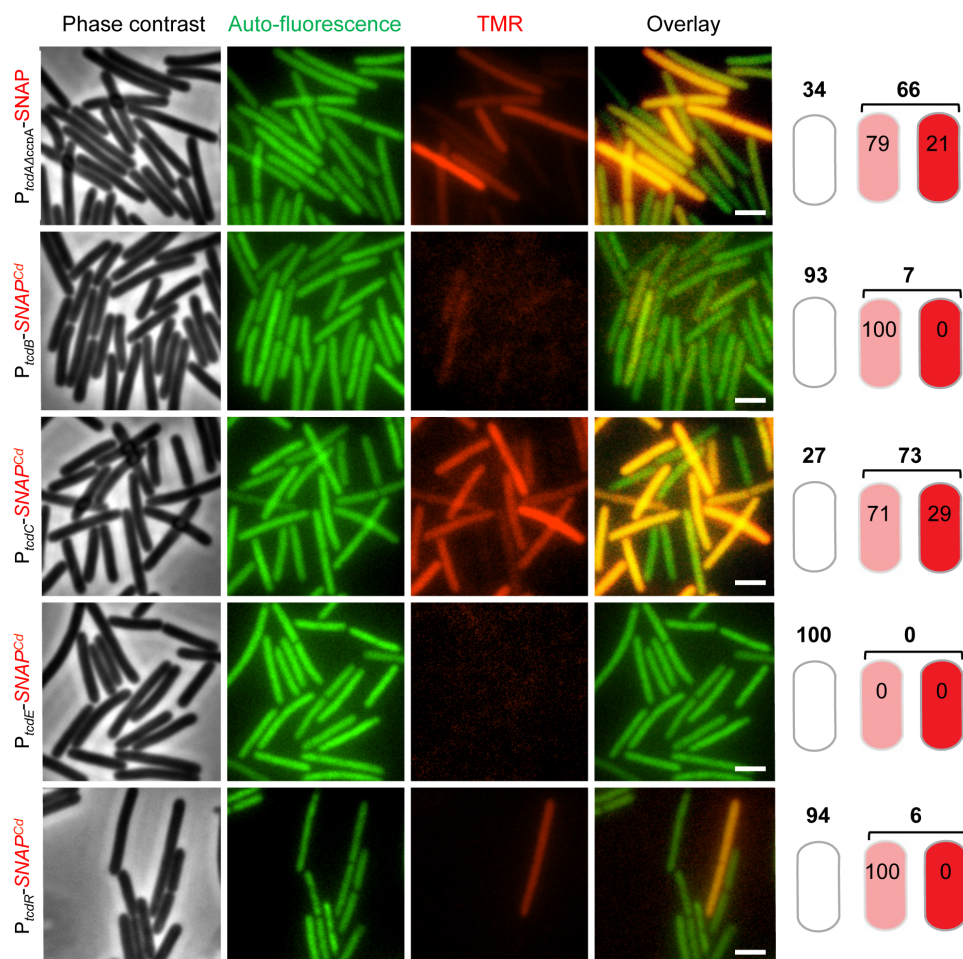


Figure 2.2. Single cell analysis of the expression of the PaLoc genes in strain 630 Δ erm. Cell type-specific expression patterns of *tcdA*, *tcdB*, *tcdC*, *tcdE* and *tcdR*. *C. difficile* 630 Δ erm cells carrying fusions of *tcdA*, *tcdB*, *tcdC*, *tcdE* and *tcdR* promoters to SNAP^{Cd} were analyzed by microscopy. The cells were

collected after 12h of growth in TY liquid medium, stained with TMR-Star and examined by phase contrast and fluorescence microscopy to monitor SNAP production. The merged images show the overlap between the TMR-Star (red) and the auto-fluorescence (green) channels. The cells are representative of the expression patterns observed for the different fusions. Scale Bar: 1 μ m. The percentage of vegetative cells with a low (light red), high (dark red) or no signal from SNAP-TMR is indicated on the right. The percentage of vegetative cells in the population is the sum of the two main classes (with and without a SNAP-TMR signal). The number of cells analyzed for each fusion, n , is as follows: $P_{tcdA\Delta ccpA}-SNAP^{Cd}$, $n = 453$; $P_{tcdB}-SNAP^{Cd}$, $n = 347$; $P_{tcdC}-SNAP^{Cd}$, $n = 376$; $P_{tcdE}-SNAP^{Cd}$, $n = 200$; $P_{tcdF}-SNAP^{Cd}$, $n = 177$.

Additionally, we examined expression of the $P_{tcdA\Delta ccpA}-SNAP^{Cd}$ fusions in the epidemic strain R20291. Interestingly, in this strain, the fraction of vegetative cells expressing *tcdA* was reduced (17%) relative to strain 630 Δerm (Figure 2.3).

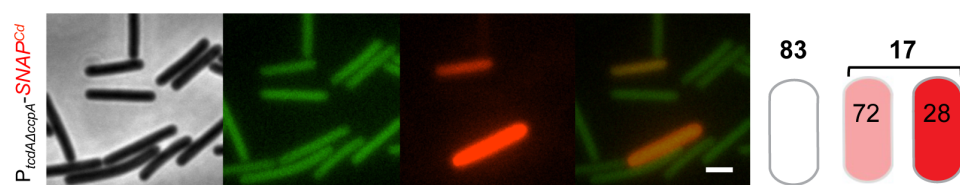


Figure 2.3. *tcdA* expression in the epidemic strain R20291. Microscopy analysis of *C. difficile* cells carrying a $P_{tcdA\Delta ccpA}-SNAP^{Cd}$ transcriptional fusion in strain R20291. The cells were collected after 12h of growth in TY and examined by phase contrast and fluorescence microscopy. The merged images show the overlap between the TMR-Star (red) and the auto-fluorescence (green) channels. The images are representative of the expression patterns observed. Scale Bar: 1 μ m. The percentage of vegetative cells in the population and the percentage of those cells with a low (light red) and a high fluorescence signal (dark red) is indicated. The number of cells analyzed, n , is as follows: $P_{tcdA\Delta ccpA}-SNAP^{Cd}$, $n = 567$.

Expression of the *tcdB* transcriptional fusion was only detected in 7% of the cells and the fluorescence levels were much lower than for *tcdA* (Figure 2.2). *tcdC* expression was detected in 73% of the vegetative cells and from these, 71% showed high levels of expression.

This result argues against a role for TcdC as a toxin repressor, and supporting this inference, no differences were observed in the expression of *tcdA* in cells of a $\Delta tcdC$ mutant (Figure 2.4).

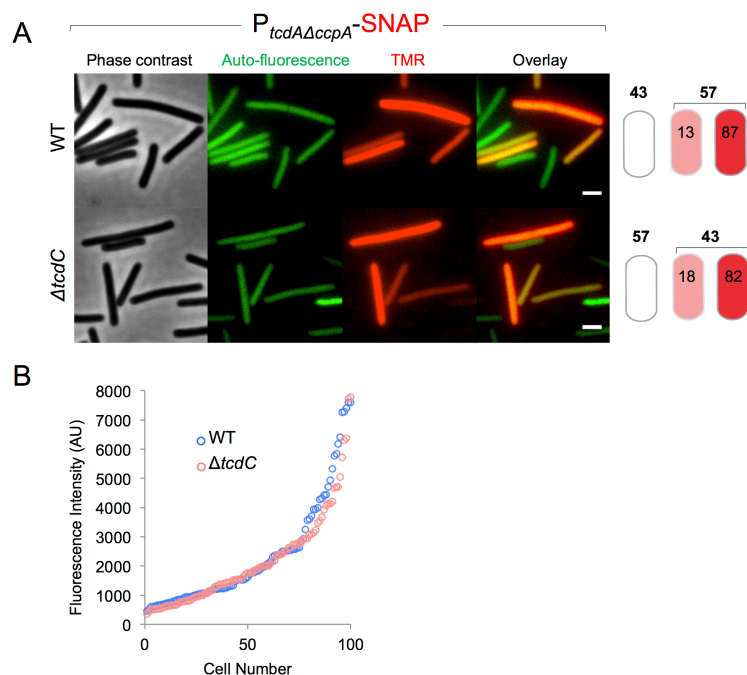


Figure 2.4. TcdC effect on toxin expression. A: Microscopy analysis of *C. difficile* cells carrying a P_{tcdA} - $SNAP^{Cd}$ transcriptional fusion (without the CcpA binding box; $P_{tcdA\Delta ccpA}$ - $SNAP^{Cd}$) in strain 630 Δerm (WT), and in the $\Delta tcdC$ mutant. The cells were collected after 12h of growth in TY liquid medium and imaged by phase contrast and fluorescence microscopy. The merged images show the overlap between the TMR-Star (red) and the auto-fluorescence (green) channels. The cells are representative of the expression patterns observed. Scale Bar: 1 μm . The percentage of vegetative cells in the population and the distribution of the SNAP signal in these cells are shown: low fluorescence signal (light red); high fluorescence signal (dark red). The number of cells analyzed for each strain, n , is as follows: WT, $n = 570$; $\Delta tcdC$, $n = 485$ **B:** Intensity of the fluorescence (FI.) signal from SNAP-TMR per cell for the *tcdA* fusion, in the wild type background (WT) and in the $\Delta tcdC$ mutant strain. The scale is in arbitrary units (AU).

We did not detect expression of the *tcdE* transcriptional fusion under our conditions (Figure 2.2). Expression of *tcdE* may be too low to be detected using a transcriptional $SNAP^{Cd}$ fusion or *tcdE* is not

expressed at significant levels in vegetative cells. *tcdR* was only expressed in 6% of the cells and at low levels (Figure 2.2.). This contrasts with the 66% of cells expressing *tcdA*, but agrees with the observation that *tcdR* is expressed at low levels (Dupuy & Sonenshein, 1998, Ransom *et al.*, 2018). In all, these results indicate that *tcdR*, *tcdA* and *tcdB* show a bistable expression pattern in vegetative cells. A bistable distribution can arise if a transcription factor is under positive auto-regulation, as stochastically, some cells will be below and others above a threshold for activation (reviewed by Alon, 2007). If the bistable expression of *tcdA* and *tcdB* comes from the auto-regulatory nature of *tcdR*, a prediction is that breaking the auto-regulatory loop, for example by replacing its promoter, would result in unimodal expression. In fact, Ransom and co-authors have placed the *tcdR* gene under the control of an inducible promoter and showed that expression of *tcdA* was now uniform and a function of the inducer concentration (Ransom *et al.*, 2018). This result supports the hypothesis that the bistable expression of *tcdA* has its origin in the *tcdR* auto-regulatory loop. In our hands, however, a similar experiment proved inconclusive because induction of *tcdR* from the nisin-responsive promoter resulted in cell lysis, which was dependent on the presence of *tcdE* (not shown). Evidence for TcdE-dependent cell lysis has been reported before (Govind & Dupuy, 2012, Govind *et al.*, 2015).

2.4.2. Regulators that respond to nutritional signals are not required for the bistable expression of *tcdR* or *tcdA*

Having characterized the expression patterns of the PaLoc genes we next wanted to determine how known regulators of the expression of the toxin-encoding genes affected expression of *tcdA* at

the single cell level. CcpA was shown to interact with *tcdB* promoter region and with a region that includes part of the coding sequence of *tcdA* (Antunes *et al.*, 2011) (Figure 2.1C). When initially studying the expression pattern of the PaLoc genes the CcpA binding box at *tcdA* was not included ($P_{tcdA\Delta ccpA}$ - $SNAP^{Cd}$). In order to access the effect of the CcpA binding box on *tcdA* expression, this region was included in a new fusion, P_{tcdA} - $SNAP^{Cd}$ (Figure 2.5). In the absence of glucose, the inclusion of the CcpA box does not have an effect on *tcdA* expression. However, when glucose was added, the presence of the CcpA box led to a repressive effect of glucose, with a significant decrease ($p < 0,0001$) in the expression levels per cell (Figure 2.5B) but without affecting the total percentage of cells expressing *tcdA* (Figure 2.5B). These results indicate that CcpA has a repressive effect only in the presence of glucose as previously described (Antunes *et al.*, 2011) but additionally show that CcpA has no significant effect on the size of the *tcdA*-expressing sub-population. Thus CcpA regulation is not a pre-requisite for bistable expression of *tcdA*. Hereafter, the transcriptional fusion $P_{tcdA\Delta ccpA}$ - $SNAP^{Cd}$ was used, as the medium used for optimal toxin production, TY, does not have glucose (see above).

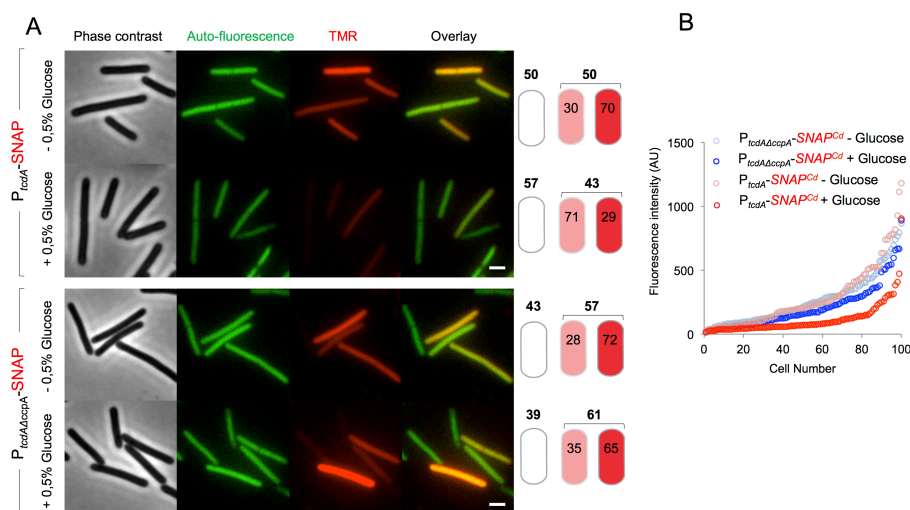


Figure 2.5. The CcpA binding box effect on *tcdA* expression. **A:** Microscopy analysis of *C. difficile* cells carrying a P_{tcdA} -SNAP^{Cd} transcriptional fusion in strain 630 Δ erm with (P_{tcdA} -SNAP^{Cd}, top) or without the CcpA binding box ($P_{tcdA\Delta ccpA}$ -SNAP^{Cd}). The cells were collected after 12h of growth in TY without or with 0.5% glucose, stained with TMR-Star and examined by phase contrast and fluorescence microscopy to monitor SNAP production. The merged images show the overlap between the TMR-Star (red) and the auto-fluorescence (green) channels. The cells are representative of the expression patterns observed. Scale Bar: 1 μ m. The percentage of vegetative cells and the percentage of those cells with a low (light red), high (dark red) or no SNAP-TMR signal is shown. The number of cells analyzed for each fusion, *n*, is as follows: P_{tcdA} -SNAP^{Cd} without glucose, *n* = 230; P_{tcdA} -SNAP^{Cd} with glucose, *n* = 285; $P_{tcdA\Delta ccpA}$ -SNAP^{Cd} without glucose, *n* = 237; $P_{tcdA\Delta ccpA}$ -SNAP^{Cd} with glucose, *n* = 175. **B:** Intensity of the fluorescence signal from SNAP-TMR per cell for P_{tcdA} -SNAP^{Cd} and $P_{tcdA\Delta ccpA}$ -SNAP^{Cd} with and without glucose.

While CcpA binds directly to the *tcdR* and *tcdB* promoters and to the 5' end of the *tcdA* coding sequence (Antunes *et al.*, 2011; Antunes *et al.*, 2012), CodY acts mainly by repressing *tcdR* through a strong interaction with its promoter region as previously demonstrated *in vitro*. A DNaseI footprinting assay identified three regions in the *tcdR* promoter region where CodY was able to bind (Dineen *et al.*, 2007). Region I is located between the -348 and -382 positions relative to the *tcdR* start codon, which includes the most upstream promoter

recognized by TcdR; region II is located between the -281 and -309 positions, *i.e.*, between the two promoters recognized by TcdR; region III is located between the -40 and -58 positions, downstream of the promoter recognized by σ^D (Figure 2.6A).

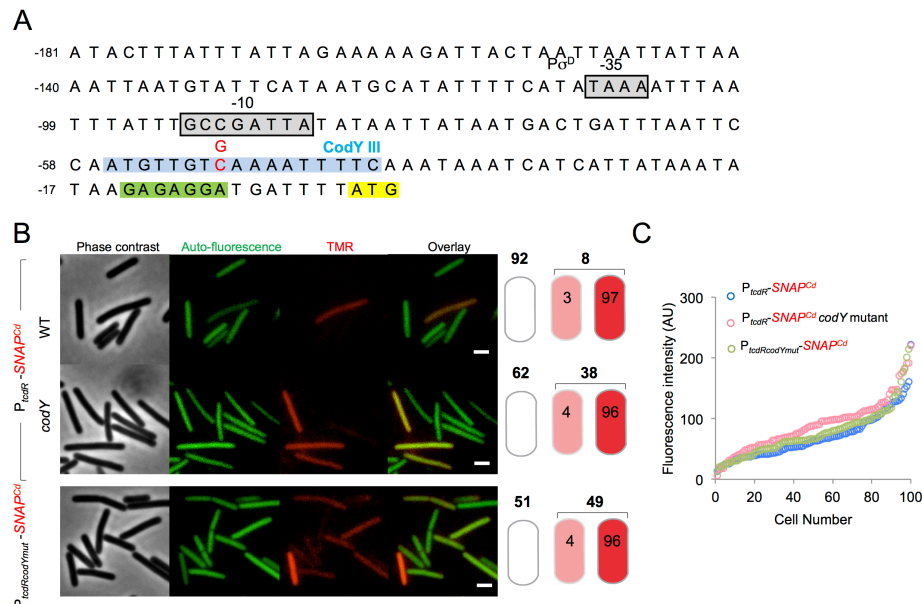


Figure 2.6. Single cell analysis of CodY role as a repressor of *tcdR* expression. **A:** Representation of the regulatory region of the *tcdR* gene, with the -35 and -10 elements of the mapped *tcdR*- σ^D -dependent promoter (grey) and the CodY binding box III (light blue). The point mutation introduced in the CodY binding box is indicated in red. The *tcdR* start codon is indicated in yellow and the predicted ribosome-binding site is indicated in green for reference. **B:** Microscopy analysis of strains carrying a P_{tcdR}^{-} $SNAP^{Cd}$ transcriptional fusion with the WT *tcdR* promoter in strain 630 Δerm (WT) and in the *codY* mutant, and of the WT strain with a $SNAP^{Cd}$ fusion to the *tcdR* promoter with point mutations at the CodY binding box III. The cells were collected after 12h of growth in TY liquid medium. The merged images show the overlap between the TMR-Star (red) and the auto-fluorescence (green) channels. The images are representative of the expression patterns observed for the different fusions. Scale bar: 1 μm . The percentage of vegetative cells and the percentage of those cells with a low (light red), high (dark red) or no SNAP-TMR signal is shown. The number of cells analyzed for each strain, *n*, is as follows: WT with P_{tcdR}^{-} $SNAP^{Cd}$, *n* = 1641; *codY* with P_{tcdR}^{-} $SNAP^{Cd}$, *n* = 278; WT with $P_{tcdRcodYmut}^{-}$ $SNAP^{Cd}$, *n* = 568. **C:** Quantitative analysis of the fluorescence intensity (in Arbitrary Units, AU) of the $SNAP^{Cd}$ signal per cell in the forespore for the *tcdR* fusions (WT and *codYmut*).

Because of the requirement for σ^D in triggering expression of *tcdR* (El Meouche *et al.*, 2013), we hypothesized that region III could have a higher influence on *tcdR* expression than regions I and II. In *B. subtilis* a 15-bp consensus sequence for CodY binding has been proposed: AATTTTCWGAAAATT with the cytosine identified as one of the most important residues for CodY binding to this region (Belitsky & Sonenshein, 2008). We constructed a transcriptional fusion between the *tcdR* promoter and the *SNAP^{Cd}* reporter where we substituted the cytosine residue at position -49 relative to *tcdR* start codon by a guanine (Figure 2.6A). We examined expression of the new fusion (*P_{tcdRcodYmut}-SNAP^{Cd}*) during growth, along with the *P_{tcdR}-SNAP^{Cd}* fusion, in a wild-type background and in a *codY* mutant (Nawrocki *et al.*, 2016). We observed an increase in the percentage of cells expressing *tcdR* both using the *P_{tcdRcodYmut}-SNAP^{Cd}* fusion (49% comparing with 8% for the wild-type fusion), as well as using the *P_{tcdR}-SNAP^{Cd}* in the *codY* background (38%) (Figure 2.6B). However, the intensity of the fluorescence signal per cell was only significantly increased in the *codY* mutant ($p < 0,05$) (Figure 2.6C). These observations indicate that box III is important for the control of *tcdR* expression *in vivo*. Furthermore, the mutations significantly affected the level of *tcdA* expression per cell ($p < 0,0001$) (Figure 2.7B), but did not affect the proportion of cells showing expression of the reporter fusion (Figure 2.7A). Our results further show that while repressing the expression of *tcdR* and *tcdA* per cell, CodY contributes but is not essential to maintain the bistable expression of *tcdR* or *tcdA*.

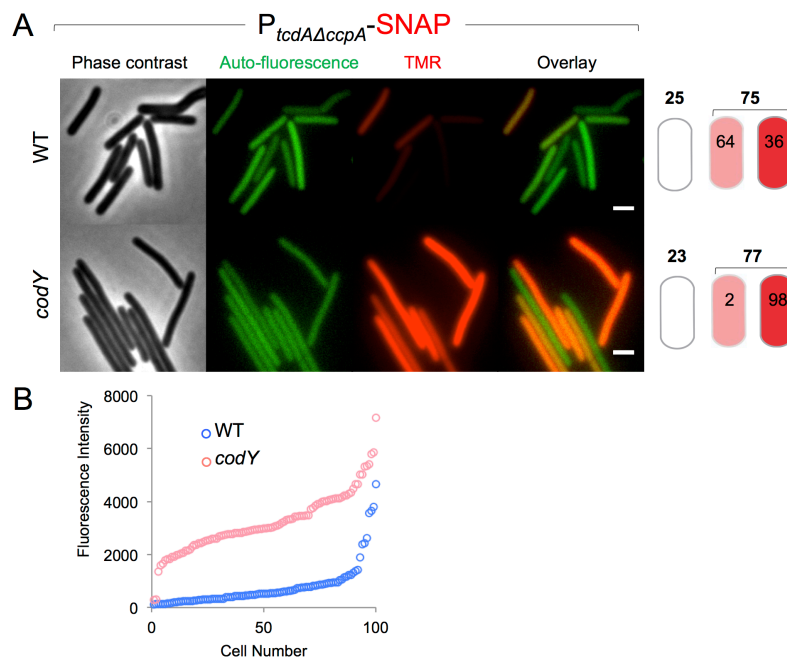


Figure 2.7. The role of CodY in *tcdA* expression. **A:** Microscopy analysis of *C. difficile* 630Δerm (WT) or the *codY* mutant bearing a P_{tcdA} -SNAP^{Cd} transcriptional fusion (without the CcpA binding box; $P_{tcdA\Delta ccpA}$ -SNAP^{Cd}). The merged images show the overlap between the TMR-Star (red) and the auto-fluorescence (green) channels. The cells are representative of the expression patterns observed. Scale Bar: 1 μm. The percentage of vegetative cells showing a low (light red), high (dark red) or no SNAP-TMR signal is shown. The number of cells analyzed for each fusion, n, is as follows: WT, n = 156; *codY*, n = 149. **B:** Quantitative analysis of the fluorescence (FI.) intensity of the SNAP signal per cell for the *tcdA* fusion, in the wild type background (WT) or in the *codY* mutant strain.

2.4.3. The activity of σ^D may directly influence the *tcdR* auto-regulatory loop

A new regulator protein affecting both toxin production and sporulation, RstA (Regulator of Sporulation and Toxins), has been recently identified. RstA upregulates sporulation through an unidentified pathway and represses motility and toxin production

through σ^D (Edwards *et al.*, 2016). We analyzed *tcdA* expression in the *rstA* mutant at the single cell level using the SNAP-tag as a reporter. In this mutant the number of cells expressing *tcdA* increases from 51% to 83% (Figure 2.8A). Furthermore, a significant increase ($p < 0.0001$) in the average expression of *tcdA* per cell is seen (Figure 2.8B). Thus, directly or indirectly, RstA controls the fraction of toxin-producing cells but is not required for the bistable pattern of *tcdA* expression.

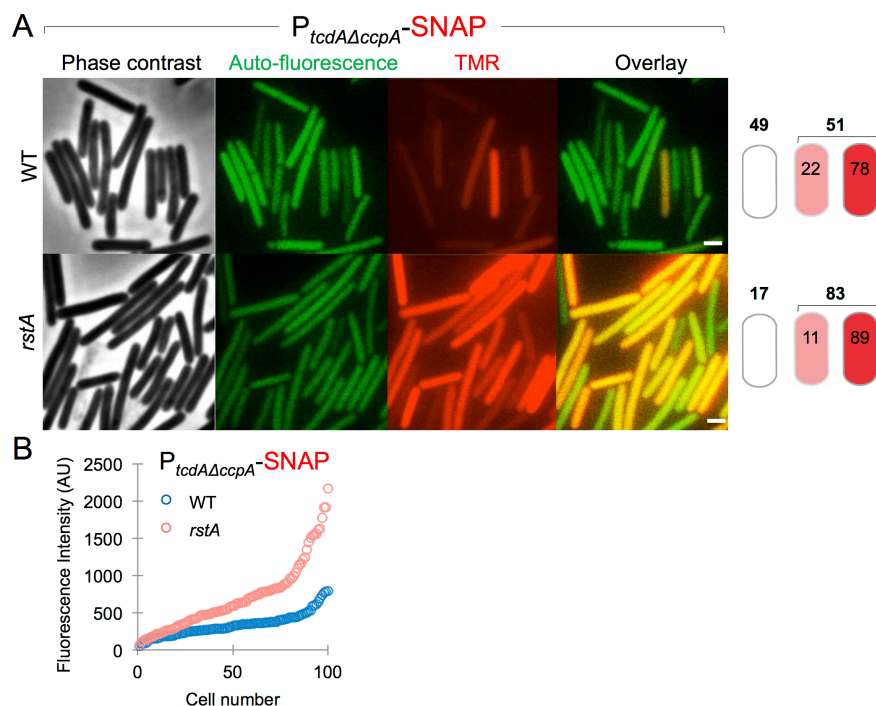


Figure 2.8. The role of RstA in *tcdA* expression. **A:** Microscopy analysis of *C. difficile* 630 Δ erm (WT) or the *rstA* mutant bearing a P_{tcdA} -SNAP^{Cd} transcriptional fusion (without the CcpA binding box; $P_{tcdA\Delta ccpA}$ -SNAP^{Cd}). The cells were imaged using phase contrast and fluorescence microscopy. The merged images show the overlap between the TMR-Star (red) and the auto-fluorescence (green) channels. The cells are representative of the expression patterns observed. Scale Bar: 1 μ m. The percentage of vegetative cells showing a low (light red), high (dark red) or no SNAP-TMR fluorescence signal is shown. The number of cells analyzed for each strain, n, is as follows: WT, n = 972; *rstA*, n = 899. **B:** Quantitative analysis of the fluorescence (Fl.) intensity of the SNAP signal per cell for the $P_{tcdA\Delta ccpA}$ -SNAP^{Cd} fusion, in the wild type background (WT) or in the *rstA* mutant strain.

Increased levels of c-di-GMP were shown to inhibit toxin production (McKee *et al.*, 2013) through the major flagellar regulator σ^D . σ^D is a positive regulator of toxin synthesis and c-di-GMP is known to repress expression of *sigD* (El Meouche *et al.*, 2013, Purcell *et al.*, 2012). However, the effect of these regulators was never observed at the single cell level. Previously, Purcell *et al.* and McKee *et al.*, showed that inducing *dccA*, which encodes a diguanylate cyclase that allows for c-di-GMP synthesis, increases c-di-GMP levels impairing motility and toxin production (McKee *et al.*, 2013, Purcell *et al.*, 2012). Therefore, we constructed a plasmid allowing for *dccA* overexpression while simultaneously monitoring *tcdA* expression. The *dccA* gene was placed under the control of the nisin-inducible *C. difficile* *cpr* promoter, P_{cpr} , (Purcell *et al.*, 2012) and the transcriptional fusion of the *tcdA* promoter to the reporter *SNAP^{Cd}* was placed in the opposite direction. Additional plasmids were constructed with a *dccA* allele encoding a catalytically inactive diguanylate cyclase that does not affect the c-di-GMP levels (*dccA^{mut}*) and with *sigD*, the latter allowing for σ^D overproduction. After 12 hours of growth in TY, increased levels of c-di-GMP resulted both in a reduction from 42% to 5% of cells expressing *tcdA* (Figure 2.9A) and in a significant reduction of the expression per cell ($p < 0,0001$) (Figure 2.9B). When the inactive form of *dccA* was induced, *dccA^{mut}*, no significant effect was observed indicating that the effect on *tcdA* expression is specific of *dccA* induction and results from increased c-di-GMP levels. When *sigD* was induced, a significant increase in the expression of *tcdA* per cell was noticed ($p < 0,0001$) but the fraction of cells expressing *tcdA* did not increase (Figure 2.9B). The effects observed result from induction of the nisin-controlled genes, as they were not observed in the absence of inducer (Data not shown).

Together, the results show that reducing the expression and/or activity of σ^D (through induction of c-di-GMP production) or deleting *sigD* itself, strongly reduce the fraction of *tcdA*-expressing cells. Under these conditions, expression of *tcdR* relies mainly if not exclusively on the auto-regulatory loop. Thus, the activity of σ^D is essential to shift the population towards a *tcdR*-ON state. Induction of *sigD* increased the proportion of cells expressing *tcdA* at high levels, but not the overall fraction of *tcdA*-ON cells. It appears that the activity of σ^D , and not the transcription of *sigD*, is important for the shift towards *tcdA*-ON cells.

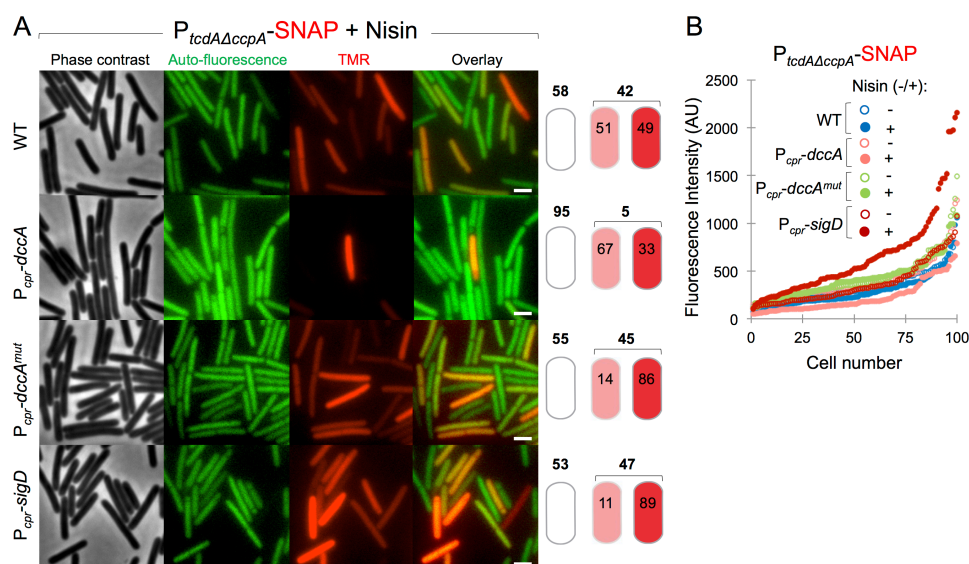


Figure 2.9. The role of c-di-GMP and σ^D in *tcdA* expression. **A:** Microscopy analysis of cells of strain 630 Δerm bearing a P_{tcdA} -SNAP^{Cd} fusion (without the CcpA binding box; $P_{tcdA\Delta ccpA}$ -SNAP^{Cd}) and a dual fusion with both $P_{tcdA\Delta ccpA}$ -SNAP^{Cd} and $P_{cpr-dccA}$, or $P_{cpr-dccA^{mut}}$ or $P_{cpr-sigD}$. These combinations allow for *dccA*, *dccA^{mut}* and *sigD* induction while monitoring *tcdA* expression. The cells were collected after 12h of growth in TY liquid medium supplemented with 1 μ g/mL nisin and examined by phase contrast and fluorescence microscopy. The merged images show the overlap between the TMR-Star (red) and the auto-fluorescence (green) channels. The patterns are representative of the expression

patterns observed. Scale Bar: 1 μm . The percentage of vegetative cells with a SNAP-TMR signal is shown: cells without signal, white; with a low fluorescence signal, light red; with a high fluorescence signal, dark red. The number of cells analyzed for each strain, n , is as follows: $P_{tcdA\Delta ccpA}\text{-SNAP}^{Cd}$, $n = 826$; $P_{tcdA\Delta ccpA}\text{-SNAP}^{Cd}/P_{cpr\text{-}dccA}$, $n = 1048$; $P_{tcdA\Delta ccpA}\text{-SNAP}^{Cd}/P_{cpr\text{-}dccA}^{mut}$, $n = 1030$; $P_{tcdA\Delta ccpA}\text{-SNAP}^{Cd}/P_{cpr\text{-}sigD}$, $n = 868$. **B**: Quantitative analysis of the fluorescence (FI.) intensity of the SNAP signal per cell in vegetative cells for the WT *tcdA* fusion, and the dual fusions $P_{tcdA\Delta ccpA}\text{-SNAP}^{Cd}/P_{cpr\text{-}dccA}$, $P_{tcdA\Delta ccpA}\text{-SNAP}^{Cd}/P_{cpr\text{-}dccA}^{mut}$ and $P_{tcdA\Delta ccpA}\text{-SNAP}^{Cd}/P_{cpr\text{-}sigD}$, with and without nisin (filled and empty circles, respectively). Analysis was performed using the Metamorph software suite version 5.8 (Universal Imaging).

We next wanted to understand which of the regulatory components, RstA or c-di-GMP, would have a more important role in the regulation of *tcdA* expression through σ^D . For this purpose we introduced the plasmids allowing for *dccA* induction and for monitoring of *tcdA* expression in the *rstA* mutant. We found that increased levels of c-di-GMP in this mutant had similar effects as in the wild-type background, *i.e.*, a reduction in the number of cells expressing *tcdA* and a significant reduction in the intensity of expression per cell ($p < 0.0001$) (Figure 2.10A, 2.10B). These results indicate that both c-di-GMP and RstA have an effect in toxin production through σ^D , but c-di-GMP might be more important in the regulatory cascade (Figure 2.10C).

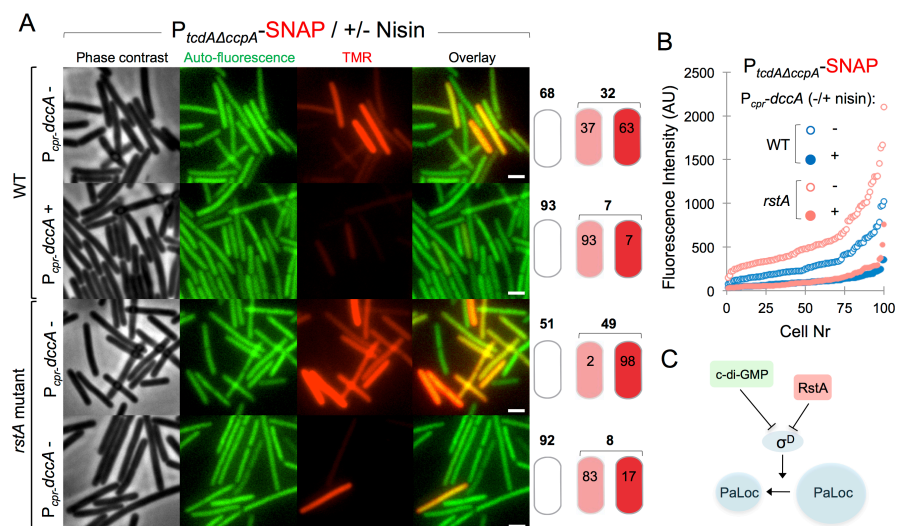


Figure 2.10. The role of c-di-GMP in *tcdA* expression in an *rstA* mutant. **A:** Microscopy analysis of *C. difficile* cells carrying a dual $P_{tcdA\Delta ccpA}$ -SNAP^{Cd} (without the CcpA binding box) and $P_{cpr-dccA}$ fusion ($P_{tcdA\Delta ccpA}$ -SNAP^{Cd}/ $P_{cpr-dccA}$) in strain 630 Δ erm (WT) and in the *rstA* mutant. The cells were collected after 12h of growth in TY liquid medium with (+) or without (-) 1 μ g/mL nisin and examined by phase contrast and fluorescence microscopy. The merged images show the overlap between the TMR-Star (red) and the auto-fluorescence (green) channels. The cells are representative of the expression patterns observed. Scale Bar: 1 μ m. The percentage of the distribution of the SNAP signal in vegetative cells is shown on the right: cells without signal (white), with a low fluorescence signal (light red) and with high fluorescence signal (dark red). The number of cells analyzed for each strain, n, is as follows: WT with $P_{tcdA\Delta ccpA}$ -SNAP^{Cd}/ $P_{cpr-dccA}$ - nisin, n = 892; WT with $P_{tcdA\Delta ccpA}$ -SNAP^{Cd}/ $P_{cpr-dccA}$ + nisin, n = 828; *rstA* with $P_{tcdA\Delta ccpA}$ -SNAP^{Cd}/ $P_{cpr-dccA}$ - nisin, n = 678; *rstA* with $P_{tcdA\Delta ccpA}$ -SNAP^{Cd}/ $P_{cpr-dccA}$ + nisin, n = 617. **B:** Quantitative analysis of the fluorescence (Fl.) intensity of the SNAP signal per cell in vegetative cells for the dual fusion $P_{tcdA\Delta ccpA}$ -SNAP^{Cd}/ $P_{cpr-dccA}$ with and without nisin (filled and empty circles, respectively) in the wild type background (WT) or in the *rstA* mutant. **C:** Schematic representation of the regulators acting on σ^D and consequently affecting the PaLoC expression. Both c-di-GMP and RstA repress σ^D but c-di-GMP acts above RstA.

Mutations in genes coding for flagellar components are known to affect toxin production. While in the *fliF*, *fliG*, *fliM* and *fliB-fliR* mutants toxin production is reduced, in the *fliC* mutant the opposite

effect is observed (Aubry *et al.*, 2012). To examine the effect of these mutations on the distribution of toxin expression throughout the population, we introduced the $P_{tcdA\Delta ccpA}$ - $SNAP^{Cd}$ transcriptional fusion in the *fliC* and in the *fliF* mutants. In the *fliC* mutant the number of cells expressing *tcdA* increased from 56% to 89%, and most of the cells in this mutant are “high-producers” (80% of the cells with expression) (Figure 2.11A). The average intensity of fluorescence per cell also significantly increased in the *fliC* mutant ($p < 0.0001$) (Figure 2.11B), indicating an increase of toxin expression both at the population and at the single cell level. In the *fliF* mutant, only 8% of the cells expressed *tcdA* and at low levels (Figure 2.11A). For reference, the percentage of cells expressing *tcdA* was reduced from 56% to 7% in the *sigD* mutant (Figure 2.11A).

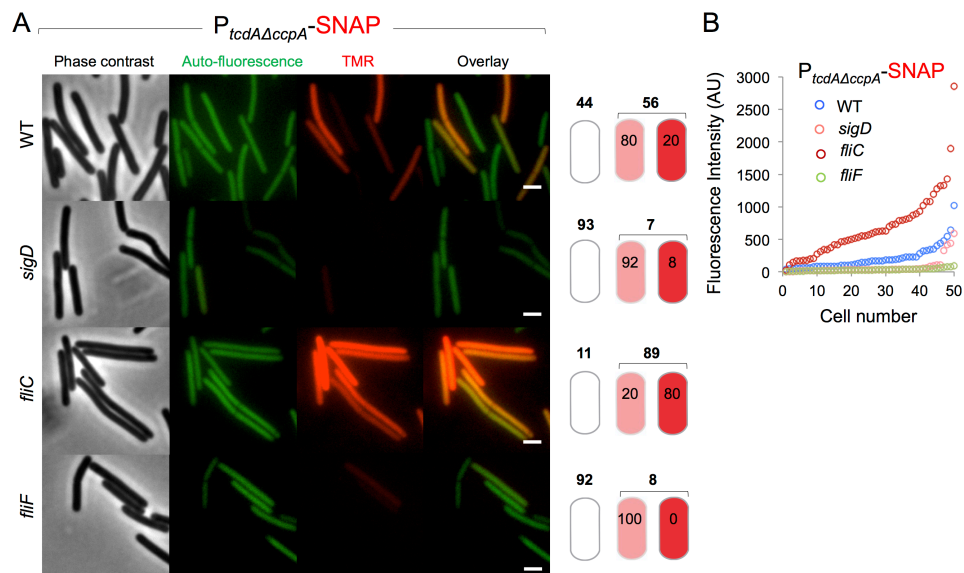


Figure 2.11. The expression of *tcdA* in flagellar mutants. **A:** Microscopy analysis of *C. difficile* cells carrying a $P_{tcdA\Delta ccpA}$ - $SNAP^{Cd}$ transcriptional fusion in strain 630 Δerm (WT) and in the congenic *sigD*, *fliC* and *fliF* mutants. The cells were collected after 12h of growth in TY liquid medium and imaged by phase contrast and fluorescence microscopy. The merged images show the overlap

between the TMR-Star (red) and the auto-fluorescence (green) channels. The cells are representative of the expression patterns observed. Scale Bar: 1 μm . The percentage of the vegetative cells showing a high (dark red), a low (light red) or no TMR-Star signal is shown on the right. The number of cells analyzed for each strain, n , is as follows: WT, $n = 284$; *sigD*, $n = 223$; *fliC*, $n = 273$; *fliF*, $n = 214$. **B**: Quantitative analysis of the fluorescence (Fl.) intensity of the SNAP signal per cell for the *tcdA* fusion, in the wild type background (WT) and in the *sigD*, *fliC* and *fliF* mutants.

An important consideration is that FliC production is regulated by σ^D and its expression is abolished in the *sigD* mutant (El Meouche *et al.*, 2013) (Figure 2.12). Still, opposite effects of the *fliC* and *fliF*/*sigD* mutations are observed. A possibility is that in the *fliC* mutant σ^D activity increases while it decreases in a *fliF* mutant. Consistent with this, expression of a P_{fliC} -SNAP^{Cd} transcriptional fusion, as a reporter for σ^D activity increased significantly in a *fliC* mutant ($p < 0.0001$), and decreased in the *fliF* mutant ($p < 0.0001$) (Figure 2.13). These results support the idea that the activity of σ^D activity is increased or reduced in these mutants, and thus that the expression of *tcdA* is affected through the σ^D -dependent transcription of *tcdR*.

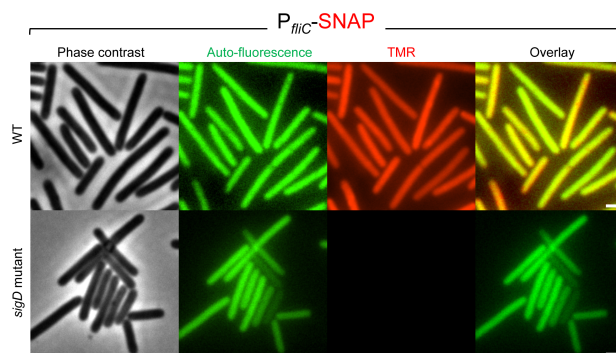


Figure 2.12. FliC regulation by σ^D . Microscopy analysis of *C. difficile* cells carrying the fusion of the *fliC* promoter to SNAP^{Cd} in the strain 630 Δ erm (WT) and in the *fliC* mutant. Microscopy was performed as indicated before. The merged images show the overlap between the TMR-Star (red) and the auto-fluorescence (green) channels. The cells are representative of the expression patterns observed. Scale Bar: 1 μm .

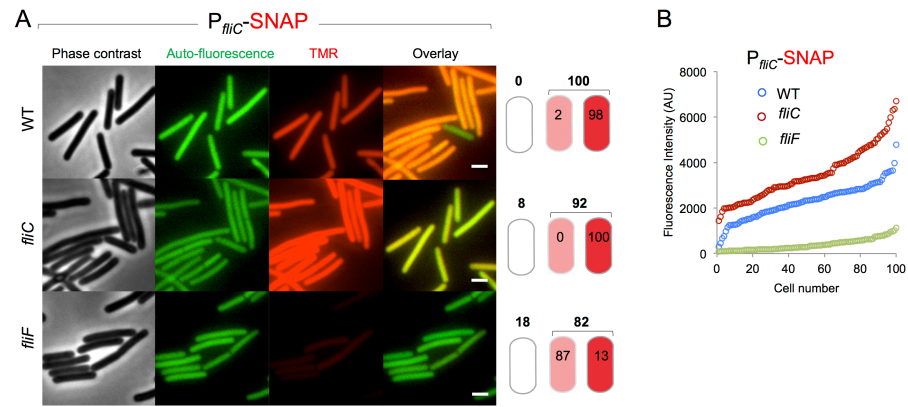


Figure 2.13. σ^D activity in flagellar mutants. **A:** Microscopy analysis of *C. difficile* cells carrying a P_{fliC} -SNAP^{Cd} transcriptional fusion in strain 630 Δ erm (WT) and in congenic *fliC* and *fliF* mutants. The cells were collected after 12h of growth in TY liquid medium and imaged by phase contrast and fluorescence microscopy. The merged images show the overlap between the TMR-Star (red) and the auto-fluorescence (green) channels. The cells are representative of the expression patterns observed. Scale Bar: 1 μ m. The percentage of vegetative cells showing a high (dark red) and a low (light red) TMR-Star signal, as well as the percentage of vegetative cells in the population is shown on the right. The number of cells analyzed for each fusion, n, is as follows: WT, n = 178; *fliC*, n = 216; *fliF*, n = 124. **B:** Quantitative analysis of the fluorescence (FI.) intensity of the SNAP signal per cell for the *fliC* fusion, in the wild type background (WT) and in the *fliC* and *fliF* mutant strains.

2.4.4. Toxin producers are a subpopulation of motile cells

As σ^D is a key regulator of flagellar assembly (Aubry *et al.*, 2012, El Meouche *et al.*, 2013, McKee *et al.*, 2013), we next wanted to examine the overlap between toxin expression and motility. With that in mind, we aimed first at distinguishing flagellated from unflagellated cells. Previously, in *Bacillus subtilis*, Blair & colleagues constructed a strain with a modified filament that allows for fluorescent staining. A tyrosine residue in the flagellin protein, Hag, was substituted for a cysteine residue. In this way, the flagella could be labelled with a membrane impermeable Alexa Fluor-conjugated Maleimide (Blair *et*

al., 2008). The tyrosine residue that was modified in the *B. subtilis* flagellin, T209, is conserved in *C. difficile* (Figure 2.14A; T195). Therefore, we constructed a strain where a modified *fliC* gene coding for flagellin in which the T195 residue was substituted by a cysteine (FliC^{T195C}) and introduced at the *pyrE* locus by allele coupled exchange (Ng *et al.*, 2013). This strain allowed for observation of the flagellum after labelling with the green Alexa Fluor Maleimide (Figure 2.14B). Using Super Resolution Structured Illumination Microscopy (SR-SIM) allowing for increased resolution (Figure 2.14C, 2.14D), we observed various flagella along the cells in a peritrichous arrangement, as previously shown (Anjuwon-Foster & Tamayo, 2017, Baban *et al.*, 2013). Furthermore some free flagella segments were observed, which may have resulted from mechanical disruption during treatment for microscopic observation or from normal loss during cell growth. Motility assays confirmed the functionality of the strain (Figure 2.14D).

We found that the majority of the cells (93%) in a population contain flagella, which is in agreement with the fraction of cells that express *fliC* in strain 630 Δ *erm* (Figure 2.12) (Anjuwon-Foster & Tamayo, 2017). In order to observe which of the flagellated cells would express *tcdA*, we used SNAP-tag as a reporter for *tcdA* transcription. Microscopy analysis showed that most of the *tcdA*-expressing cells are flagellated (Figure 2.14E). Furthermore, the presence of flagella does not seem to influence *tcdA* expression, because the percentage of cells expressing *tcdA* was similar among the unflagellated and the flagellated cells (Figure 2.14E).

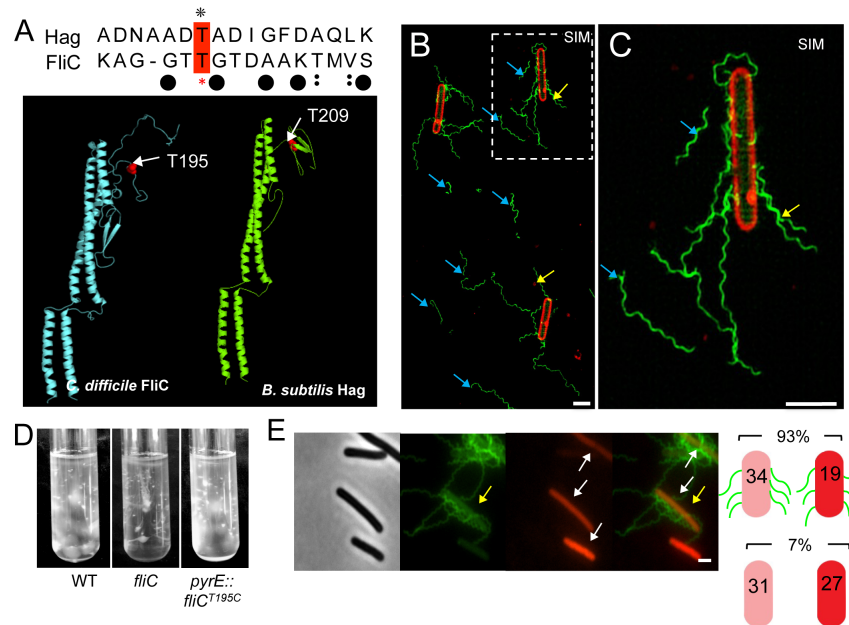


Figure 2.14. Observation of flagella in a strain producing FliC^{T195C}. **A:** Schematic representation of the amino acid sequence and structure of the flagellin proteins from *B. subtilis* and *C. difficile* highlighting the conserved tyrosine residue that was replaced by a cysteine (red). The alignment between residues 203-219 of the Hag protein from *B. subtilis* with residues 190 to 205 of the *C. difficile* FliC protein is shown at the top. The asterisk represent invariant residues, the two dots represent residues with similar properties and the circles represent chemically different residues. The structure of the Hag protein from *B. subtilis* and a model of the FliC protein from *C. difficile* are shown at the bottom. The position of tyrosine 195 in Hag and tyrosine 209 in FliC is shown. **B, C:** Microscopy analysis by SR-SIM of the *C. difficile* 630Δ*erm* strain expressing FliC^{T195C}. The cells were collected after 24h of growth in TY liquid medium and microscopy was performed as indicated before. Filaments are colored green extracellularly with Alexa Fluor 488-C₅-Maleimide. The blue arrows point to free flagella or segments of flagella and the yellow arrows point to attached flagella. Scale Bar: 1 μm (B) and 3 μm (C). **D:** Motility assays in agar soft tube (0.175%) of *C. difficile* 630Δ*erm* (WT), *C. difficile* 630Δ*erm* *fliC* and *C. difficile* 630Δ*erm* expressing FliC^{T195C} (*pyrE::fliC^{T195C}*). **E:** Microscopy analysis of *C. difficile* cells carrying a P_{tcdAΔ_{ccpA}}-SNAP^{Cd} transcriptional fusion in the 630Δ*erm* derivative producing FliC^{T195C}. Microscopy was performed as indicated above. Filaments are stained green extracellularly with Alexa Fluor 488-C₅-Maleimide. The merged images show the overlap between the TMR-Star (red) and the Alexa Fluor 488 (green) channels. The blue arrows point to free flagella, the yellow arrows point to attached flagella and the blue arrows point to cells. Scale Bar: 1 μm. The percentage of flagellated cells and the percentage cells with high (red cells) or low (pink cells) levels of SNAP signal is shown on the right.

2.5. DISCUSSION

2.5.1. Bistable expression of the toxin-encoding genes

We have shown that a population of vegetative cells bifurcates into subpopulations expressing or not the PaLoc genes *tcdR*, *tcdA*, *tcdB* and *tcdC*. Expression of these genes thus shows a bistable pattern in vegetative cells (Figure 2.2). In contrast, we did not observe expression of *tcdE* in vegetative cells. The gene coding for TcdC is the one expressed at higher levels and in a higher fraction of cells but it is unclear whether this gene is under the direct control of TcdR (Figure 2.2). Of the genes known to be under the direct control of TcdR, *tcdA*, showed the highest fraction of ON cells in the population, and a significant number of cells showed high levels of expression. Importantly, in strain R20291 the *tcdA* expression levels are lower. In this successful epidemic strain, lower toxin levels may translate into a smaller subpopulation being sacrificed when recognized by the immune system (see also below).

In contrast, *tcdR* and *tcdB* were expressed in a small (6-7%) fraction of cells and at low levels. Thus, expression of *tcdR* and *tcdB* correlated. The discrepancy between the expression of *tcdR* and *tcdA* is difficult to explain because only a TcdR-dependent promoter is known to drive expression of *tcdA* (Mani & Dupuy, 2001) and accordingly, *tcdA* expression is completely abolished in a $\Delta tcdR$ mutant (See Chapter 3). One possibility is that transcription initiation at the *tcdA* promoter is far more efficient than at the *tcdR* promoters that govern expression of *tcdR* itself and of *tcdB*. Thus, low levels of TcdR would be sufficient for robust expression of *tcdA*. The difference in numbers, however, suggests that many cells expressing *tcdA* will not show *tcdR*

expression. It is possible, however, that *tcdR* transcription does not correlate with protein levels; the expression of *tcdR* for instance, could occur in a pulsate pattern through which the protein gradually accumulates.

The bistable expression of *tcdR*, *tcdA* and *tcdB* is likely to originate in the *tcdR* auto-regulatory loop. Ramson and co-authors, however, were able to place *tcdR* under the control of an anhydrotetracycline inducible promoter and they showed unimodal expression of *tcdA* at any inducer concentration (Ransom *et al.*, 2018). This observation is consistent with a model in which the *tcdR* auto-regulatory loop is at the origin of the bistable expression of the *tcdR*, *tcdA* and *tcdB* genes.

Several other observations support this model. The level of TcdR resulting from operation of the auto-regulatory loop will depend on both the affinity of TcdR for the two TcdR-dependent promoters located upstream of the *tcdR* gene, and on the steady-state cellular concentration of TcdR. σ^D is thought to activate the loop by allowing some production of TcdR. Genetic conditions that reduce transcription and activity of σ^D , such as increased production of c-di-GMP, deletion of the *fliF* gene or of *sigD* itself (Aubry *et al.*, 2012, El Meouche *et al.*, 2013, McKee *et al.*, 2013) strongly reduce the fraction of *tcdA*-ON cells likely because production of TcdR becomes strongly or exclusively dependent on auto-regulation. In contrast, increased transcription of *sigD* does not increase the fraction of *tcdA*-ON cells but the increased activity of σ^D (as in the *fliC* mutant; see also below) raises this fraction to nearly 90% of the cells. Possibly, inducing transcription of *sigD* does not significantly increase the activity of σ^D because of the presence of an inhibitory factor (below). Deletion of *rstA* caused an expansion of

the subpopulation of high-producers (Figure 2.15), raising the possibility that this regulator has an effect on toxin production not exclusively through σ^D .

In the *fliC* mutant, however, the activity of σ^D increases, as shown by the increased expression of a P_{fliC} -*SNAP^{Cd}* fusion in a *fliC* mutant (Figure 2.13). Possibly, this results in higher levels of TcdR, shifting the auto-regulatory loop to a *tcdA*-ON state in more cells. The effects of the *fliC* and *fliF* mutations on *tcdA* expression are in agreement with previously published results (Aubry *et al.*, 2012, Dingle *et al.*, 2011b). Overall, σ^D seems the central regulator that determines the proportion of the *tcdA*-ON subpopulation (Figure 2.15).

2.5.2. The role of CodY

A single point mutation at box III is enough to relieve *tcdR* repression by CodY and in a *codY* mutant *tcdR* expression increases in the percentage of cells as well as in the intensity per cell (Figure 2.5). However, the effect on *tcdA* expression is much more drastic when comparing with the effect on *tcdR* (Figure 2.6). The box III mutation may have this effect because it directly affects the levels of TcdR, which then influences the auto-regulatory loop. Regulatory factors such as CodY may act mainly by allowing increased levels of toxin gene expression in stationary phase.

2.5.3. The role of CcpA

Toxin regulation was already shown to be multifactorial, with metabolic regulators, stress regulators and quorum sensing regulators affecting toxin expression (Figure 2.1C) (Martin-Verstraete *et al.*, 2016).

Deletion of the CcpA binding box from the *tcdA* transcriptional

fusion prevents repression in the presence of glucose in the medium. Contrarily, the inclusion of the box leads to repression of toxin expression per cell in the presence of glucose. When glucose is available as the main sugar source, the levels of toxin production per cell are maintained low. Conversely, under nutritional stress, expression of the toxins increase per cell, which might lead to increased inflammation and facilitate *C. difficile* to overcome commensal bacteria.

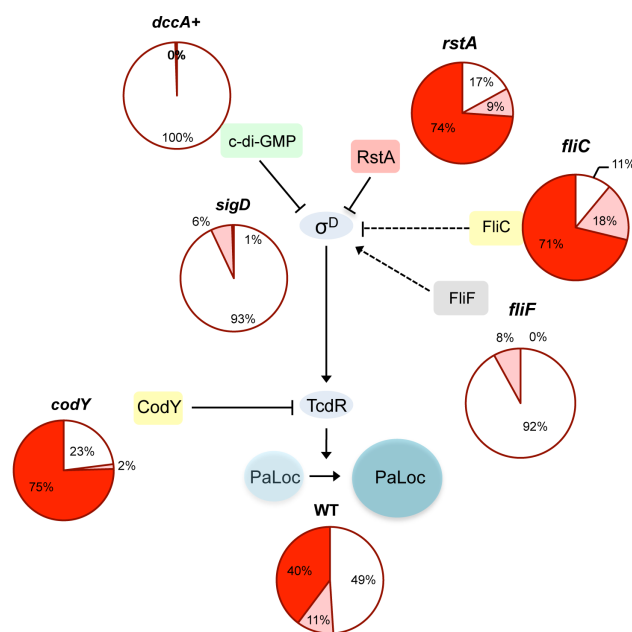


Figure 2.15. Schematic representation of the main regulators affecting toxin expression. The regulators acting on TcdR that consequently affect the PaLoC expression. Both c-di-GMP and RstA repress σ^D but c-di-GMP is more important than RstA. FliC possibly represses σ^D and FliF possibly activates σ^D . In turn, σ^D induces the PaLoC expression, by increasing the population of cells expressing the PaLoC and increasing the expression levels per cell. The regulator CodY acts directly on TcdR also affecting PaLoC expression. CodY represses TcdR reducing the population of cells expressing the PaLoC and the intensity per cell. The pie charts represent in white the percentage of cells that do not express *tcdA* and in light and dark red the percentage of cells that express low or high levels of *tcdA*, respectively.

2.5.4. Significance of the coupling between flagellar assembly and *tcdA* expression

In *B. subtilis* FlgM is an anti-sigma σ^D factor (Bertero *et al.*, 1999, Daughdrill *et al.*, 1997). Once the hook-basal body structure of the flagella is assembled FlgM is secreted and σ^D repression is relieved, leading to increased σ^D activity, a mechanism that allows separating the early steps on flagellar assembly from the late steps (Calvo & Kearns, 2015). In *C. difficile*, although it is not known whether FlgM is exported, it represses σ^D activity (El Meouche *et al.*, 2013). A similar mechanism may thus operate in *C. difficile* and if so, it could explain the differences in σ^D activity observed in these mutants: in the *fliC* mutant there is no flagellin to compete for secretion and FlgM will be continuously secreted through the basal body increasing the activity of σ^D ; on the other hand, on the *fliF* mutant assembly of the basal body is not completed and FlgM accumulates inside the cell where it binds to σ^D inhibiting its activity.

Since FliC is required for a late stage in flagellar assembly, it seems possible that the TcdR auto-regulatory loop is activated by σ^D during a window of time between assembly of the hook-basal body (and export of FlgM), and the onset of flagellin secretion. One possibility is that the toxins are exported through the flagellum secretion apparatus (as is the case for virulence factors of other intestinal pathogens) during this window of time. Alternatively, export of the toxins through the flagellar apparatus could occur if and when the flagellum is disassembled. *V. cholera* senses breakage of the flagellum caused by movement through the mucus layer, which permits export of the FlgM anti- σ^D factor, increasing expression of the virulence factors (Liu *et al.*, 2008, Tsou *et al.*, 2008). In this way, production of the virulence factors

is coordinated with the arrival of the bacteria to the sites of infection (see also the general discussion).

It has been long shown the importance in gastrointestinal pathogens of interconnecting both motility and toxin production, in an optimal combination that allows hiding from the host immune system, but still reach an optimal location for bacterial growth and toxin release. In *C. jejuni*, virulence factors are secreted through the flagella, which results in a temporal control for secretion of these proteins: the flagella polymerization possibly results in a switch of the T3SS substrate specificity that stops secreting the virulence factors and starts secreting flagellins (Barrero-Tobon & Hendrixson, 2014, Konkel *et al.*, 2004). In *V. cholerae*, a quorum sensor regulator that represses the expression of virulence genes is in turn repressed by FliA, the late stage regulator of flagellar assembly. Furthermore, this regulator is activated after flagella breakage due to mucin penetration that results in secretion of the FliA repressor. This gastrointestinal pathogen is therefore an important example of how motility and virulence are jointly regulated (Liu *et al.*, 2008, Syed *et al.*, 2009, Tsou *et al.*, 2008). In the extra-intestinal pathogenic *E. coli* (ExPEC), the high-pathogenicity island (HPI) responsible for the production of the virulence factor, yersiniabactin, also promotes flagellar motility (Magistro *et al.*, 2017).

Future studies aiming at directly testing the possibility that the TcdA and TcdB toxins are exported through the flagellar secretion system seem important. In any case, involvement of the flagellar secretion system does not exclude a role for TcdE in the export of the toxins (Govind & Dupuy, 2012, Govind *et al.*, 2015).

The visualization of the flagellum using a chemical probe shows

that most of the *tcdA*-expressing cells are flagellated (Figure 2.14), in line with the tight coupling between assembly of the flagellum and toxin production. A phase variation switch that controls the expression of flagellar genes is present in the epidemic strain R20291 but absent from 630 Δ *erm* (Anjuwon-Foster & Tamayo, 2017). This mechanism may be responsible for the reduced fraction of *tcdA*-ON cells in strain R20291 (see also below).

2.5.5. Bistable expression of the toxin-encoding genes in the context of infection

Bistability is often thought of a bet-hedging or a division of labor strategy (Davis & Isberg, 2018, Raser & O'Shea, 2005, Weigel & Dersch, 2018). Bacterial cells, especially during infection, can go through various stresses and fluctuations in the environment and alternative phenotypes, expressed by sub-populations of cells, might ensure that at least part of the population survives in the face of a changing environment (Davis & Isberg, 2018). An alternative explanation is division of labor. Only some cells will assume the burden of producing an extracellular virulence factor, for instance, while all individuals in the population benefit (Davis & Isberg, 2018). An extreme case of division of labor is self-destructive cooperation in which part of the population sacrifices itself for the survival of the others. An example comes from studies in *Salmonella typhimurium*. Virulence factors from *S. typhimurium* are secreted through a type III secretion system (TTSS-1) that is heterogeneously expressed in the population. The initial inflammation is a public good for the population since it decreases the competition with commensals. However the cells that initially invade the gut tissue and express the TTSS-1 tend to be

eliminated by the innate immune system. Therefore these invaders sacrifice themselves and give the ideal conditions for other cells to thrive (Ackermann *et al.*, 2008). Another example comes from *Streptococcus pneumonia*, in which a toxin that aids in colonization is released through cells lysis (Paton, 1996). It has also been proposed that in *C. difficile* the toxins are released through cell lysis (Ackermann *et al.*, 2008). However, this has not been demonstrated and other authors had proposed that the toxins are released by other mechanisms, including through a pore assembled by the holin-like protein TcdE (Govind & Dupuy, 2012, Govind *et al.*, 2015).

One possibility is that the cells expressing the toxin are removed by the immune system, if at least part of the toxins remain at the cell surface (Ransom *et al.*, 2018). If so, then a clear benefit of the bistable expression of the PaLoc is that part of the population survives, giving rise to new heterogeneous populations. It is tempting to suggest that part of the success of epidemic strains such as R20291 stems from variation in the fraction of toxin-producing cells.

A common feature of the scenarios described above is a cooperative behavior where the toxin producers are the cooperators for the benefit of the entire population (West *et al.*, 2002). This raises the question of how the spreading of cheaters is prevented (Ozkaya *et al.*, 2017). One possibility is that the expression of other *C. difficile* extracellular virulence factor is bistable and produced by cells that do not produce the toxins (or with little overlap with the sub-population of toxin producers). These cells that are cheaters for toxin production may thus remain cooperators for other “public goods”. It will be interesting to study the expression patterns at the single cell level of other virulence factors in order to test this possibility.

2.6. ACKNOWLEDGMENTS

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Chapter 3

**Cell type-specific production of
the *C. difficile* TcdA and TcdB
toxins**

Author contributions:

C. P. Cassona performed all experiments except the construction of the initial PaLoc transcriptional fusions (by Mónica Serrano) and the construction of the $\Delta tcdR$ mutant and the derivatives of strain R20291 (by Sara Ramalhete).

This chapter contains data from the submitted manuscript:

Expression of the toxin-encoding genes by sporulating cells of *Clostridium difficile* leads to the formation of cytotoxic spores.

3.1. SUMMARY

Clostridioides difficile causes a wide range of intestinal diseases through the action of two main toxins, TcdA and TcdB. Infection starts by the ingestion of spores. Spore germination establishes a population of vegetative cells that produces the toxins and will eventually enter sporulation. The pathogenicity locus, PaLoc, codes for TcdA/ TcdB and for TcdR, an RNA polymerase sigma factor essential for *tcdA/ tcdB* expression. The toxins cause inflammation, which in turn promotes colonization by vegetative cells. Here, we show that the PaLoc genes *tcdR*, *tcdA*, *tcdB* and *tcdE* are expressed during sporulation, which is also a stationary phase process. Expression of *tcdR*, *tcdA* and *tcdB* occurs in either the forespore or in the whole sporangium whereas expression of *tcdE* is only detected in the forespore. We show that expression of the PaLoc genes in the mother cell is σ^D -dependent and most likely results from protracted expression initiated in vegetative cells. In contrast, expression of the PaLoc genes in the forespore is not dependent on σ^D -dependent. We show that the forespore-specific regulatory proteins σ^G and SpoVT form a coherent feed forward loop, which leads to *tcdR* transcription and late *tcdA/tcdB* expression in this cell. The expression of *tcdA* and *tcdB* during sporulation points to a functional link between sporulation and toxinogenesis that might be important during infection, identifies a new regulatory mechanism for toxin production, and suggests that mother cell lysis is a route for toxin release.

3.2. INTRODUCTION

Clostridium difficile, recently re-classified as *Clostridioides difficile*, is a major nosocomial pathogen, and the leading cause of intestinal diseases that range from mild diarrhea to life-threatening illnesses, linked to the use of antibiotics in adults (Abt *et al.*, 2016, Rupnik *et al.*, 2009). In the last two decades, the emergence and spreading of epidemic clones of ribotype 027 were responsible for outbreaks worldwide with more severe symptoms, recurrence rates, morbidity and mortality (Lessa *et al.*, 2015, van Dorp *et al.*, 2016). The epidemiology of *C. difficile* is however changing, with new ribotypes spreading both in healthcare units and at the community level, which also show increased incidence among groups not previously considered at risk (Chitnis *et al.*, 2013). Moreover, the prevalence of some ribotypes in animals used for human consumption raises serious concerns of widespread dissemination through the food chain.

Infection is initiated by the ingestion of spores when gut dysbiosis, most frequently due to continued antibiotic treatment, allows spores to germinate in the colon and the outgrowing cells to originate a population of actively growing cells (Figure 3.1A). These cells produce two cytotoxins, TcdA and TcdB, which are the main factors responsible for the disease symptoms, and spores (Carter *et al.*, 2012, Deakin *et al.*, 2012, Kuehne *et al.*, 2010, Sarker & Paredes-Sabja, 2012) (Figure 3.1A). Spores are highly resistant dormant cells, hard to eradicate, and allow dissemination of this strict anaerobic pathogen; spores also allow *C. difficile* to persist in the environment and in the host and are linked to disease relapse (Carter *et al.*, 2012, Kuehne *et al.*, 2010, Sarker & Paredes-Sabja, 2012).

Spores are formed during the stationary phase of growth. Initially, an asymmetric division partitions the rod-shaped cell into a larger mother cell and a smaller forespore, the future spore (Figure 3.1B). At an intermediate stage in the process, the mother cell engulfs the forespore, which becomes isolated from the external medium. Several protective layers are then deposited around the forespore, including a peptidoglycan layer known as the cortex essential for dormancy, and a protein coat, required for resistance to noxious chemicals and containing peptidoglycan-breaking enzymes necessary for proper spore germination. *C. difficile* also has a more external layer, the exosporium, tightly associated with the underlying coat (Diaz-Gonzalez *et al.*, 2015, Paredes-Sabja *et al.*, 2014). Finally, lysis of the mother cell releases the spore into the environment (Henriques & Moran, 2007, Hilbert & Piggot, 2004) (Figure 3.1B).

Spo0A is the master regulatory protein governing entry into sporulation. Spo0A is universally conserved among sporeformers (Abecasis *et al.*, 2013, Galperin *et al.*, 2012) and, as expected, was shown to be essential for sporulation in *C. difficile* (Pettit *et al.*, 2014, Rosenbusch *et al.*, 2012). Spo0A controls a variety of other stationary phase responses. A key function of Spo0A in sporulation is that it controls placement of the division machinery close to one of the cell poles, in preparation for asymmetric division; also, it drives expression, in pre-divisional cells, of the genes coding for the first forespore and mother cell-specific sigma factors, σ^F and σ^E , respectively (Pettit *et al.*, 2014). Once the forespore and the mother cell are formed, σ^F is activated in the forespore and, soon after, σ^E is activated in the mother cell. These transcription factors govern early, prior to engulfment completion, stages of development in each of the cells that participate

in sporulation; following engulfment completion, σ^K replaces σ^E as the main factor driving developmental regulated gene expression in the mother cell, and σ^G replaces σ^F (Pereira *et al.*, 2013) (Figure 3.1B). While cell type-specific gene expression is mainly governed by the forespore and mother cell cascade of sigma factors, additional transcription factors sub-divide the sigma regulons into several temporal and epistatic classes. For example, SpoVT is produced in the forespore under the control of both σ^F and σ^G , and positively regulates the expression of a subset of σ^G -controlled genes; it also represses genes under the control of σ^F , which thus show transient expression (Rhayat *et al.*, 2009, Saujet *et al.*, 2013, Steil *et al.*, 2005, Wang *et al.*, 2006) (Figure 3.1B). Importantly, σ^E and σ^K are the main players in the morphogenesis of the spore protective structures (Fimlaid *et al.*, 2013, Fimlaid *et al.*, 2015b, Panessa-Warren *et al.*, 2007, Pereira *et al.*, 2013, Putnam *et al.*, 2013, Saujet *et al.*, 2013). These spore structures, specifically the cortex, coat and exosporium, are important for spore resistance, binding to cells, proper germination, and have been shown to dramatically influence colonization and disease-causing ability (Diaz-Gonzalez *et al.*, 2015, Janoir *et al.*, 2013, Mora-Uribe *et al.*, 2016, Phetcharaburanin *et al.*, 2014, Pizarro-Guajardo *et al.*, 2014).

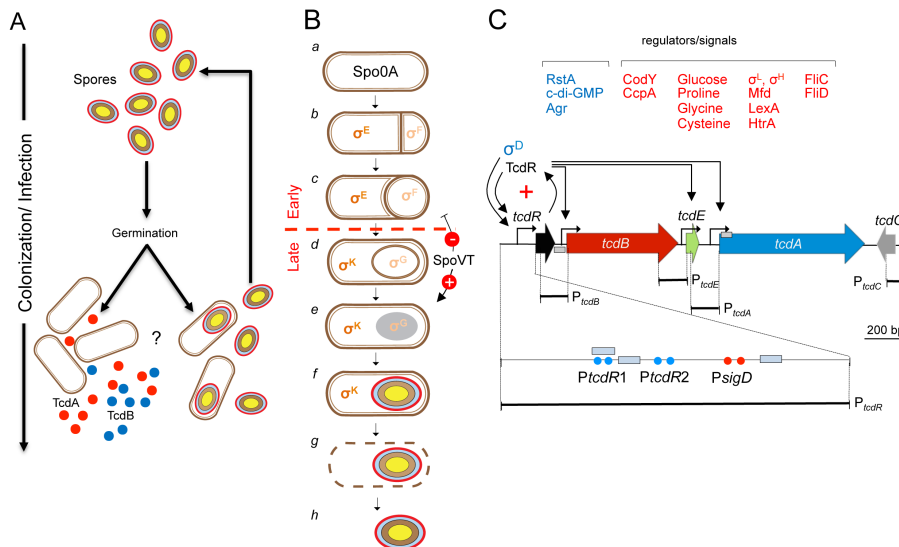


Figure 3.1. The *C. difficile* infection cycle. **A:** Infection starts with the ingestion of spores. Spores will germinate in the upper parts of the intestine, in response to bile salts. Cells will outgrow from germinated spores to establish a population of cells that will produce the TcdA and TcdB toxins and spores. It is unknown whether toxins are produced by different sub-populations. Spore shedding completes the cycle. **B:** Shown is the pathway of spore differentiation starting with vegetative (pre-divisional cells, a), asymmetric division (b), a stage in engulfment (c), engulfment completion (d), synthesis of the spore protective layers (e and f) and free mature spores (h), resulting from mother cell lysis (g). Spo0A controls several stationary phase processes in pre-divisional cells and is essential for sporulation. Cell type-specific gene expression results from the action of the indicated σ factors. The role of SpoVT in activation of a subset of σ^G -dependent genes while repressing some σ^F -controlled genes is represented. Early and late stages in development are defined as those occurring prior to or following engulfment completion, as indicated. **C:** Genetic organization of the PaLoc. Broken arrows represent promoters in the region; the "+" sign indicates positive auto-regulation of *tcdR*. The *tcdR* regulatory region is expanded below the map to show two *tcdR*-dependent promoters (P1 and P2) and a σ^D -dependent promoter. The black arrows represent direct regulation of the indicated promoters by TcdR. Known direct and indirect factors regulating PaLoc gene expression are indicated (red and blue, negative and positive regulation, respectively; see text for details). The lines below the genetic map show the position and extent of the DNA fragments used to create the indicated transcriptional *SNAP^{Cd}* fusions. Grey rectangles show the position of CcpA binding sites and blue rectangles CodY binding sites.

The genes coding for TcdA and TcdB are located in a pathogenicity locus, or PaLoc (Deneve *et al.*, 2009, Hundsberger *et al.*, 1997, Kuehne *et al.*, 2011) (Figure 3.1C). TcdA and TcdB are glucosyltransferases that belong to the family of Large Clostridial Toxins (LCTs). The PaLoc carries three other genes, *tcdR*, *tcdE* and *tcdC* (Figure 3.1C). The gene *tcdR* codes for TcdR, a Group 5 extracytoplasmic function (ECF) sigma factor, related to *C. botulinum* BotR, to *C. tetani* TetR and to UviA from *C. perfringens*. TcdR positively regulates toxin production in response to extracellular signals (Mani & Dupuy, 2001, Moncrief *et al.*, 1997). *In vitro* experiments have shown that TcdR binds to the *tcdA* and *tcdB* promoter regions and stimulates their transcription (Mani & Dupuy, 2001) (Figure 3.1C). The gene *tcdC* codes for a small acidic transmembrane protein, thought to be a negative regulator of toxin production (Hundsberger *et al.*, 1997). TcdC appears to prevent transcription of *tcdA* and *tcdB* in vitro by interacting with TcdR or with core RNA polymerase (Matamouros *et al.*, 2007). The role of TcdC *in vivo*, however, is still unclear (Bakker *et al.*, 2012, Carter *et al.*, 2011, Cartman *et al.*, 2012, Curry *et al.*, 2007, Murray *et al.*, 2009). Some authors also proposed that TcdC is able to bind to four stranded G-quadruplex DNA structures. Nevertheless no multiple G-stretches that form these structures were found at the PaLoc (van Leeuwen *et al.*, 2013). In our hands, no differences were found in toxin expression in a $\Delta tcdC$ mutant (see Chapter 2). The *tcdE* gene codes for a protein with similarity to the class I family of holins that includes the lambda phage S protein, and in line with this observation, expression of *tcdE* in *Escherichia coli* complements a lambda S mutant, and under certain conditions causes cell lysis (Tan *et al.*, 2001). While the mechanism by

which the toxins, neither of which showing recognizable secretion signals, are transported out of the producing cells is still not fully understood, at least in some strains, TcdE is required for TcdA and TcdB release (Govind & Dupuy, 2012, Govind *et al.*, 2015, Olling *et al.*, 2011).

Expression of *tcdA* and *tcdB* is strongly induced early in stationary phase and persists during this phase (Dupuy & Sonenshein, 1998, Hundsberger *et al.*, 1997). Toxin production is subject to the action of several regulatory systems that converge to prevent expression of their encoding genes during growth (Figure 3.1C). For instance, glucose or other rapidly metabolizable sugars represses transcription of *tcdR* (Antunes *et al.*, 2012, Antunes *et al.*, 2011, Dupuy & Sonenshein, 1998) as well as other metabolites like branched-chain amino acids, proline and glycine (Bouillaut *et al.*, 2015, Martin-Verstraete *et al.*, 2016) (Figure 3.1C). Toxin expression was shown to be heterogeneous and the flagellar regulator σ^D is an important positive regulator of PaLoc expression (see Chapter 2).

Although *tcdA* and *tcdB* are expressed in stationary phase, it remained unknown for a long time whether toxin producers would enter the spore differentiation pathway, or whether spores and toxins would be produced by different sub-populations of cells (Figure 3.1A). Recently, Ransom *et al.* proposed that the two processes are not mutually exclusive. Therefore, the two processes are likely to be coordinated. RstA, for instance, is a recently characterized regulatory factor that promotes sporulation while simultaneously acting as a repressor of toxin production (Edwards *et al.*, 2016). Moreover, recent work has shown that TcdR is required for efficient sporulation and normal surface layers and functional properties at least in some

strains (Girinathan *et al.*, 2017).

Using single cell analysis, we show here that both *tcdA* and *tcdB* are expressed in sporulating cells. We found that expression of the two genes occurs in the mother cell most likely as the result of protracted vegetative expression. Moreover, we found that expression of *tcdA* and *tcdB* is specifically induced in the forespore at a late stage in development, through *tcdR*, under the joint control of σ^G and SpoVT. Thus, we report a new regulatory mechanism of *C. difficile* toxinogenesis tightly connected with sporulation.

3.3. MATERIALS AND METHODS

Growth conditions and general methods. Bacterial strains and their relevant properties are listed at table 3.1. The *Escherichia coli* strain DH5 α (Bethesda Research laboratories) was used for molecular cloning, while HB101 (RP4) was used as the donor strain in *C. difficile* conjugation experiments (Hussain *et al.*, 2005). Luria-Bertani medium was routinely used for growth and maintenance of *E. coli*. When appropriate, ampicillin (100 μ g/mL) or chloramphenicol (15 μ g/mL) was added to the culture medium. The *C. difficile* strains used in this study are congenic derivatives of the wild-type strain 630 Δ *erm* (Hussain *et al.*, 2005) or R20291 (Anaerobe Reference Laboratory, Cardiff, Wales, United Kingdom) and were routinely maintained anaerobically (5% H₂, 15% CO₂, 80% N₂) at 37°C in brain heart infusion (BHI) medium (Oxoid) supplemented with L-cysteine (0.1%) and yeast extract (5 mg/ml) (BHIS) (Wilson *et al.*, 1982). For toxin assays and for sporulation assays, tryptone yeast extract, TY, (30 g/L tryptone; 20 g/L yeast extract) was used supplemented with thiamphenicol (15 μ g /mL)

when necessary. A defined minimal media (CDMM) (Karasawa *et al.*, 1997) with 1% agar was used as uracil-free medium when performing genetic selections. The minimal medium was supplemented with 5-Fluoroorotic acid (2 mg/mL) and uracil (5 µg/mL) when appropriate.

Transcriptional fusions of PaLoc promoters to the *SNAP^{Cd}* reporter. For the construction of the transcriptional *SNAP^{Cd}* fusions to the PaLoc promoters, the plasmid pFT47 previously constructed (Pereira *et al.*, 2013) was used. All the promoters were cloned between the EcoRI/ XhoI sites, upstream of *SNAP^{Cd}*. In order to construct *tcdR*-, *tcdA*-, *tcdB*-, *tcdC*- and *tcdE*- transcriptional *SNAP^{Cd}* fusions, the promoter regions of these genes were PCR-amplified using genomic DNA from *C. difficile* 630Δ*erm* with the primer pairs PtcR-SNAP-EcoRI-Fw/ PtcR-SNAP-XhoI-Rev, PtcA-SNAP-EcoRI-Fw/ PtcA-SNAP-XhoI-Rev, PtcB-SNAP-EcoRI-Fw/ PtcB-SNAP-XhoI-Rev, PtcC-SNAP-EcoRI-Fw/ PtcC-SNAP-XhoI-Rev and PtcE-SNAP-EcoRI-Fw/ PtcE-SNAP-XhoI-Rev, to produce, 815, 520, 530, 430 and 600bp products, respectively. These yielded pMS464, pMS470, pMS471, pMS472 and pMS473. The plasmids were introduced into *E. coli* HB101 (RP4) yielding the strains AHEC125, AHEC144, AHEC145, AHEC146 and AHEC147 and transferred to *C. difficile* 630Δ*erm*, producing strains AHCD608, AHCD668, AHCD669, AHCD670 and AHCD671 (Table 3.1). The plasmid pMS464 was introduced into the *C. difficile* 630Δ*erm* sporulation mutants *sigF* (AHCD533), *sigE* (AHCD532), *sigG* (AHCD534), *sigK* (AHCD535) and *spo0A* (AHCD536), yielding strains AHCD647, 648, 649, 650 and 651; pMS464 was also transferred to a *spoVT* mutant (our strain AHCD659) originating AHCD777. Furthermore, pMS464 and pMS470

were introduced into the *sigD* mutant (AHCD687) producing strains AHCD780 and AHCD779, respectively.

Deletion and mutational analysis of the *tcdR* regulatory region.

For deletion analysis of the *tcdR* promoter a fragment of 183 bp (fragment P_{tcdR-A}) was fused to $SNAP^{Cd}$ according to the general strategy outlined above for the full-length fusion (Figure 3.5). The fragment was PCR-amplified using the primer pair P_{tcdR-A}-SNAP-EcoRI-Fw/ P_{tcdR}-SNAP-XhoI-Rev and cloned between the EcoRI/XhoI sites in pFT47, yielding pCC20. As before, the plasmid was introduced into *E. coli* HB101 (RP4) yielding strain AHEC237 and transferred to *C. difficile* 630 Δ *erm*, producing AHCD795. To introduce point mutations at the -10 region of the mapped *sigD* promoter upstream of *tcdR*, overlap extension PCR was performed, initially using the primer pairs P_{tcdR}-SNAP-EcoRI-Fw/ P_{sigDmut}-Rev and P_{sigDmut}-Fw/ P_{tcdR}-SNAP-XhoI-Rev that originated fragments with 741 and 195 bp, and finally with P_{tcdR}-SNAP-EcoRI-Fw/ P_{tcdR}-SNAP-XhoI-Rev. The final PCR product was cloned between EcoRI/XhoI sites in pFT47 yielding pCC27. The plasmid was transformed into *E. coli* HB101 (RP4) yielding strain AHEC274 and then transferred to *C. difficile* 630 Δ *erm* by conjugation, producing AHCD821.

Mutant construction through allelic exchange in *C. difficile*.

Allelic exchange using the *pyrE* alleles (Ng *et al.*, 2013) was used to perform in-frame deletions of the *tcdR* gene in *C. difficile* 630 Δ *erm* and R20291. *pyrE* negative strains previously constructed (Ng *et al.*, 2013) were used in order to allow for a positive/ negative selection. The plasmids pSR3 derived from pMTL-YN3 and pSR1, derived from

pMTL-YN4, were used to construct the $\Delta tcdR$ mutant in 630 Δerm and R20291, respectively, where the allele exchange cassette composed of a left-hand homology arm (LHA) and a right-hand homology arm (RHA) was cloned between the SbfI and AscI sites. The allele exchange cassette was obtained by overlap extension PCR using the primer pair tcdR-AscI-Fw/tcdR-SOE-Rev for the LHA originating a 631 bp fragment and tcdR-SOE-Fw/tcdR-SbfI-Rev for the RHA originating a 522 bp fragment. The final PCR was performed with the primer pair tcdR-AscI-Fw/ tcdR-SbfI-Rev. DNA sequencing was performed to verify plasmid constructs. The plasmids obtained were transformed into *E. coli* HB101 (RP4) yielding strains AHEC239 for $\Delta tcdR$ in 630 Δerm and AHEC227, for $\Delta tcdR$ in R20291. These were subsequently mated with *C. difficile* 630 $\Delta erm\Delta pyrE$ and R20291 $\Delta pyrE$ (Ng *et al.*, 2013). *C. difficile* transconjugants were selected by sub-culturing on BHIS agar containing thiamphenicol (15 μ g/mL) and cefoxitin (25 μ g/mL) and re-streaked twice in this medium for 630 Δerm and four times for R20291. Colonies that appeared were screened by colony PCR using 5% chelex resin (Sigma–Aldrich) to identify single-crossover mutants. For the *tcdR* gene the primer pairs YN3-vef-Fw/ tcdR-vef-Fw and YN3-vef-Rev/ tcdR-vef-Rev for 630 Δerm or YN4-vef-Fw/ tcdR-vef-Fw and YN4-vef-Rev/ tcdR-vef-Rev for R20291 were used. The single-crossover mutants identified were streaked onto CDMM (Karasawa *et al.*, 1997) with 1% agar supplemented with 5-Fluoroorotic acid (2 mg/mL) and uracil (5 μ g/mL) in order to select for plasmid excision. The isolated FOA-resistant colonies were screened by colony PCR similarly as before using the primer pair tcdR-vef-Fw/tcdR-vef-Rev. The primers used flank the upstream and downstream homology regions. The double-crossover mutants that were thiamphenicol sensitive (AHCD811 in

630 Δ *erm* and AHCD1093 in R20291) were reverted to a *pyrE*⁺ phenotype through conjugation with the plasmid pMTL-YN1 for 630 Δ *erm* and pMTL-YN2 for R20291. The resulting colonies were streaked onto non-supplemented CDMM agar and the restoration of the *pyrE* allele was confirmed by colony PCR using the primer pair *pyrE*-vef-Fw/ *pyrE*-vef-Rev, yielding the strains AHCD820 for 630 Δ *erm* and AHCD1102 for R20291. Plasmids pMS470 and pMS464 were then introduced into Δ *tcdR* mutant in 630 Δ *erm* through conjugation with *E. coli* HB101 (RP4), which resulted in strains AHCD840 and AHCD841 for Δ *tcdR*, respectively.

Table 3.1. Bacterial strains used in this study.

Strain	Relevant properties	Origin/ reference
<i>E. coli</i>		
DH5 α	General cloning host	Invitrogen
HB101 (RP4)	Host for conjugation into <i>C. difficile</i>	Laboratory stock
AHEC068	BL21 (DE3)/ pFT36	This study
AHEC076	HB101 (RP4)/ pMTL84121	(Heap <i>et al.</i> , 2009)
AHEC125	HB101 (RP4)/ pMS464	This study
AHEC144	HB101 (RP4)/ pMS470	"
AHEC145	HB101 (RP4)/ pMS471	"
AHEC146	HB101 (RP4)/ pMS472	"
AHEC147	HB101 (RP4)/ pMS473	"
AHEC203	HB101 (RP4)/ pMTL-YN1	(Ng <i>et al.</i> , 2013)
AHEC205	HB101 (RP4)/ pMTL-YN2	"
AHEC217	BL21 (DE3)/ pCC17	This study
AHEC227	HB101 (RP4)/ pSR1	"
AHEC237	HB101 (RP4)/ pCC20	"
AHEC239	HB101 (RP4)/ pSR3	"
AHEC274	HB101 (RP4)/ pCC27	"
AHEC285	HB101 (RP4)/ pSR5	"
AHEC290	BL21 (DE3)/ pFT36/ pMS464	"
AHEC291	BL21 (DE3)/ pFT36/ pCC27	"
AHEC304	BL21 (DE3)/ pCC29	"
AHEC313	BL21 (DE3)/ pCC30	"
AHEC314	BL21 (DE3)/ pCC29/ pMS464	"
AHEC316	BL21 (DE3)/ pCC29/ pCC27	"
AHEC317	BL21 (DE3)/ pCC17/ pMS464	"
AHEC319	BL21 (DE3)/ pCC17/ pCC27	"

AHEC320	BL21 (DE3)/ pCC30/ pMS464	“
AHEC661	HB101 (RP4)/ pSR34	“
<i>C. difficile</i>		
AHCD531	<i>C. difficile</i> 630 Δ erm	(Hussain <i>et al.</i> , 2005)
AHCD532	630 Δ erm sigE::intron ermB	(Pereira <i>et al.</i> , 2013)
AHCD533	630 Δ erm sigF::intron ermB	“
AHCD534	630 Δ erm sigG::intron ermB	“
AHCD535	630 Δ erm sigK::intron ermB	“
AHCD536	630 Δ erm spo0A::intron ermB	(Saujet <i>et al.</i> , 2011)
AHCD543	630 Δ erm containing pMTL84121	(Pereira <i>et al.</i> , 2013)
AHCD608	630 Δ erm containing pMS464	This study
AHCD647	AHCD536 containing pMS464	“
AHCD648	AHCD533 containing pMS464	“
AHCD649	AHCD532 containing pMS464	“
AHCD650	AHCD534 containing pMS464	“
AHCD651	AHCD535 containing pMS464	“
AHCD659	630 Δ erm spoVT::intron ermB	(Saujet <i>et al.</i> , 2011)
AHCD668	630 Δ erm containing pMS470	This study
AHCD669	630 Δ erm containing pMS471	“
AHCD670	630 Δ erm containing pMS472	“
AHCD671	630 Δ erm containing pMS473	“
AHCD687	630 Δ erm sigD::intron ermB	(McKee <i>et al.</i> , 2013)
AHCD688	<i>C. difficile</i> R20291	Anaerobe Reference Laboratory, Cardiff, Wales, United Kingdom
AHCD772	630 Δ erm Δ pyrE	(Ng <i>et al.</i> , 2013)
AHCD777	AHCD659 containing pMS464	This study
AHCD779	AHCD687 containing pMS470	“
AHCD780	AHCD687 containing pMS464	“
AHCD795	630 Δ erm containing pCC20	“
AHCD811	630 Δ erm Δ pyrE Δ tcdR	“
AHCD820	630 Δ erm Δ tcdR	“
AHCD821	630 Δ erm containing pCC27	“
AHCD828	630 Δ erm Δ tcdR pyrE::tcdR	“
AHCD840	AHCD820 containing pMS470	“
AHCD841	AHCD820 containing pMS464	“
AHCD845	AHCD828 containing pMS470	“
AHCD846	AHCD828 containing pMS464	“
AHCD1067	R20291 Δ pyrE	(Ng <i>et al.</i> , 2013)
AHCD1093	R20291 Δ pyrE Δ tcdR	This study
AHCD1102	R20291 Δ tcdR	“
AHCD1161	R20291 Δ tcdR pyrE::tcdR	“

Table 3.2. Oligonucleotides used in this work.

Primer	Sequence (5' to 3') ^a
PtcdR-SNAP-EcoRI-Fw	CCCTTAAGC <u>GAATTCT</u> AAAAAGTAAACG
PtcdR-SNAP-XhoI-Rev	CATAAAATCCTCGAGTCTTATATTATAATG
PtcdA-SNAP-EcoRI-Fw	CACAAAGAT <u>GAATTCT</u> GGTCAGTTGGT
PtcdA-SNAP-XhoI-Rev	GTATTATTACTCGAGATAATAAATCCAC
PtcdB-SNAP-EcoRI-Fw	TATCAAAGT <u>GAATTCT</u> TTTTGAGGAAG
PtcdB-SNAP-XhoI-Rev	CTATAATACTCGAGCATCTAAATGCTAAAAAC
PtcdC-SNAP-EcoRI-Fw	ACTTCACCT <u>GAATTCT</u> GGTATATTC
PtcdC-SNAP-XhoI-Rev	CATAATACAATCCTCGAGTTATTAGAT
PtcdE-SNAP-EcoRI-Fw	CAATTGGAATTCGATGGATATATGATATG
PtcdE-SNAP-XhoI-Rev	CATTCATCATAGCTCGAGTTTTTTATTG
PtcdR _A -SNAP-EcoRI-Fw	CCCCCGAATTCTACTTTATTTATTAGAAAAA
PsigDmut-Fw	GCATATTTTCATATAAAATTTAATTTATTTGCatcTTcTATAATTATAATG
PsigDmut-Rev	CATTATAATTATAgAAgatGCAAATAAATTTATGAAAATATGC
TcdR-NcoI-Fw	CCCCCATGGAAAAAGTCTTTTTATG
TcdR-Sall-Rev	CCCGTCGACCAAGTTAAAATAATTTTC
CDSigGpET28a-Fw	TGCCTCGAGTACATATTTTCTCATATTTTTTAAAGC
CDSigGpET28a-Rev	AGGGGGTGACCCCATGGCAGCTCTTAAATC
SigD-NcoI-Fw	CCCCCATGGATAGAGAAGAATTAATAAAAG
SigD-XhoI-Rev	CCCCTCGAGTATAGAATATTTAAGTTC
SpoVT-NdeI-Fw	CCCCATATGAAAGCAACAGGTATAGTTAG
SpoVT-XhoI-Rev	CCCCTCGAGTTATTGAACTTGTTTTCC
tcdR-Ascl-Fw	CCCCGGCGCGCCATTATCTTAAGAGAGGAG
tcdR-SOE-Rev	CATAAATAAAATTTCTTGCAAATCATC
tcdR-SOE-Fw	TTGCAAGAAATTTATTTATGGAAAATTATTTAACTTG
tcdR-Sbfl-Rev	CCCCCTGCAGGTATCTATATAAATATCTG
tcdR-vef-Fw	GTATCATTTACGAAGAGG
tcdR-vef-Rev	GGGTCATTTAAGTTTTCTC
tcdR-comp-BamHI-Fw	CCCGGATCCTAAAAATATTTTGATATG
tcdR-comp-HindIII-Rev	CCCAAGCTTATTAATTTGCTCTTC
YN3-vef-Fw	CATCAAGAAGAGCGACTTCG
YN3-vef-Rev	TTCTTTCTATTGAGCACTGTTATGC
pyrE-vef-Fw	CAATAATTTTATAACATTAACATGG
pyrE-vef-Rev	GTGTTACTTAAAAAATGTAAAT
YN4-vef-Fw	CAAGAAGAGCGACTTCGCGGAGCTGG
YN4-vef-Rev	CCATTACAGACTTATCCAGGG

^a engineered restriction sites are underlined.

Table 3.3. Plasmids used in this study.

Plasmid	Relevant features	Origin/ reference
pMTL84121	<i>Clostridium</i> modular plasmid containing <i>catP</i> (Cm ^R /Tm ^R)	Laboratory stock
pMTL-YN1	Plasmid for <i>pyrE</i> reversion through ACE in 630Δ <i>erm</i>	(Ng <i>et al.</i> , 2013)
pMTL-YN1C	Plasmid for complementation through ACE in 630Δ <i>erm</i>	"
pMTL-YN2	Plasmid for <i>pyrE</i> reversion through ACE in R20291	"
pMTL-YN2C	Plasmid for complementation through ACE in R20291	"
pMTL-YN3	Plasmid for mutation through ACE in 630Δ <i>erm</i>	"
pMTL-YN4	Plasmid for mutation through ACE in R20291	"
pET16b	Expression vector/T7 <i>lac</i> promoter	Novagen
pET28a	"	"
pETDuet-1	"	"
pFT47	pMTL84121-SNAP ^{Cd} (Cm ^R /Tm ^R)	(Pereira <i>et al.</i> , 2013)
pMS464	pFT47 containing the <i>tcdR</i> promoter region	This study
pMS470	pFT47 containing the <i>tcdA</i> promoter region	"
pMS471	pFT47 containing the <i>tcdB</i> promoter region	"
pMS472	pFT47 containing the <i>tcdC</i> promoter region	"
pMS473	pFT47 containing the <i>tcdE</i> promoter region	"
pSR1	pMTL-YN4 containing a LHA and RHA from <i>tcdR</i> (R20291)	"
pSR3	pMTL-YN3 containing a LHA and RHA from <i>tcdR</i>	"
pSR5	pMTL-YN1C containing <i>tcdR</i> and its promoter for complementation	"
pSR34	pMTL-YN2C containing <i>tcdR</i> and its promoter for complementation (R20291)	"
pFT36	pET28a containing <i>sigG</i>	"
pCC17	pET16b containing <i>tcdR</i> -Strep tagII	"
pCC20	pFT47 containing 183bp from the <i>tcdR</i> promoter region	"
pCC22	pET16b containing <i>spoVT</i>	"
pCC27	pFT47 containing the <i>tcdR</i> promoter region with point mutations at the <i>sigD</i> promoter	"
pCC29	pETDuet-1 containing <i>sigD</i> and <i>spoVT</i>	"
pCC30	pET28a containing <i>sigD</i>	"

Complementation through allelic exchange at the *pyrE* locus.

In order to complement the Δ*tcdR* mutation, the coding sequence of the *tcdR* gene and its promoter region, in a total of 1460 bp, was amplified by PCR using the primer pair *tcdR*-comp-BamHI-Fw/*tcdR*-comp-HindIII-Rev and cloned between the BamHI and HindIII sites in pMTL-YN1C, yielding pSR5 for 630Δ*erm* and in pMTL-YN2C, yielding pSR34 for R20291. The plasmids obtained were transformed into *E.*

coli HB101 (RP4) yielding strains AHEC285 and AHEC661, respectively, and subsequently mated with *C. difficile* 630 Δ *erm* Δ *pyrE* Δ *tcdR* and R20291 Δ *pyrE*. The transconjugants obtained were streaked onto CDMM and tested by colony PCR using primer pair *pyrE*-vef-Fw/ *pyrE*-vef-Rev to test for both *tcdR* complementation and *pyrE* reversion; clones with the expected changes were identified and named AHCD828 for 630 Δ *erm* and AHCD1161 for R20291. As for the mutant, plasmids pMS470 and pMS464 were introduced into the complementation strain through matting with *E. coli* HB101 (RP4), resulting in strains AHCD845 and AHCD846, respectively.

Co-overexpression of *tcdR*, *sigD*, *sigG* and *spoVT* in *E. coli*. For TcdR overproduction, *tcdR* was amplified from *C. difficile* 630 Δ *erm* genomic DNA using the primer pair TcdR-NcoI-Fw/ TcdR-SalI-Rev that produced a 551bp fragment and cloned between the NcoI/SalI sites in pFN127 (pET16b-Strep tagII; Amp^R), originating pCC17. For SigG overexpression *sigG* was amplified from *C. difficile* 630 Δ *erm* genomic DNA using the primer pair CDSigGpET28a-Fw/ CDSigGpET28a-Rev that produced a 774bp fragment and cloned between the NcoI/XhoI sites in pET28a, originating pFT36. For SpoVT overexpression *spoVT* was amplified using the primer pair SpoVT-NdeI-Fw/ SpoVT-XhoI-Rev producing a 540bp fragment and cloned between the NdeI/XhoI sites in pET16b originating pCC22. For SigG and SpoVT co-induction, *sigG* was cut from pFT36 using NcoI/XhoI and cloned NcoI/SalI in pETDuet and *spoVT* was cut from pCC22 using NdeI/XhoI and cloned using the same restriction sites in pETDuet already containing *sigG*, producing pCC29. For SigD overexpression *sigD* was amplified from *C. difficile* 630 Δ *erm* genomic DNA using the

primer pair SigD-NcoI-Fw /SigD-XhoI-Rev and cloned between the NcoI/XhoI sites in pET28a, originating pCC30. All plasmids were transformed into *E. coli* BL21 (DE3) to yield strains AHEC217, AHEC068, AHEC304 and AHEC313. Additionally, pMS464 and pCC27 were co-transformed into *E. coli* BL21 (DE3) together with pFT36, pCC29 and pCC17, yielding the strains AHEC290, 291, 314, 316, 317 and 319. Furthermore, pMS464 was co-transformed with pCC30 yielding AHEC320.

SNAP induction assays in *E. coli*. In order to test for the ability of TcdR, SigD, SigG or SigG together with SpoVT to induce expression from the *tcdR* promoter, *E. coli* BL21 (DE3) strains bearing the plasmids overexpressing each of the proteins were transformed with either pMS464 or pCC27. We obtained strains containing pCC17 and pMS464 (AHEC317) or pCC27 (AHEC319); pCC30 and pMS464 (AHEC320) or pCC27 (AHEC322); pFT36 and pMS464 (AHEC290) or pCC27 (AHEC291); pCC29 and pMS464 (AHEC314) or pCC27 (AHEC316). As previously described, the strains were subjected to an auto-induction regime for the over-production of each protein (Studier, 2005). The cultures were collected by centrifugation (4000 $\times g$, for 10 minutes at 4°C) and the cell sediment was suspended in PBS with 1mM DTT. The cells were lysed using a French pressure cell (18000 lb/in²) and the extracts were labelled with 250nM TMR-Star substrate (New England Biolabs), for 30 minutes, in the dark. The labelled extracts were analyzed by fluoroimaging and immunoblotting as for the *C. difficile* extracts.

SNAP labelling of *C. difficile* extracts. Whole cell extracts were obtained by first withdrawing 10 mL samples from *C. difficile* BHIS cultures. The extracts were prepared immediately following labelling with 250nM TMR-Star substrate (New England Biolabs), for 30 minutes, in the dark. Following labelling, the cells were collected by centrifugation (4000 $\times g$, for 5 minutes at 4°C), the cell sediment was washed with phosphate-buffered saline (PBS) and suspended in 1 mL French press buffer (10 mM Tris pH 8.0, 10 mM MgCl₂, 0.5 mM EDTA, 0.2 mM NaCl, 10% Glycerol, 1 mM PMSF). The cells were lysed using a French pressure cell (18000 lb/in²). Proteins in the extracts were resolved on 15% SDS-PAGE gels. The gels were first scanned in a TLA-510 fluorimager (Fuji), and then subject to immunoblotting (Serrano *et al.*, 2011) with an anti-SNAP antibody (New England Biolabs). The antibody was used at a 1:1000 dilution, and a rabbit secondary antibody conjugated to horseradish peroxidase (Sigma) was used at a dilution 1:10000. The immunoblots were developed with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

Fluorescence microscopy and image analysis. For SNAP labelling, the TMR-Star substrate was added to cells in culture samples to a final concentration of 250 nM (New England Biolabs) and the mixture incubated for 30 minutes in the dark. Following labelling, the cells were collected by centrifugation (4000 $\times g$ for 5 minutes), washed four times with 1 mL of PBS, and finally suspended in 0.5 mL of PBS. For phase contrast and fluorescence microscopy, cells were mounted on 1.7% agarose coated glass slides and observed on a Leica DM6000B microscope equipped with a phase contrast Uplan F1 100x

objective and captured with a CCD Andor Ixon camera (Andor Technologies). Images were acquired and analyzed using the Metamorph software suite (version 5.8; Universal Imaging), cropped and adjusted using Adobe Photoshop.

Statistical analysis. Distributions obtained from quantifications of the SNAP labeling were statistically analyzed using the non-parametric Kolmogorov-Smirnov test (KS-test). The results on the quantification of a fluorescence signal are representative of three independent experiments. Only the results statistically significant by a KS-test in all three experiments were considered statistically relevant.

Sporulation assays. Overnight cultures grown at 37°C in BHI were used to inoculate BHI (at a dilution of 1:200). Once the OD₆₀₀ reached 0.4, 100 µL the cultures were plated in TY plates and incubated for 24h. After this incubation, the cells were scraped from the plates and suspended in 1 mL of TY. The suspension was serially diluted in TY and plated before and after heat treatment (30 minutes at 70°C), to determine the total and heat resistant colony forming units (CFU). The samples were plated as 20 µL spots in triplicate onto TY plates supplemented with 0.1% taurocholate (Carl Roth), to promote efficient spore germination.

3.4. RESULTS

3.4.1. The PaLoc genes are expressed in both vegetative and sporulating cells

Before, we have examined expression of the PaLoc genes at the single cell level in vegetative cells (see Chapter 2). We used the same transcriptional fusions of the *tcdA*, *tcdB*, *tcdC*, *tcdE* and *tcdR* promoter regions fused to the *SNAP^{Cd}* reporter (Cassona *et al.*, 2016, Pereira *et al.*, 2013, Saujet *et al.*, 2013) and used phase contrast and fluorescence microscopy to monitor gene expression during sporulation in the background of strain 630 Δ *erm*, across the cell population. The fragments fused to the *SNAP^{Cd}* reporter encompass all of the known promoters for each of the PaLoc genes (Figure 3.1C). The *P_{tcdR}-SNAP^{Cd}* transcriptional fusion used in these studies is termed full-length to distinguish it from a deletion construct used below. Samples were collected from cultures of strains bearing the various fusions, in the background of the wild-type (WT) strain 630 Δ *erm*, 24 hours after inoculation into TY, a medium that supports toxin production (Dupuy & Sonenshein, 1998). The cells were labelled with the red-fluorescent SNAP substrate TMR-Star and processed for microscopic examination. We found *tcdR* to be expressed in a sub-population of about 7% of the scored cells that do not present sporulation signs (Figure 3.2, yellow arrows). In addition, expression of *tcdR* was also detected in 51% of the sporulating cells, specifically in the forespore (Figure 3.2, white arrows). The forespore-specific expression of *tcdR* was mainly detected in sporangia that had completed the engulfment process and thus, at a late stage in development (Figure 3.2). Expression of *tcdA* was detected in 65% of the cells that do not have signs of sporulation (Figure 3.2,

yellow arrows) but importantly, expression of *tcdA* was also detected in sporulating cells (Figure 3.2, white arrows), but with a more complex pattern than that found for *tcdR*; while expression was confined to the forespore in 8% of the sporangia scored, 46% showed expression in both the forespore and the mother cell (Figure 3.2, blue arrows). This observation is in agreement with recent studies using a P_{tcdA} -*rfp* fusion (Ransom *et al.*, 2018).

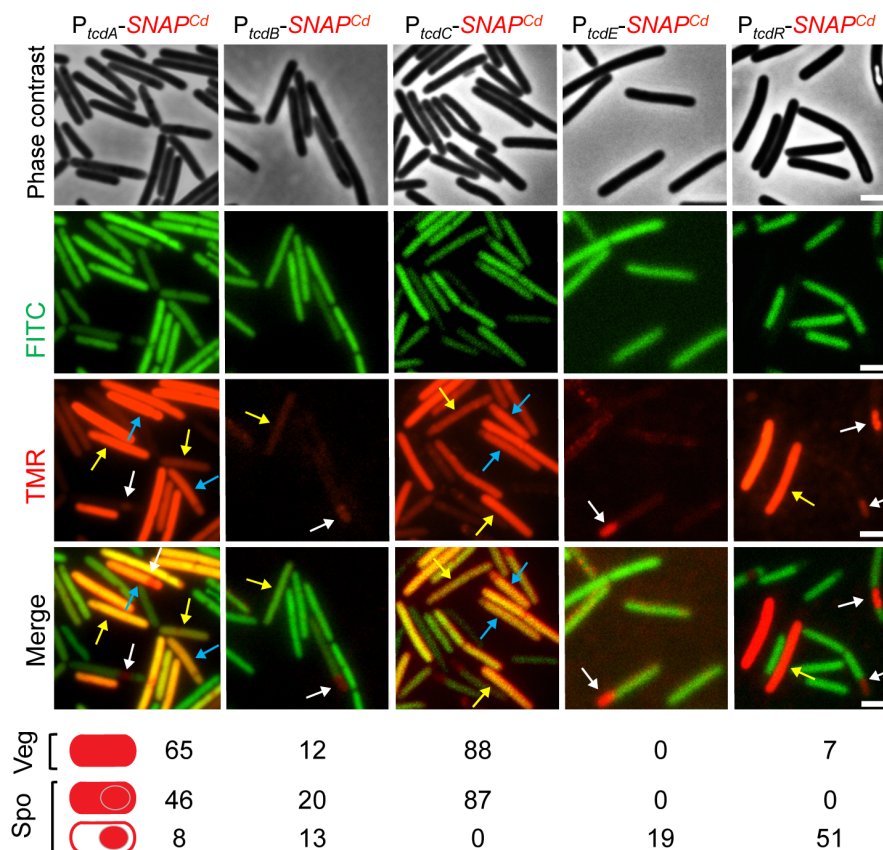


Figure 3.2. Expression of the PaLoc genes during sporulation in *C. difficile* 630 Δ *erm*. Cell type-specific expression patterns of P_{tcdA} -, P_{tcdB} -, P_{tcdE} -, P_{tcdC} - and P_{tcdR} -SNAP^{Cd} transcriptional fusions in strain 630 Δ *erm*. The cells were collected 24h after inoculation in TY liquid medium, labelled with TMR-Star and examined by phase contrast (PC) and fluorescence microscopy to monitor SNAP production. The merged images show the overlap between the TMR-Star (red)

and the auto-fluorescence (green) channels. The images are representative of the expression patterns observed for the different fusions. Yellow arrows point to vegetative cells with expression, white arrows point to sporulating cells with forespore-specific expression and blue arrows point to sporulating cells with whole sporangium expression. Scale bar: 1 μ m. The various cellular patterns of SNAP production were scored and their representation, as the percentage relative to the total number of vegetative or sporulating cells is shown. For sporulating cells the scoring includes a whole sporangium expression pattern and a forespore-specific pattern. The number of cells analyzed for each fusion, n , is as follows: P_{tcdA} -SNAP^{Cd}, $n = 245$; P_{tcdB} -SNAP^{Cd}, $n = 410$; P_{tcdC} -SNAP^{Cd}, $n = 400$; P_{tcdE} -SNAP^{Cd}, $n = 579$; P_{tcdR} -SNAP^{Cd}, $n = 353$.

Expression of *tcdB* is detected in only 12% of the vegetative cells (Figure 3.2) and as for *tcdA*, expression of *tcdB* was also detected in sporulating cells; of these, 13% showed expression only in the forespore whereas 20% showed expression in both the forespore and the mother cell (Figure 3.2). Because of the higher expression levels relative to P_{tcdB} -SNAP^{Cd}, the P_{tcdA} -SNAP^{Cd} fusion is used hereinafter to monitor toxin expression during sporulation. In contrast to *tcdR*, *tcdA* and *tcdB*, expression of *tcdE* was only detected, albeit weakly, in sporulating cells (19% of the sporangia scored), and only in the forespore (Figure 3.2, white arrows). Finally, *tcdC* expression was detected in 88% of the vegetative cells (Figure 3.2, yellow arrows). Unlike all the other PaLoc genes, however, expression of *tcdC* was not detected only in the forespore (Figure 3.2). Rather, 87% of the sporulating cells showed a whole sporangia pattern of expression, *i.e.*, expression in both the forespore and the mother cell (Figure 3.2, blue arrows). Control experiments showed very similar sporulation efficiency (around 5-10%) and kinetics for the strains bearing the various transcriptional fusions; the exception was the strain bearing the P_{tcdR} -SNAP^{Cd} fusion, which sporulated with efficiency greater than 50% (Figure 3.3A). The reason for this behaviour is not presently understood but one possibility is that the fragment fused to the

SNAP^{Cd} reporter contains a site that titrates out a negative regulator of sporulation. In any event, the increased sporulation efficiency of the strain bearing *P_{tcdR}-SNAP^{Cd}* is unlikely to introduce a bias in our scoring of expression of *tcdR* in sporulating cells. Importantly, additional control experiments, in which whole cell extracts were subject to fluoroimaging and immunoblotting following TMR-Star-labelling, suggested complete labelling of the *P_{tcdA}*-, *P_{tcdB}*-, *P_{tcdC}*- and *P_{tcdR}-SNAP^{Cd}* fusions (Figure 3.3B).

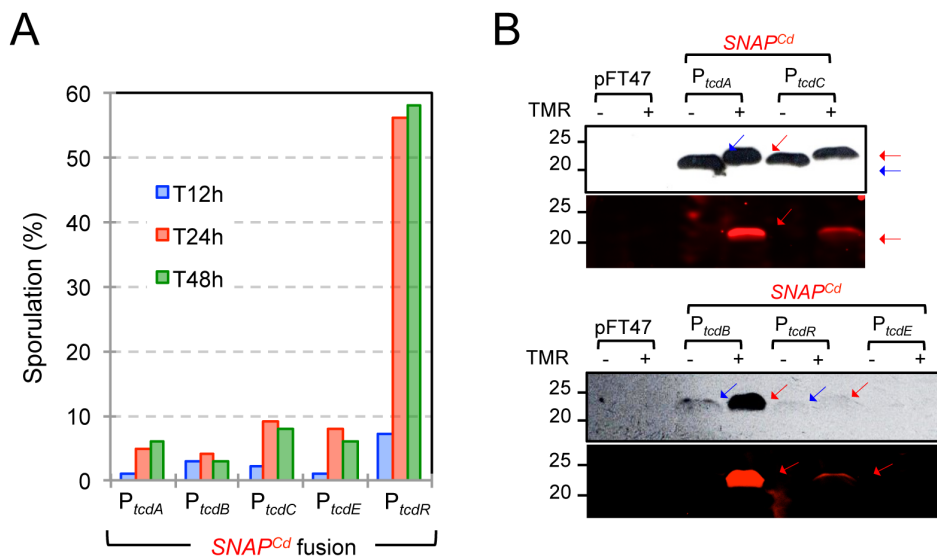


Figure 3.3. Sporulation and SNAP labelling. **A:** The panel shows the efficiency of sporulation (as the percentage of sporulating versus total cells) for strains bearing the indicated *SNAP^{Cd}* fusions. The efficiency of sporulation was measured 12, 24, and 48 hours by cell counting after inoculation in TY medium. **B:** Assessing the extent of SNAP labelling. Whole cell extracts were prepared from derivatives of strain 630Δ*erm* bearing the indicated plasmids or fusions, before (“-” sign) or immediately after (“+” sign) labelling with TMR-Star. The indicated amount of total protein was resolved by SDS-PAGE, and subject to immunoblotting with anti-SNAP antibody (top). Duplicate gels were scanned on a fluoroimager (bottom). Blue and red arrows point to unlabelled or TMR-Star-labelled, respectively, SNAP. Strain 630Δ*erm* carrying pFT47 (empty vector) was used as a negative control for SNAP production. The position of molecular weight markers (in kDa) is indicated on the left side of the panels.

In summary, we detected expression of the PaLoc genes during sporulation, which is heterogeneous: while *tcdR*, *tcdA*, *tcdB* and *tcdE* show forespore-specific expression, expression of *tcdA* and *tcdB* additionally shows a whole-sporangium pattern, and *tcdC* only shows this latter pattern. Moreover, regardless of the pattern, forespore or whole-sporangium expression is detected in only a fraction of the sporangia.

3.4.2. Expression of the PaLoc genes during sporulation is TcdR-dependent

TcdR is required for expression of the PaLoc genes *tcdA*, *tcdB* and *tcdE* (Mani & Dupuy, 2001, Moncrief *et al.*, 1997). In order to determine whether TcdR was required for the expression of the PaLoc genes during sporulation, we constructed a *tcdR* in-frame deletion mutant using Allelic Coupled Exchange (Ng *et al.*, 2013) and we monitored expression of P_{tcdR} - and P_{tcdA} -SNAP^{Cd}. We note that the insertional inactivation of *tcdR* in strain 630 Δ *erm* does not significantly affect sporulation (Girinathan *et al.*, 2017), a result that we also obtained for our in-frame deletion mutation (Table 3.4). Expression of P_{tcdA} -SNAP^{Cd} in vegetative cells was abolished in the Δ *tcdR* mutant but restored when a single copy of the *tcdR* gene was inserted at the *pyrE* locus; moreover, deletion of *tcdR* also abolished expression of *tcdA* in sporulating cells, and complementation at *pyrE* locus restored expression (Figure 3.4). Importantly, this could be observed both in the percentage of cells expressing *tcdA* (Figure 3.4A), as well as in the intensity of the fluorescence signal per cell (Figure 3.4B). Thus, TcdR is required for *tcdA* expression by sporulating cells. In sharp contrast, P_{tcdR} -SNAP^{Cd} expression was still detected in the

$\Delta tcdR$ mutant during sporulation (Figure 3.4). Thus, a factor other than TcdR is involved in *tcdR* expression in sporulating cells.

Table 3.4 – Heat resistant spore counts.

	630 Δerm			R20291		
	WT	$\Delta tcdR$	$\Delta tcdR^C$	WT	$\Delta tcdR$	$\Delta tcdR^C$
Viable	$2.1 \pm 2.2 \times 10^9$	$4.1 \pm 3.5 \times 10^9$	$2.0 \pm 1.9 \times 10^9$	$3.4 \pm 0.4 \times 10^9$	$2.8 \pm 0.1 \times 10^9$	$3.0 \pm 0.5 \times 10^9$
Heat	$3.7 \pm 3.5 \times 10^4$	$1.8 \pm 1.5 \times 10^5$	$8.6 \pm 5.6 \times 10^4$	$1.8 \pm 0.9 \times 10^4$	$7.0 \pm 8.5 \times 10^4$	$9.0 \pm 7.7 \times 10^4$

* the numbers represent the average and standard deviation of three different experiments (see the Material and Methods section) in CFU/ mL (colony forming units/ mL).

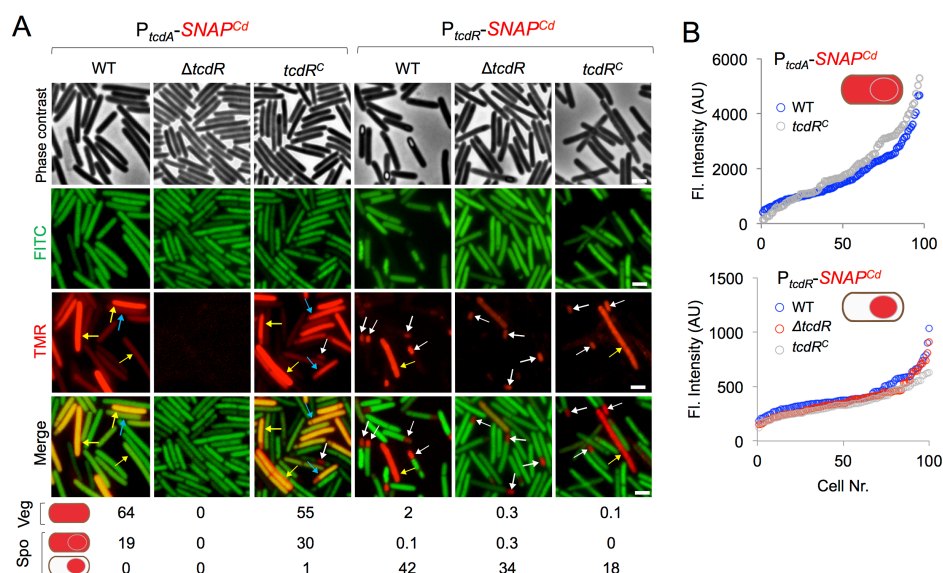


Figure 3.4. The role of TcdR in regulating toxin production during sporulation. **A:** Microscopy analysis of *C. difficile* cells carrying the fusion of the *tcdA* and *tcdR* promoters to $SNAP^{Cd}$ in strain 630 Δerm (WT), in the $\Delta tcdR$ mutant and in the $\Delta tcdR$ mutant complemented with the wild-type allele at the *pyrE* locus ($\Delta tcdR^C$). The cells were collected after 24h of growth in TY liquid medium, labeled with TMR-Star and examined by phase contrast (PC) and fluorescence microscopy to monitor SNAP production. The merged images show the overlap between the TMR-Star (red) and the auto-fluorescence (green) channels. The

patterns are representative of the expression patterns observed for the different fusions. Yellow arrows point to vegetative cells with expression, white arrows point to sporulating cells with forespore-specific expression and blue arrows point to sporulating cells with whole sporangium expression. Scale bar: 1 μm . Below is indicated the quantitative analysis, in percentage, of the distribution of the SNAP signal in vegetative cells and sporulating cells. For sporulating cells the percentages are presented for cells with fluorescence signal in the entire sporangium (red cells) and for cells with a fluorescence signal confined at the forespore (red forespore). The number of cells analyzed for each strain, n , is as follows: WT with $P_{tcdA}\text{-SNAP}^{Cd}$, $n = 918$; $tcdR^C$ with $P_{tcdA}\text{-SNAP}^{Cd}$, $n = 820$; WT with $P_{tcdR}\text{-SNAP}^{Cd}$, $n = 1456$; $\Delta tcdR$ with $P_{tcdR}\text{-SNAP}^{Cd}$, $n = 2246$; $tcdR^C$ with $P_{tcdR}\text{-SNAP}^{Cd}$, $n = 2688$. **B**: Quantitative analysis of the fluorescence intensity (FI., in Arbitrary Units, AU) of the SNAP signal per cell in the forespore for the $tcdR$ fusion and in vegetative cells for the $tcdA$ fusion, in the WT or in the $\Delta tcdR$ mutant strain. The experiment was performed in triplicate and representative results are shown.

3.4.3. Expression of the PaLoc genes in vegetative cells and in whole sporangia, but not in the forespore, is σ^D -dependent

A σ^D -dependent promoter was previously mapped upstream of *tcdR* (Aubry *et al.*, 2012, El Meouche *et al.*, 2013, McKee *et al.*, 2013) (Figure 3.6A). Before, we have observed that σ^D , and consequently its regulators, are essential to regulate the toxin expression levels in vegetative cells (see Chapter 2). To explore the relevance of this promoter for *tcdR* expression in the forespore we fused a fragment containing only this promoter to the SNAP^{Cd} reporter ($P_{tcdR-A}\text{-SNAP}^{Cd}$; Figure 3.5A). As expected, single cell analysis revealed that P_{tcdR-A} , reduced the fraction of vegetative cells in which expression was detected (Figure 3.5B), suggesting that the full-length transcriptional fusion contains elements important for expression in these cells, most likely through auto-regulation via TcdR. While no whole-sporangium expression pattern was detected using P_{tcdR-A} , the percentage of forespores showing expression, even when the regulatory region only had the σ^D promote, did not differ significantly from that obtained with

the full-length fusion (Figure 3.5B). Importantly, the intensity of the fluorescence signal in the forespore also did not differ significantly from that obtained with the full-length fusion (Figure 3.5C). Together, these results indicate that the region encompassing the σ^D promoter is sufficient for expression of *tcdR* in the forespore. σ^D , however, is not known to be directly involved in sporulation, leading us to hypothesize that other, sporulation-specific, regulators could recognize sequences within the P_{tcdR-A} fragment.

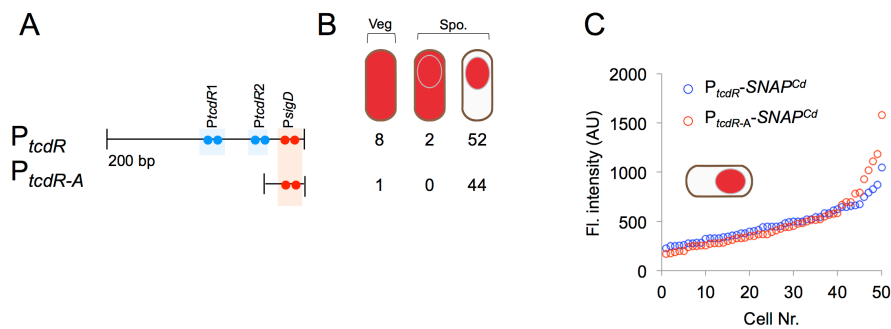


Figure 3.5. Deletion analysis of the TcdR regulatory region. **A:** Schematic representation of the fragments from *tcdR* regulatory region fused to the reporter $SNAP^{Cd}$. Each construct was inserted in strain 630 Δerm (WT). P_{tcdR1} and P_{tcdR2} represent the promoters recognized by TcdR (blue dots) and P_{sigD} represents the promoter recognized by σ^D (red dots). **B:** Quantitative analysis, in percentage, of the distribution of the SNAP signal in vegetative and sporulating cells. For sporulating cells a whole sporangium and a forespore-specific pattern were scored. The number of cells analyzed for each fusion, n , is as follows: $P_{tcdR^*}-SNAP^{Cd}$, $n = 371$; $P_{tcdR-A}-SNAP^{Cd}$, $n = 584$; **C:** Quantitative analysis of the fluorescence intensity (FI, in Arbitrary Units, AU) of the SNAP signal per cell in the forespore in strains expressing the various deletion constructs, as indicated. The experiment was performed in triplicate and representative results are shown.

To directly test whether the σ^D promoter was involved in forespore-specific expression of *tcdR*, we introduced point mutations in the -10 region of the promoter in the context of a full-length fusion ($P_{tcdR^*}-SNAP^{Cd}$) (Figure 3.6A). Additionally, we introduced the P_{tcdR-}

and P_{tcdA} -SNAP^{Cd} fusions into a previously constructed *sigD* mutant (El Meouche *et al.*, 2013). Single cell analysis showed that, as previously studied at Chapter 2 the fraction of vegetative cells expressing *tcdA* was reduced from 70% to 8% in a *sigD* mutant (Figure 3.6B). Moreover, the whole-sporangia pattern was reduced from 42% to 13% and the intensity of the signal per sporangium, measured in the mother cell, was significantly reduced as compared to the WT ($p < 0.0001$) (Figure 3.6C). In this experiment, we failed to observe the forespore-specific pattern for *tcdA*; we do not presently know if this results from an indirect effect of the *sigD* mutation on sporulation. Deletion of *sigD* abolished expression of *tcdR* in vegetative cells (Figure 3.6B). Importantly, however, forespore-specific expression of *tcdR* persisted, and the intensity of the fluorescence signal per forespore did not differ from the WT (Figure 3.6B and 3.6C). The results are consistent with the view that σ^D is a key determinant for TcdR production in vegetative cells (see Chapter 2), which in turn drives expression of *tcdA* and other PaLoc genes (El Meouche *et al.*, 2013). The results also suggest that the whole sporangia pattern of expression results from either persistent activity of σ^D in the mother cell and the forespore following asymmetric division, or that TcdR, produced under σ^D control in pre-divisional cells partitions between the two cells following division.

The point mutations introduced in the -10 region of the σ^D promoter, abolished expression in vegetative cells, consistent with the proposed role of σ^D in driving production of TcdR (Figure 3.6B). Strikingly, the forespore-specific expression of *tcdR* when expressed from P_{tcdR^*} was also abolished (Figure 3.6B). Since the expression of *tcdR* in the forespore is not affected by deletion of *sigD* (above), we

infer that the mutations affect sequences probably recognized by a forespore-specific regulatory factor or factors.

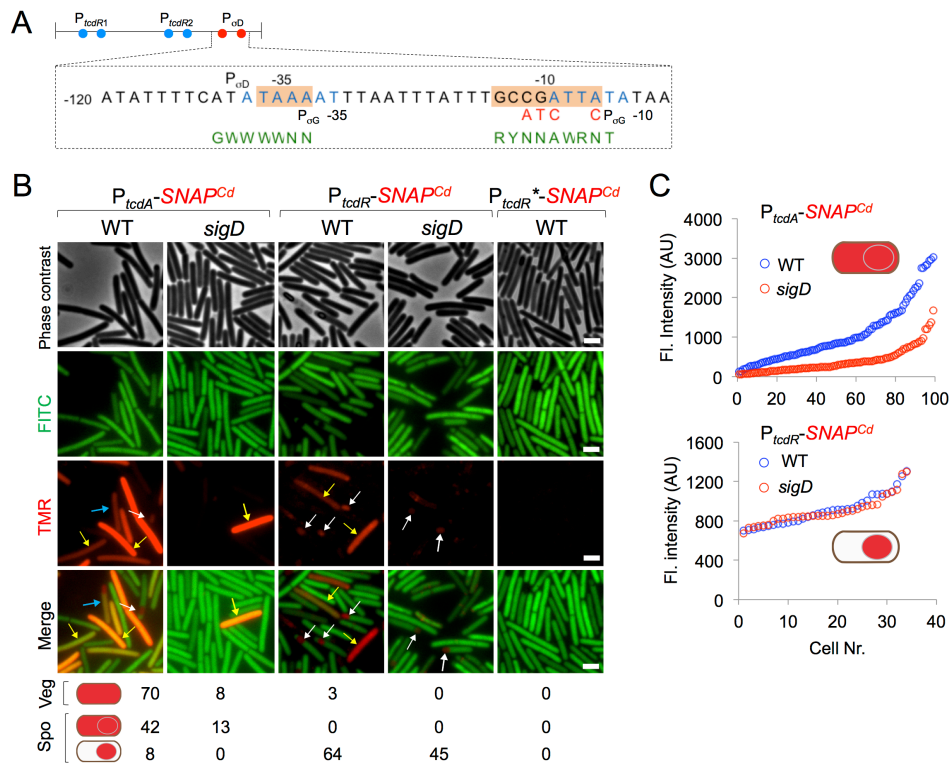


Figure 3.6. The role of σ^D in regulating toxin production during sporulation.

A: Representation of the regulatory region of the *tcdR* gene, with the -35 and -10 elements of the mapped *tcdR*-dependent promoters (blue) and the σ^D -dependent promoter (red). The point mutations introduced in the σ^D -dependent promoter are indicated in red below. The bases represented in blue indicate a putative σ^G -dependent promoter. The consensus sequence for σ^G recognition is shown in green. **B:** Microscopy analysis of *C. difficile* cells carrying the fusion of the *tcdA*, *tcdR* and *tcdR** (with point mutations in the σ^D -dependent promoter) promoters to SNAP^{Cd} in strain 630 Δ erm (WT) and in the *sigD* mutant. The cells were collected after 24h of growth in TY liquid medium, labelled with TMR-Star and examined by phase contrast (PC) and fluorescence microscopy to monitor SNAP production. The merged images show the overlap between the TMR-Star (red) and the auto-fluorescence (green) channels. The images are representative of the expression patterns observed for the different fusions. Yellow arrows point to vegetative cells with expression, white arrows point to sporulating cells with forespore-specific expression and blue arrows point to sporulating cells with whole sporangium expression. Scale bar: 1 μ m. Cells were scored according to the following

expression patterns: for vegetative cells, the percentage of cells with fluorescence signal is shown; for sporulating cells a whole-sporangia and forespore-specific pattern of expression was scored. The number of cells analyzed for each strain, n , is as follows: WT with $P_{tcdA}^{SNAP^{Cd}}$, $n = 856$; *sigD* with $P_{tcdA}^{SNAP^{Cd}}$, $n = 1550$; WT with $P_{tcdR}^{SNAP^{Cd}}$, $n = 670$; *sigD* mutant with $P_{tcdR}^{SNAP^{Cd}}$, $n = 3902$; WT with $P_{tcdR}^{SNAP^{Cd}}$, $n = 268$. **C:** Quantitative analysis of the fluorescence intensity (Fl., in Arbitrary Units, AU) of the SNAP signal per cell in the forespore for the *tcdR* fusion and in vegetative cells for the *tcdA* fusion, in the WT or in the *sigD* mutant. The experiment was performed in triplicate and representative results are shown.

3.4.4. Expression of the PaLoc genes in the forespore is under σ^G control

Having established that the region required for forespore-specific expression of *tcdR* overlaps the σ^D -recognized promoter, we next aimed at identifying the forespore-specific factors involved. We started by evaluating *tcdR* expression in *spo0A*, *sigG*, *sigE*, *sigF* and *sigK* mutants, all of which unable to sporulate (Fimlaid *et al.*, 2013, Pereira *et al.*, 2013, Saujet *et al.*, 2013) (Figure 3.7). In the *spo0A* mutant, vegetative expression is maintained, although the number of cells expressing *tcdR* was reduced to 0.6% as compared to 7% for the WT (Figure 3.7). Thus, under our conditions, Spo0A seems to positively control toxin production at least in a fraction of vegetative cells, consistent with previous reports (Saujet *et al.*, 2011, Underwood *et al.*, 2009). We note, however, that the effect of Spo0A on toxin production may differ among epidemic strains and according to growth conditions (Mackin *et al.*, 2013, Pettit *et al.*, 2014, Rosenbusch *et al.*, 2012). In the *sigF* mutant, which is arrested just after asymmetric division (Pereira *et al.*, 2013), *tcdR* expression was not detected (Figure 3.7). Unlike the *spo0A* mutation, which is pleiotropic, deletion of *sigF* only blocks sporulation. The decrease in vegetative cells expressing *tcdR* in the *sigF* mutant may suggest a contribution from cells outgrowing from

germinated spores (see also below). Importantly, no forespore-specific expression was observed in a *sigF* mutant, consistent with the requirement for a forespore-specific factor, either σ^F or a σ^F -controlled factor in *tcdR* expression in these cells.

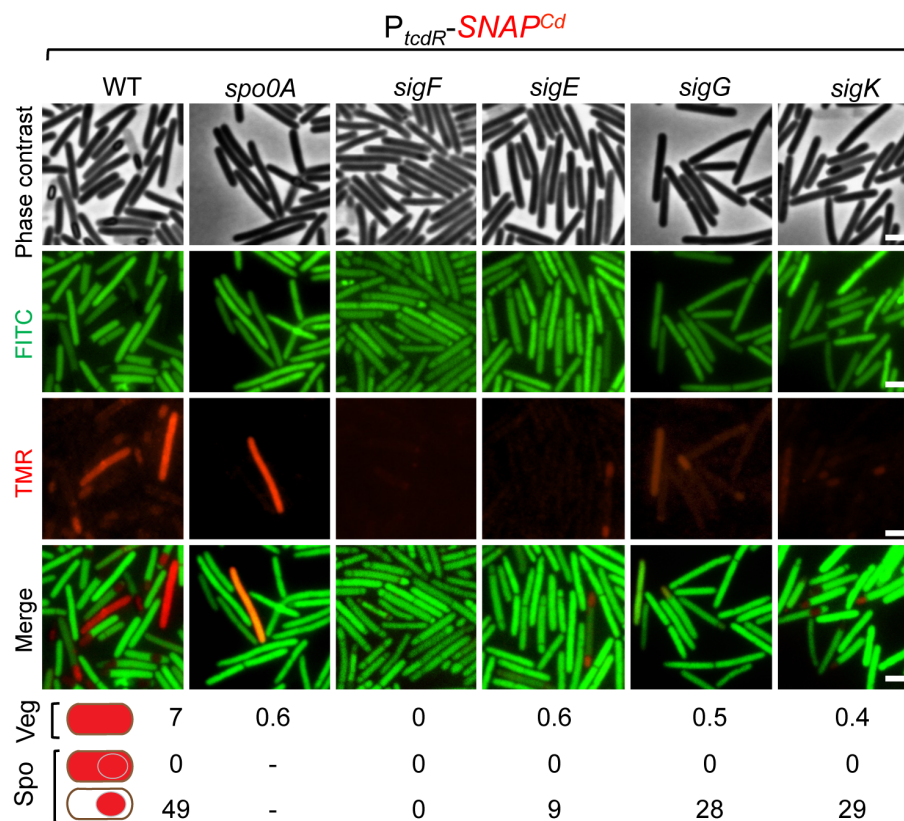


Figure 3.7. Expression of *tcdR* in sporulation mutants. **A:** Microscopy analysis of *C. difficile* cells carrying the fusion of the *tcdR* promoter to SNAP^{Cd} in strain 630 Δ *erm* (WT) and in the *spo0A*, *sigF*, *sigE*, *sigG* and *sigK* mutants. The cells were collected after 24h of growth in TY liquid medium, labeled with TMR-Star and examined by phase contrast (PC) and fluorescence microscopy to monitor SNAP production. The merged images show the overlap between the TMR-Star (red) and the auto-fluorescence (green) channels. The images are representative of the expression patterns observed for the different fusions. Scale Bar: 1 μ m. Expression patterns were scored as described in the legend for figure S2 and their percentages indicated. The number of cells analyzed for each strain, *n*, is as follows: WT, *n* = 983; *spo0A*, *n* = 1137; *sigE*, *n* = 1256; *sigG*, *n* = 1323; *sigK* = 906. The experiment was performed in triplicate and representative results are shown.

Since *tcdR* expression is mostly detected following engulfment completion (above), we anticipated that σ^G could be required for the forespore-specific expression of *tcdR*. Consistent with this inference, in cells unable to produce the early mother cell regulator σ^E , which governs assembly of a transmembrane complex required for the activity of σ^G in the forespore following engulfment completion (Fimlaid *et al.*, 2015b, Serrano *et al.*, 2016a), *tcdR* expression was reduced to 9% as compared to 49% for the wild type strain (Figure 3.7). In a *sigG* mutant, which completes the engulfment process but does not proceed further into spore morphogenesis (Pereira *et al.*, 2013), expression of *tcdR* was detected in a lower fraction of the forespores (28% as compared with 49% for the WT) and the intensity of the fluorescence signal per forespore was also lower (Figure 3.7). We presume that expression of *tcdR* is reduced but not abolished in *sigG* sporangia because σ^F can utilize promoters normally recognized by σ^G , the two sigma factors having very similar consensus recognition sequences (Pereira *et al.*, 2013). The consensus sequences for promoter recognition by σ^G are very similar in *B. subtilis* and *C. difficile*, and include the -35 and -10 elements GAATAAAAT and ATAATA, with a spacing of 15 bp (de Hoon *et al.*, 2010, Saujet *et al.*, 2013, Wang *et al.*, 2006). Sequences that approach this consensus overlap the -35 and -10 elements of the σ^D promoter upstream of *tcdR* (Figure 3.6, nucleotides in blue).

We also examined expression of *tcdR* in cells unable to produce the last, mother cell-specific regulatory protein σ^K . Unexpectedly, we found forespore-specific expression of *tcdR* to be reduced in *sigK* sporangia in which engulfment had been completed (Figure 3.7). This suggests the existence of a late signalling pathway that originates in

the mother cell to influence gene expression in the forespore (see also the Discussion). In any event, the results suggest that σ^G has a role in the late, post-engulfment expression of *tcdR* in the forespore.

3.4.5. A type II coherent feed-forward loop with AND gate logic controls expression of the PaLoc genes in the forespore

Our results suggest the involvement of σ^G in the forespore-specific expression of *tcdR*, and a possible σ^G -dependent promoter overlapping the σ^D promoter is present in the P_{tcdR-A} - $SNAP^{Cd}$ fusion, for which forespore-specific expression is seen (Figure 3.5 and 3.7; above). Similar to what occurs in *B. subtilis*, however, the expression of a subset of σ^G -dependent genes additionally requires SpoVT (Rhayat *et al.*, 2009, Saujet *et al.*, 2013). We thus monitored expression of *tcdR* in the background of a previously constructed *spoVT* mutant (Saujet *et al.*, 2013). Single cell analysis revealed forespore-specific expression of *tcdR* in 26% of the *spoVT* sporangia scored, similar to the fraction seen for the *sigG* mutant and significantly lower than for the WT (69%; Figure 3.8A and B). Moreover, the intensity of the fluorescence signal per forespore was lower than the WT in the *spoVT* mutant ($p < 0.01$), similarly to what is observed for the *sigG* mutant ($p < 0.0001$) (Figure 3.8C). These results support the idea that SpoVT, together with σ^G , is involved in regulating the expression of *tcdR* in the forespore.

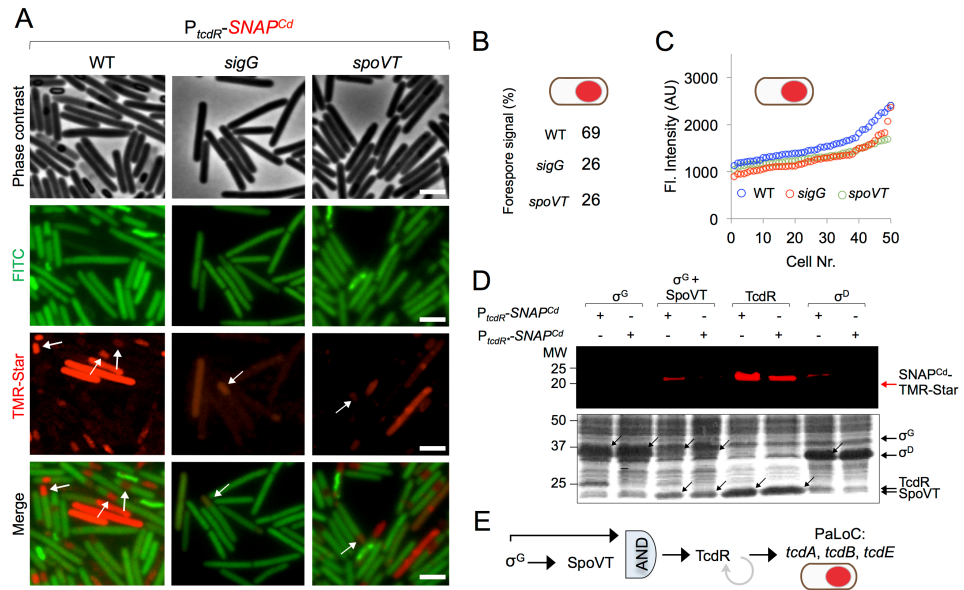


Figure 3.8. Induction of *tcdR* by σ^G and SpoVT in *E. coli*. **A:** Microscopy analysis of *C. difficile* cells carrying the fusion of the *tcdR* promoters to *SNAP^{Cd}* in strain 630 Δ *erm* (WT) and in the *spoVT* mutant. The cells were collected after 24h of growth in TY liquid medium, labeled with TMR-Star and examined by phase contrast (PC) and fluorescence microscopy to monitor SNAP production. The merged images show the overlap between the TMR-Star (red) and the auto-fluorescence (green) channels. The images are representative of the expression patterns observed for the different fusions. White arrows point to sporulating cells with forespore-specific expression. Scale bar: 1 μ m. **B:** Shows the percentage of sporangia with a forespore-specific signal for the indicated strains. The number of cells analyzed for each strain, *n*, is as follows: WT, *n* = 450; *sigG*, *n* = 878; *spoVT*, *n* = 540; **C:** Quantitative analysis of the fluorescence intensity (FI, in Arbitrary Units, AU) of the SNAP signal in the forespore for the $P_{tcdR}-SNAP^{Cd}$ fusion in the WT or congenic *sigG* and *spoVT* mutants. **D:** *E. coli* BL21(DE3) strains containing the plasmids with the *tcdR* promoter or the *tcdR** promoter fused to *SNAP^{Cd}* were transformed with plasmids for the induction of σ^G , σ^G and SpoVT, TcdR or σ^D . The sigma factors were induced through auto-induction and whole cell extracts were prepared. The extracts were resolved by SDS-PAGE, and scanned on a fluoroimager (top). Induction of the sigma factors is indicated by black arrows in the Coomassie stained gel. **E:** Representation of the coherent feed-forward loop formed by σ^G and SpoVT, with AND gate logic, that leads to delayed production of TcdR in the forespore, and the expression of the PaLoc genes *tcdA*, *tcdB*, *tcdE* and *tcdE*.

Next, we wanted to test whether σ^G and SpoVT could directly utilize a promoter in the *tcdR* regulatory region. With this goal, σ^G was overproduced alone or in combination with SpoVT, from the IPTG-inducible T7lac promoter, in *E. coli* cells bearing a compatible plasmid with either a copy of the wild-type *tcdR* regulatory region, or a copy bearing the point mutations in the -10 region of the *sigD* promoter, both fragments fused to *SNAP^{Cd}* reporter. Additionally, expression of the fusions was also monitored in *E. coli* cells overproducing TcdR or σ^D (Figure 3.8D). Accumulation of SNAP was used as a proxy for the utilization of the WT or mutant form of the *tcdR* promoter by the various regulatory proteins. As expected, induction of *tcdR* resulted in high level production of SNAP (Figure 3.8D). Also, as expected, induction of *sigD* expression led to SNAP production from the WT but not from the mutant promoter (Figure 3.8D). Furthermore, when only *sigG* was induced, SNAP could not be detected from either the WT or the mutant promoter. Strikingly, however, when *sigG* and *spoVT* were co-expressed, SNAP production was clearly increased from the WT but not from the mutant promoter (Figure 3.8D).

This *in vitro* assay suggests that together with SpoVT, σ^G is able to induce SNAP production by recognizing a promoter that overlaps the one utilized by σ^D in vegetative cells. In *B. subtilis*, σ^G and SpoVT define coherent and incoherent feed-forward loops with AND gate logic that result in a pulse of expression of early σ^G -dependent genes, and delayed expression of late genes, respectively (Wang *et al.*, 2006). σ^G and SpoVT may function in a similar way in *C. difficile* forming a coherent feed-forward loop with AND gate logic (Fimlaid *et al.*, 2013, Pereira *et al.*, 2013, Saujet *et al.*, 2013) (Figure 3.8E), which delays toxin production in the forespore.

3.5. DISCUSSION

Until now, it was unknown whether the toxin producing and spore producing cells were the same, or whether toxinogenesis and sporogenesis occurred in different sub-populations of cells. We show here that toxin- and spore-producing cells overlap because: *i*) expression of *tcdR* which initially takes place in pre-divisional cells, persists in the mother cell following asymmetric division, either because σ^D remains active and/or because sufficient TcdR is partitioned into this cell. If most of the toxin-expressing cells enter sporulation then mother cell lysis is expected to make an important contribution to toxin release. Thus, a previously proposed scenario of self-destructive cooperation in which a fraction of vegetative cells undergoes lysis, releasing the toxins for the benefit of the remaining, may be complemented by lysis of the mother cell upon completion of spore differentiation (Ackermann *et al.*, 2008); *ii*) expression of *tcdR* is induced in the forespore under the control of both σ^G and SpoVT which together utilize a promoter overlapping the σ^D -dependent *tcdR* promoter (Figure 3.1). SpoVT is conserved among sporeformers (Abecasis *et al.*, 2013, Galperin *et al.*, 2012), and positively regulates a subset of σ^G -dependent genes in both *B. subtilis* and in *C. difficile* (Bagyan *et al.*, 1996, Saujet *et al.*, 2013). SpoVT belongs to a family of transcription factors that recognizes DNA sequences with no apparent consensus (Asen *et al.*, 2009, Dong *et al.*, 2004, Sullivan *et al.*, 2008) and thus the sequence or sequences it binds to in the *tcdR* regulatory region remain unknown.

The population of sporulating cells is heterogeneous with respect to expression of the *tcdA* and *tcdB* genes. In some sporulating cells, expression of the toxin genes is detected in both the forespore and

the mother cell, while in others expression is only detected in the forespore or in none of the two cells. The repression exerted over transcription of *tcdR* may not be relieved in all of the pre-divisional cells, as discussed above (Figure 3.2), and in those cells, expression of *tcdR*, *tcdA* and *tcdB* will not take place in the mother cell. Thus, expression of the toxin-encoding genes in the mother cell relies on memory of a previous state, the vegetative state, referred to as hysteresis (Veening *et al.*, 2008). In the forespore, σ^G and SpoVT may only activate expression of *tcdR* in a fraction of the forespores, where a threshold for transcriptional activation is reached. In *B. subtilis*, σ^G and SpoVT define a coherent feed-forward loop with AND gate logic that delays expression of some genes (Eichenberger *et al.*, 2004), reviewed by (Alon, 2007). A similar network motif is likely to operate in *C. difficile* (Fimlaid *et al.*, 2015b, Pereira *et al.*, 2013, Saujet *et al.*, 2013). This regulatory scheme is expected to delay toxin production until the late stages in forespore development are reached.

SpoVT is a dimer of dimers, with each monomer formed by an N-terminal DNA binding domain and a C-terminal GAF (cGMP-specific and cGMP-stimulated phosphodiesterases, *Anabaena* adenylate cyclases, and *Escherichia coli* FhlA) domain. GAF domains act as sensory modules that can bind linear and cyclic nucleotides, porphyrin rings, as well as small signalling molecules such as homoserine lactones (Asen *et al.*, 2009). The GAF domain of *B. subtilis* CodY, and most likely that of its *C. difficile* counterpart, binds GTP and the branched-chain amino acids isoleucine and valine serves as a sensor of the nutrient state of the cell (Levdikov *et al.*, 2017). Whether SpoVT binds a specific ligand is unclear, as some GAF domains may function exclusively to mediate dimerization (Asen *et al.*, 2009). In any

event, it is tempting to speculate that yet unknown signals control the activity of SpoVT and thus the level of toxin production in the forespore. No cell-cell signalling pathway is known that links the activity of the late mother cell-specific regulator σ^K to the activity of σ^G in the forespore, and yet we found a reduction in the forespore expression of *tcdR* and *tcdA* in *sigK* sporangia. It is also tempting to suggest that the activity of σ^K may be necessary to convey to the forespore signals that influence the activity of SpoVT.

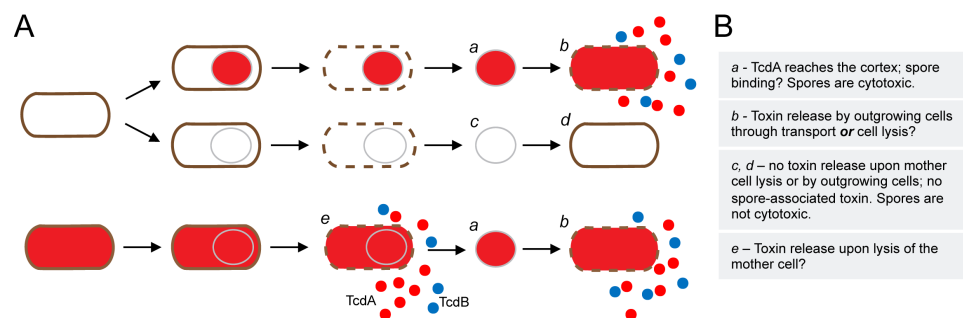


Figure 3.9. Functionally differentiated spore populations. **A:** Schematic representation of vegetative cells expressing the toxin-encoding genes (red filling) or not and their patterns of expression during sporulation (whole sporangium or forespore-specific). Five different possible functional classes are highlighted (*a* to *e*). The functional properties of classes *a* to *e* are summarized in panel **B** (see also the Discussion).

The different patterns of *tcdR* and *tcdA* expression in sporulating cells suggest the existence of functionally different populations of cells and spores (Figure 3.9). It seems likely that mother cell expression provides an important route for toxin release upon lysis of this cell, coupling toxin release to completion of spore development. Forespore-specific expression will likely result in the accumulation of the toxins in the forespore cytoplasm. Possibly, following spore

germination, the toxins can be quickly released from at least some of the outgrowing cells and we speculate that *tcdE* which, as we show, is expressed in the forespore, might be involved (Figure 3.9). Also possibly, cells outgrowing from spores expressing TcdE in the forespore may lyse. It has been proposed that low levels of toxin cause inflammation, which in turn promotes colonization by vegetative cells during infection (Kasendra *et al.*, 2014). Toxin release by outgrowing cells could thus play a role in colonization by newly formed vegetative cells (Figure 3.9). Additional studies are needed to test whether the heterogeneity in toxin production detected during sporulation *in vitro* also occurs *in vivo* and if so, how it influences colonization and disease progression by different strains.

There are other pathogens where toxin production is related with sporulation. Production of enterotoxin by *C. perfringens*, for example, is under the control of σ^E and σ^K , which leads to accumulation of the toxin in the mother cell and its release into the intestinal lumen when this cell lyses (Harry *et al.*, 2009, Li & McClane, 2010, Zhao & Melville, 1998). Expression of toxin genes by sporulating cells may be a widespread strategy both for toxin delivery and release through lysis of the mother cell.

3.6. ACKNOWLEDGMENTS

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Chapter 4

Spore localization of the *C. difficile* TcdA and TcdB toxins

Author contributions:

C. P. Cassona performed all experiments except the construction of the $\Delta tcdR$ and the $\Delta tcdA\Delta tcdB$ mutants (by Sara Ramalhete).

This chapter contains data from the submitted manuscript:

Expression of the toxin-encoding genes by sporulating cells of *Clostridium difficile* leads to the formation of cytotoxic spores

4.1. SUMMARY

C. difficile is an important nosocomial pathogen responsible for a spectrum of intestinal diseases mainly associated with antibiotic treatment. *C. difficile* is an obligate anaerobe that produces oxygen resistant spores that are essential for persistence and transmission of the organism and linked to disease recurrence. Ingested spores germinate in the intestine, which allows the establishment of a population of cells that produces two toxins, TcdA and TcdB. These two toxins have glucosyltransferase activity and are responsible for the disease symptoms. Here we show that TcdA associates with the surface of mature spores, a localization it can reach from either the mother cell or the forespore. We further show that wild type but not $\Delta tcdR$ spores or $\Delta tcdA\Delta tcdB$ spores are cytotoxic to HT29 and Vero cells, indicating that the toxins associate with spores in an active form. The production of toxins by sporulating cells and the association of toxins with spores suggest that spores act as toxin-delivery vehicles, a strategy that may be widespread among pathogenic sporeforming bacteria.

4.2. INTRODUCTION

Clostridium difficile, recently reclassified as *Clostridioides difficile* (Lawson *et al.*, 2016) is an obligate anaerobic endospore-forming bacterium (Burns *et al.*, 2010). *C. difficile* infection (CDI) is the main cause of health-care associated diarrhea but community-associated dissemination is an increase concern (Abt *et al.*, 2016, Rupnik *et al.*, 2009). *Clostridia*, like *Bacillus* species, have the ability to form highly resistant, metabolic inert endospores (McKenney *et al.*, 2013, Paredes-Sabja *et al.*, 2014). The oxygen-resistant spores are the main infectious vehicles for *C. difficile*. After ingestion, spores will germinate in the intestine, in close association with the mucosa (Sorg & Sonenshein, 2008, Sorg & Sonenshein, 2009). Spore adherence is likely to be mediated through proteins at the spore surface and may also involve a spore polar appendage (Calderon-Romero *et al.*, 2018, Panessa-Warren *et al.*, 2007). Germination will give rise to a population of cells that will produce the toxins responsible for the disease symptoms, and at least a fraction of these cells will enter sporulation ((Ransom *et al.*, 2018); Chapter 3) and produce spores.

C. difficile spores allow for environmental dissemination. In fact the spores are persistently maintained in health care facilities due to the difficulties in their elimination (Barra-Carrasco & Paredes-Sabja, 2014, Lawley *et al.*, 2009). Strains that are not able to sporulate are not able to persist in the host and cannot be horizontally transmitted (Deakin *et al.*, 2012), in line with the idea that spores are the main persistence, transmission and recurrence factor.

The bacterial spore is a dormant, highly resistant cell able to withstand harsh environmental conditions and to persist in the

environment in a viable form for long periods of time. The resistant properties of spores are in part conferred by its layered architecture (Setlow, 2006, Setlow, 2014b) (Figure 4.1). The spore is composed of a core compartment, where the genetic material is contained, surrounded by an inner membrane with low permeability to small molecules due to lipid immobility, protecting the DNA (Cowan *et al.*, 2004); the core is surrounded by a germ cell wall that will become the cell wall of the outgrowing cells; the cortex, a thick layer of modified peptidoglycan (Diaz *et al.*, 2018, Popham & Bernhards, 2015, Popham *et al.*, 1996) that is important to maintain spore dehydration and dormancy (Warth & Strominger, 1972); and the coat, which is a proteinaceous layer assembled at the forespore outer membrane important for chemical and heat resistance and that also influences the environmental interactions of spores, including the ability to respond to germinants (Setlow, 2006). Some *Bacillus* and *Clostridium* species have an extra external layer, the exosporium (Stewart, 2015). An exosporium is also present in spores of *C. difficile*. Recent work has implicated this spore surface layer in cell adhesion and pathogenesis (Barra-Carrasco *et al.*, 2013, Calderon-Romero *et al.*, 2018, Mora-Uribe *et al.*, 2016, Pizarro-Guajardo *et al.*, 2014).

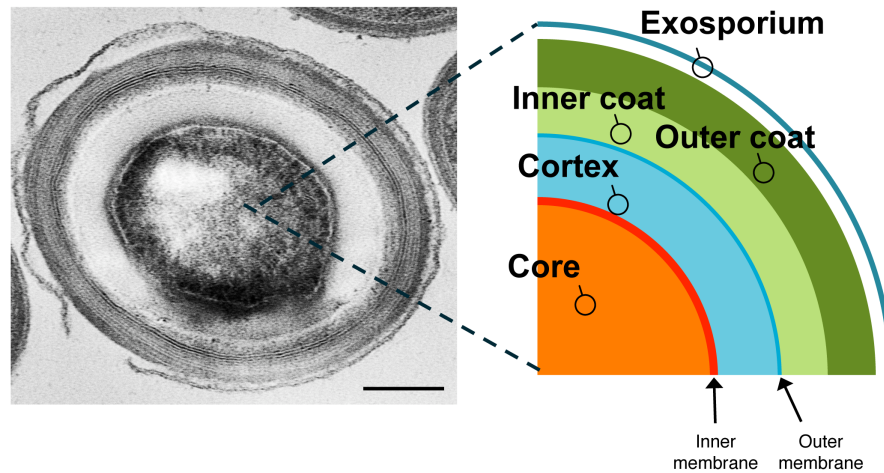


Figure. 4.1. Spore ultrastructure. **A:** TEM image of a *C. difficile* spore. Scale bar: 200nm. **B:** Schematic representation of the *C. difficile* spore. Adapted from (Setlow, 2003).

In *B. subtilis* the coat is a complex protenaceous layer formed by over 80 proteins (Henriques & Moran, 2007). Only 18 orthologs of these proteins have been found in *C. difficile* (Henriques & Moran, 2007, Lawley *et al.*, 2009). An increase number of studies have given clues about how this structure is assembled in *C. difficile*, with some morphogenetic proteins being conserved like SpoIVA and an analog of SpoVID, designated SipL (Putnam *et al.*, 2013). Furthermore, proteomic studies from *C. difficile* spore coat have identified proteins with important enzymatic activities, including manganese catalases, rubrerythrins and oxireductases (Abhyankar *et al.*, 2013, Permpoonpattana *et al.*, 2013, Permpoonpattana *et al.*, 2011b).

While the spore is the main transmission and persistence factor, the two cytotoxins produced by *C. difficile*, TcdA and TcdB, are the main virulence factors. The genes coding for TcdA and TcdB are located in a pathogenicity locus, or PaLoc (Deneve *et al.*, 2009,

Hundsberger *et al.*, 1997, Kuehne *et al.*, 2011). TcdA and TcdB are glucosyltransferases that belong to the family of Large Clostridial Toxins (LCTs). Like other LCTs, TcdA and TcdB show three main functional regions: the N-terminal glucosyltransferase domain (GTD) that acts by monoglucosylating, thereby inactivating, small Rho GTPases, which interferes with essential signaling pathways in the cell (Jank & Aktories, 2008, Voth *et al.*, 2005); an hydrophobic central region required for translocation across the eukaryotic cell membrane; and the C-terminal region which comprises repetitive oligopeptides (CROPs), important for receptor binding (Frisch *et al.*, 2003, Genisyurek *et al.*, 2011, von Eichel-Streiber & Sauerborn, 1990). The toxins enter host cells via receptor-mediated endocytosis (Florin & Thelestam, 1983, Mitchell *et al.*, 1987) and several TcdA receptors have been identified (Na *et al.*, 2008, Pothoulakis *et al.*, 1996).

Sporulation and toxinogenesis are thus two essential processes for *C. difficile* pathogenesis. Having found that the regulator TcdR is expressed during sporulation in the forespore (Chapter 3), we investigated here whether the toxins associated with mature spores. We show that TcdA associates with the spore surface layers. We also show that wild-type spores, including those of an epidemic strain of ribotype 027, are cytotoxic when assayed against HT29 and Caco-2 cells, suggesting that both TcdA and TcdB associate with mature spores in an active form. Thus, the infectious spores of *C. difficile* are toxin delivery vehicles. We discuss the implications of these findings for the role of spores during infection.

4.3. MATERIALS AND METHODS

Growth conditions and general methods. Bacterial strains and their relevant properties are listed at table 4.1. The *E. coli* strain DH5 α (Bethesda Research laboratories) was used for molecular cloning, while HB101 (RP4) was used as the donor strain in *C. difficile* conjugation experiments (Hussain *et al.*, 2005). Luria-Bertani medium was routinely used for growth and maintenance of *E. coli*. When appropriate, ampicillin (100 μ g/mL) or chloramphenicol (15 μ g/mL) was added to the culture medium. The *C. difficile* strains used in this study are congeneric derivatives of the wild-type strain 630 Δ erm (Hussain *et al.*, 2005) or R20291 (Anaerobe Reference Laboratory, Cardiff, Wales, United Kingdom) and were routinely maintained anaerobically (5% H₂, 15% CO₂, 80% N₂) at 37°C in brain heart infusion (BHI) medium (Oxoid) supplemented with L-cysteine (0.1%) and yeast extract (5 mg/mL) (BHIS) (Wilson *et al.*, 1982) and with cefoxitin (25 μ g/mL) and thiamphenicol (15 μ g/mL) when necessary. For toxin assays, tryptone yeast extract (TY, 30 g/L tryptone; 20 g/L yeast extract) was used supplemented with thiamphenicol (15 μ g/mL) when necessary. A defined minimal media (CDMM) (Karasawa *et al.*, 1997) with 1% agar was used as uracil-free medium when performing genetic selections. The minimal medium was supplemented with 5-Fluoroorotic acid (2 mg/mL) and uracil (5 μ g/mL) when appropriate.

Transcriptional fusions of the *tcdA* promoter to the *SNAP^{Cd}* reporter. For the construction of a transcriptional P_{*tcdA*}-*SNAP^{Cd}*, fusion, the *tcdA* promoter region was PCR-amplified using genomic DNA from *C. difficile* 630 Δ erm with the primer pair P_{*tcdA*}-*SNAP*-

EcoRI-Fw/ P_{tcdA}-SNAP-XhoI-Rev to produce a 520bp product. The promoter was cloned between the EcoRI/ XhoI sites of pFT47 (Pereira *et al.*, 2013), upstream of *SNAP^{Cd}*, and this yielded pMS470. The resulting plasmid was introduced into *E. coli* HB101 (RP4) yielding strain AHEC144 and subsequently transferred to *C. difficile* 630 Δ *erm*, producing strain AHCD668 (Table 4.1).

Δ tcdR mutant construction through allelic exchange. Allelic exchange using the *pyrE* alleles (Ng *et al.*, 2013) was used to construct in-frame deletions of the *tcdR* gene in *C. difficile* 630 Δ *erm* and R20291. *pyrE* negative strains previously constructed (Ng *et al.*, 2013) were used in order to allow for a positive/negative selection. The plasmids pSR3, derived from pMTL-YN3, and pSR2, derived from pMTL-YN4, were used to construct the Δ *tcdR* mutant in 630 Δ *erm* and R20291, respectively, where the allele exchange cassette composed of a left-hand homology arm (LHA) and a right-hand homology arm (RHA) was cloned between the SbfI and AscI sites. The allele exchange cassette was obtained by overlap extension PCR using the primer pair tcdR-AscI-Fw/tcdR-SOE-Rev for the LHA originating a 631 bp fragment and tcdR-SOE-Fw/tcdR-SbfI-Rev for the RHA originating a 522 bp fragment. The final PCR was obtained with the primer pair tcdR-AscI-Fw/ tcdR-SbfI-Rev. DNA sequencing was performed to verify all plasmid constructs. The plasmids obtained were transformed into *E. coli* HB101 (RP4) yielding strains AHEC239 (Δ *tcdR* in 630 Δ *erm*) and AHEC227 (Δ *tcdR* in R20291). These strains were subsequently mated with *C. difficile* 630 Δ *erm* Δ *pyrE* and R20291 Δ *pyrE* (Ng *et al.*, 2013). *C. difficile* transconjugants were selected by subculturing on BHIS agar

containing thiamphenicol (15 µg/mL) and cefoxitin (25 µg/mL) and re-streaked twice in this medium for 630 Δ *erm* and four times for R20291. Colonies that appeared were screened by colony PCR using 5% chelex resin (Sigma–Aldrich) to identify single-crossover mutants. For the *tcdR* gene the primer pairs YN3-vef-Fw/ *tcdR*-vef-Fw and YN3-vef-Rev/ *tcdR*-vef-Rev for 630 Δ *erm* or YN4-vef-Fw/ *tcdR*-vef-Fw and YN4-vef-Rev/ *tcdR*-vef-Rev for R20291 were used. The single-crossover mutants identified were streaked onto CDMM (Karasawa *et al.*, 1997) with 1% agar supplemented with 5-Fluoroorotic acid (2 mg/mL) and uracil (5 µg/mL) in order to select for plasmid excision. The isolated FOA-resistant colonies were screened by colony PCR using the primer pair *tcdR*-vef-Fw/ *tcdR*-vef-Rev, which flank the upstream and downstream homology regions. The double-crossover mutants that were thiamphenicol sensitive (AHCD811 in 630 Δ *erm* and AHCD1093 in R20291) were reverted to a PyrE⁺ phenotype through conjugation with plasmid pMTL-YN1 (for 630 Δ *erm*) and pMTL-YN2 (for R20291). The resulting colonies were streaked onto non-supplemented CDMM agar and the presence of a restored *pyrE* allele was confirmed by colony PCR using the primer pair *pyrE*-vef-Fw/*pyrE*-vef-Rev. This screen led to the isolation of strains AHCD820 (for 630 Δ *erm*) and AHCD1102 (for R20291).

Δ *tcdA* Δ *tcdB* mutant construction through allelic exchange. The same methodology used to construct the Δ *tcdR* deletion mutant was used to construct the double mutant Δ *tcdA* Δ *tcdB*. The plasmid pSR7 and pSR8 were used to delete *tcdA* and *tcdB*, respectively. For pSR7 the allele exchange cassettes were obtained by overlap extension PCR

using the primer pair *tcdA*-AscI-Fw/*tcdA*-SOE-Rev for the LHA originating a 626 bp fragment and *tcdA*-SOE-Fw/ *tcdA*-SbfI-Rev for the RHA originating a 544 bp fragment. The final PCR was obtained with the primer pair *tcdA*-AscI-Fw/ *tcdA*-SbfI-Rev. For pSR8 the allele exchange cassettes were obtained by overlap extension PCR using the primer pair *tcdB*-AscI-Fw/ *tcdB*-SOE-Rev for the LHA originating a 635 bp fragment and *tcdB*-SOE-Fw/ *tcdB*-SbfI-Rev for the RHA originating a 497 bp fragment. The final PCR was obtained with the primer pair *tcdB*-AscI-Fw/ *tcdB*-SbfI-Rev. DNA sequencing was performed to verify all plasmid constructs. The plasmids obtained were transformed into *E. coli* HB101 (RP4) yielding strains AHEC498 (for $\Delta tcdA$) and AHEC499 (for $\Delta tcdB$). The *E. coli* strain for the $\Delta tcdA$ mutant construction was subsequently mated with *C. difficile* 630 $\Delta erm\Delta pyrE$. *C. difficile* transconjugants were selected by subculturing on BHIS agar containing thiamphenicol (15 μ g/mL) and cefoxitin (25 μ g/mL) and restreaked twice in this medium. Colonies that appeared were screened by colony PCR using 5% chelex resin (Sigma–Aldrich) to identify single-crossover mutants. For the *tcdA* gene the primer pairs YN3-vef-Fw/*tcdA*-vef-Fw and YN3-vef-Rev/ *tcdA*-vef-Rev were used. The single-crossover mutants identified were streaked onto CDMM (Karasawa *et al.*, 1997) with 1% agar supplemented with 5-Fluoroorotic acid (2 mg/mL) and uracil (5 μ g/mL) in order to select for plasmid excision. The isolated FOA-resistant colonies were screened by colony PCR using the primer pair *tcdA*-vef-Fw/ *tcdA*-vef-Rev, which flank the upstream and downstream homology regions. The double-crossover mutants that were thiamphenicol sensitive (AHCD999) was used to mate with the *E. coli* strain to construct the $\Delta tcdB$ mutant. The same methodology as for the

single mutant was used yielding the strain AHCD1046. This strain was reverted to a PyrE⁺ phenotype through conjugation with plasmid pMTL-YN1. The resulting colonies were streaked onto non-supplemented CDMM agar and the presence of a restored *pyrE* allele was confirmed by colony PCR using the primer pair pyrE-vef-Fw/ pyrE-vef-Rev. This screen led to the isolation of the strain AHCD1066.

Complementation at the *pyrE* locus. In order to complement the $\Delta tcdR$ mutation, the coding sequence of the *tcdR* gene and its promoter region, in a total of 1460 bp, were amplified by PCR using the primer pair tcdR-comp-BamHI-Fw/ tcdR-comp-HindIII-Rev and cloned between the BamHI and HindIII sites of pMTL-YN1C, yielding pSR5 for 630 Δ *erm* and in pMTL-YN2C, yielding pSR34 for R20291. The plasmids obtained were transformed into *E. coli* HB101 (RP4) yielding strains AHEC285 and AHEC661, respectively, and subsequently mated with *C. difficile* 630 Δ *erm* Δ *pyrE* Δ *tcdR* and R20291 Δ *pyrE*. The transconjugants obtained were streaked onto CDMM and tested by colony PCR using primer pair pyrE-vef-Fw/ pyrE-vef-Rev to test for both *tcdR* complementation and for *pyrE* reversion; clones with the expected changes were identified and named AHCD828 (for 630 Δ *erm*) and AHCD1161 (for R20291).

Placing *tcdR* under the control of forespore- or mother cell-specific promoters. In order to construct strains where *tcdR* was under the control of the *sspA* or the *spoIIIAA* promoter, the allelic exchange using the *pyrE* allele method was employed. P_{sspA} -*tcdR* or $P_{spoIIIAA}$ -*tcdR* fusions were then stably integrated in the $\Delta tcdR$ mutant.

Overlap extension PCR was used to obtain the fusions. To amplify *tcdR* from *C. difficile* 630 Δ *erm* genomic DNA and add the RBS GGAGGA the primer pair TcdR-RBSopt-Fw/ TcdR-comp-HindIII-Rev was used, producing a PCR fragment with 723bp. The PCR fragment was cloned between the XhoI/HindIII sites in pMTL-YN1C, yielding pCC44. In order to amplify the promoter regions, the primer pairs PsspA-BamHI-Fw/ PsspA-SNAP-XhoI-Rev and PspoIIIAA-BamHI-Fw/ PspoIIIAA-SNAP-XhoI-Rev were used, to produce fragments with 482 and 494 bp in length. The final PCR products were cloned between the BamHI/XhoI sites in pCC44 yielding pCC45 and pCC46. The plasmids were transformed into *E. coli* HB101 (RP4) yielding strains AHEC391 and AHEC389, respectively, and subsequently mated with *C. difficile* 630 Δ *erm* Δ *pyrE* Δ *tcdR*. The transconjugants obtained were streaked onto CDMM and tested by colony PCR using the primer pair PsspA-BamHI-Fw/ TcdR-comp-XhoI-Rev and PspoIIIAA-BamHI-Fw/ TcdR-comp-XhoI-Rev to test for integration and with the primer pair *pyrE*-vef-Fw/ *pyrE*-vef-Rev to test for *pyrE* reversion. The clones with the expected changes were identified and named AHCD900 and AHCD926, respectively. The plasmid pMS470 was introduced into the new strains through mating with *E. coli* HB101 (RP4), resulting in strains AHCD901 and AHCD927, respectively.

Table 4.1. Bacterial strains used in this study.

Strain	Relevant properties	Origin/ reference
<i>E. coli</i>		
DH5α	General cloning host	Invitrogen
HB101 (RP4)	Host for conjugation into <i>C. difficile</i>	Laboratory stock
AHEC076	HB101 (RP4)/ pMTL84121	(Heap <i>et al.</i> , 2009)
AHEC144	HB101 (RP4)/ pMS470	This study
AHEC203	HB101 (RP4)/ pMTL-YN1	(Ng <i>et al.</i> , 2013)
AHEC205	HB101 (RP4)/ pMTL-YN2	"
AHEC227	HB101 (RP4)/ pSR1	This study
AHEC239	HB101 (RP4)/ pSR3	"
AHEC285	HB101 (RP4)/ pSR5	"
AHEC389	HB101 (RP4)/ pCC46	"
AHEC391	HB101 (RP4)/ pCC45	"
AHEC498	HB101 (RP4)/ pSR7	
AHEC499	HB101 (RP4)/ pSR8	
AHEC661	HB101 (RP4)/ pSR34	"
<i>C. difficile</i>		
AHCD531	<i>C. difficile</i> 630Δ <i>erm</i>	(Hussain <i>et al.</i> , 2005)
AHCD668	630Δ <i>erm</i> containing pMS470	This study
AHCD688	<i>C. difficile</i> R20291	Anaerobe Reference Laboratory, Cardiff, Wales, United Kingdom
AHCD772	630Δ <i>erm</i> Δ <i>pyrE</i>	(Ng <i>et al.</i> , 2013)
AHCD811	630Δ <i>erm</i> Δ <i>pyrE</i> Δ <i>tcdR</i>	This study
AHCD820	630Δ <i>erm</i> Δ <i>tcdR</i>	"
AHCD828	630Δ <i>erm</i> Δ <i>tcdR</i> <i>pyrE::tcdR</i>	"
AHCD900	630Δ <i>erm</i> Δ <i>tcdR</i> <i>pyrE::PsspA-tcdR</i>	"
AHCD901	AHCD900 containing pMS470	
AHCD926	630Δ <i>erm</i> Δ <i>tcdR</i> <i>pyrE::PspollIAA-tcdR</i>	"
AHCD927	AHCD927 containing pMS470	
AHCD999	630Δ <i>erm</i> Δ <i>pyrE</i> Δ <i>tcdA</i>	
AHCD1046	630Δ <i>erm</i> Δ <i>pyrE</i> Δ <i>tcdA</i> Δ <i>tcdB</i>	
AHCD1066	630Δ <i>erm</i> Δ <i>tcdA</i> Δ <i>tcdB</i>	
AHCD1067	R20291 Δ <i>pyrE</i>	(Ng <i>et al.</i> , 2013)
AHCD1093	R20291 Δ <i>pyrE</i> Δ <i>tcdR</i>	This study
AHCD1102	R20291Δ <i>tcdR</i>	"
AHCD1161	R20291 Δ <i>tcdR</i> <i>pyrE::tcdR</i>	"

Table 4.2. Oligonucleotides used in this work.

Primer	Sequence (5' to 3') ^a
PtcdA-SNAP-EcoRI-Fw	CACAAAGAT <u>GAATTCT</u> GGTCAGTTGGT
PtcdA-SNAP-XhoI-Rev	GTATTATTACTCGAGATAATAAATCCAC
tcdR-Ascl-Fw	CCCCGGCGCGCCATTATCTTAAGAGAGGAG
tcdR-SOE-Rev	CATAAATAAAATTTCTTGCAAATCATC
tcdR-SOE-Fw	TTGCAAGAAAATTTTATTTATGGAAAATTATTTAACTTG
tcdR-Sbfl-Rev	CCCCCTGCAGGTATCTATATAAATATCTG
tcdR-vef-Fw	GTATCATTTTACGAAGAGG
tcdR-vef-Rev	GGGTCATTTAAGTTTTCTC
tcdR-comp-BamHI-Fw	CCCGGATCCTAAAAATATTTTGATATG
tcdR-comp-HindIII-Rev	CCCAAGCTTATTAATTTGCTCTTC
tcdA-Ascl-Fw	CCCCGGCGCGCCGGTAGTATATCAAACATTGG
tcdA-SOE-Rev	CTCATTTTCTCTTGGTCTAATGCTATATGCGAG
tcdA-SOE-Fw	CCAAGAGAAAATGAGCCTGGGATATATGGC
tcdA-Sbfl-Rev	CCCCCTGCAGGGATAAGGTTGTACTATGTAG
tcdA-vef-Fw	GATGGTGCATGGTCAGTTGG
tcdA-vef-Rev	GAAGATGGTGATGAGGTGC
tcdB-Ascl-Fw	CCCCGGCGCGCCCAAAGTAAGTCTGTTTTTGAGG
tcdB-SOE-Rev	CAATATTGCAACATATTCATCTTCTTG
tcdB-SOE-Fw	GAATATGTTGCAATATTGCAATTAGTG
tcdB-Sbfl-Rev	CCCCCTGCAGGGTCTTAAAAAATTGATAC
tcdB-vef-Fw	GAC AAG CTG TTA ATA AGG C
tcdB-vef-Rev	CTG GTA ATC CAC ATA AGC AC
YN3-vef-Fw	CATCAAGAAGAGCGACTTCG
YN3-vef-Rev	TTCTTTCTATTACAGCACTGTTATGC
pyrE-vef-Fw	CAATAATTTTATAACATTAACATGG
pyrE-vef-Rev	GTGTTACTTAAAAAATGTAAAT
YN4-vef-Fw	CAAGAAGAGCGACTTCGCGGAGCTGG
YN4-vef-Rev	CCATTACAGACTTATCCAGGG
TcdR-RBSopt-Fw	GCGCGCCTCGAGGGAGGAactactATGCAAAAGTCTTTTTATG
TcdR-comp-HindIII-Rev	CCCAAGCTTATTAATTTGCTCTTC
PsspA-BamHI-Fw	AGATGAGGAGGATCCGGATAAAAGAGTTC
PsspA-SNAP-XhoI-Rev	CTTCCTTCTCTCGAGTTTATTTTGTTTGC
PspolIIA-BamHI-Fw	TAGATGGTGGGATCCCTAGGGCTTACCAAAAAAC
PspolIIA-SNAP-XhoI-Rev	GTTTATTCATCTCTTGCTCGAGTCCTTG
TcdR-comp-XhoI-Rev	CGCGCTCGAGATTAATTTGCTCTTC

^a engineered restriction sites are underlined.

Table 4.3. Plasmids used in this study.

Plasmid	Relevant features	Origin/ reference
pMTL84121	<i>Clostridium</i> modular plasmid containing <i>catP</i> (Cm ^R /Tm ^R)	Laboratory stock
pMTL-YN1	Plasmid for <i>pyrE</i> reversion through ACE in 630Δ <i>erm</i>	(Ng <i>et al.</i> , 2013)
pMTL-YN1C	Plasmid for complementation through ACE in 630Δ <i>erm</i>	"
pMTL-YN2	Plasmid for <i>pyrE</i> reversion through ACE in R20291	"
pMTL-YN2C	Plasmid for complementation through ACE in R20291	"
pMTL-YN3	Plasmid for mutation through ACE in 630Δ <i>erm</i>	"
pMTL-YN4	Plasmid for mutation through ACE in R20291	"
pFT47	pMTL84121-SNAP ^{Cd} (Cm ^R /Tm ^R)	(Pereira <i>et al.</i> , 2013)
pMS470	pFT47 containing the <i>tcdA</i> promoter region	"
pSR1	pMTL-YN4 containing a LHA and RHA from <i>tcdR</i> (R20291)	"
pSR3	pMTL-YN3 containing a LHA and RHA from <i>tcdR</i>	"
pSR5	pMTL-YN1C containing <i>tcdR</i> and its promoter for complementation	"
pSR7	pMTL-YN3 containing a LHA and RHA from <i>tcdA</i>	"
pSR8	pMTL-YN3 containing a LHA and RHA from <i>tcdB</i>	"
pSR34	pMTL-YN2C containing <i>tcdR</i> and its promoter for complementation (R20291)	"
pCC44	pMTL-YN1C containing <i>tcdR</i> with optimized RBS	"
pCC45	pCC44 containing the <i>sspA</i> promoter	"
pCC46	pCC44 containing the <i>spoIIIAA</i> promoter	"

Fluorescence microscopy and image analysis. For SNAP labelling, the TMR-Star substrate was added to cells in culture samples to a final concentration of 250 nM (New England Biolabs) and the mixture incubated for 30 minutes in the dark. Following labelling, the cells were collected by centrifugation (4000 *xg* for 5 min), washed four times with 1 mL of PBS, and finally resuspended in 0.5 mL of PBS. For phase contrast and fluorescence microscopy, cells were mounted on 1.7% agarose coated glass slides and observed on a Leica DM6000B microscope equipped with a phase contrast Uplan F1 100x objective and captured with a CCD Andor Ixon camera (Andor Technologies). Images were acquired and analyzed using the

Metamorph software suite (version 5.8; Universal Imaging), cropped and adjusted using Adobe Photoshop.

Spore production, purification and fractionation. For spore production 10 mL of BHI media was inoculated with an isolated colony of *C. difficile* and cultured overnight at 37°C in anaerobic conditions. The next day, 150 mL of fresh BHI media was inoculated with 1.5 mL of the overnight culture and incubated for 7 to 10 days at 37°C in anaerobic conditions. Cells were collected by centrifugation at 4800 $\times g$, suspended in cold water and stored for 24 to 48 h at 4°C. The pellet was suspended in PBS with 0.1% Tween-20 and spores were purified with a 42% Renografin (Bayer) step gradient, as previously described (Henriques *et al.*, 1995). The pellet was suspended in PBS with 0.1% Tween-20, washed twice with the same buffer and twice with cold water. The final spore suspension was stored at -20°C until further use. For spore fractionation, the spore coat/exosporium was removed by suspending the purified spores in 50 μ L of decoating buffer (10% glycerol, 4% SDS, 10% β -mercaptoethanol, 1mM DTT, 250 mM Tris pH 6,8) to a final OD_{580nm} of 4.0. Spores were then boiled for 5 minutes and collected by centrifugation. The supernatant, corresponding to the coat/exosporium fraction, was further analyzed by SDS-PAGE and immunoblotting. The spore sediment was washed twice with PBS with 0.1% Tween-20, and incubated with 50mM Tris-HCl pH 8,0 with 2mg/mL lysozyme for 2 h at 37°C to digest the spore cortex peptidoglycan and release cortex/core-associated proteins. The lysozyme fraction was further analyzed by SDS-PAGE and immunoblotting with anti-CotD (Permpoonpattana *et al.*, 2011b), anti-

TcdA (Santa Cruz Biotechnology) and anti-TcdB (Abcam) antibodies.

Immunofluorescence microscopy. For immunofluorescence analysis the spores were fixed with 3% paraformaldehyde (pH 7.4) for 20 minutes in poly-L-lysine-coated glass cover slides. The fixed spores were rinsed three times with PBS and blocked with 1% bovine serum albumin (BSA) for 30 minutes. The slides were incubated overnight at 4°C with a monoclonal anti-TcdA primary antibody (Santa Cruz Biotechnology) (at a dilution of 1:5000). The slides were then incubated for 2 hours at room temperature with Alexa Fluor 594 goat anti-mouse IgG secondary antibody (Life Technologies) (1:500) in PBS–1% BSA and washed three times with PBS and once with distilled water. The samples were dried for 30 minutes at room temperature and observed at the microscope (adapted from (Barra-Carrasco *et al.*, 2013)). For immunofluorescence of germinated spores, the spores were suspended in PBS to a final OD_{600nm} of 1 and heat activated for 10 minutes at 80°C. A 10% solution of taurocholic acid (TA) (Carl-Roth) was added for a final concentration of 0,5% TA to induce germination. The cells were incubated for 3h at 37°C in the anaerobic chamber in order to allow for outgrowth. Cells were collected by centrifugation at 4000 *xg*, for 5 minutes and suspended in 1 mL Histochoice (Sigma-Aldrich) for cell fixation and incubated 15 minutes at room temperature and 30 minutes on ice. The cell suspension was washed three times with PBS-2% BSA and incubated with GTE (50mM Glucose, 20mM Tris-HCl pH 7.0, 10mM EDTA pH 8.0) with 2,5 mg/mL lysozyme for 5 minutes at room temperature. The cell suspension was washed three times with GTE and blocked with PBS-1% BSA for 30 minutes at room

temperature. The cell suspension was incubated overnight at 4°C a monoclonal anti-TcdA primary antibody (Santa Cruz Biotechnology) (at a dilution of 1:500). The suspension was washed three times with PBS-2% BSA for 5 minutes each wash and incubated for 1 hour at room temperature with Alexa Fluor 594 goat anti-mouse IgG secondary antibody (Life Technologies) (1:500) in PBS-1% BSA, washed three times with PBS and finally suspended in 50 µL PBS. The samples placed in poly-L-lysine-coated glass cover slides and dried for 30 minutes at room temperature and observed at the microscope

Cell cultures and spore cytotoxicity assays. Vero and HT29 cells were cultured in Dulbecco's modified Eagle's medium (Gibco) with GlutaMAX™ supplemented with, 4,5 g/L Glucose, 5 or 10% (v/v) for Vero or HT29 cells respectively of bovine fetal serum and 1% (v/v) Pen-Strep (Sigma), at 37°C in a 5% CO₂ atmosphere. Cells were grown until confluence in 96-wells plates and incubated with 2-fold serially diluted spores in DMEM (starting by 5x10⁶ spores). Spores were purified as previously described (Pereira *et al.*, 2013). The cytopathic effect was monitored after 24h incubation at 37°C in a 5% CO₂ atmosphere using a Nikon HCS microscope equipped with a 10x objective and a Andor Zyla 4.2 sCMOS 4.2Mpx camera.

Immunostaining for cell cytotoxicity assays. Vero and HT29 cells with or without spores were fixed in PBS containing 4% (w/v) paraformaldehyde (PFA) for 15 min. For immunostaining, the Texas Red-Phalloidin antibody (Thermo Fisher Scientific) that stains F-actin was diluted 1:100 in PBS-0,1% saponin containing 10% (v/v) horse

serum. After immunostaining cells were consecutively washed with PBS-0,1% saponin, PBS and ddH₂O. The coverslips were assembled using Aqua-poly/Mount (Polysciences) on microscopy glass slides and the cells were examined by fluorescence microscopy on a Leica DM6000B microscope equipped with a phase contrast Uplan F1 63x objective and captured with a CCD Andor Ixon camera (Andor Technologies).

4.4. RESULTS

4.4.1. TcdA associates with mature spores

Since expression of the PaLoc genes, with the exception of *tcdC*, was detected in sporulating cells (see Chapter 3), we wanted to test whether the toxins associate with mature spores. *C. difficile* spores were purified and utilized in immunofluorescence assays using an anti-TcdA monoclonal antibody. We found that TcdA was accessible to the anti-TcdA antibody, which strongly decorated a fraction of purified WT spores but not those of $\Delta tcdR$ and $\Delta tcdA\Delta tcdB$ mutant spores (Figure 4.2A). The accessibility of TcdA to the anti-TcdA antibody in 630 Δerm suggests that the toxin is associated with the coat and/or exosporium layers of spores, as components of these structures have been detected by immunofluorescence (Permpoonpattana *et al.*, 2013, Phetcharaburanin *et al.*, 2014, Pizarro-Guajardo *et al.*, 2016, Pizarro-Guajardo *et al.*, 2014). In the epidemic ribotype 027 strain R20291, however, spore-associated TcdA is not detected by immunofluorescence, suggesting that the toxin is either not part of the spore or not accessible to the antibody (Figure 4.2A). For 630 Δerm

spores, TcdA was also detected by immunofluorescence shortly after germination (Figure 4.2B). Interestingly, TcdA was also detected after germination of R20291 spores, suggesting that in this strain the toxin might be at the spore surface although not accessible to antibodies (Figure 4.2B). Using commercially available antibodies (see Material and Methods), we were unable to detect TcdB by immunofluorescence in spores of both strains.

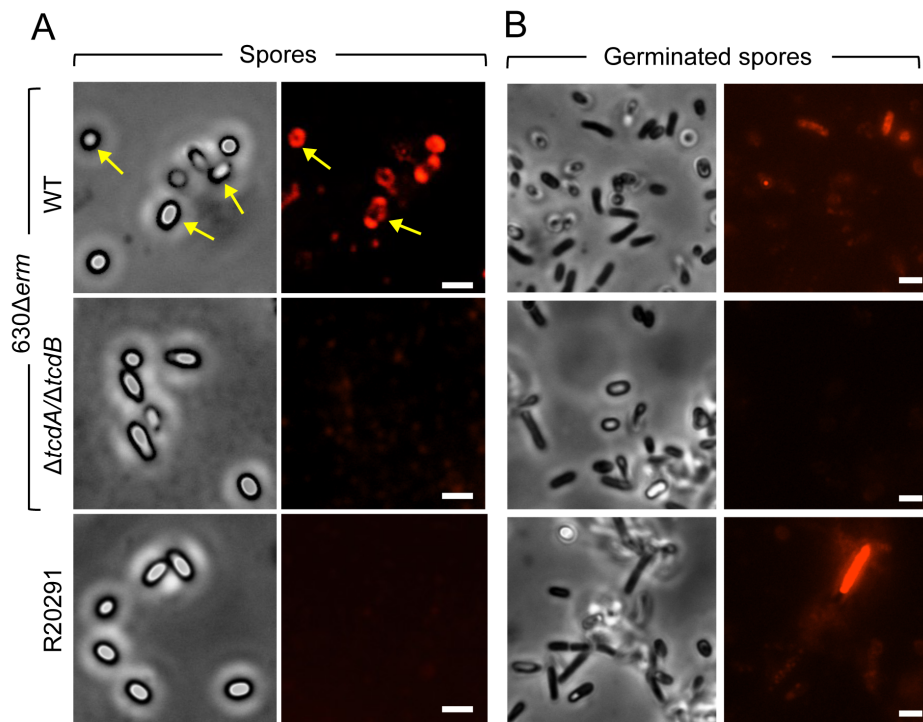


Figure 4.2. TcdA localization in mature spores. **A:** The surface accessibility of TcdA on *C. difficile* 630Δerm, 630Δerm ΔtcdAΔtcdB and R20291 fixed spores was analyzed by immunofluorescence with mouse anti-TcdA monoclonal antibody as described in Materials and Methods. Yellow arrows indicate spores displaying toxin at the surface. Scale bar: 1 μm. **B:** The presence of TcdA at the surface of outgrowing cells from spores germinated for 3 hours was analyzed by immunofluorescence with a mouse anti-TcdA monoclonal antibody as described in the Materials and Methods. Scale bar: 1 μm.

In an attempt to independently confirm the immunofluorescence results and to define the localization of TcdA we used biochemical fractionation of mature spores. Spores formed by 630 Δ *erm* and R20291 (WT), by a Δ *tcdR* congenic derivative of the two strains, by the corresponding Δ *tcdR* complemented strains (*tcdR*^C) and formed by a Δ *tcdA* Δ *tcdB* double mutant in the background of 630 Δ *erm* (as an additional control) were boiled in the presence of SDS and DTT to extract proteins present in the coat and exosporium layers (Fernandes *et al.*, 2018). Under our conditions the spores of all strains remained phase bright after extraction, indicating that no major structural alterations occurred during coat/exosporium extraction (Pereira *et al.*, 2013). Following extraction of the coat and exosporium, the spores were treated with lysozyme; this treatment is likely to release proteins that are in close proximity with or within the cortex, but also proteins present in the spore core. Proteins in the coat/exosporium and lysozyme fractions were then resolved by SDS-PAGE, the gels stained with Comassie (Figure 4.3A, top panel; loading control) and subject to immunoblotting with an anti-TcdA antibody (bottom panel). TcdA was detected in both the coat/exosporium and lysozyme fractions of 630 Δ *erm* spores, although to a higher level in the coat/exosporium (Figure 4.3A, middle panel). TcdA was not detected in the coat/exosporium and lysozyme fractions of a Δ *tcdR* mutant, but complementation with *tcdR* at the *pyrE* locus partially restored its association with both fractions (Figure 4.3A; middle panel). We note that CotD, which associates with both the coat and exosporium (Permpoonpattana *et al.*, 2013, Permpoonpattana *et al.*, 2011), was only detected in the coat/exosporium fraction. This suggests that our

extraction procedure effectively separated coat/exosporium proteins from those, presumably associated with the cortex and/or spore core (Figure 4.3A, bottom panel). In contrast to 630 Δ *erm* spores, immunoblot analysis of the coat/exosporium and lysozyme fractions obtained from R20291 spores revealed the presence of TcdA in the lysozyme fraction but not in the coat/exosporium fraction (Figure 4.3B; middle panel). Importantly, TcdA was not detected in the fractions prepared from Δ *tcdR* spores (R20291 background), but complementation with the wild-type allele at the *pyrE* locus partially restored its association with the lysozyme fraction (Figure 4.3B; middle panel).

Although not excluding the presence of TcdA in the spore core, these results suggest that TcdA associates with the coat/exosporium layers and is accessible to antibodies in 630 Δ *erm* spores, whereas in spores of the epidemic strain R20291, TcdA has a more internal localization, within the cortex and/or spore core.

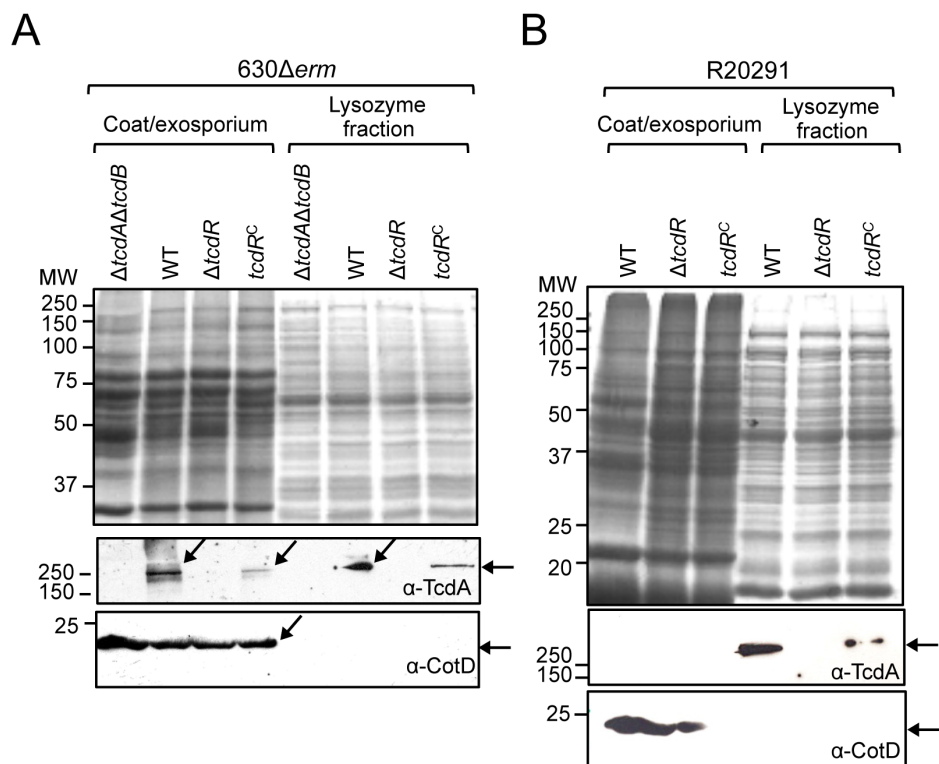


Figure 4.3 A and B: Fractionation of spores from the indicated strains in the background of 630 Δ erm (A) and R20191 (B). Δ tcdA Δ tcdB and Δ tcdR are mutants for the indicated genes; tcdR^c represents a Δ tcdR mutant complemented with the wild-type allele at the *pyrE* locus. Intact mature spores were fractionated into a coat/exosporium and a lysozyme fraction. The proteins in the various fractions were resolved by SDS-PAGE (top) and subject to immunoblotting (bottom) with anti-TcdA or anti-CotD antibodies (CotD is a *bona fide* coat protein; bottom panel).

4.4.2. Both the forespore or mother cell-specific expression of *tcdR* results in the association of TcdA with mature spores

Since *tcdA* expression was detected both in the forespore and the mother cell (see Chapter 3), we wondered whether TcdA could reach the coat/exosporium layers from the mother cell, the forespore, or both. To test this, the *tcdR* deletion mutant was complemented *in trans* at the *pyrE* locus using a *tcdR* allele expressed from a forespore specific promoter, that of the *sspA* gene, or from the mother cell-specific *spoIIIAA* promoter (Pereira *et al.*, 2013, Saujet *et al.*, 2013). We first examined expression of the P_{tcdA} -SNAP^{Cd} fusion in the P_{sspA} -*tcdR* (*tcdR*^{FS} for simplicity in Figure 4.4) and $P_{spoIIIAA}$ -*tcdR* (*tcdR*^{MC}) strains by fluorescence microscopy. In the *tcdR*^{FS} strain, expression of the fusion in vegetative cells was reduced, as expected (Figure 4.4A). The weak signal detected, however, suggests that TcdR persists in cells outgrowing from germinated spores, and contributes to the expression of the PaLoc genes in those cells (see also above). Consistent with this interpretation, a lower fluorescence signal was observed for P_{tcdA} -SNAP^{Cd} in vegetative cells of the *tcdR*^{MC} strain (Figure 4.4A). Importantly, the forespore-specific expression of the fusion is maintained in the *tcdR*^{FS} strain (Figure 4.4A, white arrows). Conversely, the mother cell-specific expression of *tcdA*-SNAP^{Cd} is increased in the *tcdR*^{MC} strain relative to the WT (Figure 4.4A), where the accumulation of SNAP is σ^D -dependent and most likely results from the activity of this sigma factor in pre-divisional cells and/or persistent activity in the mother cell (see Chapter 3).

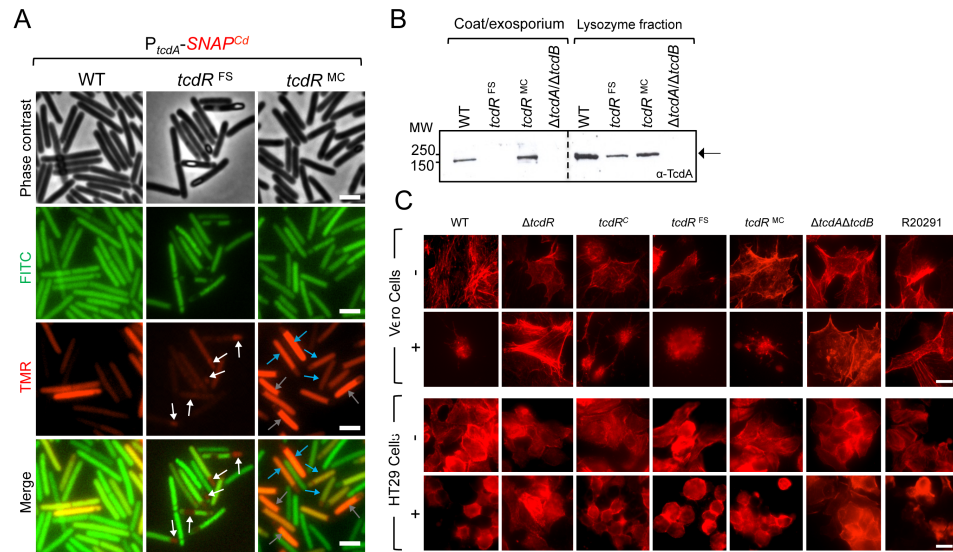


Figure 4.4. Spores are cytotoxic. **A:** The pattern of P_{tcdA} -SNAP^{Cd} expression driven by the forespore-specific *sspA* promoter ($tcdR^{FS}$) or the mother cell-specific *spoIIIAA* promoter ($tcdR^{MC}$). The cells were collected after 24h of growth in TY liquid medium, labeled with TMR-Star and examined by phase contrast (PC) and fluorescence microscopy to monitor SNAP production. The merged images show the overlap between the TMR-Star (red) and the auto-fluorescence (green) channels. White arrows point to sporulating cells with forespore-specific expression, blue arrows point to sporulating cells with whole sporangium expression and grey arrows point to sporulating cells with mother cell-specific expression. Scale bar: 1 μ m. **B:** Fractionation of spores from strains 630 Δerm and $tcdA/tcdB$ into a coat/exosporium and a lysozyme fraction. The proteins in the various fractions were resolved by SDS-PAGE (top) and subject to immunoblotting (bottom two panels) with anti-TcdA. **C:** Immunostaining of monolayers of Vero and HT29 cells. The cells were exposed to 5×10^6 spores purified from sporulating cultures of the indicated strains. Following incubation for 24h with spores, the cells were immunostained with Texas Red-Phalloidin to detect cell rounding. Scale bar: 10 μ m.

Biochemical fractionation of mature spores produced by the $tcdR^{MC}$ strain shows that TcdA is present in the coat/exosporium fraction, at a level slightly higher than for the WT, consistent with the

increased expression of *tcdA* when *tcdR* is under the control of the *spoIIIAA* promoter (Figure 4.4B). TcdA is also present in the lysozyme fraction of *tcdR^{MC}* spores, but at levels lower than for the WT (Figure 4.4B). In spores of the *tcdR^{FS}* strain, however, TcdA is present in the lysozyme fraction, but seems to be less extractable than from WT or *tcdR^{MC}* spores, and it seems absent in the coat/exosporium fraction (Figure 4.4B). This suggests that forespore-produced TcdA most likely cannot reach the coat and exosporium (or it does so but below our detection level), and that most of the toxin detected in spores by immunofluorescence (Figure 4.4; see also above), results from the fraction of cells in which *tcdA* expression takes place in the mother cell. We further infer that TcdA can associate with the coat/exosporium and also with more internal layers of spores when produced in the mother cell.

4.4.3. The spore-associated toxins are functional

The finding that at least TcdA associates with mature spores begged the question of its functionality. To test whether spore-associated TcdA and possibly TcdB, the latter of which we were not able to detect in spores by immunoblotting (above), were functional, we conducted cytotoxicity assays using HT29 and Vero cell lines known to be differentially sensitive to TcdA and TcdB, respectively (Lyras *et al.*, 2009, Torres *et al.*, 1992). The toxins induce cell rounding, which can be monitored and quantified by microscopy (Lyras *et al.*, 2009, Torres *et al.*, 1992). HT29 and Vero cells, grown as monolayers, were exposed to spores and cell rounding was monitored as a function of the spore concentration. We found that WT spores, but not those of the $\Delta tcdR$

mutant and the $\Delta tcdA\Delta tcdB$ double mutant, caused cell rounding of both HT29 and Vero cells, in a dose-dependent manner (Figure 4.4C and 4.5), suggesting that the observed effect is specifically caused by the toxins. Importantly, cell culture medium and cell culture supernatant did not induce spore germination (data not shown), indicating that the spores cause the cell rounding. Complementation with the wild-type *tcdR* allele at *pyrE* locus partially restored the cell-rounding ability to $\Delta tcdR$ spores (Figure 4.4C and 4.5). Thus, TcdA and most likely also TcdB are associated with spores in an active form. R20191 spores were less cytotoxic than 630 Δerm spores (Figure 4.5), possibly because of the more internal localization of TcdA, found mainly in the cortex and/or core of the spores formed by this strain (Figure 4.3).

Spores produced by the *tcdR^{FS}* and *tcdR^{MC}* strains were as cytotoxic against Vero cells and HT29 cells as those of the WT strain (Figure 4.4). This suggests that the toxins can associate in an active form with the surface of spores when produced in either the forespore or the mother cell. That the cytotoxicity observed for *tcdR^{FS}* spores was similar as for *tcdR^{MC}* or WT spores is intriguing since in this strain TcdA shows a more internal localization, within the cortex and/or spore core. It seems possible that differences in the structure of the spore surface layers between 630 Δerm and R20291 spores influence the exposure and activity of the spore-associated toxins (Paredes-Sabja *et al.*, 2014).

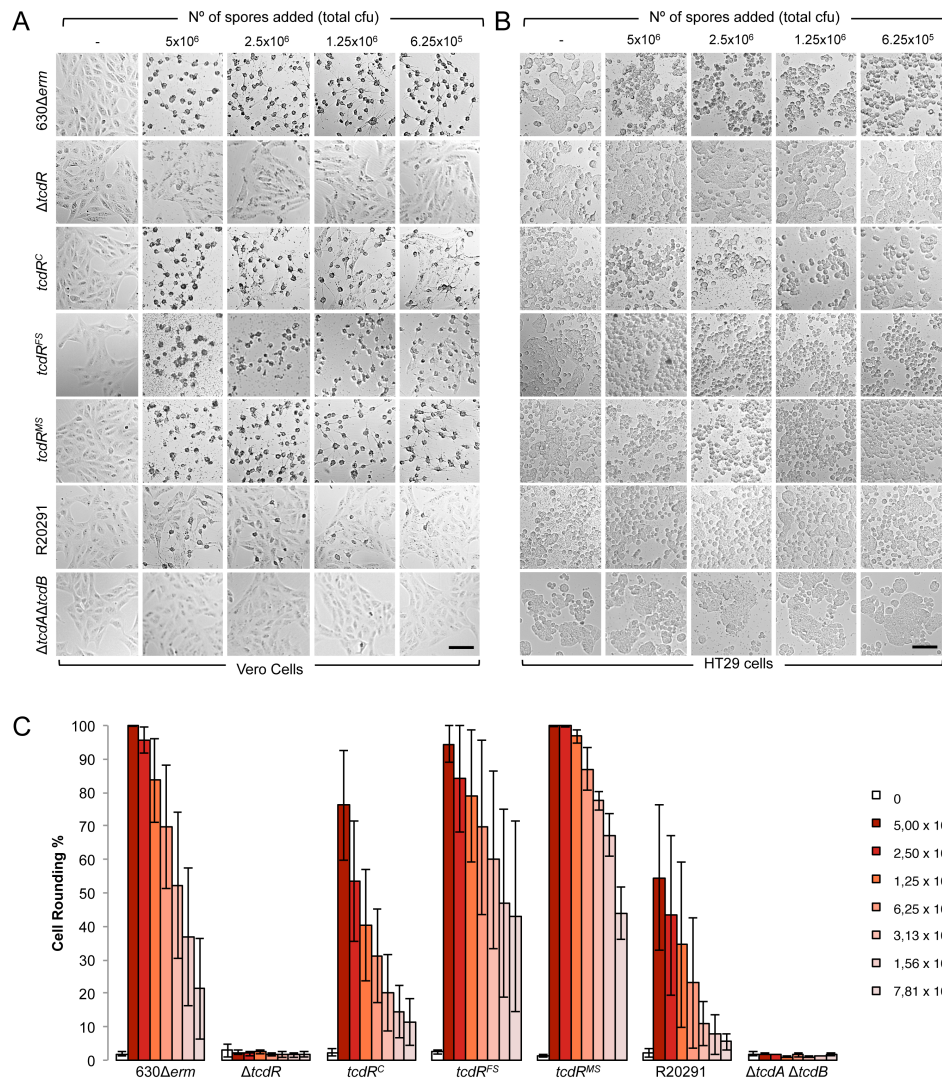


Figure 4.5. Mature spores are cytotoxic in a dose-dependent manner. Monolayers of Vero (A) and HT29 (B) cells were exposed to the indicated number of spores from the indicated strains. Following incubation, the cells were examined by phase contrast microscopy to detect cell rounding. Scale bar: 100 μ m. The percentage of cell rounding was quantified in the phase bright pictures of Vero cells (n=220-1094). The experiment was performed in triplicate and error bars indicate the standard error.

4.5. DISCUSSION

We show that TcdA associates with the spore surface, with distinct localization in different strains. We further show that the toxin present at the spore surface is functional, as WT spores but not those of a *tcdR* mutant, have a cytotoxic effect in susceptible cell lines.

We detected TcdA by immunoblotting at the coat/exosporium and cortex and/or core of purified spores (Figure 4.3). In a previous study, antibodies raised against a fragment of TcdA (residues 26-39 within the CROPS region) recognize species of about 100, 60, 50 and 20 kDa at the surface of *C. difficile* spores, and two of these species were identified as AdhE1 and CdeC (Hong *et al.*, 2017b). While this study raised the possibility that coat/exosporium proteins cross-reacted with anti-TcdA antibodies, we note that the proteins detected by Hong and co-authors were not detected in our study, in which we used a monoclonal antibody that recognizes the TcdA CROPS region (Frey & Wilkins, 1992). In addition, we detected a species of about 250 kDa, close to the expected size for TcdA, in WT but not in $\Delta tcdA\Delta tcdB$ mutant spores.

Spores of both 630 Δerm and R20291 are cytotoxic when assayed against Vero or HT29 cells. Thus, both TcdA and TcdB appear to associate in an active form with mature spores. In particular, fractionation and immunofluorescence studies showed the association of TcdA with the coat/exosporium and cortex/core of 630 Δerm spores, whereas in spores of the epidemic R20291 strain, TcdA mainly associated with the spore cortex and/or core. Additionally, for both strains, immunofluorescence showed that TcdA is present in cells outgrowing from spores shortly after germination is induced. We

further show that in strain 630 Δ *erm*, TcdA can associate with the coat/exosporium and cortex/core in active form whether production is confined to the mother cell or the forespore (Figure 4.4). Since assembly of both the cortex and coat layers is largely a function of the mother cell, it is conceivably that some TcdA produced in the mother cell can associate with these layers during their formation. TcdA may associate with the cortex peptidoglycan via its CROPs region, and may localize at the cortex/coat interface. In fact, glycan motifs with the core structure Gal β 1-4 GlcNAc were identified as having high-affinity for TcdA (Gerhard, 2016). Although it seems unlikely, we cannot rule out the possibility that mother cell-produced TcdA reaches the spore core. The forespore-produced TcdA, however, has to cross the forespore inner membrane to become in contact with the cortex, if it associates with this layer, and possibly also the forespore outer membrane, if it associates with the coat/exosporium. No mechanism is presently known that could explain this re-localization. How TcdA and TcdB are released from producing cells is still debatable but the holin-like protein TcdE appears to be required for TcdA and TcdB release at least in some strains (Govind & Dupuy, 2012, Govind *et al.*, 2015, Olling *et al.*, 2011). The expression of *tcdE* in the forespore raises the possibility that TcdE may be involved in the localization of TcdA and possibly also of TcdB to the cortex/core and coat/exosporium (see also Chapter 5).

It was recently shown that disruption of *tcdR* by integration of a *erm* resistance determinant affects the efficiency of sporulation in R20291 and the structure and functional properties of the spores (Girinathan *et al.*, 2017). In contrast, disruption of *tcdR* in the background of 630 Δ *erm*, did not affect sporulation (Girinathan *et al.*,

2017); this work). While it is tempting to speculate that the more internal localization of TcdA in R20291 relative to 630 Δ *erm* is responsible for the altered ultrastructure and properties of the *tcdR* insertional mutant (Girinathan *et al.*, 2017), we note that in our hands, the in-frame deletion of *tcdR* in R20291 did not affect sporulation (Table 4.4). Girinathan and co-authors used spores purified from plates of a 70-30 medium, rather than produced in solid TY medium as in this study (Girinathan *et al.*, 2017). Possibly, the discrepancy may be explained both by the nature of the *tcdR* allele and culturing conditions used in the two studies.

Regardless of their production in the mother cell or the forespore, active toxins associate with the spore surface layers, make infectious spores toxic delivery vehicles and we speculate that this spore-associated toxin may promote colonization by vegetative cells, even without active toxin production. Spore germination seems to occur in close contact with the epithelial cells (Panessa-Warren *et al.*, 1997, Panessa-Warren *et al.*, 2007) and evidence suggests that spores interact directly with proteins found at the surface of Caco-2 cells (Sarker & Paredes-Sabja, 2012). While the existence of spore-specific receptors may promote spore attachment to the colonic mucosa, no spore-specific receptor, however, is presently known. In contrast, sucrose-isomaltase, only found in rabbit intestinal cells (Pothoulakis *et al.*, 1996) and gp96, a glycoprotein found in the human colonic mucosa (Na *et al.*, 2008), have been identified as TcdA receptors (Hansen *et al.*, 2013). It is tempting to hypothesize that spore-associated toxins may interact with their cognate receptors, thus contributing to spore retention, completion of germination and outgrowth of the cells, while

multiple rounds of sporulation in the gut would endow the spores with cytotoxicity and binding ability upon infection of a new host. Interestingly, spore coat protein CotE, which has a C-terminal chitinase domain, was recently shown to allow directly binding of spores to mucus and to contribute to mucin degradation (Hong *et al.*, 2017b). Moreover, *cotE* mutant spores are impaired in colonization and virulence (Hong *et al.*, 2017a). It is tempting to suggest that the mucus-hydrolytic activity of CotE may allow exposure of host cells to the spore-associated toxin. Several mechanisms may thus contribute to a direct participation of spores in establishment and promotion of disease, in addition to their role as persistence and dissemination vectors ((Hong *et al.*, 2017b), this work).

Importantly, in spores of the epidemic strain R20291, TcdA associates mainly with the cortex/core, and thus has a more internal localization as compared to 630 Δ *erm* spores (Figure 4.3). This differential localization may explain the lower cytotoxicity of R20291 spores as compared with 630 Δ *erm* spores (Figures 4.4 and 4.5). The more internal localization of the toxins in spores of strain R20291, together with the presence of a more robust exosporium in spores of this strain (Pizarro-Guajardo *et al.*, 2016), may however afford protection during transit through the gastro-intestinal tract, allowing intact spore-associated toxin to reach sites of spore germination.

There are other pathogenic sporeformers where toxins have been found to be associated with the spores with relevance for the success of the infectious cycle. The insecticidal crystal toxins (Cry) from *Bacillus thuringiensis* were found at the surface of purified spores (Du & Nickerson, 1996). The presence of the toxins at the spore surface

allows in this insect pathogen to provide toxicity even before spore germination that only occurs when the spores bind to cell receptors and the pH of the gut drops sufficiently. Furthermore, it was suggested in *B. thuringiensis* the exosporium might have a protective role of the spore surface associated toxin (Du & Nickerson, 1996). Proteins involved in pathogenicity were also found at the surface of *Bacillus cereus* spores (Ruiu *et al.*, 2015) as well as in spores of *Brevibacillus laterosporus*, a pathogen of invertebrates that has atypical spores surrounded by a firmly attached canoe-shaped parosporal body (CSPB). In *B. laterosporus* proteins with a pathogenic action were found not only associated with the coat, but also the exosporium and the CSPB (Marche *et al.*, 2017). Importantly in the human pathogen *Bacillus anthracis*, the protective antigen, which is the receptor-binding component of the lethal toxin and the edema toxin, was found both at the coat and exosporium layers of the spore (Cote *et al.*, 2005). Therefore, toxin or proteins with pathogenic effects are found at the surface of spores of a variety of pathogenic species, with different hosts and pathogenic actions, which might suggest a general mechanism for toxin delivery.

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4.7. REFERENCES

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Chapter 5

Discussion

We have studied how the main regulators of toxin production affect the overall pattern of expression across the population of vegetative cells. We also found that expression of the toxin-encoding genes takes place in sporulating cells, both in the mother cell and in the forespore. Surprisingly, we also detected TcdA at the spore surface, and we provide evidence that both TcdA and TcdB associate with mature spores in active form.

5.1. THE HETEROGENOUS EXPRESSION PATTERN OF THE *C. DIFFICILE* TOXIN-ENCODING GENES – A STRATEGY FOR SUCCESSFUL INFECTION?

Within a clonal population of bacterial cells there are cell-to-cell variations in gene expression that are maintained through time (Spudich & Koshland, 1976). This phenotypic heterogeneity is noticed at the level of metabolism, stress responses or other important cellular functions (reviewed at (Ackermann, 2015, Davis & Isberg, 2018, Raj & van Oudenaarden, 2008)). Phenotypic heterogeneity affects the overall fitness of the community and for pathogenic bacteria seems to be important for virulence, as virulence factors often show heterogeneous expression (Arnoldini *et al.*, 2014, Burton *et al.*, 2014, Davis & Isberg, 2018, Hautefort *et al.*, 2003, Spickett *et al.*, 2017, Weigel & Dersch, 2018).

The TcdA and TcdB toxins, and in some strains the binary toxin CDT, are the main *C. difficile* virulence factors (reviewed at (Aktories *et al.*, 2017)), together with motility, cell surface proteins, biofilm and spore development, which are also important for pathogenesis (reviewed at (Janoir, 2016)). We used single cell analysis to study the

expression patterns of the PaLoc genes in vegetative and sporulating cells (Chapters 2 and 3). We show that *tcdA* is expressed at higher levels when comparing with *tcdB*, an observation that is in agreement with batch analysis and with a recently published analysis using FACS (Dupuy & Sonenshein, 1998, Ransom *et al.*, 2018). Most importantly, both genes are expressed heterogeneously with a subpopulation of cells producing the toxins (“ON” cells) while in others toxin expression is not detected (“OFF” cells). A heterogeneous pattern is also seen in the epidemic strain R20291, of ribotype 027 (Chapter 2; (Ransom *et al.*, 2018)).

Positive feedback loops slow down response times because the production of a certain protein is only activated when the regulator that is under positive auto-regulation reaches the activation threshold (Alon, 2007)). Strong positive auto-regulation can lead to bistable expression in which two states exist: one where the concentration of the regulator is high and therefore the state is considered “ON” and is maintained by itself in this state; another where the concentration of the regulator is below the threshold and therefore is considered “OFF”. This leads to phenotypic heterogeneity. Two functional consequences of phenotypic heterogeneity are “bet-hedging” in which sub-populations of cells may expand when faced with environmental challenges to which they are prepared, or division of labor and cooperation, as sub-populations will produce extracellular common goods that can benefit the entire population (Ackermann, 2015, Ackermann *et al.*, 2008, Arnoldini *et al.*, 2014, Burton *et al.*, 2014, Davis & Isberg, 2018, Davis *et al.*, 2015, Raj & van Oudenaarden, 2008, Weigel & Dersch, 2018).

In *C. difficile*, the main toxin regulator TcdR is under positive

auto-regulation (Mani *et al.*, 2002), which is most likely the cause of the observed bistability in *tcdA* expression, as recently proposed by Ransom and co-workers (Ransom *et al.*, 2018). In the case of *C. difficile* the benefits of having only part of the population producing the toxins is unclear. Heterogeneity in virulence gene expression may be regulated both stochastically and spatially (Davis & Isberg, 2018). Since *C. difficile* is faced with dramatic environmental changes during its infectious cycle (Koenigskecht *et al.*, 2015), it would be of interest to examine the expression of the toxin-encoding genes *in vivo*, in order to determine if: *i*) bistability is maintained; *ii*) if different subpopulations are segregated to different microenvironments in the host.

If virulence is cooperative, is there actually a subpopulation benefiting from the toxins produced by another subpopulation? Overall, the population benefits from toxin production as it can subvert the host defenses, facilitating *C. difficile* growth. However, there should be differences in benefits and costs for the individual cells if producing or not the toxins. It has been proposed that in *Salmonella* Typhimurium the heterogeneously expressed virulence factor T3SS-1, the type-III secretion system that inject bacterial proteins into the host cell, has a fitness cost, reducing the growth rate of the cells producing it (Sturm *et al.*, 2011). Furthermore, *in vivo*, the emergence of avirulent mutants, or cheaters, that benefit from the production of the virulence factor by others, suggests that there is a cost associated with the production of T3SS-1 (Diard *et al.*, 2013). In *C. difficile*, *in vitro*, no differences in the growth rate were observed for strains that do not produce the toxins, both of which are large proteins that could have a

high energetic cost for the bacteria (data not shown). However, it would be of interest to analyze possible growth differences within a heterogeneous culture (Fievet *et al.*, 2015).

We have proposed (Chapter 2) that the bistable production of the TcdA and TcdB toxins suggests division of labor and cooperative virulence; studies on the expression of other virulence factors at the single cell level would be an important aspect of a demonstration that this is indeed the case (Davis & Isberg, 2018), and if so, how the populations overlap with the toxin-producers. For example, it would be of interest to study, in strains that code for the binary toxin CDT, such as R20291, whether heterogeneous production of the binary toxin is also observed (Chapter 2, (Ransom *et al.*, 2018)), and if so, to what extent CDT production overlaps with TcdA/TcdB production, as CDT is thought to potentiate the effects of TcdA/TcdB (Gerding *et al.*, 2014).

Additionally, it would be interesting to test whether heterogeneity in the production of the toxins (and other virulence factors) is important for the success of colonization and infection. A strain in which the TcdR auto-regulatory loop is disrupted could be tested for colonization and infection in competition with a strain in which bistability is maintained.

5.2. THE CONNECTION BETWEEN MOTILITY AND TOXINOGENESIS IN *C. DIFFICILE*

In *S. Typhimurium*, production of the virulence factor T3SS-1 is an example of a bet-hedging mechanism, and also an example of division of labor in conjunction with *fliC*. The combination of the two virulence factors originates three types of cells: cells producing only

T3SS-1, others only expressing *fliC* and others both. In this way, the cells that express *fliC* are motile and therefore inside the host are able to move to nutrient rich environments, while the cells that produce T3SS-1 induce inflammation and invade host cells. Expressing both factors might be advantageous as the bacteria can move to optimal locations while causing inflammation; on the other hand, they can also be more easily detected by the host immune system (Ackermann *et al.*, 2008, Weigel & Dersch, 2018). By labeling the *C. difficile* flagellum, we found that in a population of cells, at least in the case of the laboratory strain 630 Δ *erm*, only a negligible fraction is not flagellated. This differs from *B. subtilis* where some cells have the flagellar operon ON and other OFF (Kearns & Losick, 2005). While motility is a common feature to all cells, various regulatory components constrain toxin production to a subpopulation of cells.

The connection between flagellar assembly and toxin production, through the σ^D -dependent transcription of *tcdR*, raises the question of why is this important for the cells. In *Vibrio cholera*, flagella are broken by mucin penetration, which increases the expression of virulence factors due to the secretion of a repressor (Liu *et al.*, 2008). It has been proposed that in this way, secretion of the virulence factors coincides with the arrival of cells to a colonization site. In another example, secretion of virulence factors by *Campylobacter jejuni* depends on hook assembly, while later, flagellin polymerization represses this secretion (Barrero-Tobon & Hendrixson, 2014, Konkel *et al.*, 2004). In preliminary experiments, we found that *C. difficile* flagella integrity is compromised by mucins (protocol adapted from (Liu *et al.*, 2008)) (Figure 5.1A). However, we could not

observe differences in toxin expression in cells exposed to mucins (Figure 5.1A and 5.1B). One possibility is that toxin secretion is increased by flagella breakage; once in the gut, motile cells would reach the mucosa and upon mucin penetration, flagella breakage could allow for toxin release from the flagellar secretion system. However, detection of TcdA/TcdB in the extracellular environment is affected by the presence of mucins, and we have not yet succeeded in overcoming this technical difficulty.

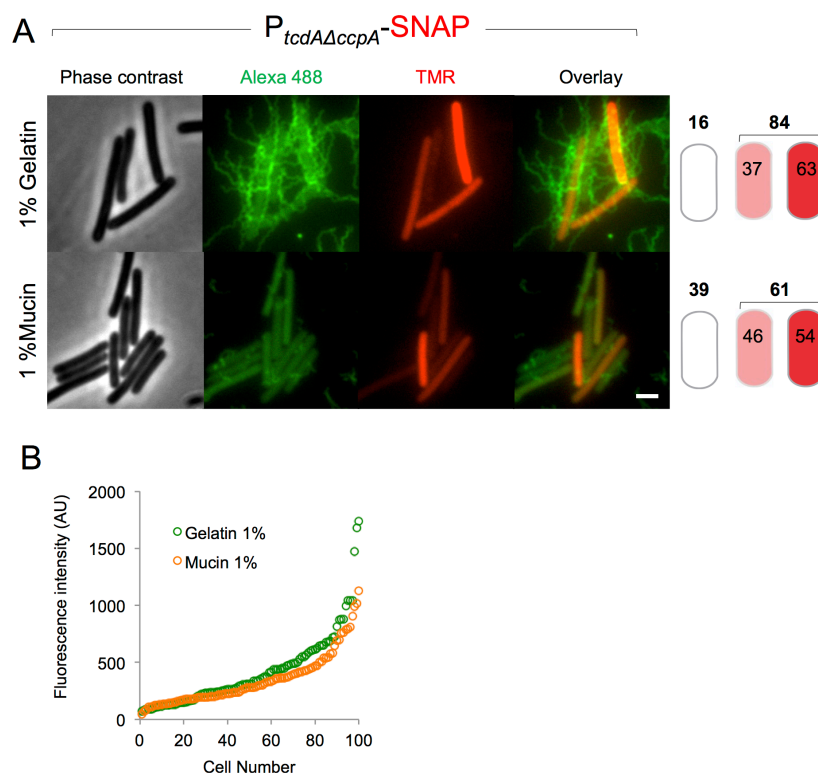


Figure 5.1. The effect of mucins on flagella integrity. **A:** Microscopy analysis of *C. difficile* cells collected after 12h of growth in TY liquid medium after incubation in a 1% gelatin or 1% mucin column. The cells carry a fusion of the *tcdA* promoter without the CcpA binding box to SNAP^{Cd} in the 630Δ*erm* strain producing FliC^{T195C}. The cells migrated through columns of 1 ml volume

containing 800 μ l of gelatin or mucin to which 200 μ l of cells were added and collected in the bottom after 30 minutes. Microscopy was performed as indicated before. Filaments are colored green extracellularly with Alexa Fluor 488 C₅ Maleimide. The merged images show the overlap between the TMR-Star (red) and the Alexa Fluor 488 (green) channels. Scale Bar: 1 μ m. On the right is the percentage of vegetative cells with no SNAP accumulation (white) or with low (light red) or high (dark red) levels of SNAP accumulation. The percentage of vegetative cells is also indicated. **B**: Analysis of the fluorescence (Fl.) intensity of the SNAP-TMR signal per cell for the *tcdA* fusion, after cells migrate through a gelatin or mucin column. The analysis was performed using the Metamorph software suite version 5.8 (Universal Imaging).

5.3. THE LINK BETWEEN TOXINOGENESIS AND SPORULATION IN *C. DIFFICILE*

Both toxin production and sporulation are stationary phase phenomena ((Hundsberger *et al.*, 1997, Pereira *et al.*, 2013) and references therein). Importantly, the two processes are not antithetical, in that expression of at least *tcdA* is detected in many sporulating cells ((Ransom *et al.*, 2018); Chapter 3). Moreover, at least TcdA and most likely also TcdB, associate with mature spores in an active form (Chapter 4).

While *tcdA* expression in the mother cell may result from protracted expression by vegetative cells, expression in the forespore results from activation of *tcdR* expression in this cell. The forespore-specific expression of *tcdR* requires σ^G and SpoVT; this feed forward loop, together with the auto-regulatory *tcdR* loop, may delay toxin production until the late stages in spore morphogenesis (Alon, 2007). Moreover, the positive auto-regulatory loop may also determine that *tcdA* is not expressed in all of the forespores. In addition, since not all of the sporulating cells exhibit expression in the mother cell, the population is highly heterogeneous with respect to *tcdA* expression. We

have suggested (Chapter 3) that *tcdA* expression in the mother cell may provide a route for toxin release from the cell as this cell lysis at the end of spore differentiation. Conversely, the toxins that accumulate in the forespore may be released shortly after spore germination (Chapter 4). The toxins induce inflammation (Abt *et al.*, 2016, Solomon, 2013) and there is a correlation between the inflammation levels and the severity of infection (El Feghaly *et al.*, 2013). It seems possible that the toxins carried by some of the spores or released after germination may induce inflammation early in infection, facilitating the progression of the *C. difficile* infectious cycle.

Several receptors have been described for both TcdA and TcdB (see Chapter 1, section 1.4.3.1. Toxin cell receptors). One possibility is that spore-associated toxins participate in spore binding to the mucosa. We note that the antibodies used to localize the toxin at the spore surface recognize an epitope at the CROPs domain (Frey & Wilkins, 1992), indicating that at least this domain, one of the domains recognized by host cell receptors (See Chapter 1, section 1.4.3.1 Cell receptors), is exposed at the spore surface.

The association of toxinogenesis and sporogenesis has been noticed in other spore-formers. In the enteropathogen *Clostridium perfringens*, the enterotoxin CPE is produced during sporulation, in the mother cell, and associates with the spore coat (Friebe & Duncan, 1973, Labbe & Duncan, 1977). Furthermore, in *C. perfringens*, CPE is produced exclusively under the control of the sporulation sigma factors σ^E and σ^K (Harry *et al.*, 2009, Zhao & Melville, 1998). Since in this case the toxin is produced in the mother cell, lysis of this cell will lead to toxin release (Paredes-Sabja & Sarker, 2009). In *Clostridium*

botulinum type E, the production of the neurotoxin BoNT was also shown to be interconnected with sporulation, as Spo0A positively regulates toxin production. Furthermore, maximal BoNT release was only reached through mother cell lysis (Mascher *et al.*, 2017).

In the genus *Bacillus* other examples can be found of a connection between toxin production and sporulation. In *Bacillus thuringiensis* sporulation is essential for toxin production. In this insect pathogen the *cryIA* gene that encodes the insecticidal toxin is also regulated by σ^E and σ^K and depending on the strains, crystalline inclusions of the toxin may associate with the coat, the exosporium, or are trapped in the interspace between the two structures (Aronson, 2002, Aronson *et al.*, 1982, Baum & Malvar, 1995, Lereclus *et al.*, 2000). Similarly to *C. perfringens*, mother cell lysis will lead to toxin release (Minnich & Aronson, 1984, Sedlak *et al.*, 2000). *Bacillus anthracis* produces two toxins formed by three polypeptides: the lethal toxin and edema toxin that have a common receptor-binding component, the protective antigen (Moayeri *et al.*, 2015). Although the anthrax toxins are associated with the late stages of infection, the protective antigen is found soon after germination and at the spore surface by immunoelectron microscopy (Cote *et al.*, 2005). Protective antigen is mainly produced during the transition between the exponential and stationary phases, but contrarily to *B. thuringiensis* it is not regulated by sporulation-specific sigma factors (Koehler, 2002). A more distant example is *Brevibacillus laterosporus*, a pathogen of invertebrates, which is characterized by its insecticidal action and by the formation of a unique spore surrounded by a firmly attached canoe-shaped parasporal body (CSPB) on one side of the spore. In *B.*

laterosporus proteins important for virulence were found at the spore coat, the exosporium and the CSPB (Marche *et al.*, 2017).

Thus, different toxins, without homology, with different cell tropisms and from pathogens with different hosts, associate with the spore surface and in some cases sporulation-specific regulators regulate their production. Spores acting as toxin delivery vehicles seem a widespread strategy among pathogenic spore-formers.

5.4. TOXIN LOCALIZATION AT THE SPORE SURFACE IN *C. DIFFICILE*

Previous studies were not able to detect the toxin at the spore surface, but this may reflect differences in spore purification and extraction methods (Diaz-Gonzalez *et al.*, 2015, Ketley *et al.*, 1984). Still, an important question is how these large proteins localize at the spore surface. In a wild-type population of spores, the toxin that is detected in association with the coat/exosporium may result mainly from expression in the mother cell. One possibility is that the toxins are recruited during assembly of the spore protective layers through interactions with other proteins that form these structures (Henriques & Moran, 2007). A Far Western blotting assay suggests an interaction between different domains of TcdA and a protein of about 35 kDa that may be part of the coat/exosporium (Figure 5.2). This interaction is not due to unspecific detection by the secondary antibody, which was not able to detect any protein if the membrane was not incubated with TcdA or its fragments (Figure 5.2B). The protein or proteins with which TcdA interacts remain(s) to be elucidated.

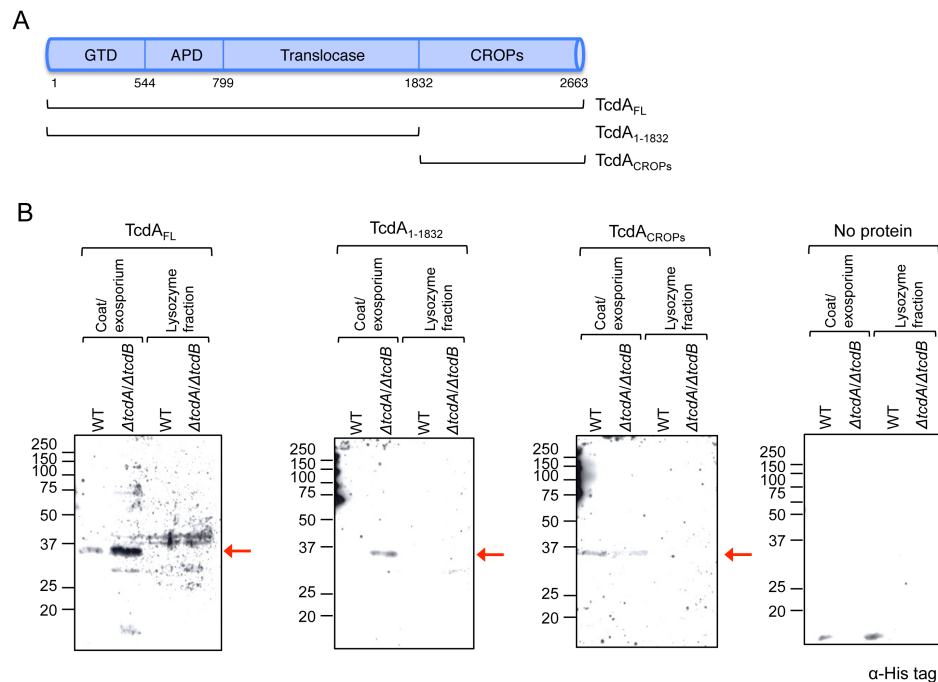


Figure 5.2. Toxin interaction with spore coat proteins. A: Domain organization of TcdA. The toxin is composed of four domains, the glucosyltransferase domain at the N-terminal, and auto-protease domain, the translocase domain and the combined repetitive oligopeptides (CROPs). The numbers represent the amino acid residues at the boundaries between the various domains; the fragments that were overproduced and purified are represented below the diagram. **B:** Far Western assay to detect interactions between the TcdA fragments and spore surface components. Spores of *C. difficile* 630Δ*erm* wild-type (WT) or the double mutant Δ*tcdA*Δ*tcdB* were purified and were fractionated into a coat/exosporium and a lysozyme fraction (see also Chapter 4). The proteins in the various fractions were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with purified versions of TcdA-His₆ (Kroh *et al.*, 2017, Pruitt *et al.*, 2012): full-length (TcdA_{FL}), a fragment with the GTD, APD and translocase domains (TcdA₁₋₁₈₃₂) and a fragment just with the CROPs domain (TcdA_{CROPs}), or with no protein as a negative control. Possible interactions with spore proteins immobilized in the membrane were detected using anti-His₆ polyclonal antibody. The red arrow points to a possible interactor, of about 35 kDa.

However, in a strain engineered to express *tcdR* only in the

forespore, at least a fraction of TcdA is also associated with the coat/exosporium. How the toxin reaches the spore surface layers from the forespore is presently unknown. Also unknown is how the toxins that associate with the spore surface have a cytotoxic effect, since the toxins need to be internalized by the host cells to reach their targets. In future work, we will determine whether the cytotoxic activity is due to the glucosyltransferase activity from the spore-associated toxin and whether Rac glucosylation is detected in cells that were in contact with purified spores (Gerhard *et al.*, 2008, Papatheodorou *et al.*, 2010).

We do not presently know whether the toxins are released from the spore surface or whether the spores are internalized and if so, whether the toxin is released at any stage. It has been shown that *C. difficile* spores can be internalized by macrophages. Once inside macrophages, *C. difficile* spores, unlike *C. perfringens* and *B. anthracis* spores are not able to germinate but still have cytotoxic activity (Paredes-Sabja *et al.*, 2012). The spores are phagocytosed and lysosomes fuse with the phagosomes containing the *C. difficile* spores. There, the spore surface contacts with the membrane, compromising the phagolysosome membrane integrity. Most likely, proteins at the spore surface interact with the membranes, and these interactions may involve the spore-associated toxins. Therefore, another role for the spore-associated toxins may be to counteract the immune cells system. Nevertheless, it is important to note that macrophages are immune cells that have the ability to phagocytize foreign bodies and the same might not be occurring for epithelial cells where the toxins act. Spore internalization could be tested with fluorescently labeled spores in contact with colonic cells. If the spores enter the cells, most likely they

will enter through endocytosis. Once inside the endosome, acidification might facilitate toxin release from the spore surface that will fuse with the endosome membrane and continue its normal mode of action.

Interestingly, *tcdE* that codes for a possible holin like protein suggested to be involved in toxin release (Govind & Dupuy, 2012, Govind *et al.*, 2015) is expressed in the forespore during sporulation (see Chapter 3). Whether TcdE has any role in the localization of the toxins in mature spores or in toxin release could be addressed by evaluating the cytotoxicity of $\Delta tcdE$ spores.

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