Molecular characterization of pathogenic Clostridium difficile strains

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Introduction

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General introduction and outline of the thesis

1 General introduction

Clostridium difficile is an enteropathogenic anaerobic Gram-positive spore forming rod, which can cause a wide variety of symptoms. *Clostridium difficile* infection (CDI) is recognized as the leading cause of infectious nosocomial diarrhoea. In the last decade the incidence and severity of CDI has increased and currently *C. difficile* is also frequently recognized as an important cause of community-acquired diarrhoea in humans. Finally, *C. difficile* may be present in the intestinal tract of asymptomatic and diarrhoeal (farm) animals.

Clostridiaceae

A major part of the human gut microbiome consists of Firmicutes (1). The phylum Firmicutes was traditionally classified to include all Gram-positive bacteria, but recently it was re-classified as a bacterial core-group with low-G+C genomic DNA content (2). Many Firmicutes are able to form (endo)spores, which are highly resistant to disinfectants and extreme environmental conditions (2). The phylum of Firmicutes can be roughly divided into two main classes; the *Bacilli*, which are aerobic, and the *Clostridiaceae,* which are obligate anaerobic (3).

The genus *Clostridium* belongs to the class of *Clostridiaceae* which consists of approximately 100 species (4). *Clostridium spp.* are ubiquitously found in the environment, soil, water and in the human and mammalian gastro intestinal tract as part of the commensal microbial flora (5). The genus *Clostridium* comprises several well characterized important human pathogens such as: *Clostridium tetani*, *Clostridium botulinum*, *Clostridium perfringens*, *Clostridium sordellii* and *Clostridium difficile* (2,4,6). All diseases caused by *Clostridium spp*. are mediated by the secretion and action of the secreted (exo)toxins (2,4,6,7).

Clinical disease and epidemiology

Almost eighty years ago (1935), *Clostridium difficile* was first described by Hall and O'Toole as *Bacillus difficilis* (8,9). *Clostridium difficile* was isolated from faeces of new-born infants and described as an actively motile, heavy bodied Gram-positive rod with elongated subterminal or nearly terminal non-bulging spores (8). Injection of whole or filtrated cultures in guinea pigs and rabbits resulted in rapid death (8). Survival of the animals injected with boiled and filtrated cultures led to the conclusion that *C. difficile* was able to produce a soluble heat labile toxin (8).

More insights in the pathogenicity of *C. difficile* were obtained by investigations in germfree mice and rats (10). In addition, a prospective study by Tedesco *et al*, evaluating 200 patients, subsequently reported that clindamycin administration was associated with development of diarrhoea (21%) and pseudomembranous

colitis (PMC) (10%) (11). In retrospect this was the first study to describe *C. difficile* as a nosocomial human pathogen (11). In 1977, Larson and Price isolated a clostridial toxin from faeces of patients with antibiotic-associated colitis and PMC (12). Subsequently, Bartlett and colleagues reported that the causative agent of antibiotic-associated PMC was indeed a toxin producing *C. difficile* (13). The lack of sensitive and reliable detection and identification methods has delayed the recognition of *C. difficile* as an important nosocomial pathogen for a long time (14). However, nowadays *C. difficile* is recognized as the most frequent cause of infectious nosocomial diarrhoea worldwide (9,15,16).

Several reports indicate that up to 3% of healthy individuals and up to almost 40% of hospitalized patients are colonized with toxinogenic *C. difficile* strains (17-19). Healthy individuals are usually protected by their normal colonic microflora (17). However, disturbance of the colonic microflora by for instance antibiotic usage leads to loss of the colonization resistance, enabling *C. difficile* to colonize, overgrow and cause disease (17). Besides antibiotic usage, other risk factors for CDI are advanced age (>60 years), underlying diseases and infection pressure (20). During infection *C. difficile* can produce three toxins which affect the integrity of the colon epithelium by destruction of the tight junctions, disrupt the cytoskeleton and lead to the formation of protrusions (21-24).

The typical clinical features of CDI range from mild diarrhoea to fulminant colitis, which can be life-threatening (25-27). Endoscopic examination of patients suffering from CDI may reveal minor abnormalities to extensive pseudomembranes (25). The formation of these pseudomembranes is caused by deep haemorrhagic ulcerations of the colon epithelium and a massive host inflammatory response (28). Symptoms of pseudomembranous colitis are cramping, fever, hypo-albuminemia, leukocytosis, nausea and general malaise besides the typical lesions observed by endoscopy (25,26). Eventually, pseudomembranous colitis may evolve into a syndrome called toxic megacolon, the most serious form of disease caused by CDI (29). Toxic megacolon is a toxic dilatation of the colon causing paralysis of the peristaltic movement and systemic cytotoxicity (29) accompanied by sepsis and organ dysfunction and resulting in high (33%) mortality rates (29,30).

In the last decade the epidemiology of CDI has changed radically. North-America was the first to report numerous hospital outbreaks with an increased incidence of CDI cases with a more severe course of the disease (27,31,32). Later on reports of outbreaks in Europe followed (33-35). These epidemics were primarily due to *C. difficile* PCR Ribotype (RT) 027, also known as North America Pulse-field type (NAP) 1 and Restriction Endonuclease Analysis (REA) type B1 (027/NAP1/B1) strain. *Clostridium difficile* RT 027 strains were referred to as hypervirulent strains, due to reports of higher toxin production, higher relapse

rates, more severe course of the disease and increased mortality rates (19,27,36-39). Certain other *C. difficile* types (e.g. RT017 and 078) have also been reported to be associated with outbreaks and a more severe course of disease in a hospital setting (34,39-44). Besides the increase of CDI incidence rates in a health care setting, an increased incidence rate in a community setting was reported (45-47). In some studies *C. difficile* RT 078 was more frequently associated with community acquired CDI than other ribotypes and affected a younger population than for instance *C. difficile* RT 027 strains (48,49). The course of *C. difficile* RT 078 associated disease can be as severe as observed for the hypervirulent *C. difficile* RT 027 strain (48) and the incidence of *C. difficile* RT 078 has increased in the Netherlands and other European countries (34,50). Interestingly recent studies have demonstrated that *C. difficile* RT 078 is the predominant type in cattle and pigs, suggesting a zoonotic potential (51-54).

Diagnostic and Typing methods

Diagnosis of CDI

The emergence of *C. difficile* strains as an important nosocomial human pathogen has stimulated the development of better diagnostic, detection and typing methods. The diagnosis of CDI is primarily based on clinical symptoms in combination with laboratory assays (55). Culturing *C. difficile* and the detection of the toxin(s) with a cytotoxicity assay is regarded as the gold standard for diagnosis of CDI (28,55,56). However, in many diagnostic laboratories culturing and cell cytotoxicity assays are not performed routinely, as they are labour intensive and costly (55,57,58). Most diagnostic laboratories depend on rapid and easy-to perform enzyme immuno assays (EIAs) that are generally designed to detect *C. difficile* Toxins A and/or B (55,57). Unfortunately, EIAs have limited specificity and/or sensitivity in an endemic situation (55). The limitations of EIAs have tempted clinicians into testing multiple samples per patient and to develop two-step algorithms for CDI diagnosis (55,59). However, for prevention and management of CDI rapid and more accurate assays are essential.

Quantitative real-time PCRs (qPCRs) are potential rapid assays with better performances than EIAs (56,60-62). Additionally, molecular tests such as qPCR have high sensitivities and specificities, comparable to the cell cytotoxicity assays for diagnosis of CDI (56,60,63-66). Both commercial and in-house developed qPCRs for the detection of *C. difficile* are primarily based on the detection of the toxin B and/or toxin A genes (56,60,63-66). In some cases the detection of the toxin genes is combined with the detection of point mutations in the *tcdC* gene, or the binary toxin genes, i.e. *cdtA* and *cdtB* (56,60,63-66).

The disadvantage of molecular tests compared to conventional phenotypic tests such as the cytotoxicity assay is the lack of discrimination between asymptomatic and symptomatic carriership. Despite the improved performances (sensitivity and specificity), molecular tests have in general low positive predictive values and therefore cannot be applied as stand-alone test (64). However, due to the high negative predictive values molecular tests, such as qPCRs, are valuable as first screening method in a two-step testing algorithm for diagnosis of CDI (56,60).

Typing of *Clostridium difficile* strains

Typing methods have been developed to study the epidemiology of *C. difficile* for several reasons; for detection and investigation of outbreaks, to recognize new emerging strains, to investigate local and global transmission routes, and to study the phylogeny (67,68). One of the most crucial properties of a typing method is the capability to differentiate between strains (67-69). Other important features are reproducibility and ease of use (69,70). Typing methods can be differentiated into two groups: phenotypic assays and genome based assays. Phenotypic assays are based on gene expression products, whereas genotyping methods are based on the DNA content of the strain (67,69,70).

Until the 1980s, phenotypic assays were standard to determine relatedness of strains (28,69). The simple and rapid serotyping assay was the most commonly used phenotypic assay (69,71). Eventually the assay was able to discriminate 23 different serotypes (69). Several of these (A, G, K, S1 and S4) were associated with (severe) CDI and PMC caused by CDI (72). Other serotypes (D and Cd-5) were suggested to be associated with asymptomatic carriership (72). Other typing methods were the Radio PAGE (25 types) and immunoblotting assay (26 types) (69). Eventually, the phenotypic typing methods were abandoned due to low reproducibility and a low discriminatory power for epidemiological studies with a larger -than local- scope (69).

The emergence of the important global epidemic *C. difficile* RT 027 strain has stimulated the development of genotyping methods. Molecular typing methods rely on purified DNA of a single cultured *C. difficile* clone. Often these techniques are performed in national reference centres, since culturing of *C. difficile* is not routinely performed in most diagnostic labs (69). Molecular methods are generally more reproducible and sensitive compared to the phenotypic assays used in the 1980s (69). The most common genotyping methods to include PCR ribotyping, Multi Locus Variable number of tandem repeat Analysis (MLVA), Multi Locus Sequence Typing (MLST) and single nucleotide polymorphisms (SNP) typing (60).

PCR ribotyping is the most frequently used molecular typing method in Europe (67,73). This PCR band-size based typing method exploits the variability of the

intergenic spacer region between 16S and 23S ribosomal DNA, as well as rDNA copy numbers (73-75). In comparison to other DNA based typing assays PCR ribotyping is superior in discriminatory power, (inter laboratory) reproducibility and its low hands on time (67,70). Although PCR ribotyping is the preferred typing method for monitoring outbreaks, it is not able to differentiate between strains in an outbreak situation (70).

MLVA is a highly discriminatory (sub)typing method to discriminate between strains within one Ribotype to identify outbreak transmission routes (60,68,70,76). MLVA is based on the amplification of short tandem repeats that vary in size and are spread throughout the genome (77). Marsh *et al.*, were the first to describe an automated analysis of the number of tandem repeats per locus followed by van den Berg *et al.,* (78,79). The two independently developed MLVA typing methods contained four out of seven identical loci (78,79). Based on MLVA results, a minimum spanning tree can be constructed to determine genetic relationships among strains. Genetic relationships are based on the number of Summed Tandem Repeat Differences (STRD) (41,78). Despite the lack of variability of two loci in RT027, MLVA has been reported to be the most discriminatory subtyping method to investigate outbreaks with the epidemic *C. difficile* RT 027 strain (79,80).

The availability of multiple genome sequences has made whole genome sequencing (WGS) accessible as a typing method (77,81-83). Recently, major advances have been made with WGS-based typing of *C. difficile* strains (81,84). WGS is able to reveal the natural history, phylogeny and global spread of epidemic strains (81,82,84). In addition, WGS is a reliable sequencing method that can distinguish strains at a single nucleotide level (81,84). The identification of single nucleotide polymorphisms (SNPs) across sequenced genomes has the potential to improve the discriminatory power over the more traditional MLVA genotyping method (81,85). Although MLVA and WGS analyse different aspects of the *C. difficile* genome, a recent publication on the performance of both techniques resulted in similar discriminatory power in an outbreak situation (85). WGS is also capable of detecting mixed infections, which offers prospects of screening for mixed infections in transmission studies (86). To date, the costs of WGS are still relatively high compared to other typing methods (67,86). However, the ability of WGS to extrapolate PCR ribotyping, MLST, sequences of toxin and resistance genes and other additional data, combined with standardized computational pipelines, could balance the cost benefit towards WGS in the future (38,67,77,81-83).

Mobile elements

WGS as a typing method is based on the non-repetitive core genome (82,84,87). However, comparative genomics has demonstrated that *C. difficile* has a highly

mosaic genome which consists of a large proportion (11%) of mobile elements (77,88). These mobile elements from phage, plasmid or transposon origin constitute the so called accessory genome (83,89). Mobile DNA elements can be transferred between *C. difficile* strains and may be passed on to other bacteria (77,90). Many *C. difficile* conjugative transposons (CTn) contain genes or other functions that could potentially contribute to the fitness and or virulence of strains harbouring them (76,77,90-92).

Conjugative transposons are defined as "specific DNA elements that can integrate into one or more sites in one or more genomes" (93). In general, CTns consist of a core region (conjugation and regulation module), a recombination module and a module with accessory gene(s) (91,93,94). After excision from the genome, intermediates are formed that can be transferred through conjugation into a recipient strain, where they can integrate via specific target site recombination (91,93,94). The accessory module can be diverse, and the genes primarily present encode determinants for antibiotic resistance or metabolic functions (76,91,93-98), often accompanied by dedicated transcriptional regulators (94).

The genomic sequence of *C. difficile* strain 630 revealed the presence of six putative CTns in addition to the extensively investigated Tn*5397* element (76,77,95,99). The putative CTns are named based on their locations in the 630 genome (77). Four of the identified transposons are closely related to Tn*916* (77). The Tn*916*-like family of conjugative transposons generally encode tetracycline resistance determinants and is broadly found in Firmicutes (94). The other CTns present in *C. difficile* strain 630 are more closely related to Tn*1549*, a conjugative transposon responsible for vancomycin resistance (77,100). Transposons in other (non 630) *C. difficile* strains encode resistance to chloramphenicol, spectinomycin, streptomycin and erythromycin (81,96).

It is easy to envisage how the presence of antibiotic resistance determinants could increase the fitness of strains, but the contribution of other genes in the accessory genome to virulence remains largely unexplored. A recent study suggested that the accessory genome could be involved in the increased virulence and mortality of a sub-group of *Clostridium difficile* RT 015 strains (39). Furthermore, some virulence associated genes, including the *C. difficile* pathogenicity locus (PaLoc) have been suggested to be of phage origin (101-103). Moreover, it has been demonstrated that phages can affect toxin production levels in *C. difficile*, thereby modulating virulence (103,104).

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Clostridium difficile virulence factors

Clostridium difficile toxins

The symptoms of CDI are mainly caused by the two main virulence factors, Toxin A (TcdA) and Toxin B (TcdB) (23,105-107). All naturally disease causing (toxinogenic) *C. difficile* strains produce Toxin B, and most of the strains also Toxin A (23,34,108,109). No naturally occurring Toxin B negative strains have been reported to date. Toxin A (308 kDa) and Toxin B (270 kDa) belong to the family of large clostridial cytotoxins (21,24). The members of this family are single chain toxins consisting of at least four domains (Figure 1): a receptor binding domain (110,111), a cysteine protease domain, a translocation domain (112,113) and a glycosyltransferase domain (21,23,24).

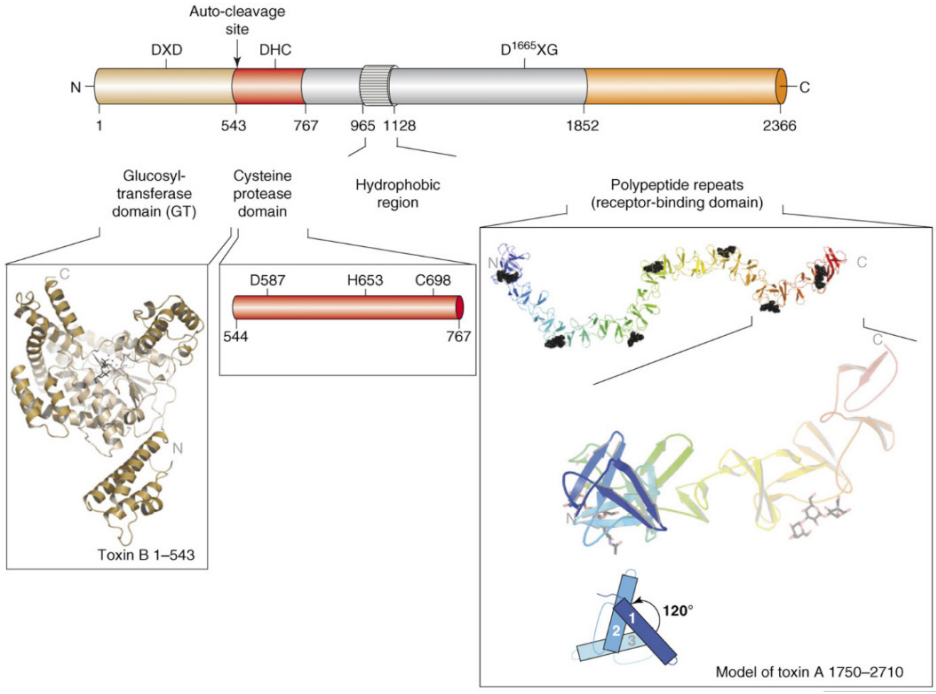

Figure 1: Schematic view of the domain structure of clostridial toxins. The catalytic domain is located at the N-terminus and the CROP region, which serves as receptor binding domain, is situated at the C-terminus. In between the N- and C-terminus the translocation domain (hydrophobic region) and the cysteine protease domain are located (113). Jank, T and Aktories K., Structure and mode of action of clostridial glycosylating toxins: the ABCD model.Trends Microbiol.16:222-229, Copyright © 2014 Elsevier Masson SAS. All rights reserved.

The receptor binding domain is located at the C-terminus and covers almost one third of the toxin, in the case of Toxin A (23,24). The C-terminus is characterized by repetitive peptide elements that are called combined repetitive oligopeptides (CROPS) (23,24,111). The crystal structure of the CROPs of Toxin A shows a solenoid like structure (110). Solenoid structures are widely present on bacterial surfaces and play a role in protein-protein or protein-carbohydrate interactions. The crystal structure of the CROP region in complex with a carbohydrate demonstrated a specific interaction (114). However, the carbohydrates used in the crystal structure are absent in sensitive cells and in human tissue (113,115) and other (human) glycan structures need to be tested for their role in toxin binding.

Despite a similar structure of the C-terminus of Toxin B, experimental data suggest that binding of Toxin A and B is mediated by different types of receptors (113,116). Interestingly, it has recently been shown that toxins can enter eukaryotic cells via a CROP independent mechanism (117). Although the biological relevance of this observation is not yet clear, it is suggestive of an alternative binding motif within the toxin (117).Binding of the toxin to the eukaryotic cell receptor results in receptor-mediated endocytosis (21,24). Internalization of the toxins occurs through the clathrin mediated pathway (118,119). After the clathrin coated vesicle is fused to an endosome (118), the low pH in the endosome results in a conformational change of the toxin characterized by an increased exposure of the hydrophobic region (21,24,115). This, in turn, allows translocation of the catalytic domain and the cysteine protease domain into the cytosol (115). Cytosolic inositol hexaphosphate (InsP6) can subsequently induce the autocleavage activity of the cysteine protease domain, resulting in the release of the catalytic domain in the cytosol (21,24,115). Interestingly, it has recently been shown that cytotoxicity of Toxin B can be independent of catalytic domain cleavage (120). The catalytic domain is a glycosyltransferase which inactivates Rho GTPases (Rho, Rac and Cdc42) in the cytosol by glycosylation (21,24,115,121). This results in the inhibition of multiple effectors that ultimately results in apoptosis of the targeted cell (21,23,24,113). A subsequent inflammatory response and further degradation of the intestinal epithelial cell layer eventually leads to the development of the clinical symptoms of CDI (23,24,122).

Transcriptional regulation of the Pathogenicity Locus

The genes encoding the major clostridial toxins, *tcdA* and *tcdB*, are located on a 19-kb genomic region called the Pathogenicity Locus (PaLoc) (23,24). In between the toxin genes the *tcdE* gene is situated, which encodes a holin-like protein (101,123,124). TcdE is a membrane associated protein (23,101) but its involvement in the release of the toxins is disputed (123,124). Besides the toxin genes and *tcdE*, the PaLoc also contains two regulatory genes, *tcdR* and *tcdC* (23,125,126).

The *tcd*R gene encodes the alternative RNA polymerase sigma (**σ**) factor, TcdR that positively regulates toxin production. It mediates binding of RNA polymerase core enzyme to the *tcdA*, *tcdB* and *tcdR* promoter regions (125-127). TcdR belongs to the sub-family of extra cytoplasmic factor (ECF) σ^{70} factors (125). In other bacteria, members of this family have also been demonstrated to positively regulate potent toxins, such as the tetanus neurotoxin of *Clostridium tetani* (128). TcdC has been reported to act like an anti-sigma factor for toxin production by destabilizing the TcdR-RNA polymerase core enzyme complex in a way that is not yet fully understood (126). Besides the PaLoc encoded regulators, toxin levels are also directly influenced by the nutritional regulators CodY and CcpA, and the sigma factors σ^{H} and σ^{D} (129-134).

In strain VPI10463, the exponential growth phase of *C. difficile* is associated with a high transcription level of the *tcdC* gene and low transcription levels of *tcdR* and the toxin genes, whereas the stationary growth phase is associated with a low transcription level of the *tcdC* gene and high transcription levels of *tcdR* and the toxin genes (135). The synthesis and secretion of the toxins is increased upon entry into the stationary growth phase (135-138). The decreasing transcription of *tcdC* correlates with diminishing TcdC protein levels in stationary growth phase (135,138,139). Interestingly, mutations in the *tcdC* gene in the hypervirulent strains RT 027 (**Δ117**) and RT 078 (C184T) have been linked to increased virulence (43,140). These mutations result in the absence of a functional TcdC caused by a frame shift mutation (Δ117 bp) or a premature stopcodon (C184T) in *tcdC* which is linked to an increased toxin production (19,140). Together, the reported inverse correlation between the transcription of *tcdC* and the toxin genes and the expression patterns of the corresponding proteins (TcdC, TcdA and TcdB), have led to the prevailing model that TcdC is an important repressor of toxin expression (125,126,135,139). However, recently some doubts have been raised about the importance of TcdC in the regulation of toxin expression. (137,138).

Some strains that are associated with an increased mortality and morbidity (e.g. RT 027 and RT 078) also produce a third toxin, called the binary toxin since it consists of two polypeptides encoded by the *cdtA* and *cdtB* genes (27,43). The binary toxin genes are located on a 6.2 kb region called the Cdt locus, or CdtLoc (141). A gene (*cdtR*) encoding for a regulatory protein is located alongside the binary toxin genes on the CdtLoc (141). It has been suggested that binary toxin may contribute to disease in hamsters (106) and *in-vitro* assays have demonstrated that the binary toxin affects adhesion of *C. difficile* to cells through induction of protrusion formation of the target cells (22,142).

Non-toxin virulence factors of *Clostridium difficile*

Clostridium difficile sporulation

Clostridium difficile is transmitted through the faecal-oral route. Spores are believed to form the primary vehicle for infection and sporulation is recognized as a crucial factor in the transmission route and as a persistence factor for *C. difficile* (143). Ingested spores germinate in the small bowel upon exposure to bile acids (144). The newly formed vegetative cells start to produce toxins and can cause disease (144).

The primary purpose for sporulation is survival of the bacteria under harsh environmental conditions for prolonged periods of time. Spores have a high tolerance against disinfectants and resistance towards antimicrobial compounds and therefore are able to persist on (hospital) surfaces and in the environment (145,146). The ability to form spores is a key factor to infect a new host, as they are able to survive the oxygenated environment outside the host as well as the harsh conditions in the host. Moreover, they confer the ability to persist whereas the commensal flora is eliminated by an antibiotic treatment (144,147).

The regulation of spore formation in *C. difficile* is still poorly characterized compared to the extensively studied spore forming *Bacillus subtilis* (148). Though the pathways downstream of the master regulator for sporulation, Spo0A, seem to a large extent conserved between *B. subtilis* and *Clostridia*, this is less so for the pathways upstream Spo0A activation (149-152). As expected, *spo0A* is required for sporulation in *C. difficile* (153). The sporulation pathway in *Bacillus subtilis* is induced under conditions of nutrition-starvation (148). Under these conditions phosphorylation of the master regulator Spo0A occurs (150,154). Phosphorylated Spo0A (Spo0A-P) is an essential activator of the sporulation signalling cascade in *Bacilli* which ultimately leads to the formation mature spores (149,150). Importantly, in other organisms Spo0A regulates many other processes than sporulation, such as stress responses, biofilm formation, competence for genetic transformation, and DNA replication but also the synthesis of virulence factors (155-164).

Clostridium difficile factors affecting colonization

Spores and toxins are established virulence factors that contribute to transmission, infection and development of disease (23,122,143,144). But an essential first step in establishment of *C. difficile* in the colon is adhesion to the mucosal surfaces and subsequent colonization of the host (Figure 2) (144). Relatively little is known about adhesion and colonization factors of *C. difficile*. The surface layer of *C. difficile* contains a whole arsenal of proteins that could potentially be involved in adhesion and colonization. Presently, a few surface proteins are identified or hypothesized to

play a role in adhesion to cells or colonization of the host. Among these proteins are fibronectin-binding protein A (165), Cwp84 (166), flagellar proteins (167) CD1581 (168), and several S-layer proteins (169).

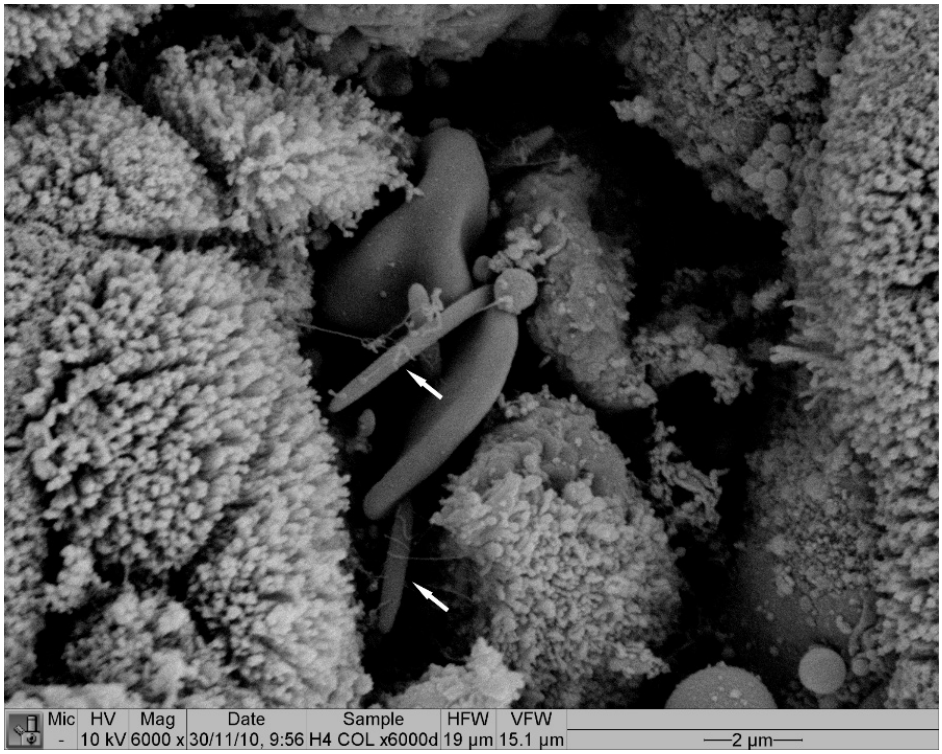

Figure 2: Visualisation (SEM picture) of an adhered vegetative *C. difficile* (arrows) to host colon epithelium (This picture was kindly provided by A.M. Buckley).

Mechanisms of stress survival

During its life cycle *C. difficile* encounters stressful environments, like antibacterial substances and proteins, elevated temperatures, extreme pH and osmotic stress. These extracellular stresses can result in the accumulation of (partially) unfolded proteins, especially in the bacterial envelope, which are non-functional or form poisonous aggregates (170). They may also affect the integrity of the bacterial cell envelope, compromising cell viability. Thus, sensing and responding to extracellular stresses is important for survival of the bacteria. Adaptation to these host-induced stresses can play a vital role in the virulence of bacteria and the establishment of an infection.

Regulated intramembrane proteolysis (RIP) is one of the mechanisms that enables bacteria to monitor and respond to extracellular stresses (Figure 3) (171).

The RIP mechanism activates regulatory pathways by releasing the sequestered sigma factors from their cognate anti-sigma factors as the result of envelope stress (171,172). In *Bacillus subtilis*, extracellular stresses are sensed by an site-1 protease, PrsW (172). Upon stress, PrsW undergoes conformational changes which allow cleavage of an anti-sigma factor at the C-terminus (172). The cleaved anti-sigma factor is recognized and further processed by extracytoplasmic proteases and a site-2 protease, RasP (171,172). The remaining part of the anti-sigma factor is further degraded in the cytoplasm by proteases like ClpX, leading to release of the sigma factor (171,172). The sigma factor then interacts with RNA polymerase to activate transcription of stress related genes (171,172).

Another well described mechanism to sense and respond to stress are two-component systems. Two component systems are composed of a sensor kinase and a response regulator (173,174). Monitoring envelope stress by the sensor kinase results in phosphorylation of the response regulator (174). This in turn leads to activation of stress related target genes (173,174). In *Bacillus subtilis* a two

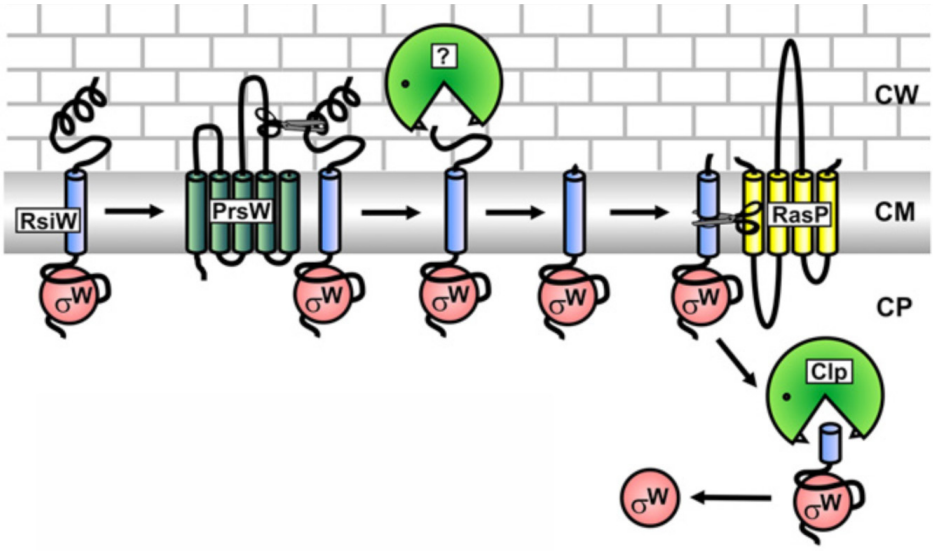

Figure 3: Schematic view of the RIP pathway in Bacillus *subtilis*. The stress induced PrsW degrades the C-terminus of the anti-sigma factor (RsiW) resulting in further processing of RsiW by extracytoplasmic proteases and RasP. The partially degraded anti-sigma factor is released into the cytoplasmic and further degraded by cytoplasmic proteases like Clp. The released sigma factor interacts with RNA polymerase which results in activation of stress related genes (171). Heinrich, J and Wiegert T. Regulated intramembrane proteolysis in the control of extracytoplasmic function sigma factors. Res. Microbiol.160:696-703

component system consisting of a sensor kinase (CssS) and a response regulator (CssR) plays a role in the detection of extracytoplasmic misfolded proteins (174). It does so primarily through the activation of expression of two homologous proteins of the High temperature requirement A (HtrA) family (174).

Bacillus subtilis HtrA and HtrB are associated with the cytoplasmic membrane and are composed of membrane spanning domain, a trypsin-like serine protease domain and one PDZ domain (174-178). Both proteins have fairly broad substrate specificity. PDZ domains are highly flexible domains and are involved in substrate recognition and/or the regulation of protease activity (177,179). Bacterial HtrA-like proteins are important for controlling protein quality and homeostasis by combining proteolytic and chaperone activities (175,176,179). Membrane anchored HtrA-like proteases are active as trimers and soluble HtrA-like proteases form larger active oligomers (176,179).

Outline of this thesis

This thesis covers a broad range of research topics varying from an applied approach to more fundamental experiments. **Chapter 1** is the current chapter where we provide a general overview.

Applied research

In **Chapter 2** we describe the evaluation of a modified in-house developed molecular assay with improved performance for diagnosis of CDI. In addition we evaluated two other in-house developed qPCRs, of which one detects the presence of both toxin genes and the other detects the frame shift mutation in the *tcdC* gene. We evaluated the qPCRs by comparing them to a commercially available molecular assay and to the gold standards. In **Chapter 3,** the relatedness between human and porcine *C. difficile* RT 078 strains is investigated using a modified and optimized MLVA. Moreover, we also investigate the susceptibility to tetracycline and determined the genetic origin of tetracycline resistance to further support the high relatedness between human and porcine RT 078 strains. In **Chapter 4** we identified a 100kb insert in a porcine RT 078 strain through whole genome sequencing and characterized this mobile element further.

Fundamental research

Chapter 5 reports that *C. difficile* Spo0A recognizes and binds to similar sequences as the well characterized *B. subtilis* Spo0A. The *in-vitro* binding assays suggest that *tcdB* may be a direct target of Spo0A. Therefore, we also characterized the toxin production of an isogenic Spo0A mutant strain. In **Chapter 6** we generated an isogenic *tcdC* mutant to investigate the possible role of TcdC in toxin production.

In **Chapter 7** we propose an alternative role for the putative anti-sigma factor TcdC. **Chapter 8** describes the identification and characterization of a *C. difficile* HtrA-like protease. The effect on the transcriptome and *in-vivo* virulence was determined by comparing the generated isogenic HtrA mutant to wild type.

Discussion

Chapter 9 provides the general discussion, including the main conclusions of this thesis and future perspectives. **Chapter 10** provides an overview of the content of this dissertation in Dutch.

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