Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

BIOLOGIA CELLULARE E MOLECULARE

Ciclo XXVI

Settore Concorsuale di afferenza: 05/E2- BIOLOGIA MOLECOLARE

Settore Scientifico disciplinare: BIO/11- BIOLOGIA MOLECOLARE

Biofilm formation by the anaerobic pathogen *Clostridium difficile*

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ABSTRACT

Clostridium difficile is an obligate anaerobic, Gram-positive, endospore-forming bacterium. Although an opportunistic pathogen, it is one of the important causes of healthcare-associated infections. *C. difficile* colonizes the gut when the normal intestinal microflora is disrupted by antimicrobial agents and causes *Clostridium difficile* infection (CDI). Recurrent clostridial infections and rapid rise of antibiotic resistant strains have made disease treatment very difficult.

While toxins TcdA and TcdB are the main virulence factors of *C. difficile*, the factors or processes involved in gut colonization during infection remain unclear. The biofilm-forming ability of bacterial pathogens has been associated with increased antibiotic resistance and chronic recurrent infections. Bacteria within biofilms, which are complex bacterial communities held together by a self-produced extracellular matrix, are protected from multiple stresses, including immune responses and antimicrobial agents. Although biofilms have been well studied for several gut pathogens, little is known about biofilm formation by anaerobic gut species. Biofilm formation by *C. difficile* could play a role in virulence and persistence of *C. difficile*, as seen for other intestinal pathogens. This work aimed to study biofilm formation by *C. difficile* and the factors that modulate this process.

We demonstrate that *C. difficile* clinical strains, 630, and the strain isolated in the outbreak, R20291, form structured biofilms *in vitro.* Biofilm matrix is made of proteins, DNA and polysaccharide. Strain R20291 accumulates substantially more biofilm. Employing isogenic mutants, we show that virulence-associated proteins, Cwp84, flagella and a putative quorum sensing regulator, LuxS, are required for maximal biofilm formation by *C. difficile*. A mutant in Spo0A, a transcription factor that controls spore formation, was also defective for biofilm formation, indicating a possible link between sporulation and biofilm formation. Moreover we demonstrate that bacteria in *C. difficile* biofilms are more resistant to high concentrations of

vancomycin, a drug commonly used for treatment of CDI, and that inhibitory and sub-inhibitory concentrations of the same antibiotic induce biofilm formation.

Surprisingly, clinical *C. difficile* strains from the same out-break, but from different origin, show differences in biofilm formation. Genome sequence analysis of these strains showed presence of a single nucleoide polymorphism (SNP) in the anti-σ factor RsbW, which regulates the stress-induced alternative sigma factor B (σ^B). We further demonstrate that RsbW, a negative regulator of alternative sigma factor B, has a role in biofilm formation and sporulation of *C. difficile*.

Our data suggest that biofilm formation by *C. difficile* is a complex multifactorial process and may be a crucial mechanism for clostridial persistence in the host.

ACKNOWLEDGEMENTS

My first debt of gratitude must go to **Dr. Meera Unnikrishnan**. She patiently provided with new visions, encourage me when most needed, and advise neccesary for me to proceed through my scientific cariere. With her enthusiasm for the science she taught me how to challenge myself, pushed me to think outside of the box and to critically think inside the box.

To **Jacqueline** and **Nigel Minton** go my most sincere thanks, not just for giving me the opportunity to be part of ClostNet family, but also for always warm welcome in Nottingham and for being like my English family. Thank you! Nevertheless, big thanks from all my heart go to my **ClostNet friends**! Every ClostNet reunion was the best one! Love you guys!

I would like to thank **Dr. Davide Serruto**, **Dr. Isabel Delany** and whole **Molecular Genetics Unit**. It was nice to work with all of you. To my guys – **Jack**, **Andreas** and **Mark** – for all the funny, interesting, useful, scientific and unscientific coffees and co.! To **Valeria Cafardi,** for sharing with me Matilde, useful ideas, and overall helping me during all three years of my PhD.
Thanks to **Prof. Vincenzo Scarlato**, who followed my project through all years of my PhD, and to the members of PhD thesis defense committee.

Buket, **Christina**, **Giuseppe**, **Hebert**, **Myra** – to have made "Siena memories" with me. Because of you they are memorable! Thank you!

Andrej, **Ninč**, **Maš**, **Mohi**, **Sta**, **Tanja** – kako že..? Godine prolaze nervoznim korakom, jesmo li sretniji i jesmo pametniji? Godine prolaze, mi gledamo… Dokler gledam z vami je meni čisto OK!

…and to the magic, stars, orion, saksofon, kontrabas and spetses.. in zemlja zapleše, tja med zvezde!

Največja hvala moji **družini** in **mami**. Za nepogojno ljubezen in podporo v uresničitvi še tako bizarnih želja! Hvala!

Sapere aude, dare to be wise.

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ADP	Adenosine diphosphate
BHI	Brain heart infusion
bp	Base pair
BF	Biofilm
BSA	Bovine serum albumin
CDI	*Clostridium difficile* infection
c-di-GMP	Cyclic diguanylate
CDM	Chemically defined medium
CDMM	Chemically defined minimal medium
Cdt	*Clostridium difficile* transferase
CdtLoc	Cdt locus
CFU	Colony-forming unit
CV	Crystal violet
CWPs	Cell wall proteins
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
ECF	Extracytoplasmic function
EPS	Extracellular polymeric substance

FC	5-fluorocytosine
Glc	D-glucose
HMW	High molecular weight
kb	Kilobase pair
kDa	Kilodalton
LB	Luria-Bertani medium
LMW	Low molecular weight
MIC	Minimal inhibitory concentration
OD	Optical density
PaLoc	Pathogenicity locus
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PMC	Pseudomembranous colitis
qRT PCR	Quantitative real-time PCR
QS	Quorum sensing
RNA	Ribonucleic acid
RT	Room temperature
SLPs	Surface layer proteins

SNP	Single-nucleotide polymorphism
U	Unit
WT	Wild-type

LIST OF PUBLICATIONS

- Dapa et al. 2014. Role of antisigma-factor RsbW in *Clostridium difficile* biofilm formation and sporulation. Manuscript under preparation.
- Dapa and Unnikrishnan. 2013. Biofilm formation by *Clostridium difficile*. Gut Microbes 4: 1-6. Invited addendum.
- Đapa et.al. 2013. Multiple factors modulate biofilm formation by the anaerobic pathogen *Clostridium difficile*. J Bacteriol. 2013 Feb;195(3):545-55.

1 INTRODUCTION

1.1 *Clostridium difficile*

Clostridium difficile is a Gram positive, rod shaped, obligate anaerobic bacterium. *C. difficile* belongs to the phylum Firmicutes, and like other genera from this phylum, exists in two forms, the vegetative cell and in the endospore (Figure 1) (Fritze, 2004). When in the vegetative form the bacterium is metabolically active and in a reproductive state, while when in the endospore form, the bacterium is a metabolically dormant, non-reproductive state. As an endospore, the bacterium can survive for long periods under unfavourable conditions, like lack of nutrients, severe physical and chemical environments, and extreme temperatures (Setlow, 2007).

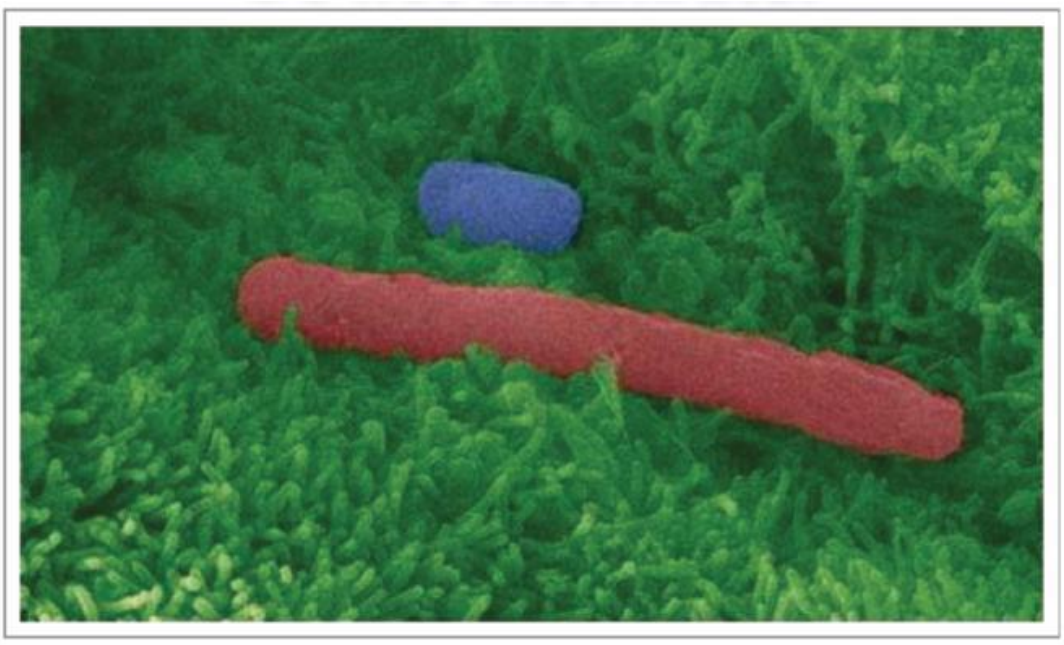


Figure 1 *C. difficile* spore and vegetative cell (Vedantam et al., 2012).

Clostridium difficile is an intestinal opportunistic pathogen of humans and animals and in the last decades was recognized as the most common cause of nosocomial diarrhea (Poxton et al., 2001); (Rupnik et al., 2009); (Kuipers and Surawicz, 2008). *C. difficile* infections (CDI) occur when the normal intestinal flora is damaged or absent after the use of antimicrobial agents. The bacterium colonizes the last part of the intestinal tract, the colon. Clinical outcomes can

range from asymptomatical colonization or mild diarrhoea to severe disorders, such as pseudomembranous colitis (PMC) (Rupnik et al., 2009). Antibiotic treatment is available, but high percentages of patients develop recurrent infections. The spores were connected with the transmission and the recurrence of the disease (Lawley et al., 2009); (Vedantam et al., 2012).

1.1.1 History

C. difficile was first reported in first half of the last century. In 1935, in an attempt to understand the development of normal bacterial flora in neonates, Hall and O'Toole (1935) discovered a new pathogenic anaerobe, which they named *Bacillus difficilis*. The name 'difficilis' (Latin 'difficile' stands for 'difficult, obstinate') was given to the newly isolated bacterium because of the difficulty involved in its isolation and study. *Bacillus difficilis*, later renamed in *Clostridium difficile* and placed in the genus *Clostridium*, did not show pathogenicity in newborns, but was pathogenic in guinea pigs.

The connection between *C. difficile* infections, and diseases caused by it, was first reported long after the first isolation. While the disease pseudomembranous colitis (PMC) was first described by Finney in 1893, the connection with the *Clostridium difficile* was not linked till second half of 20th century. In 1974 Tedesco *et al*. (Tedesco et al., 1974) published the key paper connecting the use of antibiotics to PMC (at the time named 'clindamycin-associated colitis', due to the connection of use of antibiotics with the development of the disease), and couple of years later, *C. difficile* was found responsible for causing the disease (Gerding, 2009).

Since then the bacterium *Clostridium difficile* has been well studied and characterized. Two large toxins, toxin A and toxin B, were recognized as main virulence factors (Kuehne et al., 2010); (Lyras et al., 2009); (Shen, 2012); (Voth and Ballard, 2005). However, of late, due to high percentage of recurrent and persistent infections, other virulence factors like colonization and spore formation, are coming to the forefront.

1.1.2 *Clostridium difficile* infection (CDI)

Clostridium difficile infection (CDI) is recognized as one of the main causes for healthcare associated diarrheoa in recent years. Elderly patients, undergoing treatment with broad-spectrum antibiotics or surgical procedures, in the hospitals or other healthcare institutions, are at the highest risk for developing CDI (Figure 2) (Rupnik et al., 2009). The symptoms of the infection can be mild, where the patients may present slight fever, loose stools and abdominal cramps. The disease can progress to severe colitis with typical adherent pseudomembranes, which can coalesce to obscure the mucosa. Severe colitis can result in toxic megacolon, colon perforation and progressive multiorgan failure. Mortality in patients with CDI ranges from 8–38% (Mitchell and Gardner, 2012).

Clostridium difficile, as an obligate anaerobic bacterium, can exist in aerobic conditions exclusively in the form of spores. As such, it is present in the air and on contaminated medical devices. Hence, the only way that *C. difficile* can infect the host, is in the form of spores. The most common way of transmission of *C. difficile* spores is by direct contact between patients, through the contact between healthcare workers and patients, or via contact with contaminated surfaces. Most people acquire the infection once hospitalized, or in other healthcare institutions. However, infections acquired in the community have started to increase. *C. difficile* spores are high resistant to different stresses like heat stress, low pH stress, presence of alcohol or other disinfectants. This allows *C. difficile* spores to persist in the hospital and healthcare environments for months. Around 3% of healthy adults, and about 20 % of treated patients, carry *C. difficile*, and it is known, that both, asymptotic carriers and infected patients, are sources of infection (Gould and McDonald, 2008).

Recurrences of disease are frequent and pose major problems for the clinical treatment of CDI. Recurrent CDI can arise in 20% of the cases after the first episode, and in 50% after the second episode after the treatment (Barbut et al., 2007).

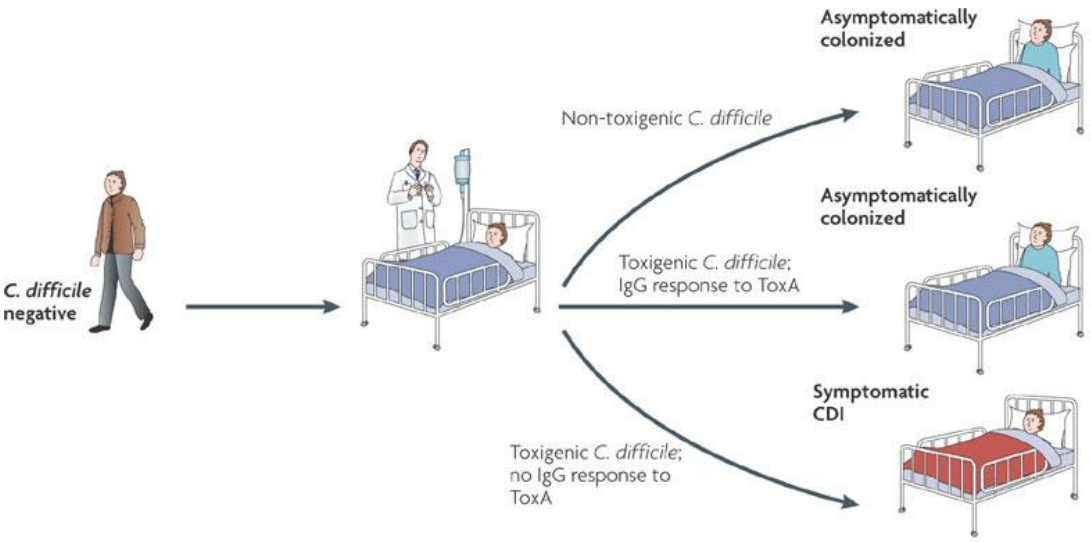


Figure 2 Hospitalized patients are at the highest risk for developing CDI. Patients come in contact with *C. difficile* spores when hospitalized and through the contact with the hospital environment or health care workers. If the acquired *C. difficile* strain is a toxigenic strain, and the patient after taking antibiotics fails to mount an anamnestic serum immunoglobulin G (IgG) antibody response to toxin A (TcdA), the patient develops CDI. In case, when the patient can mount an antibody response, or if the acquired *C. difficile* strain is a non-toxigenic strain, the colonization with *C. difficile* is asymptomatic. It has been shown that colonized patients develop protection from CDI (Rupnik et al., 2009).

1.1.2.1 Emergence of 'hypervirulent' strains

The rates and the severity of CDI is drastically increasing since 2000. The parts of the world where the CDI causes biggest problems are the developed countries, where the antibiotic treatments are most common, like in North America and big part of Europe. The increasing incidence and severity of CDI has been attributed to the emergence of ribotype 027 (McDonald et al., 2005); (Loo et al., 2005); (O'Connor et al., 2009); (Warny et al., 2005). The factor thought to contribute to incidence is the strain's resistance to fluoroquinolones, and enhanced virulence, which was associated with increased toxin production *in vitro* (Warny et al., 2005). Emerging strains belonging to PCR-ribotype 017 and 078, that are also linked with severe disease, have been isolated in parts of Asia and Europe (Drudy et al., 2007); (Kim et al., 2008). At the moment there is a prevalent interest in understanding the underlying factors that have led to the emergence and the apparent increased virulence of such *C. difficile* strains.

1.1.2.2 Transmition and colonization of the colon

Since the spores are resistant to the low pH, they can pass through the stomach acid environment, and reach the small intestine. Favourable conditions of the small intestine (presence of specific germination-promoting factors e.g. bile salts) promote spore germination (Burns, 2010). Vegetative bacteria can then colonize the last part of the intestinal tract, the colon. Colonization can occur only when the normal intestinal microflora of the host is absent or altered, which usually occurs in the presence of risk factors, such as prolonged antibiotic therapy, advanced age, or hospitalization (Poxton et al., 2001); (Rupnik et al., 2009); (Kuipers and Surawicz, 2008). Thus, a strong association between antimicrobial therapy and CDI has been almost generally recognized. Once the bacteria proliferate in the colon of the host, they are able to produce toxins, which results in the characteristic pathology of the colon (Figure 3) (Vedantam et al., 2012).

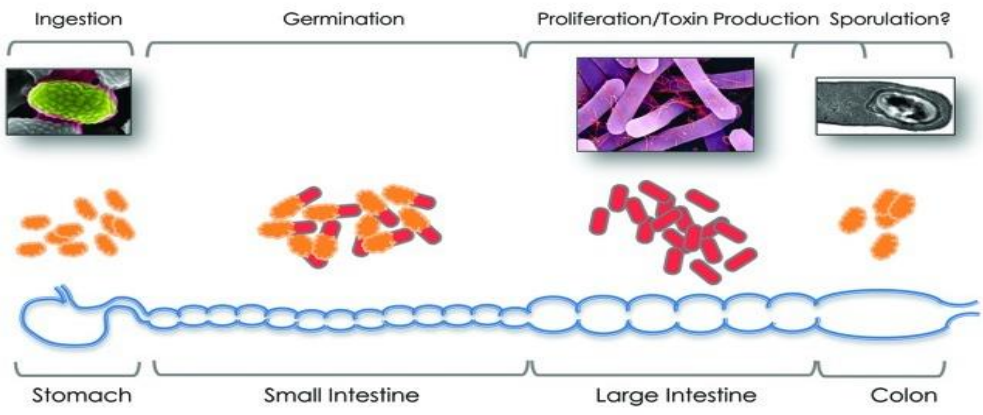


Figure 3 Schematic of human *C. difficile* infection. Bacterial factors that contribute to the colonization of the human intestine are spores, vegetative cells, tolerance to the bile salts, cell-wall proteins resistance to cathelicidin and O-nitrosylation. Resistant barrier mechanisms from the side of the host are gastric acid, bile salts, innate and adaptive immune responses and normal gut microbiota (Vedantam et al., 2012).

Initial studies correlating antibiotic treatment and CDI were focused on use of antibiotics clindamycin and cephalosporins (Aronsson et al., 1985); (Gerding, 2009). Subsequently the use of fluoroquinolones have arisen as possibly the major inducing agents. Use of fluoroquinolones was connected with several outbreaks of the CDI (Pepin et al., 2005). The

only way to control the emerging outbreaks has been to restrain, or completely prohibit, the use of the entire class of these antibiotics (Gerding, 2004); (Kallen et al., 2009).

1.1.2.3 Recurrence and the treatment of CDI

Recurrence and severe complications of CDI are the main problems in the treatment of CDI. Antibiotics of choice for the treatment of CDI are metronidazole and vancomycin. Resistance to antibiotics used for treatment does not pose a significant threat so far. Nonetheless, in the case of multiple recurrences, the CDI typically respond to treatment with vancomycin or metronidazole, but the diarrheoa symptoms resume within days to weeks after treatment is stopped. Between 20% and 50% of these recurrences are caused by a reinfection rather than a relapse of the original infection (Johnson et al., 1989); (O'Neill et al., 1991). Till recently no highly effective treatment for multiple recurrent CDI have been recognise as efficient. Prolonged pulse dosing of vancomycin was used in hope of preventing *C. difficile* from regrowth, while the normal microflora recovers. Lately was demonstrated that treatment with antibiotic fidamoxin is not inferior compared to current available antibiotics, but has minimal effect on the normal colonic microflora, which is the reason why less patients develop a recurrent CDI (Cornely, 2012). Moreover treatment with the replacement of the normal bacterial flora with a faecal transplant, delivered either by nasogastric tube or by enema, shown to be very effective (Aas et al., 2003); (Di Bella et al., 2013). In cases of severe complicated CDI no highly effective cure has been found and in some cases surgical removal of the colon can be the only remaining life-saving treatment (Lamontagne et al., 2007). Novel methods and agents, for the treatment of multiple relapsing and complicated CDIs, are needed. Several approaches are under investigation, including vaccine development, toxin-binding agents and passive antibodies.

1.1.3 Virulence factors

The majority of *Clostridium difficile* strains, associated with CDI are toxigenic strains. This made two large clostridial toxins, toxin A and toxin B (TcdA and TcdB), the best studied *C. difficile* virulent factors (Kuehne et al., 2010); (Lyras et al., 2009); (Voth and Ballard, 2005); (Schwan et al., 2009). However, other virulence factors are needed during the pathogenesis for other steps e.g. adaptation to the host, adhesion to the gut mucosa, colonization and dissemination. Lack of available molecular tools for *C. difficile* made studies on clostidial pathogenesis more complicated (Kuehne et al., 2011). In recent years the available sequence for several *C. difficile* genomes (Sebaihia et al., 2006); (He et al., 2013), and the development of molecular tools that allow *C. difficile* genetic studies (Cartman et al., 2012); (Kuehne et al., 2011), have led to a increase in knowledge of the molecular basis of *C. difficile* pathogenesis and non-toxin virulence factors involved in the CDI. Yet, still very little is known about non-toxin virulence factors and their role in pathogenesis.

1.1.3.1 Toxins

1.1.3.1.1 Toxin A and toxin B

Two main toxins, produced by *C. difficile*, are toxin A (TcdB) and toxin B (TcdB) (Voth and Ballard, 2005). TcdA and TcdB cause symptoms of the disease, triggering intestinal damage, and the release of nutrients from the damaged colonic epithelium. TcdA and TcdB target the Ras superfamily of GTPases, modifying glycosylation, and ultimately leading to the destruction of intestinal epithelial cells (Carter et al., 2012); (Voth and Ballard, 2005).

Toxins are encoded in the Pathogenicity Locus (PaLoc) (Figure 4a), which is approximately 19.6 kb big and is very stable and conserved among toxigenic strains (Cohen et al., 2000); (Voth and Ballard, 2005). Nontoxigenic strains lack the PaLoc, but isolates with a defective PaLoc can still colonize the gut (Cohen et al., 2000); (Curry et al., 2007).

PaLoc carries five genes: *tcdA*, *tcdB*, *tcdC*, *tcdE* and *tcdR*. The two toxin genes, *tcdA* and *tcdB*, are closely aligned, separated by an intervening sequence (*tcdE*) (Voth and Ballard, 2005).

The proposed function for a *tcdE*, which encodes a holin, a protein with pore-forming activity, is to allow the release of TcdA and TcdB from the cell. Recently however, it was shown that TcdE does not exhibit pore-forming function in *C. difficile* (Olling et al., 2012); (Voth and Ballard, 2005).

TcdR, found upstream of *tcdB*, is a major positive regulator of toxin expression. TcdR responds to the environmental conditions, and increases during the stationary phase (Voth and Ballard, 2005). TcdC, found downstream of *tcdA*, has been long time considered a negative regulator of toxin production, but a recent report showed that functionality of this gene does not affect toxin production (Cartman et al., 2012).

All the genes of the locus, except *tcdC*, are expressed during stationary phase. Additionally, the expression of the toxin genes has been shown to be regulated by the global gene regulator CodY, which acts by monitoring environmental nutrient factors (Dineen et al., 2007). In the presence of sufficient nutrients, CodY binds to the promoter region of *tcdR*, and represses toxin gene expression. When nutrients in the environment are lacking, toxin gene expression is de-repressed (Voth and Ballard, 2005); (Carter et al., 2012); (Dineen et al., 2007).

TcdA and TcdB are part of the large clostridial toxin family, along with *Clostridium sordellii* lethal and hemorrhagic toxin, and *Clostridium novyi* alpha toxin (Carter et al., 2012). Both toxins, TcdA and TcdB, are glucosyltransferases. They transfer a UDP-glucose to small GTPases, such as Rho, Rac, and Cdc42, in the host cell. These small proteins are important in regulating signalling pathways. Glycosylation disrupts these pathways, which results in morphological changes of the cell, inhibition of cell division and membrane trafficking, and eventually cell death (Dawson et al., 2009); (Voth and Ballard, 2005).

Toxins A and B are large single-stranded proteins. Recent X-ray crystallography and small angle X-ray scattering models (SAXS) of TcdB suggested four structural domains (Figure 4a) (Albesa-Jove et al., 2010), including: (i) a biologically active N-terminal glucosyltransferase protruding from the core of the protein; (ii) a cysteine protease domain; (iii) a middle translocation section that contains a hydrophobic region implicated in toxin delivery; and (iv) a C-terminal receptor-binding domain (Albesa-Jove et al., 2010).

Toxin activity is located in the N-terminal domain. This portion is delivered into the cytosol of host cells (Jank and Aktories, 2008). Cleavage of the biologically active segment occurs by autoproteolysis via the cysteine protease domain (Albesa-Jove et al., 2010).

The C-terminal domain has short combined repetitive oligopeptides (CROPs) for receptor binding. In animal models of TcdA, carbohydrate structures play a role in toxin binding (Rupnik et al., 2009); (Voth and Ballard, 2005). These carbohydrates are not present in humans and the glycoprotein gp96 present in the human colon is the receptor for TcdA (Rupnik et al., 2009).

Toxin A binds to the apical side of the cell. After the internalization in the cell, TcdA, causes cytoskeletal changes, what results in disruption of tight junctions and loosening of the epithelial barrier. This causes the cell death or the production of inflammatory mediators, which subsequently attract neutrophils. With the disruption of tight junctions toxins TcdA and TcdB are now able to cross the epithelium. Unlike TcdA, TcdB binds to the basolateral part of membrane.

Presence of the two cytotoxic toxins causes inflammation and accumulation of neutrophils as a result to the induced response of various immunomodulatory mediators from epithelial cells, phagocytes and mast cells (Figure 5) (Rupnik et al., 2009).

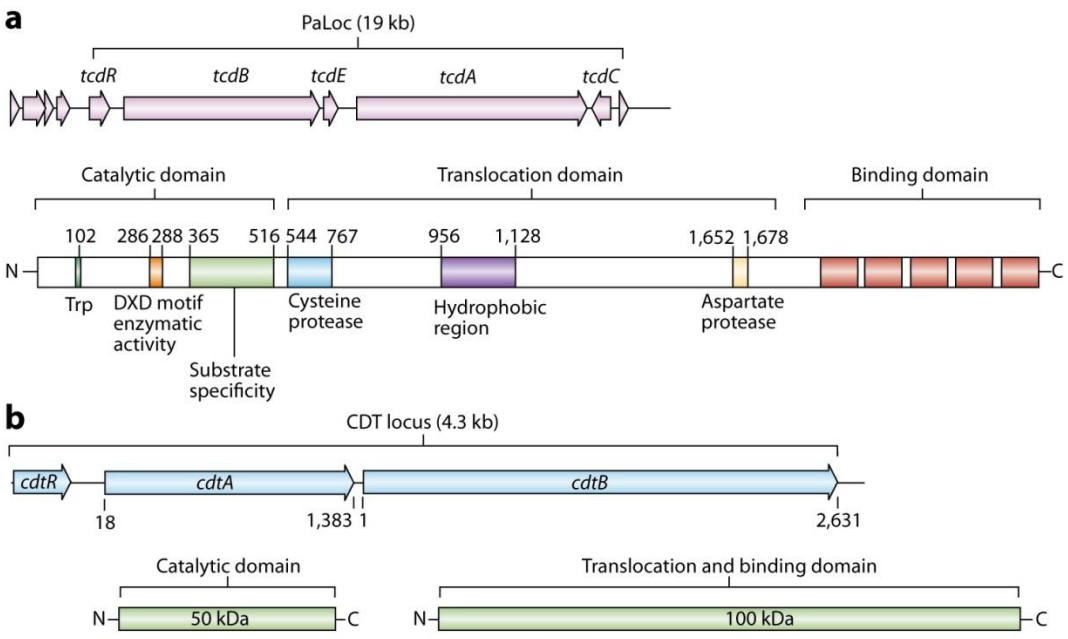


Figure 4 Toxin A and toxin B, produced by *C. difficile*. (**A**) The pathogenicity locus (PaLoc) encodes two large toxins, toxin A and toxin B (TcdA and TcdB), together with other three genes. Toxins are single-chain proteins with several functional domains and motifs. TcdB is shown in detail below the PaLoc. (**B**) The binary toxin or Cdt (*C. difficile* transferase), is encoded on a separate region of the chromosome (CdtLoc), and comprises three genes. The binary toxin is composed of two unlinked proteins, CdtB and CdtA. CdtB has a binding function and CdtA is the enzymatic component (Carroll and Bartlett, 2011).

The importance of two toxins in the clostrial pathogenesis has been long under question. First reports indicated that TcdA (308 kDa), an enterotoxin, was toxin associated with disease, and therefore necessary for virulence. This hypothesis was rejected when TcdA− TcdB+ strains were associated with outbreaks of severe *C. difficile* infection (Freeman et al., 2010). A report by Lyras *et al*. showed that TcdB (270 kDa), a cytotoxin, is 100- to 1000-fold more toxic to culture cells than TcdA, and that TcdB, and not TcdA, essential for clostridial virulence (Lyras et al., 2009). However, Kuehne *et al*. re-established the observation that both toxins can cause significant disease (Kuehne et al., 2010). In that study TcdA+ TcdB− isolates were as likely to cause disease as the wild-type strains.

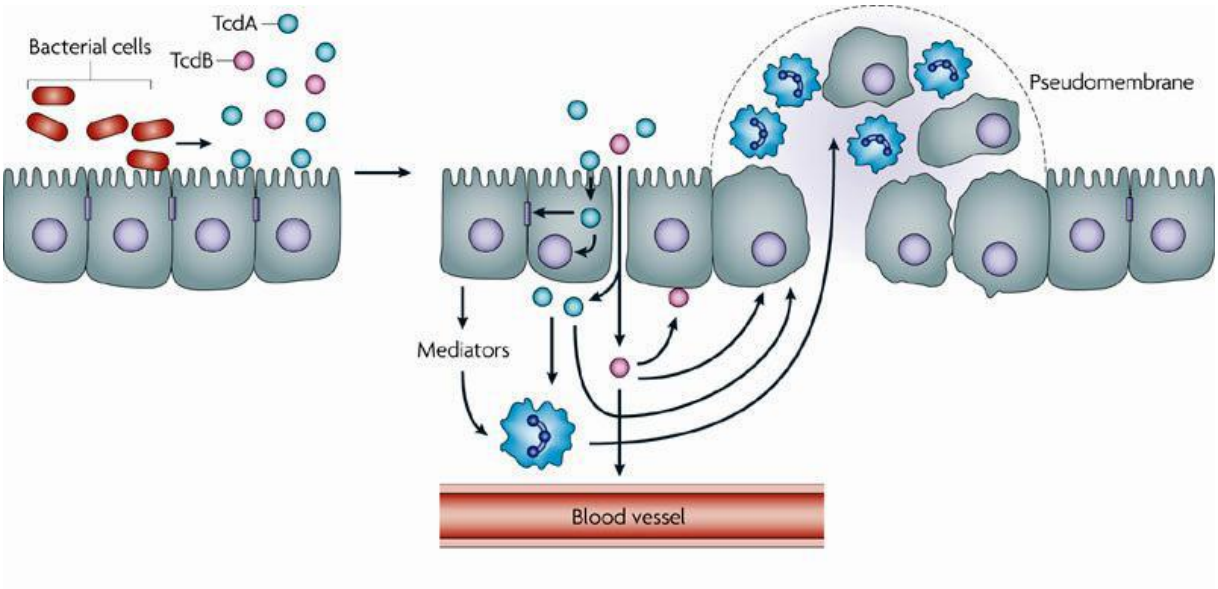


Figure 5 *Clostridium difficile* pathogenesis. After disruption of the normal intestinal flora *C. difficile* can colonizes the last part of intestine, the colon. Bacterial cells are presented as free and attached cells (red). TcdA (blue) binds to the apical side of the cell and can internalize in the cell, causing cytoskeletal changes, which results in disruption of tight junctions and loosening of the epithelial barrier, cell death or in the production of inflammatory mediators that attract neutrophils. Disruption of tight junctions enables toxins to cross the epithelium. TcdB (pink) binds preferentially to the basolateral cell membrane. Toxins are cytotoxic and induce the release of various immunomodulatory mediators from epithelial cells, phagocytes and mast cells, resulting in inflammation and the accumulation of neutrophils (Rupnik et al., 2009).

1.1.3.1.2 *C. difficile* Transferase (binary toxin Cdt)

C. difficile transferase (Cdt), also known as binary toxin, is encoded on the Cdt locus (CdtLoc) (Figure 4b), and it is found in approximately 6%–12.5% of all *C. difficile* strains (Schwan et al., 2009); (Sundriyal et al., 2010). While the role of the toxins TcdA and TcdB in disease has been extensively studied, the role of the binary toxin is less certain and many information are still missing.

Cdt is an ADP-ribosylating toxin that disrupts the cytoskeleton of the cell, leading to excessive fluid loss, rounding of the cell, and eventual cell death (Sundriyal et al., 2010). Cdt is composed of two subunits, CdtA and CdtB (Figure 4b). Each component alone is not cytotoxic, while together they cause cytotoxicity *in vitro*. The fact that the incidence of Cdt is

higher in some strains isolated from the outbreaks, suggests that Cdt contributes to the severity of disease (Sundriyal et al., 2010); (Geric et al., 2006).

Cdt induces the formation of novel thin, dynamic, microtubules on the surface of epithelial cells, leading to increased adherence of bacteria *in vitro* and *in vivo* (Schwan et al., 2009). Electron microscopy showed that these protrusions increase adherence to the epithelial cell surface approximately five-fold *in vitro* and four-fold in the mouse large intestine, indicating an important role for Cdt in intestinal colonization (Schwan et al., 2009).

1.1.3.2 Sporulation

C. difficile spores are highly resistant, metabolically inactive, dormant cells. When bacteria exist as spores they can survive in the environments where heat, chemical stress and pH extremes are present. Spores are moreover resistant to mechanical forces and aerobic conditions (Borriello et al., 1990). Because of their morphological and physiological characteristics, spores can persist on hospital surfaces and in the air, where they can then survive for long periods. Spores are also resistant to most conventional disinfection methods (Lawley et al., 2009). The ability of spores to persist in the environment, is one of the key factors in acquisition and transmission of CDI (Borriello et al., 1990).

Once ingested by the patient, the spores can pass through the stomach and survive low pH environment, until they reach the small intestine, where they can start to germinate. Patients with CDI episode can excrete high levels of infectious spores to the environment. Moreover, a fraction of spores are believed to adhere to the surface of the intestine. These spores, which are not affected by antibiotic therapies commonly used for CDI, can then germinate, outgrow and recolonize the host gastrointestinal tract before the normal microflora recovers after the antibiotic treatment, causing relapse of the disease (Sarker and Paredes-Sabja, 2012). Spores are therefore one of the main reasons for recurrence of CDI.

Endosporulation is a complex, ancient bacterial process, which aim is to form a highly resistant endospore. In this process the vegetative cell type of bacterium is transformed into a resistant endospore. External signals are needed to induce the sporulation and to trig the signaling cascade of sigma factors, leading to spore formation. For the spore forming bacteria, like bacilli and clostridia, the most common signal for initiation of sporulation is starvation. Moreover, in the case of *C. difficile*, an obligate anaerobe, oxygen can cause the initiation of sporulation. Recently has been shown that the initial levels of the endosporulation network are conserved in evolution between *Bacillus subtilis* and *C. difficile*. The sigma factors are the key regulators of the pathway, established 2.5 billion years ago upon its emergence at the base of the Firmicutes phylum (Pereira et al., 2013). Endosporulation is a highly regulated and extremely complex irreversible pathway. Once vegetative bacteria start the sporulation, in no case can the pathway be stopped (Piggot and Coote, 1976).

Spo0A, a master regulator which among other controls the entry of bacteria into the developmental process of sporulation, in present in the cell in a phosphorylated and dephosphorylated form. Concentration of phosphorylated Spo0A in the cell of *B. subtilis* determines which process will be activated, sporulation or other processes, like biofilm formation (Vlamakis et al., 2013). Higher concentration of Spo0A~P presents in the cell activates sporulation and represses vegetative cell functions (Piggot and Hilbert, 2004). Mutant in Spo0A in *C. difficile* is unable to form spores (Underwood et al., 2009) and to form biofilm (Đapa et al., 2013); (Dawson et al., 2012).

Until recently, little was known about molecular mechanisms of *C. difficile* sporulation process, and all hypotheses were based on the knowledge from the well-known and well-understood *B. subtilis* sporulation pathway (Figure 6a). In this model organism, gene expression in the forespore, and in the mother cell, is governed by cell type-specific RNA polymerase sigma (σ) subunits. Sporulation is controlled by a cascade of σ-factors. When in the vegetative *B. subtilis* pre-divisional cell high concentrations of Spo0A~P are present, the

σ^H is activated, what leads to an asymmetric division and creation of the prespore, dividing prespore from mother cell by a septum (Piggot and Hilbert, 2004). σ^F is activated in the prespore, and σ^E in the mother cell. σ^E is responsible for the production of coat proteins for the spore and, by communication with the forespore, for the activation of σ^G in the forespore. σ^G starts a signaling cascade that activates σ^K, what finally leads to the assembly of the outer spore coat (Piggot and Hilbert, 2004). During the formation of the septum, the chromosome replicates and forms an axial filament. The remaining section of the chromosome is actively transported into the forespore and segregated from the mother cell with the completion of the septum. This results in two distinct cells, both with a complete chromosome. The mother cell surrounds the forespore and compresses the membrane off in the way to completely envelop the forespore. In the forespore the chromosome is now reconstructed into a circular structure, the cortex forms a thick layer of cell wall material, and the protective protein spore coat forms around, completing in this way the synthesis of the spore. Once spore is completely synthesized, the mother cell is lysed, liberating the mature spore.

For *C. difficille* it was recently shown that the main periods of activity for the four cell type-specific σ-sigma factors are conserved relative to the *B. subtilis* model (Figure 6b) (Pereira et al., 2013). σ^F and σ^E control the early stages of spore development, and σ^G and σ^K are responsible for the late developmental stages in spore formation. The main observed differences with *B. subtilis* model were that σ^E activity was partially independent from σ^F, and that σ^G or σ^K did not require σ^E or σ^G, respectively (Pereira et al., 2013). Results by Pereira and coauthors suggest that the connection between the forespore and mother cell lines of gene expression are weaker compared to those observed in *B. subtilis*.

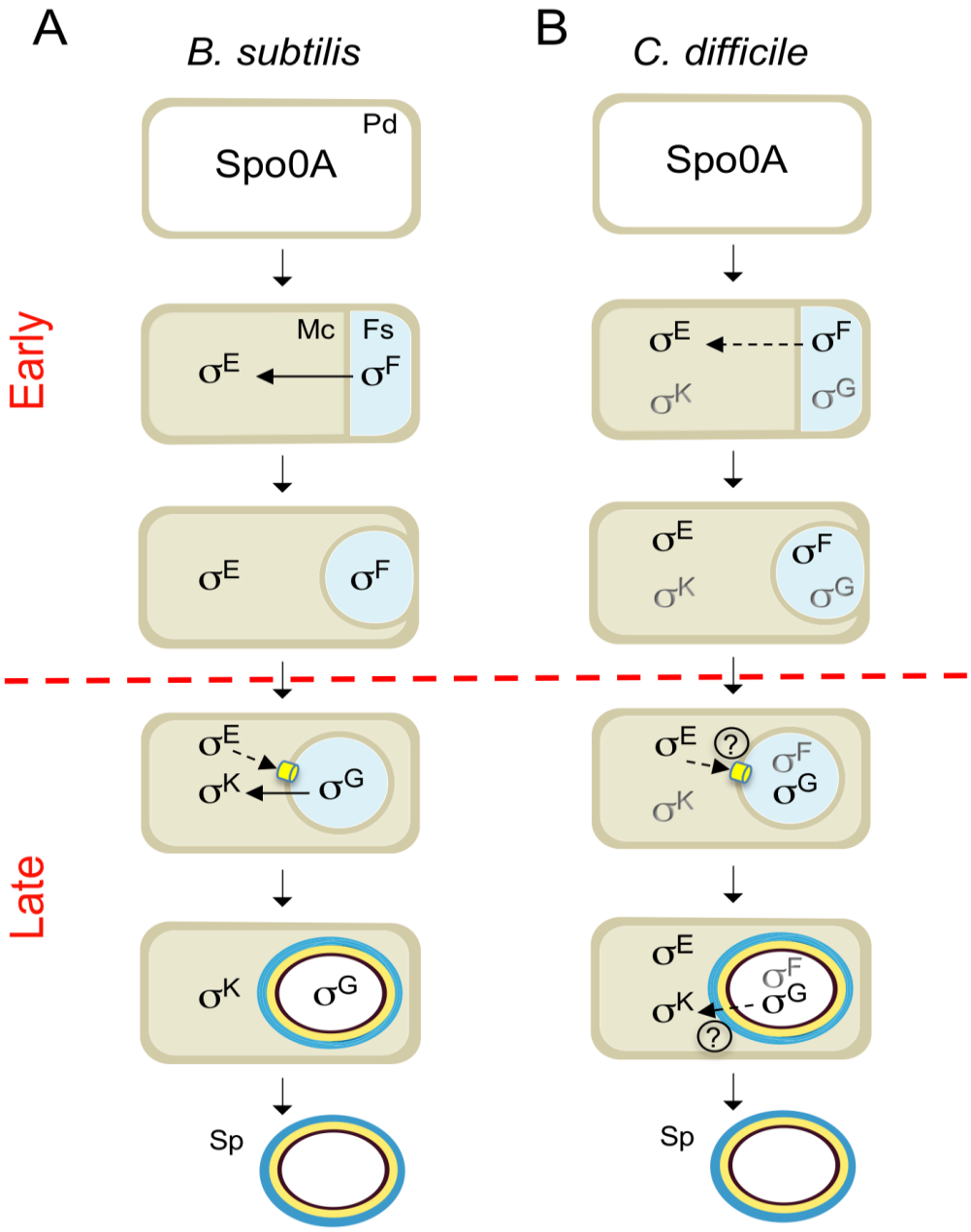


Figure 6 Activity of sigma factors in different stages. Periods of activity for σ^F, σ^E, σ^G and σ^K are presented for *B. subtilis* (**A**) and *C. difficile* (**B**). Solid or broken arrows represent dependencies or partial dependencies, respectively. In *C. difficile* black indicates the main period of activity. With the broken line and the question mark the possible cell-cell signaling pathways are shown. Yellow represents the SpoIIIA-SpoIIQ/CD0125 channel. PD: predivisional cell; MC: mother cell; FS: forespore. The early (prior to engulfment completion) from late (post-engulfment completion) development is distinguished with the red horizontal broken line (Pereira et al., 2013).

1.1.3.3 Surface virulence factors

The colonization of the colon is a key factor in the course of CDI. The bacterium can colonize exclusively when the normal intestinal microbiota is absent, which makes this step of pathogenesis crucial. After the ingestion of the spores, and their germination in the small intestine, *C. difficile* vegetative bacteria can colonize the gut. Just bacteria that adhere to the gut, and colonize it, are afterwards able to produce high quantities of the toxins, which cause the symptoms of the disease and colonic tissue damage. However non-toxigenic strains of *C. difficile* are still able to colonize the gut and it has been shown that colonization of the animals with non-toxigenic strains can prevent colonization with toxigenic strains and in this way prevent the disease (Wilson et al., 1982).

First step in the colonization is the adhesion to the mucosal epithelium. Until now little is known about the interactions between *C. difficile* and the gut. It is known that *C. difficile* can associate with intestinal mucosa in humans and hamsters, and the positive correlation between virulence and mucosal adherence *in vivo* has been observed (Borriello et al., 1988). *C. difficile* can persist in the gut regardless of physical stresses, e.g. shed of the colonic mucosa from the epithelial surface during the infection and the flow of luminal contents in the colon, which creates an environment with considerable mechanic force. This suggests that the adhesion of *C. difficile* to the gut, and surface-associated virulence factors, play an important role during CDI. In the last few years several surface factors, involved in adhesion and colonization of the bacteria, have been identified and partially characterized.

1.1.3.3.1 Surface layer proteins (SLPs)

Surface layer proteins (SLPs), also called S-layer, are surface-associated crystalline or paracrystalline arrays, formed by identical subunits of glycoproteins or proteins. SLPs are present in many prokaryotes (Sara and Sleytr, 2000).

Functions including acting as molecular sieves, protective factors against parasitic attack, virulence factors and adhesion sites for extracellular proteins, were attributed to bacterial S-layers (Sara and Sleytr, 2000). *C. difficile* S-layer is composed of two superimposed and structurally different S-layer lattices, a high-molecular-weight (HMW) SLP (40 kDa) and a low-molecular-weight (LMW) SLP (35 kDa) (Figure 7a) (Cerquetti et al., 2000); (Calabi et al., 2001); (Karjalainen et al., 2001). Both, HMW and LMW are transcribed by the same gene, *slpA*. Proteins are then subject to a post-translational cleavage of a common precursor, with the N-terminal portion coding for the LMW SLP, and the C-terminal portion coding for the HMW SLP (Calabi et al., 2001). The translated gene product, *slpA*, then undergoes two post-translational cleavages. In first, the signal sequence is removed, following secretion, and then internally to release the two mature SLPs (Figure 7). HMW and LMW are tightly linked by a non-covalent complex (Fagan et al., 2009). HMW SLP, localized in the internal surface of the bacterium, is immunologically conserved. LMW SLP, localized at the external surface of the bacterium, is immunologically variable (Cerquetti et al., 2000); (Takeoka et al., 1991); (Fagan et al., 2009), and suggests a role for LMW SLP in evasion of the immune system (Vedantam et al., 2012). Chemical treatment, or treatment of *C. difficile* bacterial cells with anti-SLP antibodies, cause the removal of the SLPs. Bacteria with removed SLPs showed an abolished adherence of *C. difficile* in the animal model and human HeLa cells, indicating the role of SLPs in adhesion to the host (Takeoka et al., 1991). Purified SLPs can bind to intestinal tissues and several proteins of the extracellular matrix (Calabi et al., 2002), but the precise host receptor remains unknown.

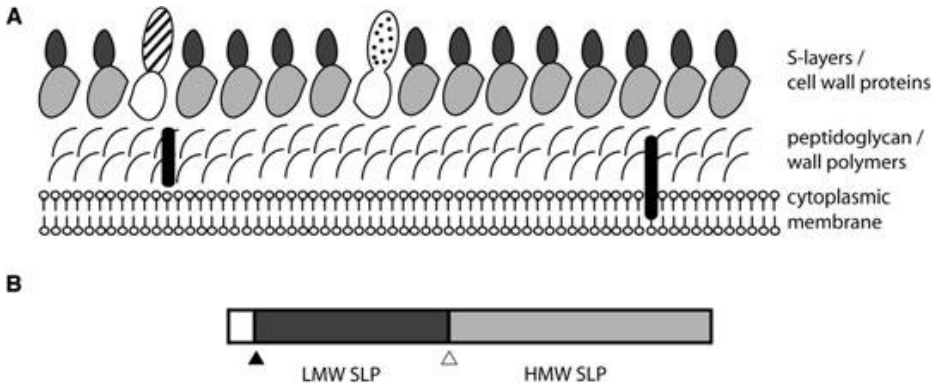


Figure 7 ***Clostridium difficile*** **cell wall model**. (**A**) Two types of surface layer proteins are presented. High-molecular-weight (HMW) SLP are presented as light grey molecules, and low-molecular-weight SLP as dark grey. Two-lobed structures (white) indicate other minor cell wall proteins. Vertical black bars represent putative cell wall polymers. (**B**) The precursor protein SlpA showing the cleavage sites generating the signal peptide (△) and the mature HMW SLP and LMW SLP (▲) (Fagan et al., 2009).

1.1.3.3.2 Cell wall proteins (CWPs)

C. difficile 630 genome contains 28 paralogs of the HMW SLP, which are known as the family of cell wall proteins (CWPs) (Sebaihia et al., 2006). All CWPs contain up to three copies of the cell wall-binding motif (PF01422), a motif characteristic of the SLPs, which is involved in anchoring the protein to the outer surface of the bacterium, and a variable motif, that might be function-specific (Sebaihia et al., 2006).

Cwp84 is a cysteine protease conserved in various *C. difficile* strains (Savariau-Lacomme et al., 2003). It is localized in the locus containing the *slpA* gene. Presence of Cwp84 was detected in antisera from patients with CDI, suggesting Cwp84 may play a role in *C. difficile* pathogenesis (Pechine et al., 2005a). Cwp84, a cysteine protease, is likely synthesized as an inactive proprotein of 80 kDa, which then autoprocesses itself to a 47 kDa mature Cwp84 (Janoir et al., 2007); (ChapetonMontes et al., 2011). Using chemical and genetic techniques it was shown that cysteine protease Cwp84 mediates the cleavage of the mature SlpA precursor (Kirby et al., 2009); (Dang et al., 2010). SlpA in a *C. difficile cwp84* mutant weakly binds the bacterial cell wall due to the lack of formation of the mature S-layer (Kirby et al., 2009). Correct processing of SlpA is important for the maturation of S-layer of the bacteria. Despite

this, a *C. difficile cwp84* mutant is able to cause infection (Kirby et al., 2009). Cwp84 is also involved in degradation of fibronectin, vitronectin, and laminin,and hence it is likely that in this way it induces a loss in the integrity of the host's colonic epithelium and increases the diffusion of toxins (Janoir et al., 2007).

1.1.3.3.3 Flagella

Mobility of the bacterium is an important physiological factor. For *Campylobacter jejuni* and *Legionella pneumophila* flagella plays an important role in internalization of the bacterium (Dietrich et al., 2001); (Grant et al., 1993), and in adherence and colonization of *Campylobacter jejuni* (McSweegan and Walker, 1986) and *Helicobacter pylori* (Eaton et al., 1996). Nonflagellated strains of *C. difficile* showed a tenfold reduced adherence to tissue in the mouse cecum, compared to flagellated strains belonging to the same serogroup, indicating an important role of flagella in adherence (Tasteyre et al., 2001a); (Tasteyre et al., 2001b).

Sera from patients with CDI have shown the presence of two important proteins in synthesis of flagella, FliC and FliD, associating them as important virulence factors during the CDI (Pechine et al., 2005a); (Pechine et al., 2005b).

Inactivation of either the flagellin (*fliC*) or flagellar cap (*fliD*) genes results in complete loss of flagella and motility of *C. difficile* (Dingle et al., 2011). Unexpectedly, both mutants, *fliC* and *fliD*, adhere more to enterocyte-like Caco-2 cells and were more virulent in the hamsters compared to the wild-type *C. difficile* (Dingle et al., 2011). Microarray and real-time PCR results suggest that when the vegetative cell *C. difficile* enters in contact with Caco-2 cells there is a downregulation of the genes involved in assembly of flagella (i.e., *fliH* and *flgG*) (Janvilisri et al., 2010). From these results we can conclude that the flagellum is adequately regulated to aid motility when in the colonic lumen and adherence when in contact with Caco-2 cells (Vedantam et al., 2012).

1.2 BIOFILMS

Biofilms are communities of surface-associated microorganisms, encased in a self-produced extracellular matrix. Formation of the biofilm is a common way of bacterial life cycle, and biofilms are found on practically all natural and artificial surfaces. Biofilms are well studied because they represent an important example of microbial lifecycle (Vlamakis et al., 2013). In the last decades, the role of biofilms was pushed to the forefront because of its function during an infection. Majority of bacterial infections is caused by bacteria in form of biofilms. Biofilms are ubiquitous in the nature, and are a prevalent form of microorganisms living in natural, industrial, and hospital settings (Hall-Stoodley et al., 2004). Bacteria in the biofilm are enclosed in self-produced extracellular matrix, which is essential for the integrity of the biofilms, and which holds the communities together (Flemming and Wingender, 2010).

It is well known that formation of biofilms influences the ability of several pathogens to colonize and establish an infection (Nobbs et al., 2009); (Allsopp et al., 2010). Biofilms provide an enclosed environment to escape immune responses and resist to antibiotics (Beloin et al., 2008); (Mah and O'Toole, 2001). We recently showed that *C. difficile* is able to form biofilms *in vitro*, an ability which could be crucial for clostridial colonization and persistence in the host (Đapa et al., 2013); (Dawson et al., 2012).

1.2.1 History

After inventing the microscope, Antonie van Leewenhoek, in 1684, published the first record describing biofilm structures. His report in the Royal Society of London was describing the vast accumulation of microorganisms observed in dental plaque: "The number of these animalcules in the scurf of a man's teeth are so many that I believe they exceed the number of men in a kingdom."

However, for long time after the first biofilm report, microorganism were primarily studied as planktonic, freely suspended cells, and their characteristics were described on the basis of

their growth in nutritionally rich culture media (Donlan, 2002). It was in 1973 when Characklis reported a study on microbial slimes in industrial water systems and showed that attached microbial growths are very tenacious and highly resistant to disinfectants such as chlorine. In 1978, Costerton *et al*. (Costerton et al., 1978), based on observations of dental plaque and sessile communities in mountain streams, put forward the theory of bacteria that stick together as biofilms, and explained the mechanisms by which microorganisms adhere to living and non-living materials, and the benefits accrued by this ecologic niche.

Since then, number of research and article published on the topic of biofilms increased dramatically. Not just studies relevant for public health, but knowledge on biofilms in industrial and ecologic settings also greatly improved. Tools for studying standard microbiologic culture techniques for biofilm characterization and biofilm genetics were developed (O'Toole and Kolter, 1998). Much of the work has relied on microscopy, e.g. scanning electron microscopy (SEM), and recently confocal microscopy and confocal scanning laser microscopy (CSLM). All these tools had a dramatically impact on our understanding of biofilms.

1.2.2 Biofilm structure

Bacteria in the biofilm are organized in three-dimensional bacterial communities, surrounded and entrenched by a self-produced extracellular matrix (Costerton et al., 1999). Biofilms are usually attach to the surface, either to a living or a non-living surface (Stoodley et al., 2010); (Yang et al., 2008); (Fux et al., 2004). Bacterial cells in the biofilm are morphologically and physiologically different from planktonically cultured cells.

Genetically identical bacteria within the monoculture biofilm express different genes at the same time, and in this way produce subpopulations of functionally different cell types (Vlamakis et al., 2008). Bacteria in the biofilms exist in heterogeneous communities (Hall-

Stoodley et al., 2004), and consequently show community behavior and functions, specialized for biofilm form of life (Vlamakis et al., 2008).

1.2.3 Biofilm formation and development

The switch between a planktonic cell and a biofilm community lifestyles brings a change in gene expression, which leads to varying phenotypes. The first step in a formation of a biofilm multistructural community is attachment of planktonic cells to a surface, creating in this way a microcolony. Bacteria in the microcolony communicate through quorum sensing (QS). QS is a form of cell-cell communication between bacteria, based on a system of stimulus and response correlated to population density. QS allows bacteria to analyse and sense population density through a series of secreted signals and receptors, and enable the bacteria itself to coordinate the behavior and respond to environmental factors (Hall-Stoodley et al., 2004); (Hall-Stoodley and Stoodley, 2005).

After the initial attachment (Figure 8) and formation of the microcolony, the microcolony starts to expands through recruitment of additional planktonic cells and cell divisions. Bacteria start to produce extracellular matrix, which later encase the attached bacteria. In biofilms the microorganisms account for less than 10% of the dry mass, 90% is surrounding self-produced extracellular matrix that covers and protects the bacteria. The extracellular matrix, hydrated biopolymers, composed of protein, nucleic acid, and polysaccharides, is called extracellular polymeric substance (EPS) (Flemming and Wingender, 2010).

During the next steps, aggregation and maturation of the biofilm (Figure 8), the microorganisms encapsulated in the biofilm start to actively replicate and increase the total density and complexity of the biofilm. Biofilm architecture matures as a result of interactions between the microbial colonies and extracellular substances (Davies et al., 1998). The gene expression and synthesis of proteins is different compared to planktonic growth (Sauer et al., 2002); (Whiteley et al., 2001). Mature biofilms, 3D structures, form porous and in some cases

contain channels which help the distribution of nutrient and signaling molecules (Costerton et al., 1999); (Hall-Stoodley et al., 2004); (Fux et al., 2004); (Hall-Stoodley and Stoodley, 2005). The last step in biofilm development is dispersion of the biofilm (Figure 8). Numerous environmental conditions determine the moment when biofilms reach its critical mass and when the dispersion of biofilm starts. Such conditions are availability and perfusion limitation of nutrients and wastes. With the dispersion of motile cells from a mature biofilm bacteria can now colonize new surfaces. Disassembly of the biofilm causes continuous release of single cells, spores, or small clusters, over an extended period. Biofilm dispersal can releases large portions of the biofilm late in biofilm development. Dispersal is a rapid method of spreading and releasing single cells or small clusters. Several matrix-degrading enzymes, e.g. proteases, deoxyribonucleases, DNases, and glycosidases, can cause the dispersal and disassembly of biofilm (Kaplan, 2010). It has been shown that nitric oxide and D-amino acids can cause the dispersal of *B. subtilis* biofilm (Schreiber et al., 2011); (Kolodkin-Gal et al., 2010), however recent data called into question the role of D-amino acids in biofilm disassembly, as it seems D-amino acids inhibit growth of *B. subtilis* itself (Leiman et al., 2013).

With the maturation of the biofilms, many morphological changes occur to the cells within the biofilms. The number of motile cells decreases with the progression of biofilm formation, although a small subpopulation of motile cells remains in the mature *Bacillus subtilis* biofilms (Vlamakis et al., 2008). The role of these motile cells in *B. subtilis* biofilms varies depending on growth conditions. Mutants defected in motility, which do not have flagella, are delayed in forming *B. subtilis* pellicle biofilms (Kobayashi, 2007b), and are defective in the formation of submerged, surface-adherent biofilms (Chagneau and Saier, 2004).

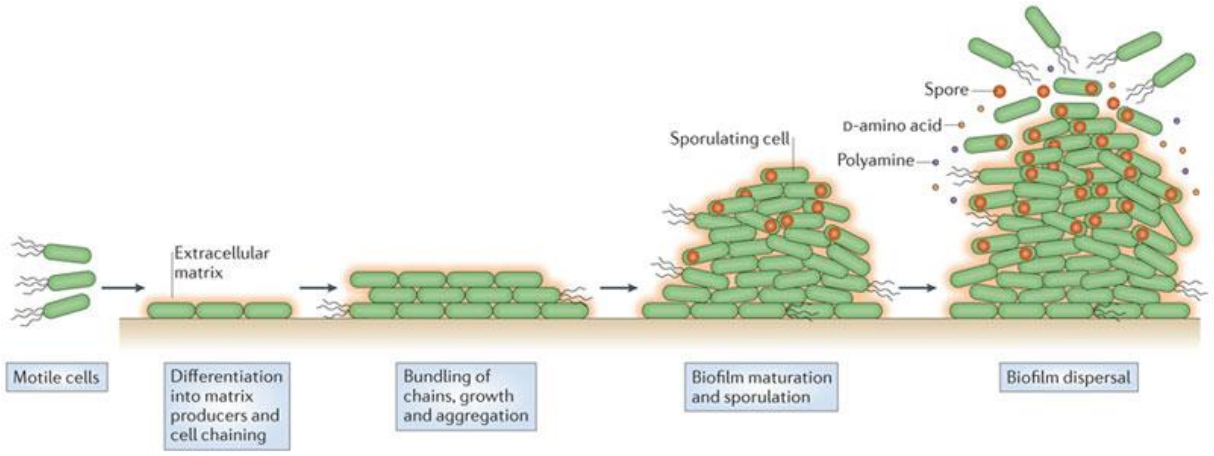


Figure 8 The life cycle of a *Bacillus subtilis* biofilm. Different stages of biofilm formation. Main stages are development, maturation and disassembly of the bacterial community. After the initial attachment to the surface motile cells differentiate into non-motile and matrix-producing cells. In this stage cells stop separating and form chains, surrounded by self-produced extracellular matrix. Next step is maturation of biofilms, where *B. subtilis* cells start to sporulate, followed by biofilm disassembly. In biofilms, different functionally distinct cell types exist, but these cells are genetically identical, and differentiation into a specific cell type is not terminal and can be altered when environmental conditions change (Vlamakis et al., 2013).

1.2.4 Role of biofilms during the infections

Bacteria within biofilms are protected and more resistant to different environmental stresses, e.g. antibiotic or oxygen stress (Davey and O'Toole G, 2000). But biofilms protect bacteria in myriad of other ways. Bacteria in the biofilms are more resistant to antibiotics, biofilms help bacteria to avoid phagocytosis, support bacterial adherence to the epithelial tissues, and exclude host defense molecules like antibodies and anti-microbial peptides (Hall-Stoodley and Stoodley, 2009); (Parsek and Singh, 2003). Resistance to the antibiotics can be up to thousand times higher for bacteria in the biofilm compared to bacteria in the planktonic growth (Stewart and Costerton, 2001). These are the reasons why majority of human infections is caused by bacteria in biofilms.

Common diseases as dental caries and periodontitis are caused by bacteria in biofilm, and biofilm formation has been connected with persistent tissue infections such as chronic otitis media, chronic rhinosinositis, recurrent urinary tract infections, endocarditis and cystic

fibrosis-associated lung infections (Costerton, 1999). Recently, biofilms were also associated with chronic inflammatory diseases as Crohn's disease (Claret et al., 2007), and acute infections (Hannan et al., 2012); (Kumagai et al., 2011). Moreover, biofilms represent a big problem when formed on artificial devices used in medicine, as catheters, stents, orthopaedic implants, contact lenses and implantable electronic devices (Costerton, 1999); (Lynch and Robertson, 2008). Resistance, of bacteria within biofilms to the antimicrobials, makes treatment of disease difficult and unsuccessful. Furthermore, mature biofilms are highly resilient to the action of the innate and adaptive immune defense systems, and for several pathogens recurrent and persistent infections have been associated with the ability to form biofilms (Romling and Balsalobre, 2012). Therefore due to enhanced antibiotic resistance, and evasion of host defenses, biofilm infections can be especially difficult to treat.

Biofilms are found in many niches in the mammalian host, including the surface of the gut mucosa (Bollinger et al., 2007); (Palestrant et al., 2004); (Probert and Gibson, 2002), however, biofilm formation by individual gut species, particularly anaerobic species, has not been well characterized. In addition to spore formation, a known means of adaptation to stress, it is likely that *C. difficile* forms microcolonies *in vivo* to survive the unfavorable environment of the human gut.

1.2.5 Antibiotics and biofilms

One of the main functions of a biofilm is to protect bacteria from unfavorable conditions like antibiotics, particularly during the infections. The role of bacterial biofilms in resisting antibiotics has been well demonstrated for many pathogenic species, including methicillin-resistant *Staphylococcus aureus* (Olson et al., 2011). Resistance to antibiotics in biofilm can increase from 10- to 1,000-fold more compared to planktonic bacteria (Mah and O'Toole, 2001). An increase in the number of recurring clostridial infections (Kelly, 2012), and a rise in *C. difficile* strains resistant to drugs currently used for the treatment, such as metronidazole

and rifampicin, have made management of CDI difficult (Carman et al., 2012); (Surawicz and Alexander, 2011).

Antibiotics at minimal concentrations, on the other hand, could also act as stress signals and biofilm formation could be a defensive reaction to the presence of antibiotics, as shown for Gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* (Boehm et al., 2009); (Hoffman et al., 2005). Induction of biofilms in presence of sub-inhibitory concentrations of antibiotics has been previously attributed to alterations in the level of c-di-GMP (Hoffman et al., 2005). Such induction *in vivo* could be clinically relevant when there is exposure to low doses of antibiotics, like at the beginning or at the end of antibiotic therapy, which could perhaps explain ineffective treatment (Boehm et al., 2009).

1.3 EXAMPLES OF REGULATION OF BIOFILM FORMATION

1.3.1 *Bacillus subtilis*

Bacillus subtilis is a non-pathogenic, aerobic, spore forming, Gram-positive bacterium. In the past years this bacterium arose as a model Gram-postive organism for studying the molecular basis of different pathways, e.g. sporulation and biofilm formation. *B. subtilis*, as *C. difficile*, belongs to the phylum Firmicutes, which makes the comparison of two bacteria more relevant, even though there are many differences between them.

In natural settings *B. subtilis* forms biofilm on plant roots, where provides benefits to the plant. *B. subtilis* requires a nutrient source, such as decaying organic material or plant roots, in order to proliferate in the soil. On the other hand, bacteria in the rhizoshere can benefit the plant with promoting the growth and protect plants from pathogenic bacteria, fungi and nematodes. In response to the presence of *B. subtilis*, the plant induced systemic resistance, and coupled with the secret antimicrobial compounds by *B. subtilis*, enhances the capacity of the plants to resist various pathogens (Vlamakis et al., 2013). It has been shown that biofilm formation on plant roots requires, as the biofilm formation *in vitro*, expression of exopolysaccharide matrix. Extracellular matrix holds the cells together. Plant polysaccharides, the major components of the plant's cell wall, triggers biofilm formation by *B. subtilis* and serves as a carbon source used to produce the extracellular matrix (Figure 9) (Beauregard et al., 2013).

First step in biofilm formation is the expression of matrix genes in response to an external signal, e.g. lipopeptide surfactin. Short motile rod cells turn into long chains of non-motile cells, which adhere to each other and to the surface with the development of biofilm. Bacteria secret self-produced extracellular matrix (Vlamakis et al., 2013). In addition to the cells expressing genes for matrix production, motile cells and spores are present in *B. subtilis* biofilm. Different cell types together form a mature biofilm. The presence and the localization of this different cell types within the biofilm is dynamic and there are evidences of an order

sequence of differentiation. Motile cells become matrix-producing cells, which then eventually start the sporulation and turn into spores (Vlamakis et al., 2008).

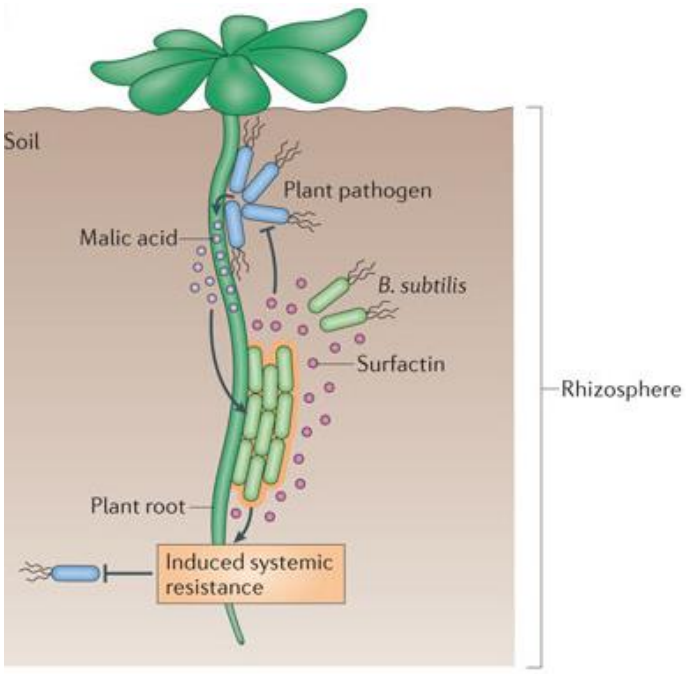


Figure 9 *Bacillus subtilis* biofilm in natural settings. For the *B. subtilis* colonization of plant's roots the production of surfactin is required. Surfactin is an antimicrobial lipopreptide, and together with other lipopetides produced by *B. subtilis* is one of the main mechanisms for plant biocontrol. These molecules can induce systemic resistance and inhibit the growth of plant pathogens as *Pseudomonas syringae*. In case, when the plant is infected with *P. syringae*, the plant secretes malic acid, what enhances *B. subtilis* biofilm formation on the root (Vlamakis et al., 2013).

In laboratory conditions *B. subtilis* biofilm can form different types of biofilm structures (Figure 10). The most studied forms of biofilm for *B. subtilis* are colony biofilms at the air–agar interface, floating biofilms at the air–liquid interface (also termed pellicles) and, in the case of certain domesticated strains, submerged, surface-adhered biofilms at the liquid–solid interface. Colony biofilm forms a complex wrinkled colony within a few days. Formed wrinkles are a consequence of localized death cell, coupled with the rigidity provided by the extracellular matrix (Branda et al., 2001); (Asally et al., 2012). In the liquid media the formation of the biofilm will occur on the surface of the liquid, in case when the cells will

float to the surface of the liquid, produce extracellular matrix and form a pellicle at the air–liquid interface, or they will form biofilm at the side of the culture vessel, in case when the cells remain under the surface of the liquid, where they form a submerged biofilm. Type of the formed biofilm from *B. subtilis* varies depending on the strain and the experimental conditions used (Vlamakis et al., 2013).

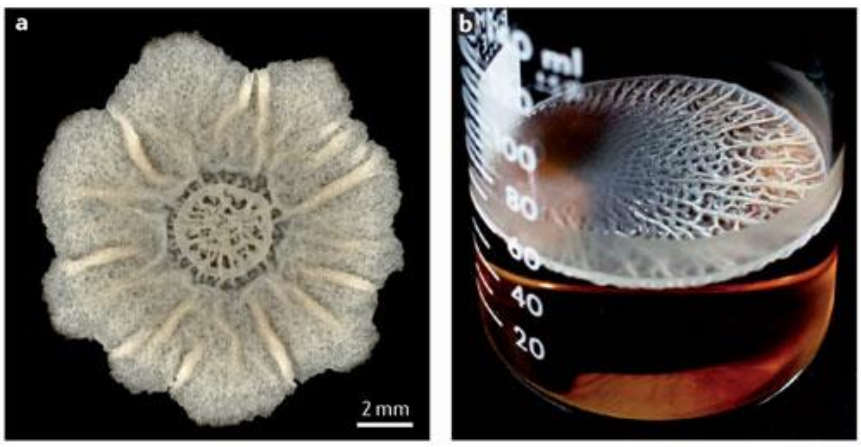


Figure 10 *B. subtilis* biofilm in laboratory conditions. (**A**) Seven days old *B. subtilis* colony, grown on biofilm inducing medium (MSgg medium). (**B**) Five days old *B. subtilis* pellicle, grown at room temperature (Branda et al., 2001).

Extrapolysaccharide matrix is major component in *B. subtilis* biofilm. EPS is synthesized by the products of the *epsABCDEFGHIJKLMNO* operon. Two additional characterized structural proteins, present in *B. subtilis* biofilm matrix, are translocation-dependent antimicrobial spore component (TasA), and biofilm surface layer protein BslA (Vlamakis et al., 2013) TasA protein assembles into long amyloid-like fibres, which are attached to the cell wall by another protein, TapA (Romero et al., 2010); (Romero et al., 2011). TasA was the first described protein component of the extracellular matrix of colony and pellicle biofilms (Branda et al., 2006). BslA, another secreted protein, is important for surface hydrophobicity, complex colony morphology and pellicle formation (Kobayashi and Iwano, 2012); (Kobayashi, 2007a); (Kovacs and Kuipers, 2011); (Verhamme et al., 2009).

One of main regulator of *B. subtilis* biofilm formation is master regulator Spo0A. Spo0A, central transcriptional regulator, controls the expression of more than 100 genes, including genes for biofilm matrix expression and sporulation (Fujita et al., 2005); (Molle et al., 2003). Spo0A is regulated by phosphorylation, concentration of phosphorylated or unphosphorylated forms of Spo0A (Spo0A~P) determinates gene expression. Spo0A is activated by phosphorylation as response to nutrient limitation (Burbulys et al., 1991); (Fujita and Losick, 2005). Both forms, phosphorylated and unphosphorylated form, are always present in the cell. Intermediate levels of Spo0A~P induce matrix gene expression, and higher levels induce the sporulation of *B. subtilis*. With maturation of the biofilm, and accumulation of Spo0A~P in the cell, sporulation is activated.

In *B. subtilis* at least four kinases (KinA, KinB, KinC and KinD) are responsable for phosphorylation of Spo0A~P. The pathway starts with phosphorylation of Spo0F by KinA, KinB, KinC or KinD. Spo0F~P then pass the phosphoryl group to Spo0B, which goes on to phosphorylate Spo0A. Depending on the enviromental signals different kinases are responsible for matrix gene expression (Hamon and Lazazzera, 2001); (McLoon et al., 2011); (Lopez et al., 2009). In *C. difficile* it has been suggested that phosphorylation of Spo0A occurs directly by a sporulation-associated sensor kinase. Kinase HK CD1579 was able to directly, and specifically, phosphorylate Spo0A (Underwood et al., 2009).

Spo0A~P in *B. subtilis* regulates several genes responsible for matrix production. The main regulatory pathway, governed by Spo0A~P, is by controlling the activity of the master regulator SinR. SinR is a repressor of the *eps* and *tapA–sipW–tasA* operons (Vlamakis et al., 2013). SinR antirepressor, SinI, is under the control of Spo0A~P. When SinI is expressed, it forms a SinI-SinR complex, which renders SinR incapable of binding to DNA (Lewis et al., 1996). Regulator SinR is expressed in all cells, but is inactivated just in the cells producing antirepressor SinI. As a result, only a subpopulation of cells expresses the the *tapA–sipW–tasA* and *eps* operons (Vlamakis et al., 2008); (Chai et al., 2008). Moreover, Spo0A~P

represses a second matrix gene repressor, AbrB (Strauch et al., 1990). AbrB represses *tapA–sipW–tasA* and *eps* operons as well (Vlamakis et al., 2013).

AbrB represses the *abh* gene (Strauch et al., 2007), however, the *abh* gene transcription is controlled by several extracytoplasmic function (ECF) RNA polymerase σ-factors, including σ^M, σ^W and σ^X. This ECF σ-factors are activated by enviromental stimuli, e.g. cell wall stress and specific antibiotic stresses (Helmann, 2002). In this way the bacterium can respond to enviromental changes in a Spo0A independent way (Vlamakis et al., 2013).

The regulators described are just few mechanisms that control biofilm formation by *B. subtilis* (Figure 11), and suggest the complexity and multilayered regulatory mechanisms that control biofilm formation in *B. subtilis*.

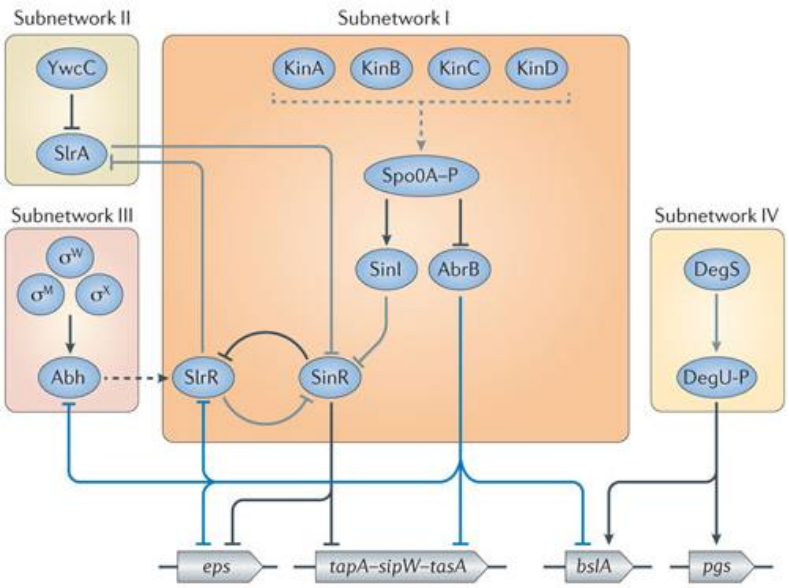


Figure 11 Shematic regulatory network that controls biofilm formation in *Bacillus subtilis*. Many different pathways regulate biofilm formation by *B. subtilis* (subnetworks I-IV). Main regulatory pathway is the Spo0A dependent pathway. However, different enviromental conditions may activate different Spo0A independent pathways. The role of all pathways is repression or activation of expression of biofilm matrix genes: the *eps* (exopolysaccharide synthesis) operon, the *tapA–sipW–tasA* (TasA anchoring and assembly–type I signal peptidase W–translocation-dependent antimicrobial spore component) operon, the *bslA* (biofilm surface layer) gene and the *pgs* (γ poly dl glutamic acid synthesis) operon. In the schematic presentation, dark grey and blue lines indicate transcriptional regulation, and light grey lines indicate protein–protein interactions. Direct and indirect regulation is presented with solid and dashed lines, respectively (Vlamakis et al., 2013).

1.3.2 *Staphylococcus epidermis*

Staphylococcus epidermis is an important representative of staphylococci and is one of main commensal skin bacterium, but is also recognized as an opportunistic pathogen. Majority of *S. epidermis* infections are associated with the formation of biofilm on medical devices, e.g. vascular catheters and prosthetic graft materials (Otto, 2012).

S. epidermis, as an important skin commensal, plays an important role in maintaining a healthy skin flora, providing so-called colonization resistance (Stecher and Hardt, 2008). It is believed that *S. epidermis* competes with potentially harmful bacteria (Otto, 2012). For example, it has been suggested that *S. epidermis* inhibits the *agr* quorum sensing reponse of *Staphylococcus aureus* (Otto et al., 2001), and it has been shown that extracellular serine protease (Esp), produced by *S. epidermidis*, inhibits *S. aureus* biofilm formation (Iwase et al., 2010).

The pathogenesis of *S. epidermidis* is dependent on its ability to form biofilms on polymeric surface (Anglen et al., 1994); (Ziebuhr et al., 1997). First step in *S. epidermidis* biofilm formation is attachment of bacterial cells to a polymer surface. It is a complex process, dependent by multiple different factors, e.g. hydrophobic interactions, the presence of host proteins, specific staphylococcal factors like the capsular polysaccharide adhesin, the autolysin AtlE, and other staphylococcal surface proteins (Knobloch et al., 2004). In the second phase, after the attachment, accumulation of bacteria in a multilayered biofilm embedded in an amorphous glycocalyx occures. For *S. epidermidis*, cell accumulation is essential for the synthesis of the polysaccharide intercellular adhesin (PIA), which mediates cell-to-cell adhesion of the proliferating cells (Mack et al., 1996a); (Mack et al., 1996b); (Mack et al., 1994); (Mack et al., 1992).

It has been shown that operon of alternative sigma (σ) factor B plays an important role in biofilm formation by *S. epidermidis* (Knobloch et al., 2004). The function and regulation of alternative σ-factor B is well studied and understood in other Gram positive bacteria like

Bacillus subtilis and *Staphylococcus aureus*. The initiation of transcription in bacteria depends on the sigma factors that associate with the RNA polymerase core enzyme. Several alternative sigma factors are associated with different promoter specificities, and binding of σ-factors to the promotors results in a changes in the patterns of gene expression.

Numerous environmental stresses, e.g. signals of energy stress like carbon, phosphate or oxygen starvation, and salt, heat, acid, or ethanol shock stresses, can induce σ-factor B in *B. subtilis* (Hecker et al., 1996). The alternative σ-factor B in *B.subtilis* is controlled by a complex signal transduction pathway (Boylan et al., 1992); (Wise and Price, 1995). RsbVW partner-switching controls σ-factor B activity in *B. subtilis* and is highly conserved among Gram positive bacteria that contain σ-factor B operon (Figure 12) (de Been et al., 2011). In normal conditions σ-factor B is in an inactive state, forming a complex with the anti-sigma factor RsbW. When stress conditions occurs, the release of σ-factor B from RsbW is accomplished by anti-anti σ-factor RsbV, which upon dephosphorylation, sequesters RsbW. Anti σ-factor RsbW in addition acts as a kinase of RsbV, providing a negative feedback on activation of σ-factor B. RsbV is dephosphorylated during the stress conditions, sequestring in this way RsbW, and activating σ-factor B (Hecker et al., 2007). In addition to RsbV, RsbU was shown to be a positve regulator of σ-factor B activity (Palma and Cheung, 2001).

σ-factor B positvily controls many different genes. In *B. subtilis* more than 100 genes are under σ-factor B control, which are induced by different stresses (Palma and Cheung, 2001). In *S. aureus* several different factors were shown to be included in σ-factor B regulon (Kullik et al., 1998); (Nicholas et al., 1999).

Knobloch and co-workes (2004) were studying biofilm formation for *Staphylococcus epidermis* in mutants *rsbU*, *rsbV*, *sigB*, *rsbW*, *rsbUVW* and *rsbUVWsigB*, in two different backgrounds. Significant decrease in biofilm formation has been observed in mutants *rsbU*, *rsbV*, *sigB* and *rsbUVWsigB*. In mutants for anti sigma-factor *rsbW*, and in mutant *rsbUVW*, however, a significant increase in biofilm formation was observed. Deletion of the positive

regulators of σ-factor B, and σ-factor B itself, represses the transcription of genes in σ-factor B regulon. In contrast, the lack of anti sigma-factor RsbW, enable the binding of σ-factor B with the RNA polymerase core enzyme, and allows the transcription of genes in σ-factor B regulon. This suggests that gene or several genes in σ-factor B regulon are involved in biofilm formation by *S. epidermidis* (Knobloch et al., 2004).

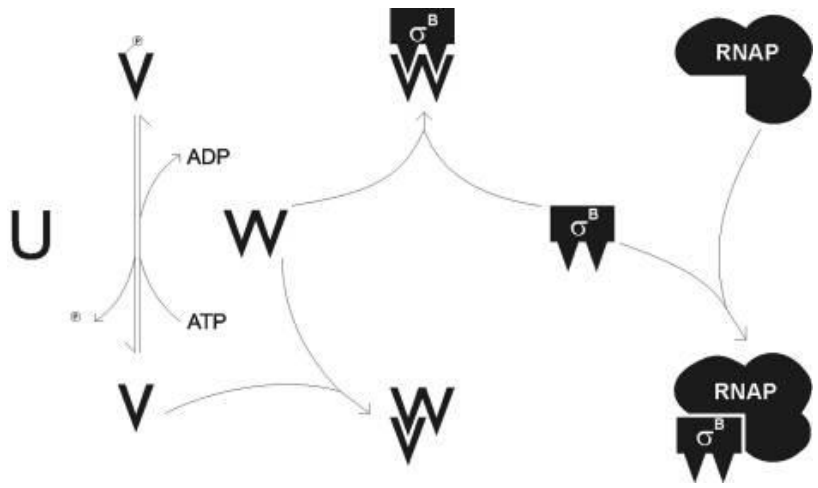


Figure 12 Regulatory pathway for the activity of the alternative σ-factor B. The pathway is highly conserved among Gram positive bacteria that contain σ-factor B operon. RsbW is an anti-σ factor and negatively regulates σ-factor B activity. Moreover RsbW act as a specific kinase for the anti-anti-σ factor RsbV, which, once phosphorylated, is unable to bind to RsbW. RsbV can be activated by dephosphorylation by specific phosphatase RsbU. Active, dephosphorylated, RsbV can bind anti-σ factor RsbW, what derepresses σ-factor B (Knobloch et al., 2004).

2 MATERIALS AND METHODS

2.1 BACTERIAL STRAINS AND CULTURE CONDITIONS

Two *C. difficile* strains, 630 and strain B1/NAP1/027 R20291 (isolated from the Stoke Mandeville outbreak in 2004 and 2005), were used in our studies. Bacteria were grown in liquid or solid media at 37°C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2), in a Don Whitley workstation (Yorkshire, United Kingdom).

Bacteria from glycerol stocks were grown overnight on BHIS plates. One single colony was inoculated in 5ml of liquid BHIS and grown overnight to stationary phase for biofilm assay, or to logarithmic phase for growth curve experiments.

For the second selection in the genetic manipulation of *C. difficile* a minimal medium was used (CDM), prepared according to Cartman and co-workers (Cartman et al., 2012).

Where appropriate the medium was supplemented with 15μg/ml thiamphenicol (Sigma-Aldrich, USA) or 20μg/ml of lincomycin (Sigma-Aldrich, USA). All medium was pre-incubated before use for at least 12hrs under anaerobic conditions.

All *Escherichia coli* strains were grown in Luria-Bertani (LB, Bacto, USA) at 37°C, supplemented with 20μg/ml chloramphenicol when required. *E. coli* strain DH5α (Invitrogen) was used for cloning, and *E. coli* strain CA434 was used as a conjugative donor.

Growth curves for bacteria were performed in BHIS containing 0.1M glucose. Overnight cultures of *C. difficile* R20291 and mutants were diluted to a starting OD_{600} of 0.05 and re-grown to mid-exponential phase (0.4-0.8). Cultures were then diluted to OD_{600} 0.01, and OD_{600} was measured every hour for 12hrs. All measurements were performed in triplicates.

Compositions of all the culture media used in this study are reported in Appendix 1, and list of all bacterial strains and plasmids in Appendix 2.

2.2 BIOFILM FORMATION ASSAY AND MEASUREMENT OF BIOFILM BIOMASS

For generation of biofilms, overnight cultures of *C. difficile* were diluted 1:100 into fresh BHIS, containing 0.1M glucose, and incubated in the tissue culture treated 24-well polystyrene plates (Costar, USA), 1ml per well, in anaerobic conditions at 37°C for 1hr to 120hrs. 24- or 48-well cell culture plates were pre-incubated in anaerobic conditions for 48hrs prior to use. To prevent evaporation of liquid, plates were wrapped with parafilm. Measurement of biofilm biomass with crystal violet was done as described in previously published methods (Varga et al., 2008). After the required incubation, wells of the 24-well plate were gently washed with sterile phosphate buffered saline (PBS) and then allowed to dry for 10min. The biofilm was stained with 1ml of filter-sterilized 0.2% crystal violet and incubated for 30min at 37°C, in anaerobic conditions. Crystal violet was removed from the wells, followed by two washes with sterile PBS. The dye was extracted by adding 1ml methanol to each well and incubation for 30min at room temperature (RT) in aerobic conditions. The methanol-extracted dye was diluted 1:1, 1:10 or 1:100 and *A*570 was measured with spectrophotometer Ultrospec 500 *pro* (Amersham Biosciences).

For bacterial cell counts from biofilms, the planktonic phase was first removed and wells were washed with sterile PBS. The adherent biofilms were then detached by scraping with a sterile pipette tip, washed in PBS, and plated on BHIS for determination of the number of CFU present in the biofilm.

2.3 ENZYMATIC INHIBITION OF BIOFILMS

Proteins, extracellular DNA and polysaccharides are known to be an important part of extracellular biofilm matrix. To study the presence and the role in biofilm formation of proteins and extracellular DNA in *C. difficile* biofilms, 0.1mg/ml of proteinase K and 2U/ml DNase I was added to biofilm at time 0. To see if these enzymes affect preformed biofilms, biofilms were allowed to form in 24-well plate as described above and washed with sterile

PBS. Fresh BHIS 0.1M Glc with 0.1mg/ml proteinase K or 2U/ml DNase I was added and the biofilms were incubated for a further 24hrs, followed by staining with 0.2% crystal violet as described above.

2.4 CONFOCAL MICROSCOPY ANALYSIS OF BIOFILM FORMATION

C. difficile strains were grown in 4-well glass chamber slides (BD Falcon, USA), in BHIS 0.1M glucose, at 37°C in anaerobic conditions for 24hrs or 72hrs, and stained with various staining reagents. For biofilm staining with fluorescent BacLight Live/Dead stain mixture (Molecular Probes, Invitrogen), which contains the nucleic acid stains Syto 9 and propidium iodide (staining live bacteria green and dead bacteria red, respectively), wells were gently washed twice with PBS 0.1% saponin to remove unattached cells and incubated with dye for 15min at 37°C. The wells were additionally washed twice, and before removing from the anaerobic chamber, bacteria were fixed with 4% PFA (paraformaldehyde) for 15min.

For immunoflourescent staining bacteria were fixed with 4% PFA for 15min, washed three times with 2% bovine serum albumin (BSA)/PBS and blocked with 2% BSA and 0.1% saponin in PBS for 10min. Samples were then incubated with mouse serum against fixed R20291 strain or against a synthetic *C. difficile* PSII polysaccharide (synthetic phosphorylated hexasaccharide repeating unit) (Adamo et al., 2012), diluted 1:1000 for 1hr, followed by incubation with Alexa Fluor 568 goat anti-mouse antibodies diluted 1:500 for 1hr. Anti *C. difficile* antibodies were in house generated against paraformaldehyde-fixed whole bacteria in mice.

Biofilm matrix was labeled by SYPRO Ruby biofilm matrix stain (Molecular Probes, Invitrogen) which stains matrices of biofilms (labels most classes of proteins, including glycoproteins, phosphoproteins, lipoproteins, calcium binding proteins and fibrillar proteins). After two washes with sterile PBS 0.1% saponin, samples were incubated for 30min with 1ml of Ruby biofilm matrix stain at room temperature.

All chamber slides were mounted with ProLong Gold Antifade Reagent with DAPI (Molecular Probes, Invitrogen) and analyzed with a Zeiss Observer LSM 710 confocal scanning microscope.

2.5 BIOFILM RESISTANCE TO ANTIBIOTICS

To determine the resistance capabilities of planktonic and biofilm cells we used antibiotic of choice for CDI treatment, antibiotic vancomycin (Sigma-Aldrich, USA), at 200 times the MIC, 20μg/ml. Resistance was determined to the both parts of biofilm, sessile and the planktonic populations, from the same *C. difficile* culture. The supernatant (planktonic fraction) from 1 or 3 day biofilm cultures in 6-well plates was removed, washed once in sterile PBS, re-suspended in BHIS 0.1M glucose with 20μg/ml vancomycin (Sigma-Aldrich, USA), and incubated in fresh 6-well plates. In parallel, to the 6-well plate where supernatants were removed, fresh BHIS 0.1M glucose containing 20μg/ml vancomycin was added to the adherent biofilms (sessile part). Treatment with vancomycin was performed for 6hrs or 24hrs. Colony forming units (CFU) from planktonic and adherent phases were determined by plating serial dilutions on BHIS agar. Biofilm cell counts were determined as described above. Percent survival was calculated by dividing the number of CFU post-treatment by the initial number of CFU.

To investigate if the protection of bacteria in the biofilm from antibiotic vancomycin is a result of biofilm structure (matrix), or is a physiological attribute of the cells in the biofilm, we perform experiment as described above, but prior to the incubation with antibiotic we disrupted the biofilm matrix. The sessile part of biofilm was disrupted gently by pipetting and incubated with BHIS with 0.1M Glc and 20μg/ml vancomycin. The planktonic growth from the same well was washed and incubated with BHIS with 0.1M Glc and 20μg/ml vancomycin. CFU counts were determined after 6hrs and 24hrs, and counts before and after different times of treatment were compared.

2.6 INDUCTION OF BIOFILM FORMATION WITH ANTIBIOTICS

Formation of biofilms can be stimulated by different environmental stresses, including antibiotic stress. To investigate if antibiotic could induce biofilm formation, sub-inhibitory and inhibitory concentrations of antibiotic vancomycin (sub-inhibitory concentrations 0μg/ml, 0.05μg/ml, 0.1μg/ml, 0.25μg/ml, and inhibitory concentration 0.5μg/ml) were added to biofilm at time point 0. Biofilms were allowed to form in 24-well plate for one and three days, followed by staining with 0.2% crystal violet as described above.

2.7 MEASUREMENT OF *C. difficile* SPORES IN BIOFILM

C. difficile spores were measured as previously described (Burns et al., 2010). At day 1, 3 and 5 of biofilm formation samples (separated two biofilms parts, adherent and planktonic part, and as a control *C. difficile* non-biofilm planktonic culture, grown in a tube) were removed from the anaerobic chamber and heated at 65°C for 25min to kill the vegetative cells but not the spores. Serially diluted were then plated onto BHIS agar and BHIS supplemented with 0.1% taurocholate (Sigma-Aldrich, USA) in anaerobic contitions. Bile acid taurocholate stimulates the germination of *C. difficile* spores (Burns et al., 2010). BHIS agar was used as negative control, and BHIS supplemented with 0.1% taurocholate was used for germination of spore. Plates were incubated for 24hrs to 48hrs at 37°C under anaerobic conditions. As a control, total counts of vegetative cells and spores were determined by plating dilutions of untreated culture on the same medium.

2.8 SWIMMING ASSAY

For swimming assay semi-solid 0.3% BHIS agar plates were employed. The plates were solidified overnight at room temperature and dried at 37˚C for 60 minutes. Single *C. difficile* colonies on BHIS plates were picked with toothpick and stabbed into the middle of the semi-solid agar of the motility plates. Plates were incubated for at least 48hrs at 37°C under

anaerobic conditions. Plates were not inverted. After incubation photographs of the plates were taken.

2.9 GENETIC MANIPULATION OF *C. DIFFICILE*

2.9.1 Generation of *C. difficile* deletion mutants with allelic exchange method

C. difficile R20291Δ*cwp84* (Ng et al., 2013) and *C. difficile* R20291Δ*rsbW* (this study) were generated in R20291 background employing the allele exchange strategy, described by Cartman and co-workers (Figure 13) (Cartman et al., 2012). Positions of in frame deletions are reported in Appendix 2.

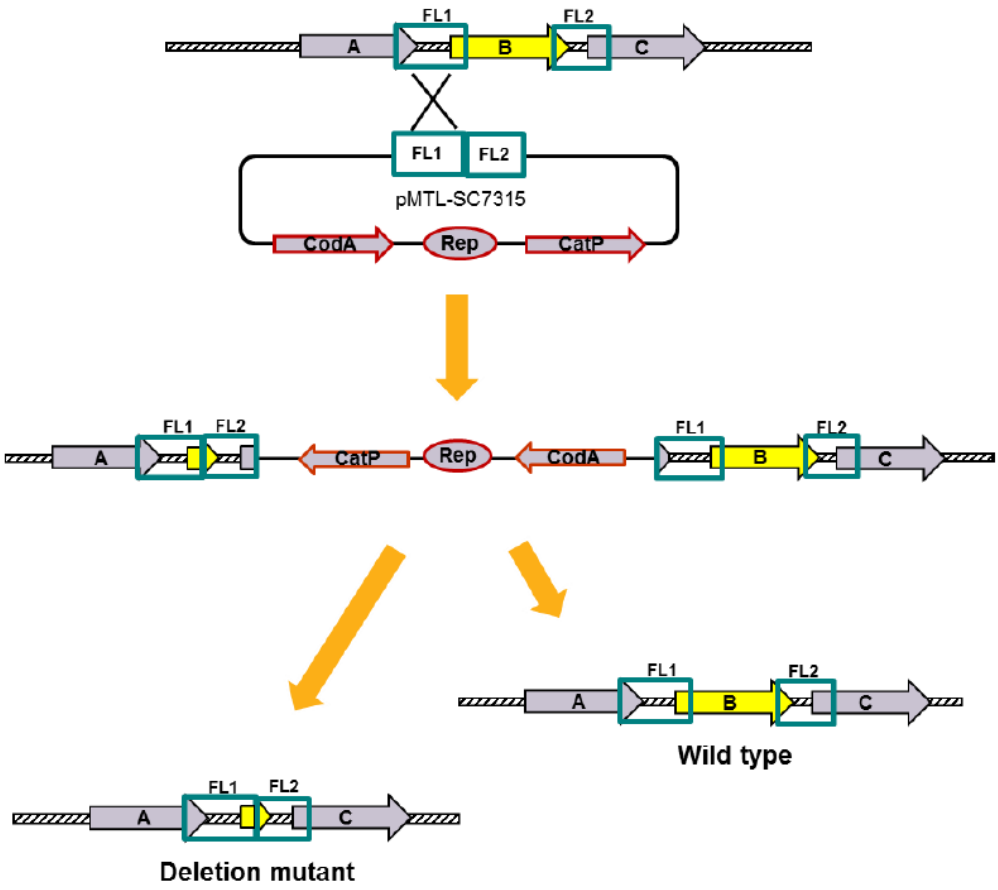


Figure 13 Scheme representing the allelelic exchange strategy used to generate deletion mutants in *C. difficile*.

Two allele exchange cassettes were cloned into the pMTL-SC7315 vector (Figure 14).

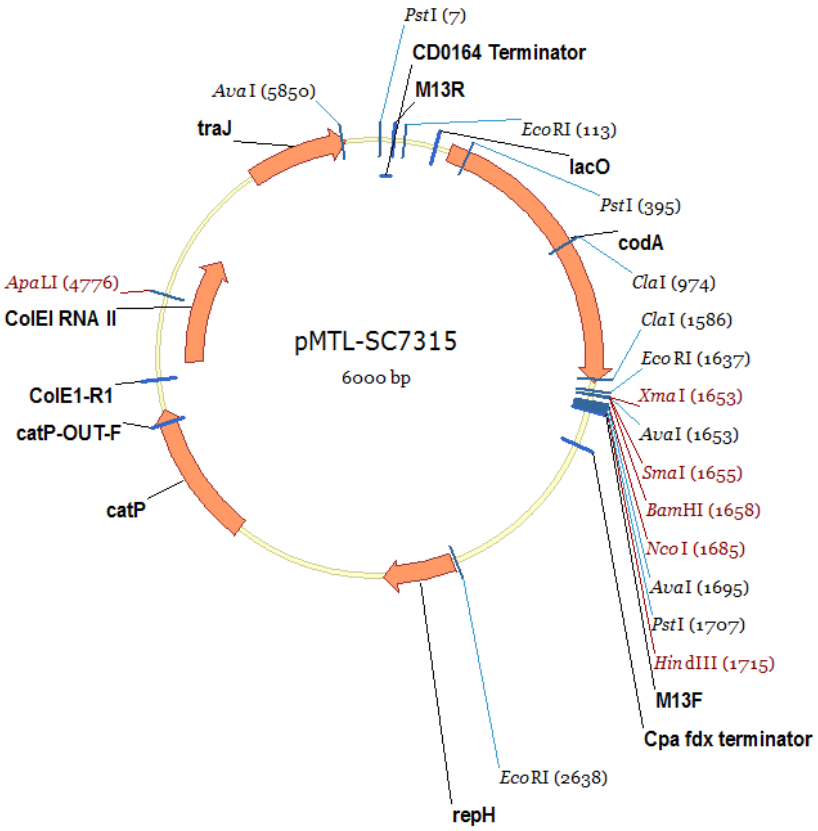


Figure 14 Map of pMTL-SC7315 vector for allelic exchange.

Approximately 500 bp fragments of the flanking regions of the target gene were amplified by PCR from R20291 genomic DNA (isolated using a DNeasy Blood and Tissue kit, Qiagen). Amplification reaction was carried out with Phusion High-Fidelity DNA polymerase (New England BioLabs) using the primers couples F1_*rsbW* and R1_*rsbW* for amplification of the upstream cassette and F2_*rsbW* and R2_*rsbW* for amplification of the downstream cassette (Appendix 3). Primers have ends containing the appropriate restriction sites for cloning (F1_*rsbW* and R2_*rsbW* carry a *Pme*I site, R1_*rsbW* and F2_*rsbW* an *Eco*RI site). Thermocycling conditions were 95°C for 5min, followed by 35 cycles of 95°C for 30s, an appropriate annealing temperature for 30s, 72°C for 1min/kb, and a final extension of 72°C for 10min. The upstream and downstream fragments were cloned into the *Pme*I site of pMTL-

SC7315 by one-step ligation with T4 DNA ligase (Rapid DNA Ligation kit, Roche) to generate an in-frame deletion in the *C. difficile* R20291 *rsbW* open reading frame (deletion of nucleotides 10 to 401). The reaction was transformed in the *E. coli* cloning strain DH5α (Invitrogen) by heat shock. Selection of transformants was carried out on LB solid medium supplemented with 25μg/ml chloramphenicol. Several colonies were picked and inoculated in liquid LB medium supplemented with 12.5μg/ml chloramphenicol. Using standard plasmid extraction methods, plasmid was purified from each of the overnight cultures and screened by restriction analysis with *Pme*I and by PCR using primers couple SC7-F1 and SC7-R1 (Appendix 3). Then the DNA sequence was checked by Sanger sequencing using primers SC7-F and SC7-R (Appendix 3). Restriction endonucleases were obtained from New England BioLabs and used according to the manufacturer's instructions.

Knock-out plasmid was transferred into the *C. difficile* recipient by conjugation. A knockout cassette plasmid verified by sequencing was re-transformed into electrocompetent *E.coli* conjugation donor strain CA434, carrying out selection on LB plates supplemented with 25μg/ml chloramphenicol. Recipient strain *C. difficile* R20291 and donor strain *E. coli* CA434/pMTL-SC7315-*rsbW* were grown overnight in BHIS and in LB with 12.5μg/ml chloramphenicol, respectively. 1ml of the stationary overnight culture of *E. coli* donor strain was pelleted and washed with 0.5ml PBS. The conjugal donor pellet was then resuspended in 200μl of stationary overnight culture of conjugal recipient cells and the entire conjugation mixture was pipetted onto a single BHIS non-selective plate in discrete drops. After 24hrs of incubation under anaerobic conditions (to allow conjugal transfer of the knockout plasmid from the *E. coli* donor to the *C. difficile* recipient) all growth was harvested into 500μl PBS and plated onto BHIS plates containing D-cycloserine (250μg/ml), cefoxitin (8μg/ml) and thiamphenicol (15μg/ml) to select for transconjugants (i.e. *C. difficile* cells harbouring the plasmid). Plates were incubated at 37°C under anaerobic conditions for 24-72hrs. Antibiotics D-cycloserine and cefoxitin are selection markers for *E. coli* donor, while thiamphenicol is a

selection for *C. difficile* transconjugants (bacteria with pMTL-SC7315-*rsbW* plasmid carry thiamphenicol cassette, *catP*).

Transconjugant colonies were then restreaked and incubated 24-72hrs at 37°C under anaerobic conditions. Single cross-over clones were identified as faster growing colonies in amongst slower growing transconjugant colonies. In the first cross-over, mutants were isolated based on an antibiotic cassette on the pseudo-suicide plasmid, which replicates episomally slower that bacterial chromosome, which makes single cross-overs faster growing colonies in amongst slower growing transconjugant colonies. Single cross-over clones were purified by picking and restreaking, and then confirmed by PCR analysis carried out with Platinum Taq (Invitrogen) in Failsafe PCR buffer E (Epicentre), using primers pairs RsbW_contr_F and SC7_R and RsbW_cont_R and SC7_R (Appendix 3).

Single cross-over mutants are unstable, which makes the loss of plasmid easy and efficient, once selective antibiotic pressure is removed. To allow rare second recombination events to occur, single-crossover clones were restreaked onto nonselective BHIS medium and incubated for 96hrs. All growth was harvested in 500µl PBS and serial dilutions were made (from 10^{-1} to 10^{-6}). 100µl of each dilution were plated onto CDM supplemented with 50µg/ml 5-fluorocytosine (FC). Second cross-over mutants were isolated using a negative selective marker present on the plasmid. After 48hrs of incubation FC-resistant clones were patch plated onto BHIS supplemented with cycloserine, cefoxitin, and thiamphenicol, to screen for plasmid loss. Fluorocytosine-resistant and thiamphenicol-sensitive clones were analyzed by PCR reaction to distinguish double-crossover recombinant clones from wild-type revertant clones, using primers F1_*rsbW* and R2_*rsbW* (Appendix 3). Sanger sequencing was used to confirm the expected genotype.

2.9.2 Generation of *C. difficile* insertion mutants with Clostron method

Mutants in genes *sleC, spo0A* and *fliC* were generated using the insertional inactivation system, ClosTron, as previously described (Burns et al., 2010); (Heap et al., 2007); (Baban et al., 2013). Positions of ClosTron insertions are reported in Appendix 2. The *luxS*-gene was insertionally inactivated using the ClosTron system as described (Heap et al., 2007); (Heap et al., 2010). Plasmid pMTL007C-E2 was retargeted to yield pMTL007C-E2-*luxS*161a. These plasmids were conjugated into R20291 and transconjugants were selected on agar plates supplemented with thiamphenicol and cycloserine/cefoxitin. Transconjugants were then plated onto lincomycin agar plates to select for integrants. Potential integrants were verified by PCR screening and subsequent DNA sequencing using the primers *luxS*_95_F and *luxS*_262_R.

2.9.3 Construction of *C. difficile* mutants and complemented strains

For complementation studies for *C. difficile* R202911::*sleC* plasmid pMTL-DB1 (Burns et al., 2010) was introduced into the mutant, resulting in strain *C. difficile* R202911::*sleC* + pMTL-DB1 (*sleC*-C). Chromosomal complementation of *C. difficile* R20291Δ*cwp84* with *cwp84* was performed as described (Ng et al., 2013) to yield *C. difficile* R202911Δ*cwp84*-C (*cwp84*-C). A 724-bp fragment surrounding the putative *luxS* structural gene (456-bp) and 5-noncoding region (268-bp) was amplified by PCR using oligonucleotide primer F1_*luxS* and R1_*luxS* and cloned using *Not*I and *Xho*I into pMTL-84151 (Heap et al., 2009), resulting in plasmid pMTL-TD1. This plasmid was conjugated into the *luxS* mutant *C. difficile* R202911::*luxS* to yield *C. difficile* R202911::*luxS* + pMTL-TD1 (*luxS*-C). Fragments containing the native promoter and full length genes for *fliC* and *spo0A* were cloned into plasmids pMTL-84151 and pMTL-960, respectively. The *spo0A* sequence was amplified by PCR using genomic DNA from *C. difficile* 630Δ*erm* as template and primers Cdi-*spo0A*-F1 and Cdi-*spo0A*-R1. The resulting product comprised the *spo0A* open reading frame with 174-

bp of upstream sequence and 205-bp of downstream sequence. This was cloned into the pCR2.1-TOPO vector (Invitrogen) and confirmed by sequencing. The *spo0A* complementation construct was subsequently excised as a *Spe*I/*Bam*HI fragment and cloned into similarly digested pMTL960 and conjugate into *spo0A* mutant *C. difficile* R202911::*spo0A* to yield *C. difficile* R202911::*spo0A* + pMTL960-*spo0A* (*spo0A*-C).

For complementation studies for *C. difficile* R202911::*fliC*, a 973-bp fragment encompassing the *fliC* open reading frame (873bp) with the 100-bp 5' noncoding region most likely containing the *fliC* promoter, was cloned in pMTL-84151 to generate pMTL-SB1(Baban et al., 2013). Plasmid pMTL-SB1 was introduced into the mutant, resulting in strain *C. difficile* R202911::*fliC* + pMTL-SB1 (*fliC*-C).

2.10 SANGER SEQUENCING

For Sanger sequencing, genomic DNA was isolated (using a DNeasy Blood and Tissue kit, Qiagen) from strains R20291, derived from different laboratories. PCR was carried out with Platinum Taq (Invitrogen) in Failsafe PCR buffer E (Epicentre), using primers pair *rsbW*_F and *rsbW*_R (Appendix 3). Sequencing was repeated in at least three independent experiments, from three independent isolated genomic DNA preparations.

2.11 STATISTICAL ANALYSES

All experiments were performed in triplicates and at least three independent experiments were performed. A paired Student's t test was performed to determine if the differences between two groups was significant. *P* values <0.05 were considered statistically significant.

3 RESULTS

3.1 *Clostridium difficile* BIOFILM FORMATION

3.1.1 *Clostridium difficile* forms time and strain dependent biofilms *in vitro*

In order to investigate biofilm formation by *C. difficile* we studied two clinically important strains. Stain 630, a commonly studied clinical strain, and strain B1/NAP1/027 R20291, strain isolated from the Stoke Mandeville outbreak in 2004 and 2005 in United Kingdom (R20291). Formation of the biofilms was measured with different methods: by staining the biofilms with crystal violet stain (CV), by preforming colony-forming unit counts (CFU) of sessile and planktonic part of the biofilm, and by staining the biofilms with Live/Dead stain and observe it under the confocal microscopy.

In order to obtain optimal conditions for *C. difficile* biofilm formation we tested biofilm formation in several different media (chemically defined minimal media CDMM, and rich media BHIS and TYM). Moreover, we supplemented the media with different additives:

- 10mM, 20mM glucose, 40mM and 0.1M glucose
- 0.3M sodium chloride
- 0.1M glucose and 0,3M sodium chloride
- 0.1% and 1.0% Bile salts mixture (Sigma-Aldrich, USA)
- 0.1% Bile salt taurocholate (Sigma-Aldrich, USA)
- 0.1% Bile salt chenodeoxycholate (Sigma-Aldrich, USA)
- 0.1% Bile salt sodium cholate (Sigma-Aldrich, USA)
- 2.5g/L mucin (Sigma-Aldrich, USA)

We incubated the bacteria in biofilm assay for period from one to five days, in anaerobic conditions, at 37°C. For the biofilm assay 24- or 48-well cell culture plates were used. To observe the biofilms under confocal microscopy, biofilm was allowed to form in 4-well glass chamber slides (BD Falcon, USA).

Both strains form higher amounts of biofilm in rich medium BHIS supplemented with 0.1M glucose. In the absence of glucose, strain 630 forms significantly lower amounts of biofilm as compared to biofilm seen in presence of 0.1M glucose. However, the presence of glucose does not significantly affect biofilm formation for the strain R20291. Presence of 0.3M sodium chloride inhibits biofilm formation in both strains (Figure 15). We were not able to observe biofilm formation when used medium CDMM and TYM, or when supplemented with a mixture or single bile salts. Biofilms formed were adherent in nature, and were found to form only biofilm on the bottom of the wells (of 24-well plate), and not on the surface of the culture medium (Figure 15). Biofilm formed by the strain R20291 uniformly covers the entire surface area of the bottom of the wells (24-well plate) at 24hrs (day 1), while at the same time point for strain 630 we see much lesser biofilm formation. At 72hrs we observe relatively more uniform biofilms for strain 630 and uneven biofilms for strain R20291 (Figure 15).

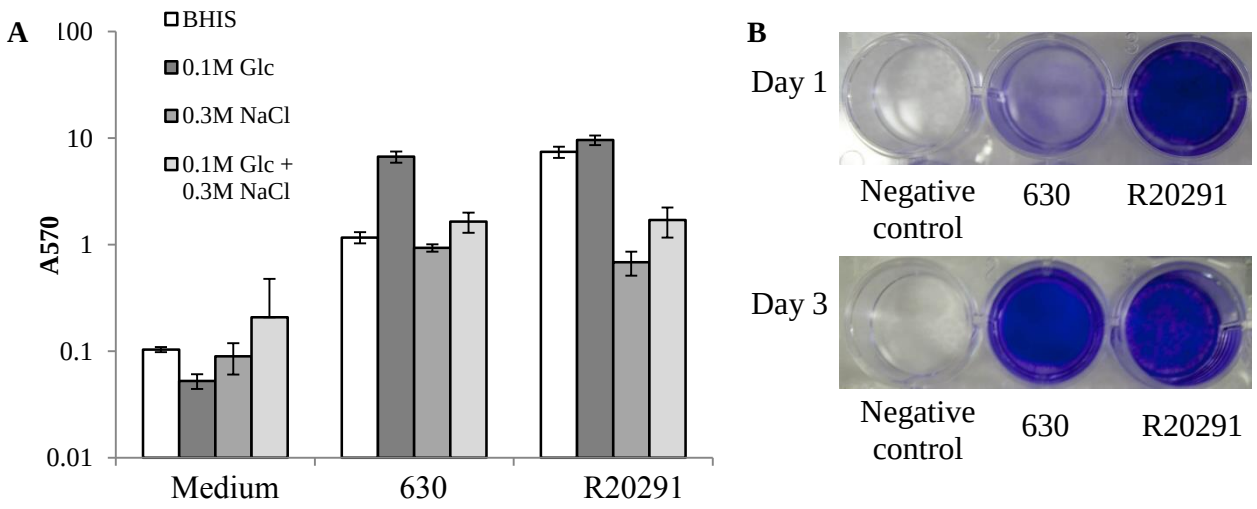


Figure 15 *C. difficile* biofilm formation *in vitro*.

(**A**) Biofilm formation by strain 630 and strain R20291, in BHIS supplemented with 0.1M glucose or 0.3M NaCl, for 3 days at 37°C in anaerobic conditions. Biofilm formation was measured by crystal violet staining. Results are presented in log scale and the error bars represent standard deviations (P<0.05). Data are representative of at least three independent experiments, each performed in triplicates. (**B**) Photographs of biofilms formed on a 24 well plate for strains 630 and R20291 on day 1 and day 3 are shown.

Biofilm formation in BHIS 0.1M glucose was monitored over a time period from 1hr to 120hrs (5 days), and quantitated by both, crystal violet staining and colony-forming unit counts (CFU) (Figure 16). *C. difficile* 630 biofilm accumulation appears to be maximal at day 5 (120hrs) (Figure 16a). Strain R20291 starts to form biofilm after 6hrs of incubation, with maximum biofilm formation after 24hrs, as measured by CV staining (Figure 16b). Bacterial counts from biofilms indicate a similar trend of increasing bacterial numbers until 24hrs and a decrease after this period for both strains. However it is clear that R20291 biofilms have significantly higher number of vital bacteria in the biofilm, as well as higher amounts of biofilms as quantitated by CV, compared with 630, under the conditions tested (Figure 16).

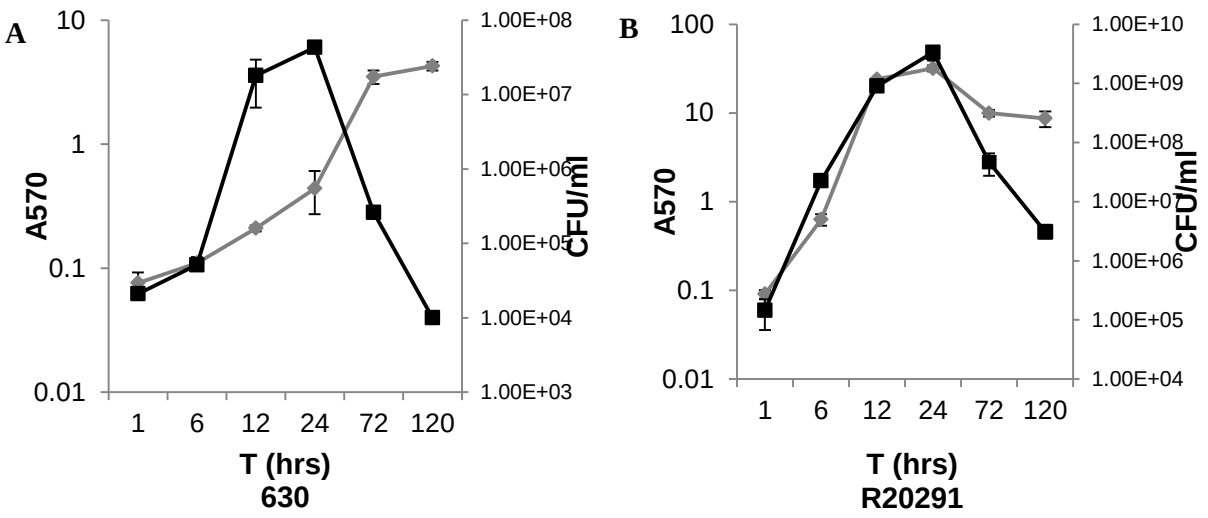


Figure 16 Time course experiment. Biofilm formation for strains 630 (**A**) and R20291 (**B**) measured by crystal violet staining presented on primary axis (gray line), and colony counts on secondary axis (CFU/ml, black line). Results are presented in log scale and the error bars represent standard deviations (P<0.05). Data are representative of at least three independent experiments, each performed in triplicates.

To examine biofilm formation by strains R20291 and 630 further, biofilms were allowed to form on glass slides in BHIS 0.1M glucose for one or three days. Live/Dead staining (Syto 9 and propidium iodide dyes, staining live bacteria green and dead bacteria red, respectively) was employed in order to evaluate the bacterial viability and biofilm thickness. We find that the majority of bacteria in both the 630 (Figure 17 a and b) and R20291 (Figure 17 c and d) biofilms were alive with a minor number of dead cells after one (Figure 17 a and c) and three days (Figure 17 b and d). Z-stack acquisitions revealed multiple layers of bacteria in the biofilm underneath a dense layer of material (Figure 17, right panel). 3D images show the presence of uniformly spread biofilms for R20291 on day 1 (Figure 17c) and with uneven secondary structures by day 3 (Figure 17d), and with a thickness ranging from 30 to 45μm. For strain 630, we observe that the live dead staining of biofilms was not homogeneous as seen for R20291 after day one (Figure 17 Confocal microscopy analysis of biofilms formed by C. difficile.a) or day three (Figure 17b). The maximum thickness of the biofilm was 30μm (72hrs). Thus microscopic analysis suggests that *C. difficile* biofilms are structured, with several layers of largely live bacteria encased within a dense matrix.

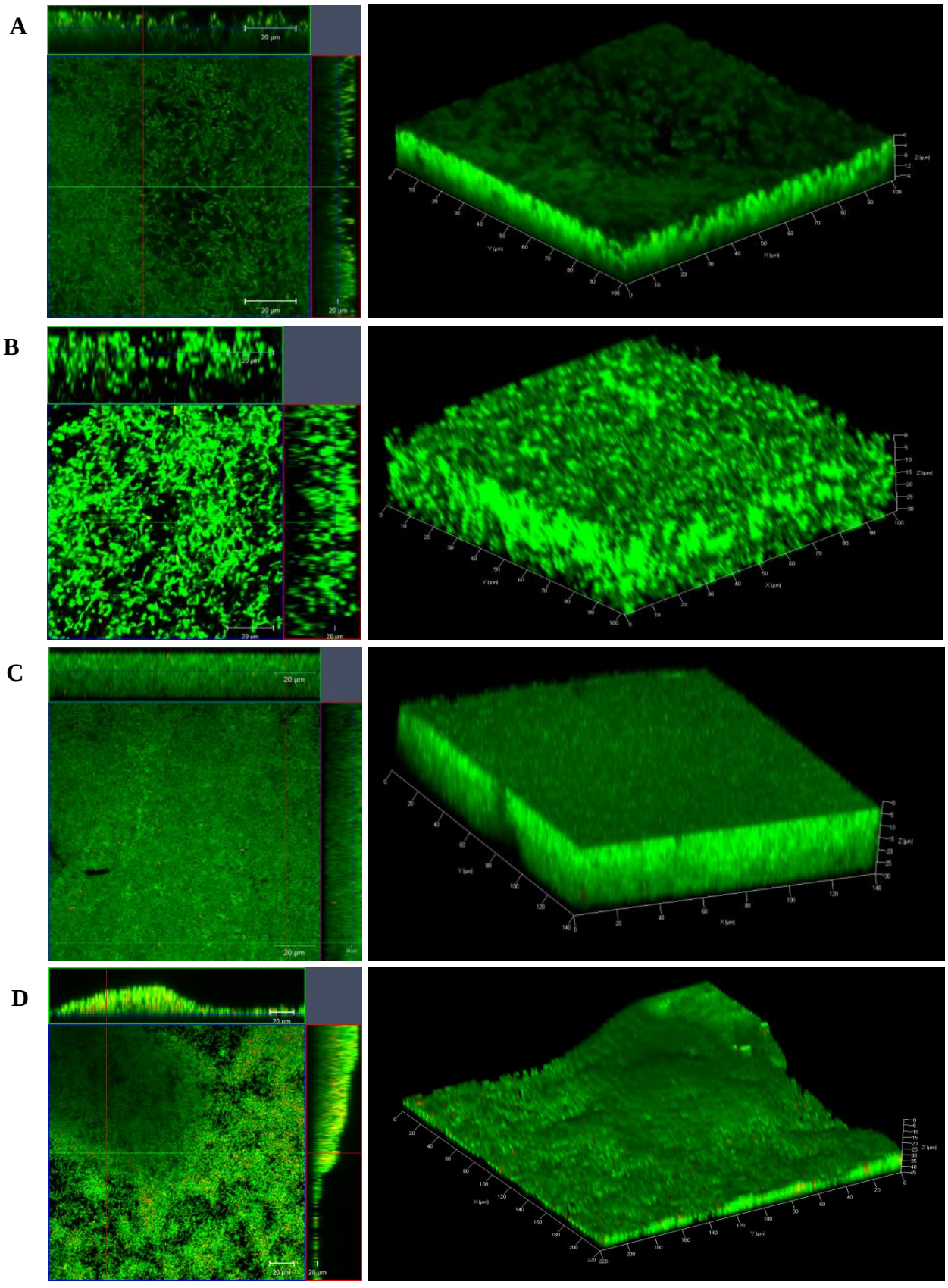


Figure 17 Confocal microscopy analysis of biofilms formed by *C. difficile*. Live/dead staining shows dead (red) and live (green) bacteria (Syto 9 and propidium iodide dye, respectively) in strains 630 (**A**, **B**) and R20291 (**C**, **D**) biofilms after incubation for 1 day (**A**, **C**) and 3 days (**B**, **D**). 3D images of biofilms depicting biofilm thickness in µM are shown in the left panels.

3.1.2 *C. difficile* biofilm matrix

Since the R20291 biofilm was more robust and reproducible, we decided that for further studies on *C. difficile* biofilm formation we focused exclusively on this strain, isolated form Stoke Mandeville outbreak. To study *C. difficile* biofilm matrix we used multiple staining to evaluate the extra-cellular matrix. Staining with antibodies against whole bacteria was able to stain a complex biofilm matrix and just few superficial individual bacteria (Figure 18), suggesting that biofilm matrix may be impermeable for the antibody. As a control we stained bacteria with preimmune serum (Figure 18, right panel).

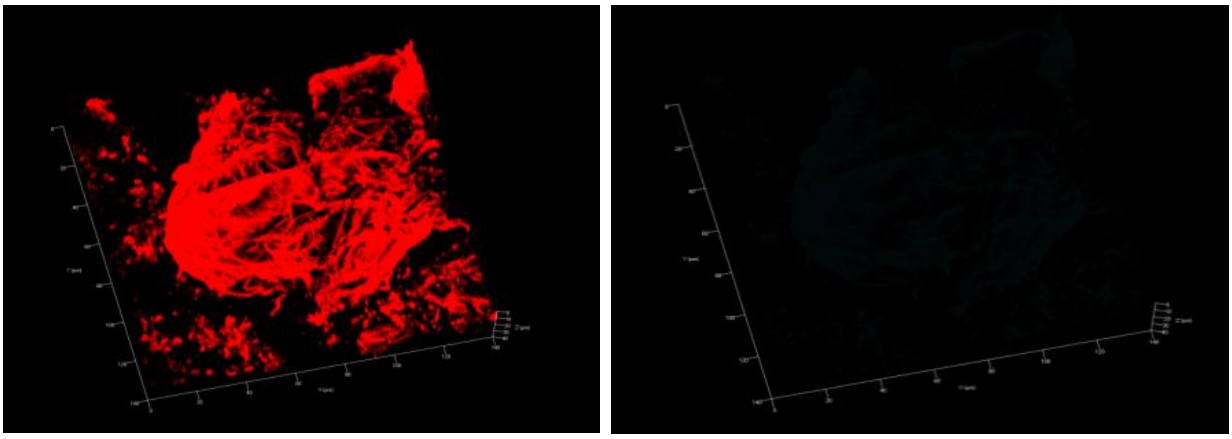


Figure 18 Staining with antibody against whole bacteria R20291. 3D confocal microscopy images are presenting stained R20291 biofilm with murine anti-R20291 (left panel), and with mouse preimmune serum (right panel), after 3 days incubation.

The formation of a thick mature biofilm was visualized by a biofilm matrix tracer Ruby stain, which labels a range of protein classes including glycol-, lipo- and phosphoproteins (Figure 19).

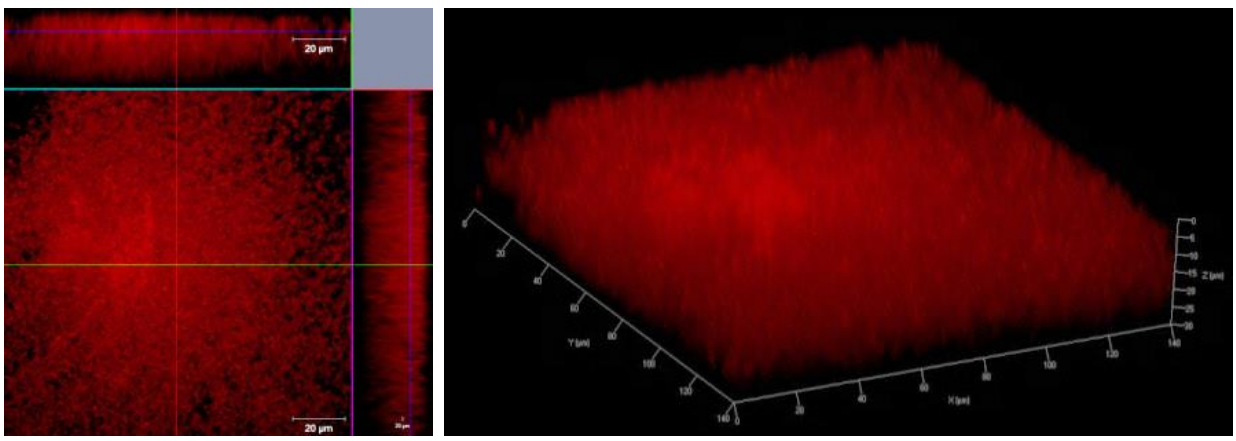


Figure 19 Biofilms stained with Ruby matrix stain after 3 days of incubation.

As last, we stained the biofilm with antibody against a synthetic *C. difficile* PSII polysaccharide (synthetic phosphorylated hexasaccharide repeating unit) (Adamo et al., 2012). Staining of biofilm with antibody against PSII showed presence of fiber-like structures on the biofilm (Figure 20a) suggesting bacterial capsule could be involved in biofilm formation. As a control we stained bacteria with preimmune serum (Figure 20b). DAPI staining stained bacterial DNA.

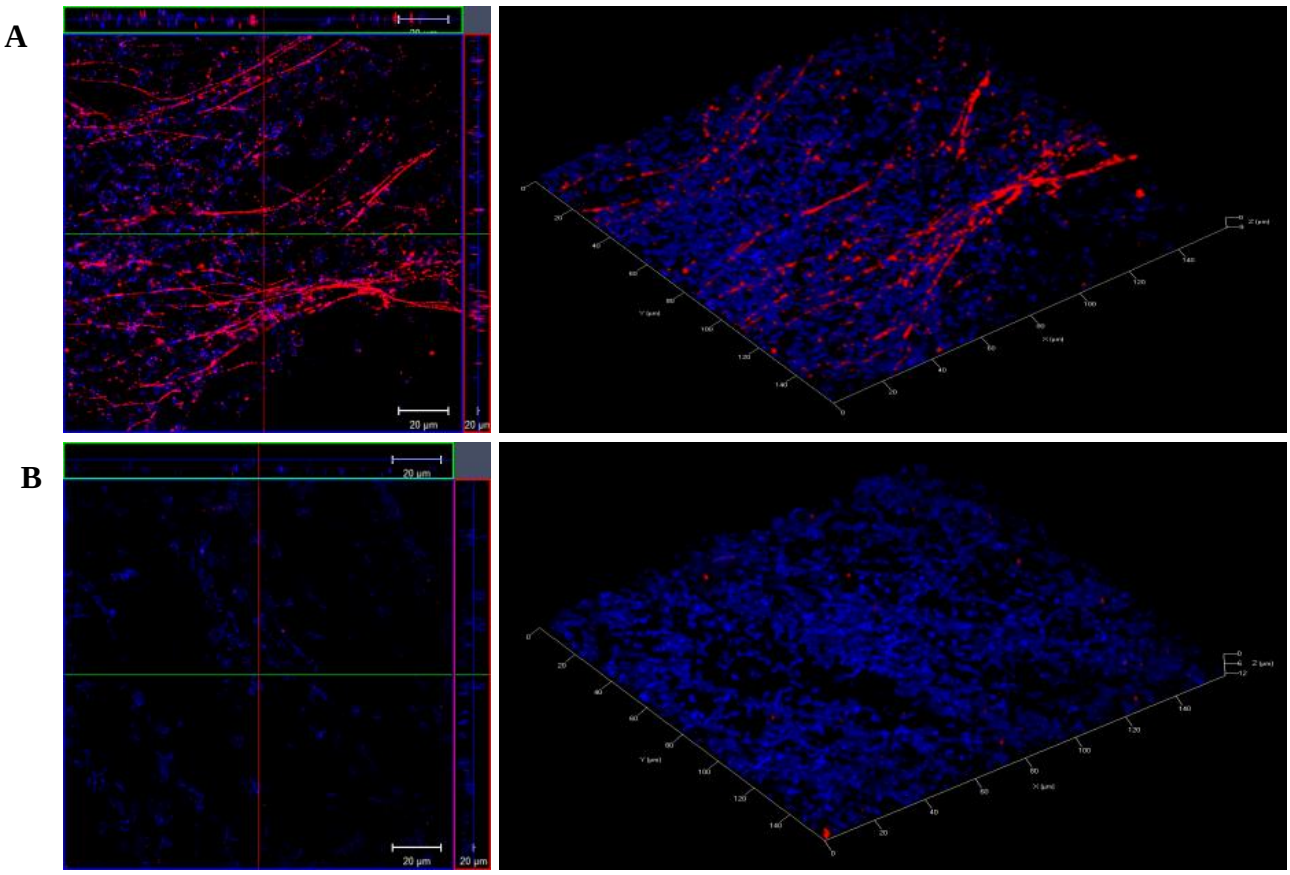


Figure 20 Staining with antibody against synthetic *C. difficile* PSII polysaccharide. Biofilms were stained with antibodies to a synthetic *C. difficile* PSII polysaccharide (red) and DAPI (blue), which stains the bacterial DNA **(A)**, or with the control mice preimmune serum and DAPI **(B).**

Biofilm matrix is composed of so called extracellular polymeric substance (EPS). Major components of EPS are protein, nucleic acid, and polysaccharides (Flemming and Wingender, 2010). To confirm that proteins are part of biofilm matrix we treated biofilms with 0.1mg/ml proteinase K. While planktonic growth was not inhibited by proteinase K (Figure 21c), incubation of the bacterial cultures with the enzyme resulted in a significant inhibition in

biofilm formation on days 1 and 3 (Figure 21a, dark bars). To examine if proteinase K can also disrupt already formed biofilm, we incubated 1 day-old biofilm with the same enzyme (Figure 21a, light bars). Results showed that addition of proteinase K to the preformed biofilm caused its disassembly compared to a non-treated control. We also performed similar experiments with DNase I treatment, and find that DNase I also inhibits biofilm formation (Figure 21b, dark bars), and is able to reduce pre-formed biofilms (Figure 21b, light bars), although to a lesser degree as compared to proteinase K. DNase I also did not affected the planktonic growth (Figure 21c).

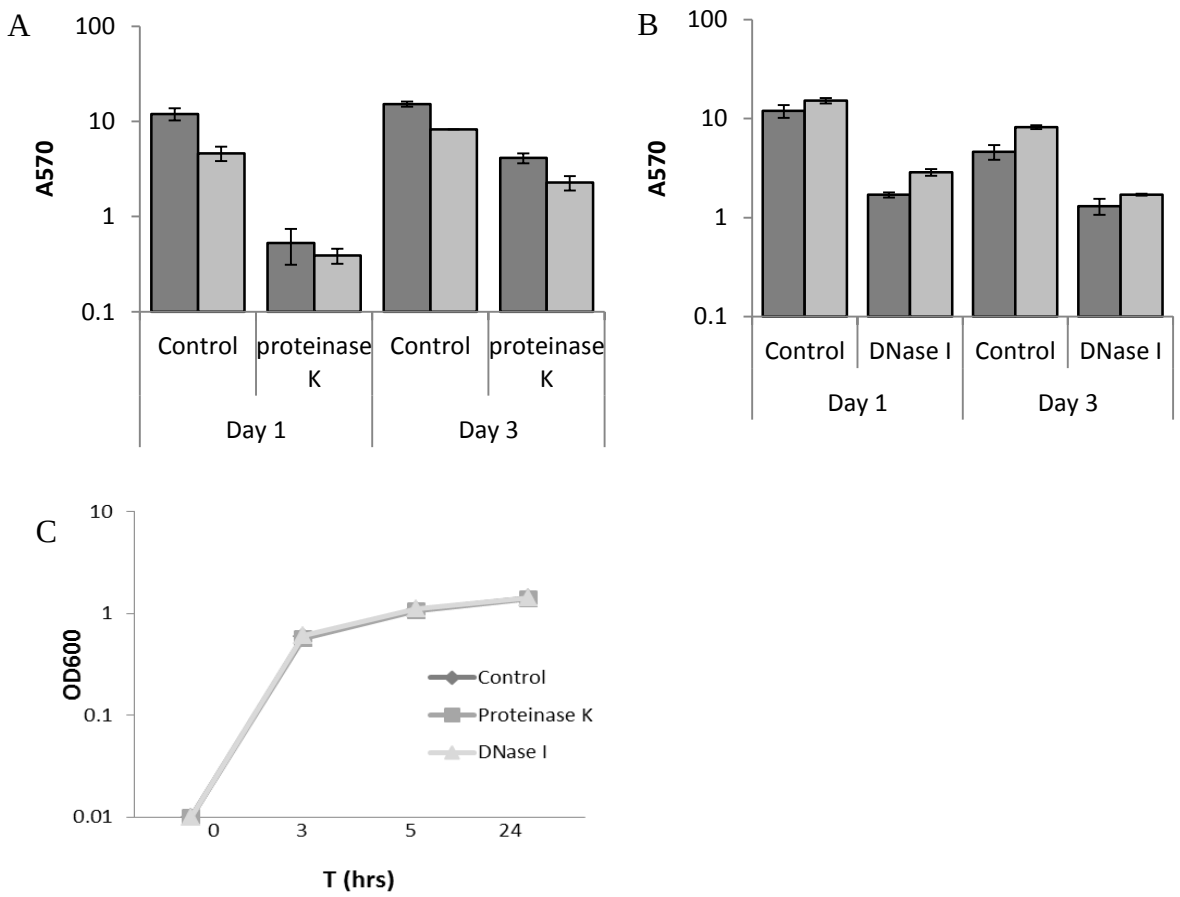


Figure 21 Proteins and DNA are part of biofilm matrix. Biofilms were incubated with proteinase K (**A**) or DNase I (**B**). The dark grey bars represent data from treatment of either enzyme at the start of incubation (inhibition of biofilm formation), and the light grey bars represent data from incubating pre-formed 1 day-old biofilms with either enzyme (disruption of biofilms). Data shown are representative of at least 2 independent experiments performed in triplicates (P<0.05). (**C**) Growth curves of R20291 in BHI in presence of Proteinase K (0.1mg/ml) or DNase I (2U/ml).

3.1.3 Genetic analysis

3.1.3.1 Cysteine protease Cwp84

Numerous cell surface proteins and surface structures such as flagella and pili have been shown to be important for biofilm formation in Gram-positive bacteria. Cwp84 is a key surface protease involved in maturation of the S-layer of *C. difficile*. We compared biofilm formation of a strain with a deletion in the *cwp84* gene, CdiR20291Δ*cwp84* (Δ*cwp84*), with wild-type (WT), using multiple methods. While there was no defect in planktonic growth (Figure 22c), we saw a dramatic decrease in biofilm accumulation for Δ*cwp84* strain as measured by crystal violet staining (Figure 22a). The *cwp84* mutant showed a more dramatic defect in biofilm formation on day 1, as compared to day 3 and day 5. Microscopic analysis showed a single layer of bacteria for Δ*cwp84* strain (Figure 22b, panel 2) as compared with WT (Figure 22b, panel 1). The biofilm defect for Δ*cwp84* strain was fully complemented by restoring the WT gene on the chromosome CdiR20291Δ*cwp8*-C (*cwp84*-C).

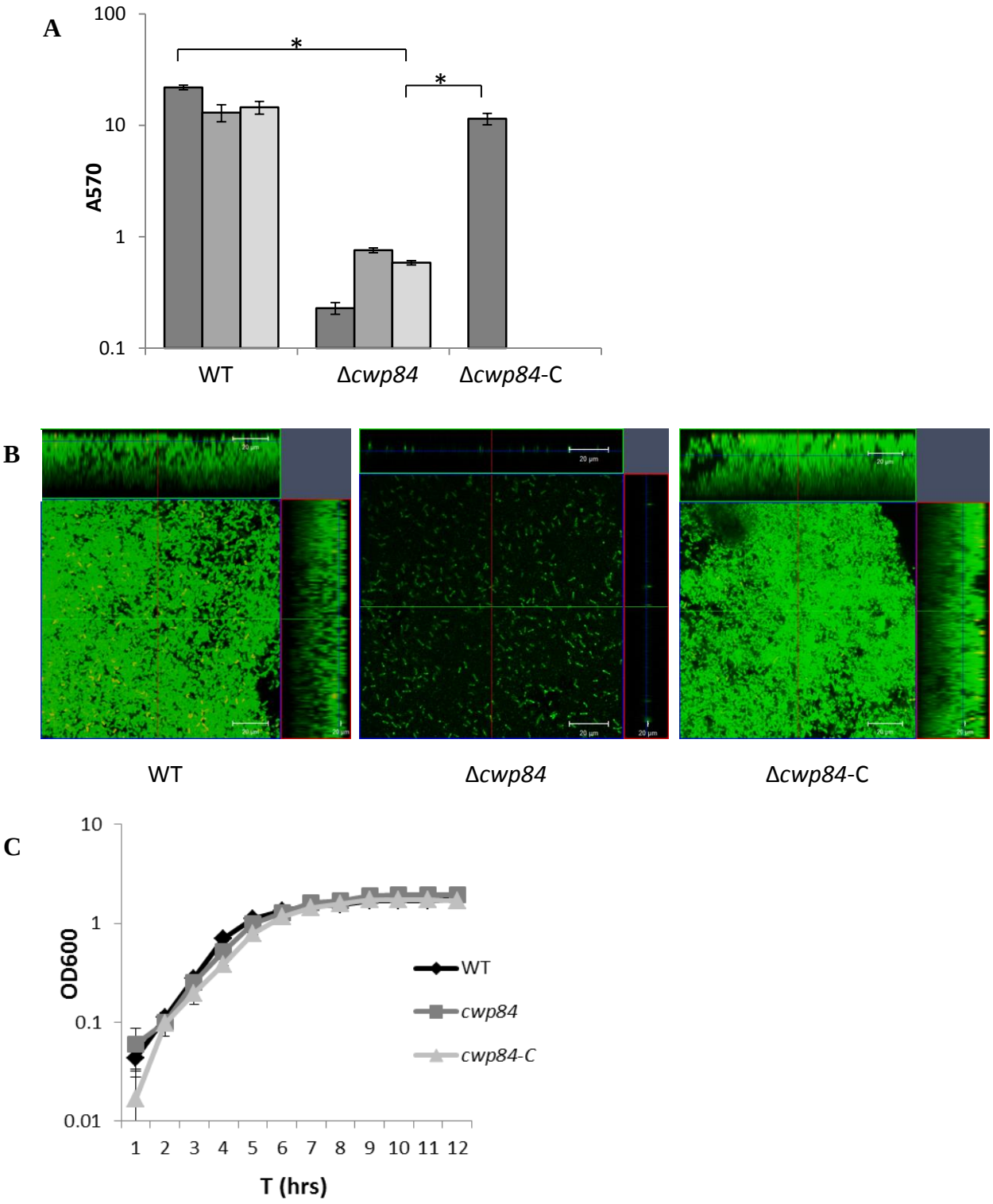


Figure 22 Role of S-layer in biofilm formation. (**A**) Biofilm formation by WT R20291, a Δ*cwp84* mutant for days one, three and five and a complemented strains (Δ*cwp84*-C) for day one *in vitro* as measured by crystal violet staining. (**B**) Confocal microscopy images of live-dead staining of biofilms formed by the WT, Δ*cwp84* and Δ*cwp84*-C. Results are presented in log scale and the error bars represent standard deviations (P<0.05). Biofilm assays were performed in triplicates, and data are representative of at least 3 independent experiments. (**C**) Growth curves for Δ*cwp84* mutant and complemented (*Δcwp*-C) strains.

3.1.3.2 Flagellin FliC

To examine the role of flagella, a mutant in the flagellin gene, *fliC*, CRG3351 (*fliC*) was tested for biofilm formation. We observed a significant decrease in biofilm accumulation for the *fliC* mutant as compared to WT on day 5, but not at earlier times, with crystal violet staining (Figure 23a), and microscopic analysis (Figure 23b, panel 2). This was reversed upon expressing the FliC protein episomally from its native promoter (*fliC*-C) (Figure 23a). No differences in planktonic growth were observed between these strains (data not shown).

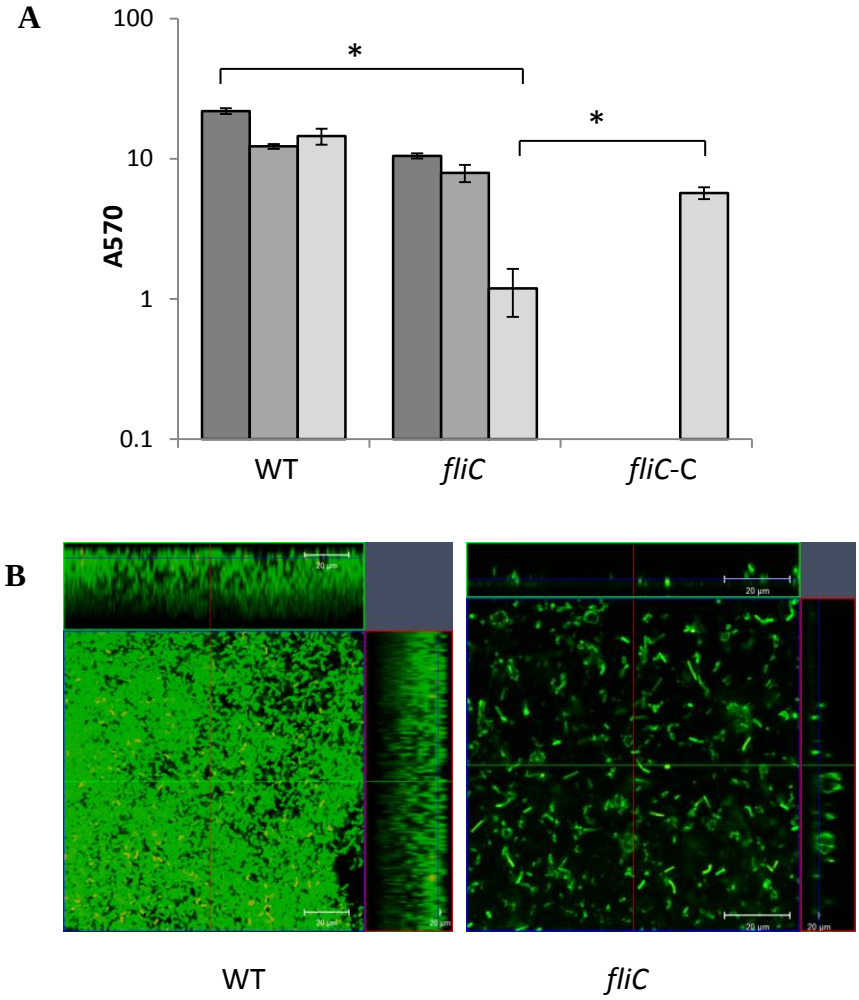


Figure 23 Role of flagella in biofilm formation. (**A**) Biofilm formation by WT R20291, *fliC* mutant (*fliC*) for days one, three and five and complemented strains *fliC* (*fliC*-C) for day five *in vitro* as measured by crystal violet staining. (**B**) Confocal microscopy images of live-dead staining of biofilms formed by the WT and *fliC*. Results are presented in log scale and the error bars represent standard deviations ($P<0.05$). Biofilm assays were performed in triplicates, and data are representative of at least 3 independent experiments.

3.1.3.3 Quorum sensing authoinducer LuxS

Quorum sensing (QS) plays a vital part in biofilm formation, and involvement of quorum sensing regulators such as *luxS* has been demonstrated for various other bacteria (Lombardia et al., 2006); (Ohtani et al., 2002); (Vendeville et al., 2005). We tested a mutant of a *luxS*, homologue in *C. difficile*, by homology to other Gram-positive bacteria. A dramatic defect in biofilm formation was observed for the *luxS* mutant, CRG1183 (*luxS*), both by crystal violet staining (Figure 24a) and by microscopy (Figure 24b, center panel), with no significant differences at different times after incubation. Examination of the *luxS* mutant showed that it is unable to form even a bacterial monolayer on glass surface. Biofilm defects of this mutant were complemented by episomal expression of the full length gene under the control of the native promoter (*luxS*-C). While the complementation did not completely restore the WT phenotype we were able to detect several layers of bacteria for the complemented strain (Figure 24b, right panel). Although the growth curves for mutant and complemented strains were similar (Figure 24c), it is possible that expressing *luxS* episomally may be toxic for the bacteria, as we observe a smaller colony size for this strain on plates.

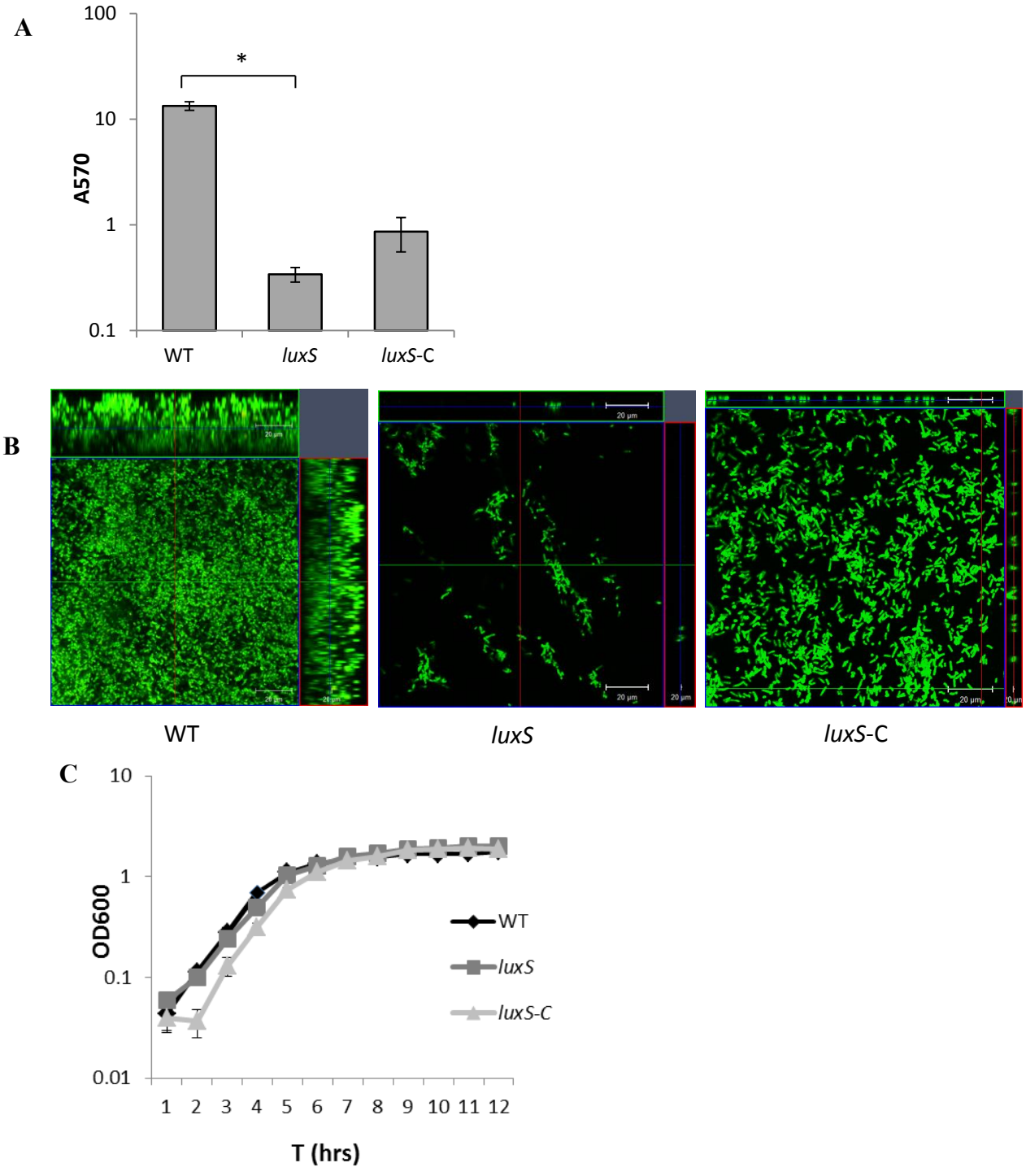
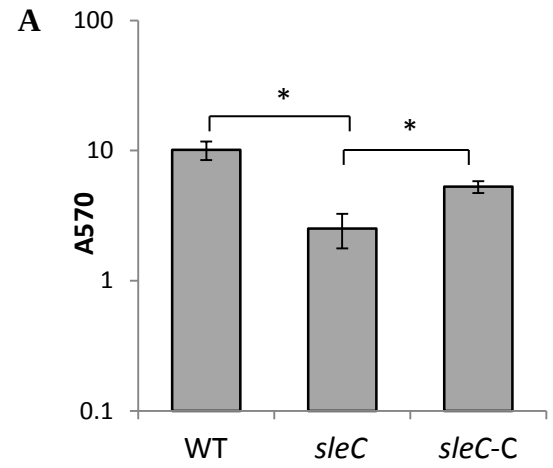


Figure 24 Potential role for quorum sensing in *C. difficile* biofilm formation. (**A**) Biofilm formation by WT R20291, putative quorum sensing gene *luxS* mutant (*luxS*) and complemented strains (*luxS*-C) as measured by crystal violet. (**B**) Confocal microscopy analysis of WT, *luxS* and complemented strain *luxS*-C. Results are presented in log scale and the error bars represent standard deviations (P<0.05). Data from biofilm assays are representative of at least 3 independent experiments performed in triplicates. (**C**) Growth curves for *luxS* mutants and complemented *(luxS*-C) strains.

3.1.3.4 Germination factor SleC

Biofilm formation and sporulation are well known processes. Their connection has been extensively studied in *Bacillus* spp, and master regulator Spo0A was connected with biofilm formation and sporulation (Hamon and Lazazzera, 2001). About the role of Spo0A in *C. difficile* biofilm formation we will talk more in the chapter Regulation of *C. difficile* biofilm formation. SleC is a protein that was recently reported to be specifically involved in germination of *C. difficile* spores (Burns et al., 2010). The *sleC* mutant is able to form biofilm-like structures (Figure 25a), but the biofilm is uneven and the thickness of biofilm produced by this strain is never more than 20μm (Figure 25b, right panel). The cellular morphology of the bacteria in these biofilms is different to wild-type, and appears to form filamentous structures. A biofilm defect of the mutant was complemented by episomal expression of gene *sleC*, (*sleC*-C) under control of its native promoters (Figure 25a). Mutant *sleC* did not have a defect in planktonic growth (Figure 25c) and was unable to form heat-resistant colonies in a germination assay, confirming the involvement of SleC in germination of *C. difficile* spores (Figure 25d).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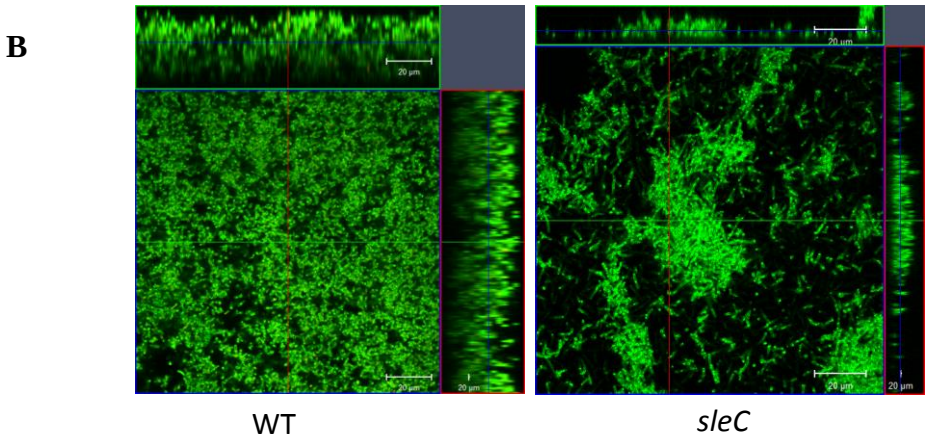
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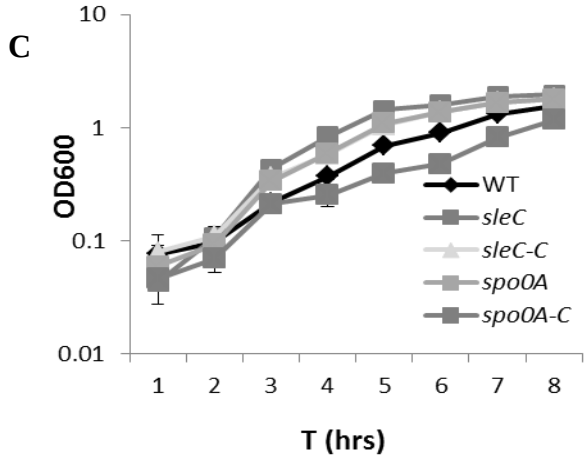
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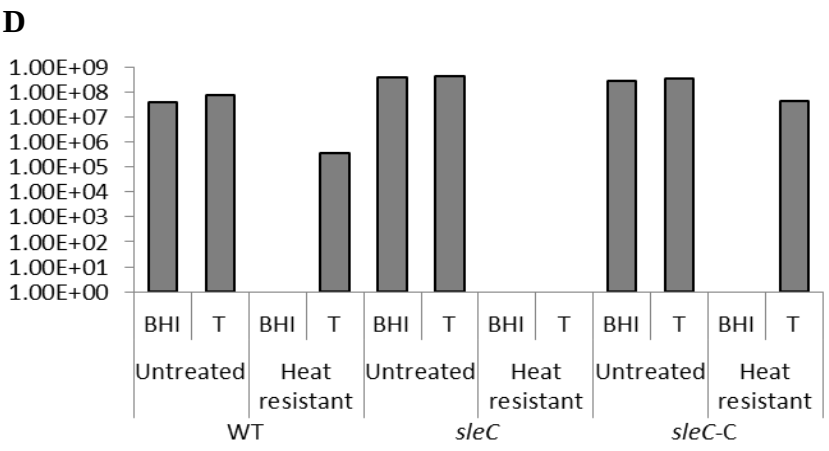
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Untreated
Heat resistant
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Figure 25 Germination proteins affect *C. difficile* biofilm formation. (**A**) Biofilm formation by WT R20291, *sleC* mutant (*sleC*) and complemented *sleC* mutant (*sleC*-C) after 3 days. (**B**) Confocal microscopy analysis of WT and *sleC* mutant. (**C**) Growth curves for sporulation and germination mutants, *spo0A* and *sleC* and complemented strains (*spo0A*-C and *sleC*-C). (**D**) Germination assays for *sleC* mutant. Numbers of colony forming units CFU/ml obtained from sporulating cultures, with and without heat treatment of *C. difficile* R20291, *sleC* and *sleC* complemented strain (*sleC*-C) after 48hrs of incubation in on BHI in presence or absence of sodium taurocholate (T). Results are presented in log scale and the error bars represent standard deviations ($P<0.05$). Both, biofilm and spore quantitation experiments were performed in triplicates, and data shown are representative of at least 3 independent experiments.

3.2 EFFECTS OF ANTIBIOTIC VANCOMYCIN ON *C. difficile* BIOFILMS

3.2.1 Bacteria in the biofilms are resistant to the vancomycin

Since biofilms are known to be a means by which bacteria protect themselves from antibiotics, we studied if *C. difficile* biofilms are important in mediating antibiotic resistance. We tested the resistance of bacteria in biofilms to the antibiotic vancomycin, which is commonly used for the treatment of CDI. *In vitro*, vancomycin, has excellent activity against *C. difficile*; a MIC of 0.75–2.0µg/ml is sufficient to inhibit 90% of strains (Surawicz and Alexander, 2011). Both sessile and planktonic phases, from the same *C. difficile* biofilm culture, 1 day-old (Figure 26a) and 3 day-old biofilms (Figure 26b) were exposed to 20µg/ml vancomycin (200 times the MIC of strain R20291, MIC determinate in our conditions) for 6hrs and 24hrs.

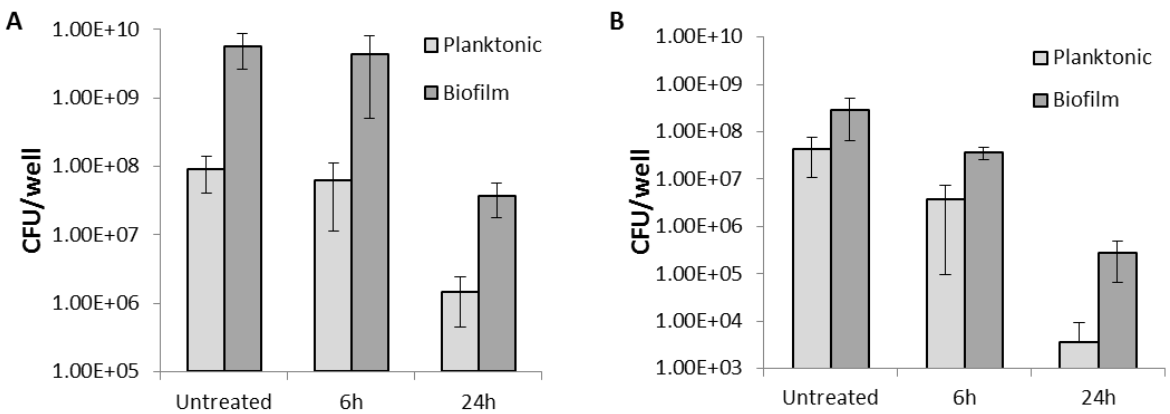


Figure 26 Antibiotic resistance of *C. difficile* biofilms. *Clostridium difficile* 1-day-old (**A**) to 3-day-old (**B**) biofilms, and the corresponding planktonic growth, were exposed to 20µg/ml vancomycin (200 times the MIC) for 6hrs and 24hrs. CFU counts before (untreated) and after (6hrs and 24hrs) treatment with vancomycin are shown. Results are presented in log scale and are representative of results from 3 independent experiments, performed in triplicates ($P<0.05$).

The percentage of surviving bacteria after treatment with antibiotics for 24hrs for 1 day and 3 day old biofilms is presented in Figure 27. Bacteria in 1 day-old and 3 day-old biofilms survived 5 fold and 12 fold more respectively as compared to planktonic bacteria. These data support a role for *C. difficile* biofilms in resisting antibiotics.

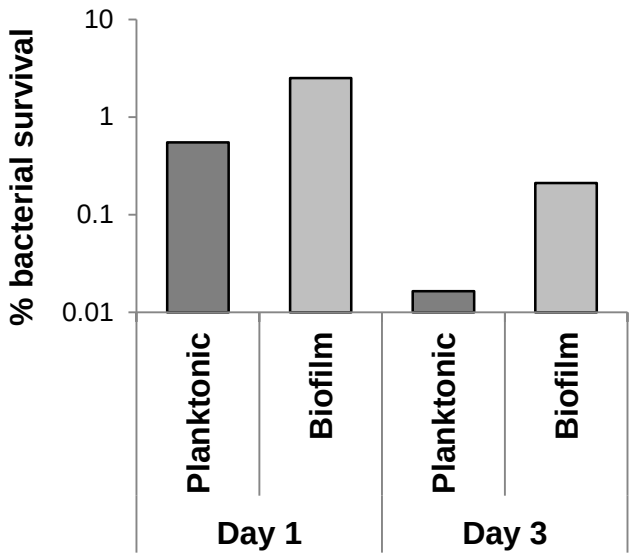


Figure 27 Effect of antibiotics on *C. difficile* biofilms. *Clostridium difficile* 1-day-old to 3-day-old biofilms and the corresponding planktonic growth were exposed to 20μg/ml vancomycin (200 times the MIC) for 24hrs. Data are presented as percentage of surviving bacteria after treatment with antibiotics for 24hrs for 1 day and 3 day biofilms. Results are presented in log scale and the error bars represent standard deviations ($P<0.05$).

3.2.2 Biofilm matrix protects bacteria from the vancomycin

Different mechanism can be responsible for the resistance of the bacteria to the antibiotics in the biofilms. To try to understand if the resistance to the vancomycin in case of *C. difficile* biofilms is due to protection conferred by biofilm matrix structure, or is an inherent property of the bacteria in biofilm, we studied the effect of vancomycin on adherent biofilms which were disrupted by pipetting (Figure 28). Disrupted sessile biofilm and the planktonic phase from one-day old biofilm were incubated for 6hrs and 24hrs with 20μg/ml vancomycin. Bacteria from the disrupted adherent biofilms were not more resistant to high concentrations of antibiotics of biofilm compared with bacteria from the planktonic phase (Figure 28). Although the bacteria from disrupted biofilms do not form new adherent biofilms after the incubation with antibiotics, we observed unstable, thread-like structures in the wells (which were disrupted by pipetting before performing CFU counts). These data indicate a lack of genetic changes in the bacteria within biofilms, and may suggest that the biofilm matrix, and/or other epigenetic mechanisms are involved in mediating vancomycin resistance.

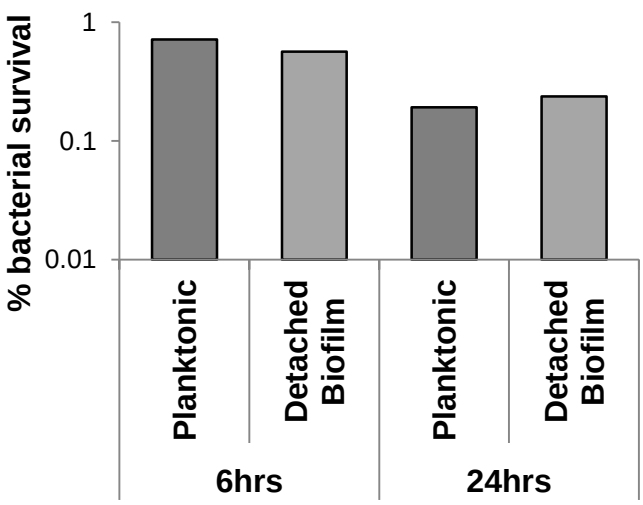


Figure 28 Biofilm matrix protects the bacteria. *Clostridium difficile* 1-day-old biofilms were disrupted by pipetting. Disrupted biofilm and the corresponding planktonic growth were exposed to 20μg/ml vancomycin (200 times the MIC) for 6hrs and 24hrs. Data are presented as percentage of surviving bacteria after treatment with antibiotics for 6hrs and 24hrs for 1 day old biofilms. Results are presented in log scale and the error bars represent standard deviations (P<0.05).

3.2.3 Subinhibitory and inhibitory conentrations of vancomyin induce biofilm formation

Biofilm formation is a common response to the stress conditions, e.g. environmental stresses, starvation, and exposure to low concentrations of antibiotics. It has been reported that sub-inhibitory concentrations of antibiotics can stimulate biofilm formation (Hoffman et al., 2005). To study if vancomycin stimulates the biofilm formation *in vitro,* bacteria were treated with a range of concentrations of vancomycin (0 – 0.5µg/ml), both lower and higher than the tube growth MIC (0.2µg/ml) and biofilm formation was measured at day 1 and day 3. No significant induction of biofilm was observed for any of the vancomycin concentrations after 1 day. Inhibition of biofilm formation was evident for concentrations of vancomycin 0.5µg/ml and higher (Figure 29). Interestingly, after 3 days incubation, a significant induction of biofilms was observed with 0.5µg/ml vancomycin and to a lesser extent with a sub-inhibitory concentration (0.25µg/ml) of vancomycin (Figure 29). These results suggest that exposure to sub-inhibitory and inhibitory concentrations of vancomycin can stimulate biofilm formation *in vitro*.

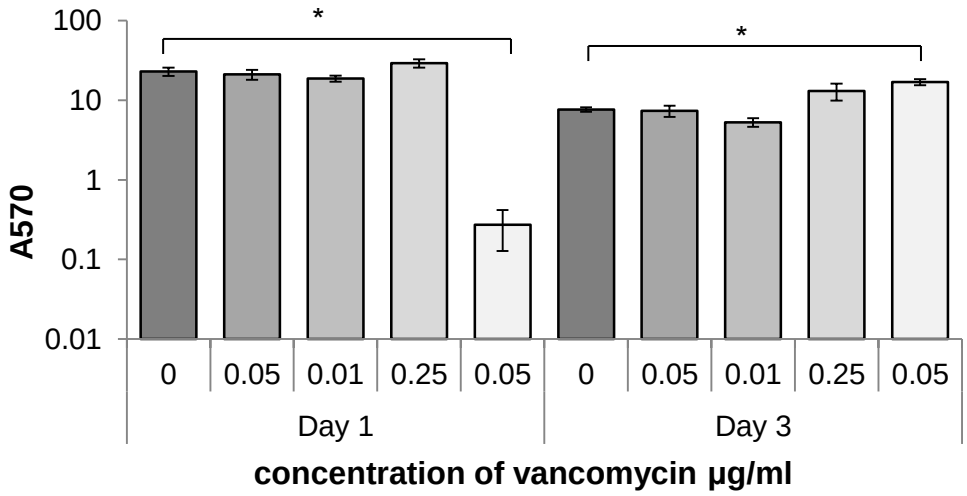


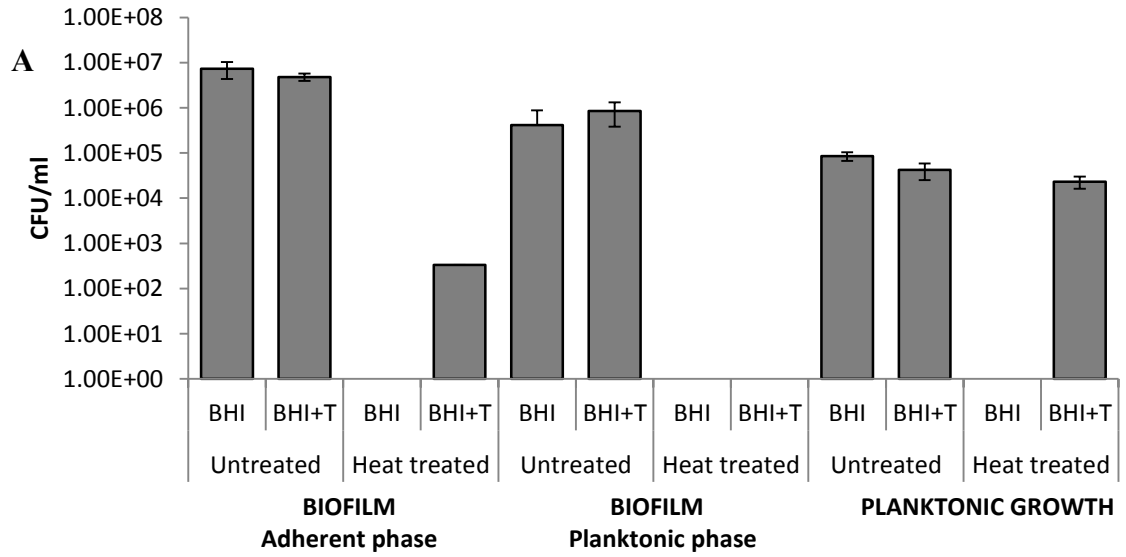
Figure 29 Sub-inhibitory and inhibitory concentrations of antibiotic stimulate biofilm formation by *C. difficile*. Biofilm formation measured by crystal violet staining at day 1 and day 3 after treatment with sub-inhibitory and inhibitory concentration of antibiotic vancomycin (MIC for R20291 was 0.2µg/ml). Results are presented in log scale and the error bars represent standard deviations ($P<0.05$). * denotes significant differences as compared with biofilm formation in absence of vancomycin (0µg/ml).

3.3 REGULATION OF *C. difficile* BIOFILM FORMATION

3.3.1 Master regulator Spo0A

3.3.1.1 Spores in *C. difficile* biofilms

Sporulation it is a key pathway that is initiated when *C. difficile* is under stress conditions. Regulators of sporulation like Spo0A are also involved in formation of biofilms in other Gram-positive bacteria (Hamon and Lazazzera, 2001). We first studied if spore formation occurs in *C. difficile* biofilms in our growth conditions, for adherent biofilms and planktonic phase from the same well of the 24-well plate (Figure 30a). We found that there are very few spores in the biofilm (0.0001%) and in planktonic phases (none detectable) on day 3 and day 5. However, in the control, which was bacteria cultured in a tube (where biofilm formation did not occur) spores were formed by day 3 (40-50%) (Figure 30a). We also confirmed that a spo0A mutant is defective in sporulation (Figure 30b).



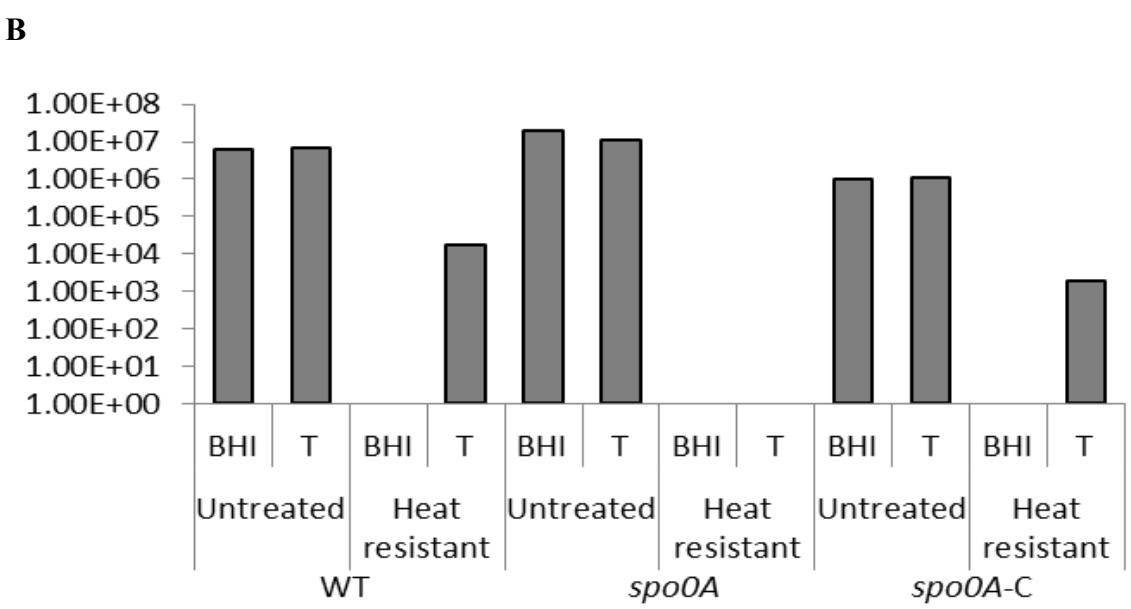


Figure 30 Sporulation in *C. difficile* biofilms. (**A**) Quantitation of the number of spores present in the adherent, planktonic phases of biofilm and in planktonic tube culture, in brain heart infusion media (BHI) with sodium taurocholate (BHI+T) and heat treatment (65^0C). (**B**) Germination assays for *spo0A*. Numbers of colony forming units CFU/ml obtained from sporulating cultures, with and without heat treatment of *C. difficile* R20291, *spo0A* and *spo0A* complemented strain (*spo0A*-C) after 48hrs of incubation in on BHI in presence or absence of sodium taurocholate (T). Results are presented in log scale and the error bars represent standard deviations ($P<0.05$). Experiments were performed in triplicates, and data shown are representative of at least 3 independent experiments.

3.3.1.2 Mutant in *spo0A* is unable to form biofilm *in vitro* conditions

Master regulator Spo0A is a central transcriptional regulator, which controls the expression of more than 100 genes, including genes for biofilm matrix expression and sporulation (Fujita et al., 2005); (Molle et al., 2003). Concentration of phosphorylated or unphosphorylated forms of Spo0A (Spo0A~P) determines gene expression. Both forms, phosphorylated and unphosphorylated form, are always present in the cell. Intermediate levels of Spo0A~P induce matrix gene expression and with it biofilm formation, and higher levels induce the sporulation in the bacterium *B. subtilis*. With maturation of the biofilm, and accumulation of Spo0A~P in the cell and the process of sporulation is activated (Vlamakis et al., 2013).

We demonstrated that the mutant in *spo0A*, which was unable to sporulate (Figure 30b), forms significantly less biofilm compared with WT both by CV staining (Figure 31a) and microscopy (Figure 31b, panel 2). This phenotype was restored by episomal expression of genes *spo0A* (*spoA*-C) under control of its native promoters (Figure 31).

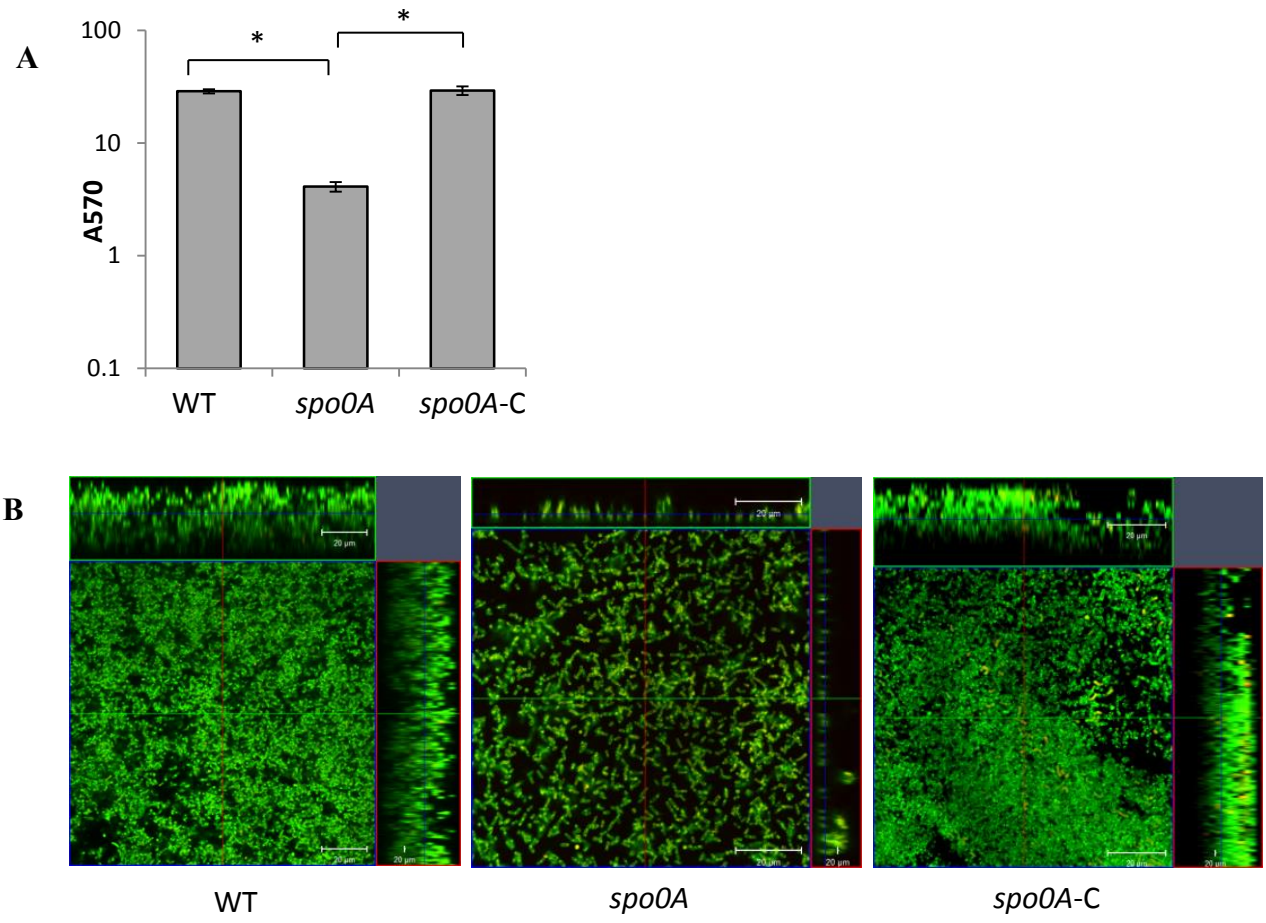


Figure 31 Sporulation protein affect *C. difficile* biofilm formation. (**A**) Biofilm formation by WT R20291, sporulation transcription factor *spo0A* mutant (*spo0A*) and complemented *spo0A* mutant (*spo0A*-C) after 1 day. (**B**) Confocal microscopy analysis of WT and mutants *spo0A* and complemented *spo0A* mutant (*spo0A*-C). Results are presented in log scale and the error bars represent standard deviations ($P<0.05$). Both biofilm and spore quantitation experiments were performed in triplicates, and data shown are representative of at least 3 independent experiments.

Our results indicate that Spo0A could have a similar role in *C. difficile* as it has in *B. subtilis*. We were unable to detect significant number of spores in the biofilms in the R20291 strain under our culture conditions, while the mutant in *spo0A* was defective in its ability to form biofilm. This could indicate that the intermediate levels of Spo0A~P are present in the cells, which induces matrix gene expression and with it biofilm formation.

3.3.1.3 Inhibitory concentrations of vancomyicin restore biofilm formation in *spo0A* mutant

Regulation of biofilm formation through the master regulator Spo0A is the best studied biofilm pathway in *B. subtilis*, but not the only one (Vlamakis et al., 2013). Several different pathways for biofilm formation are Spo0A-independent. The *abh* gene transcription is controlled by several extracytoplasmic function (ECF) RNA polymerase σ-factors, including σ^{M}, σ^{W} and $\sigma^{X,}$ which are are activated by enviromental stimuli, e.g. cell wall stress and specific antibiotics stresses (Helmann, 2002). Abh can indirectly repress the repressor SinR, which leads to the transcription of biofilm matrix genes. In this way the bacterium can respond to enviromental changes in a Spo0A-independent way (Vlamakis et al., 2013).

To study potential Spo0A-independent pathways in *C. difficile* biofilm formation we incubated the mutant *spo0A* (which was unable to form biofilm) with sub-inhibitory and inhibitory concentrations of antibiotic vancomycin (MIC for *spo0A* mutant was 0.5µg/mL). Inhibitory concentration of vancomycin, 1.0µg/mL, was able to restore the biofilm formation to a wild-type R20291 level (Figure 32). This indicates that the regulation of *C. difficile* biofilm formation is a complex, and as in case of *B. subtilis*, biofilm formation may be subject to several layers of regulation, including Spo0A-independent pathways.

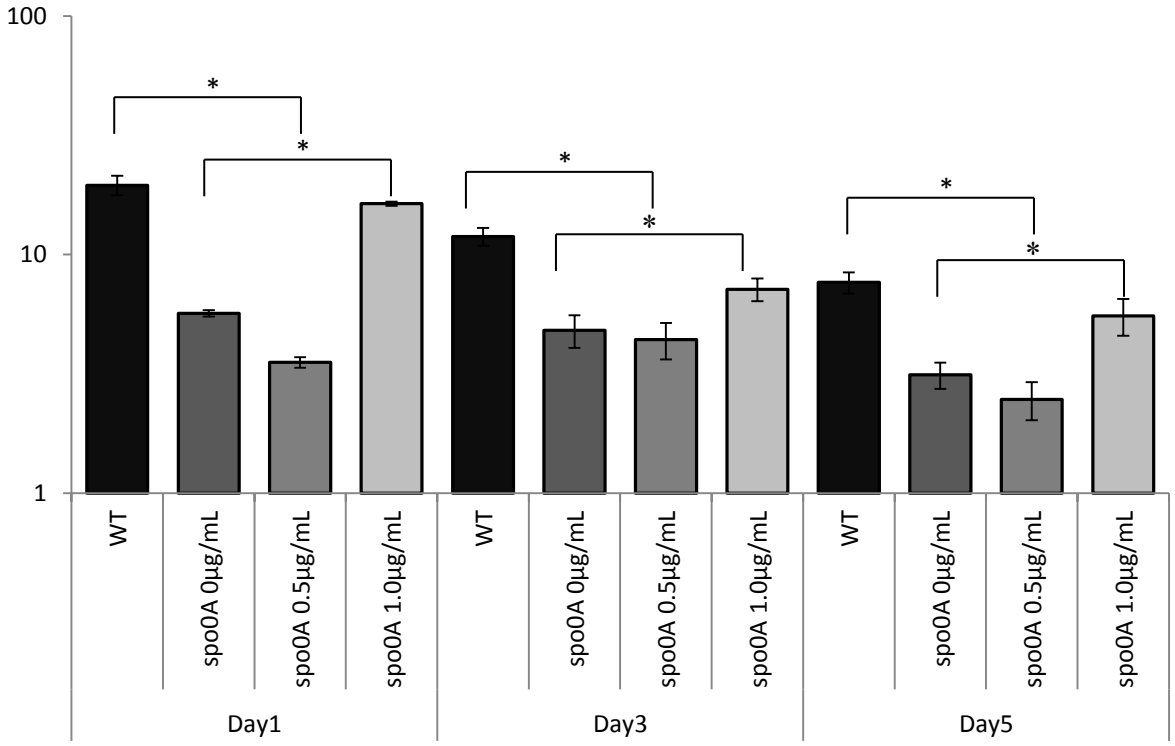


Figure 32 Spo0A-independent biofilm formation. Mutant *spo0A*, which is unable to form biofilm in normal conditions, was complemented with addition of inhibitory concentrations of antibiotic vancomycin (1.0µg/mL). Mutant *spo0A*, supplemented with antibiotic vancomyicin, was able to form biofilms comparable with the wild-type, R20291.

3.3.2 Anti sigma-factor RsbW

3.3.2.1 Single-nucleotide polymorphism (SNP) in *C. difficile*

Many contradictions within studies on *Clostridium difficile* can be found in the literature. Recently, two different stories, based on similar experiments and animal models, were published. Lyras and co-authors published in Nature Letters (2009) results showing that only toxin B is essential for the pathogenesis of *C. difficile* (Lyras et al., 2009). Just a year later (2010), Kuehne and co-authors published that toxin A and toxin B are both needed for the pathogenesis by *C. difficile* (Kuehne et al., 2010).

In our initial studies we found that clinical *C. difficile* strains, originally coming from the same out-break, but stored in different laboratories around United Kingdom, showed differences in biofilm formation, sporulation, minimal inhibitory concentration to the antibiotic vancomycin, and motility.

Whole genome sequencing was performed in collaboration with Nigel Minton laboratory, from University of Nottingham, Clostridia research group (CRG). Sequences of these strains showed the presence of single nucleoide polymorphisms (SNPs) in different regulatory genes. The strain, in which we performed our previous studies, carries a SNP in the anti-sigma factor RsbW and in the gene *vncR*.

SNP for the gene *rsbW*, detected with whole genome sequencing, was confirmed with Sanger sequencing with the primers *rsbW*_F and *rsb*_R (Appendix 3). Nucleotide in the position 245 changed from a nucleotide 'G' to a nucleotide 'T', compared to a reference genome R20291. The SNP is a nonsynonymous mutation in the genome, and caused a change in amino acid sequence, changing a glycine to valine.

RsbW is an anti-sigma factor which regulates the stress response induced by alternative sigma factor (σ^B). As its role in biofilm formation was previously shown in other bacteria, e.g. *Staphylococcus epidermidis* (Knobloch et al., 2004), we investigated the potential role of this regulator in biofilm formation by *C. difficile*.

3.3.2.2 Characterization of role of *rsbW* in biofilms

3.3.2.2.1 Generation of a Δ*rsbW* deletion mutant in R20291 background

To investigate the function of anti-sigma factor RsbW, and the effect of the SNP in this gene, a deletion mutant was generated in strain R20291 strain that lacks SNP in *rsbW*, taking advantage of an allele exchange strategy set up by Cartman and co-workers (Cartman et al., 2012).

A suitable construct was generated cloning "in frame", in the pMTL-SC7315 vector (Cartman et al., 2012), the two regions flanking the gene of interest, in order to substitute the functional endogenous gene with a short sequence coding for a non-functional short peptide. The construct was transformed in *C. difficile*, where two steps of selection where carried out, as described in Methods. First, clones which had integrated the entire plasmid were selected for antibiotic resistance and checked by PCR (data not shown); then, clones which had undergone excision of the plasmid (resulting either in deletion of the gene or in recovery of the wild-type genotype) were positively selected on fluorocytosin and the genotype (wild-type or deletion mutant) was determined by PCR (Figure 33). Abolishment of the expression of the gene *rsbW* in a deletion mutant, the expression of the gene *sigB*, which is upstream of *rsbW*, and predicted gene *rsbV*, which is downstream, were checked by qRT PCR (data not shown).

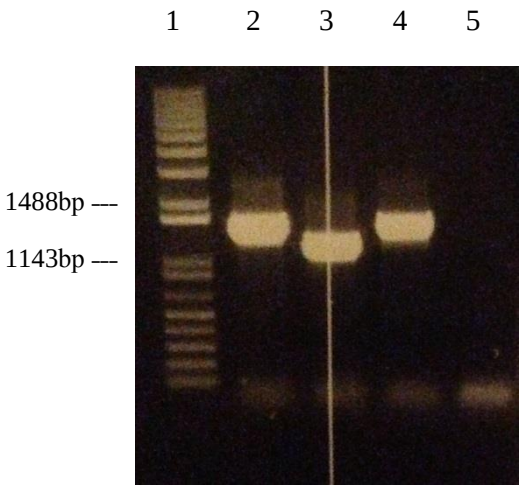
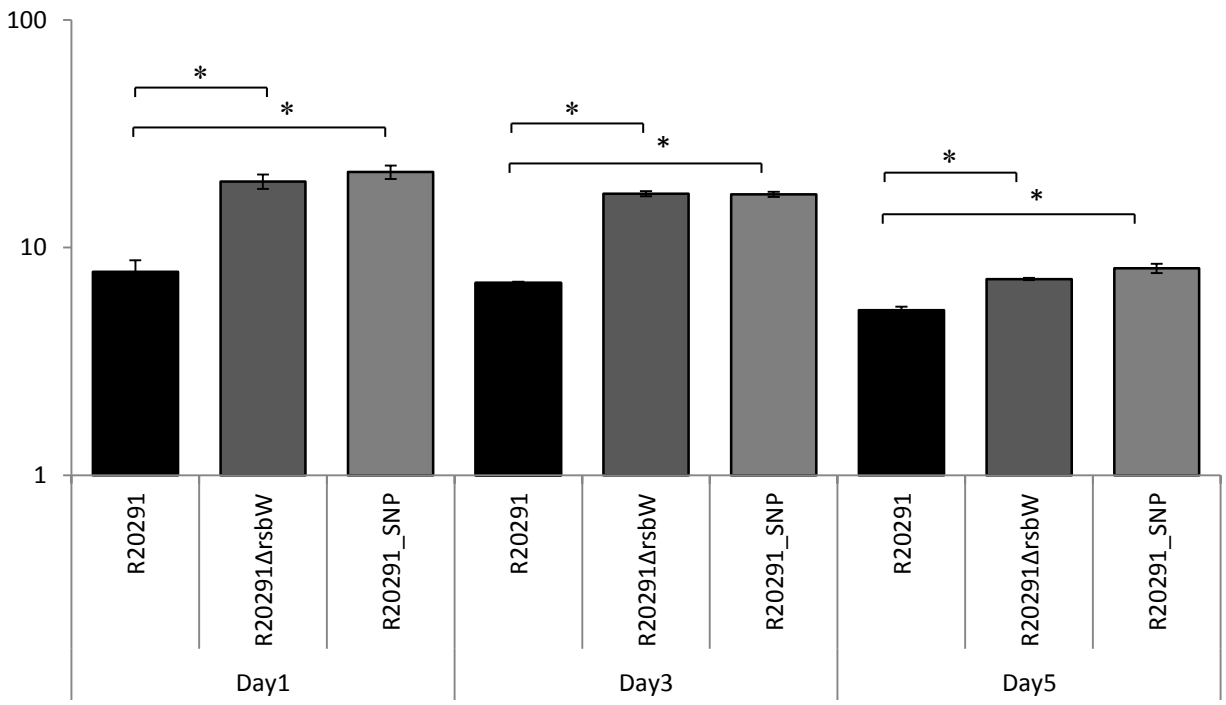


Figure 33 PCR selection of R20291Δ*rsbW* deletion mutant. Primers external to the *rsbW* coding sequence were used. Expected molecular weight is 1488 bp for the wild-type, 1143 bp for the deletion mutant. Lines: 1 – marker; 2 – R20291; 3 – R20291Δ*rsbW*; 4 – R20291_SNP; 5 – negative control.

3.3.2.2.2 Tracking bacterial growth and biofilm formation

We compared biofilm formation between three strains, wild-type R20291 without the SNP in *rsbW* (R20291), strain with a clean deletion in the gene *rsbW* (R20291Δ*rsbW*), and R20291 strain with the SNP in gene *rsbW* (R20291_SNP). In time course experiment mutants R20291Δ*rsbW* and R20291_SNP formed significantly more biofilm compared to wild-type strain (Figure 34a). Growth curve showed that strain R20291_SNP has a growth defect when grown in liquid BHIS (Figure 34b). When we preformed CFU counts on biofilm culture (sessile and planktonic part of biofilm together) we did not observe any significant difference at day one, however, at day three and five we observed a significant drop in CFU for the mutant R20291Δ*rsbW* (Figure 34c). This result suggests that even though the strain with the SNP in *rsbW* has a defect in growth in liquid media, this does not affect biofilm formation. And the drop in number of vital, colony forming, bacteria in biofilm culture in the strain R20291Δ*rsbW* does not cause a reduction of biofilms, which could suggest that majority of biomass in R20291Δ*rsbW* is composed from biofilm matrix, and not vital bacteria.

A



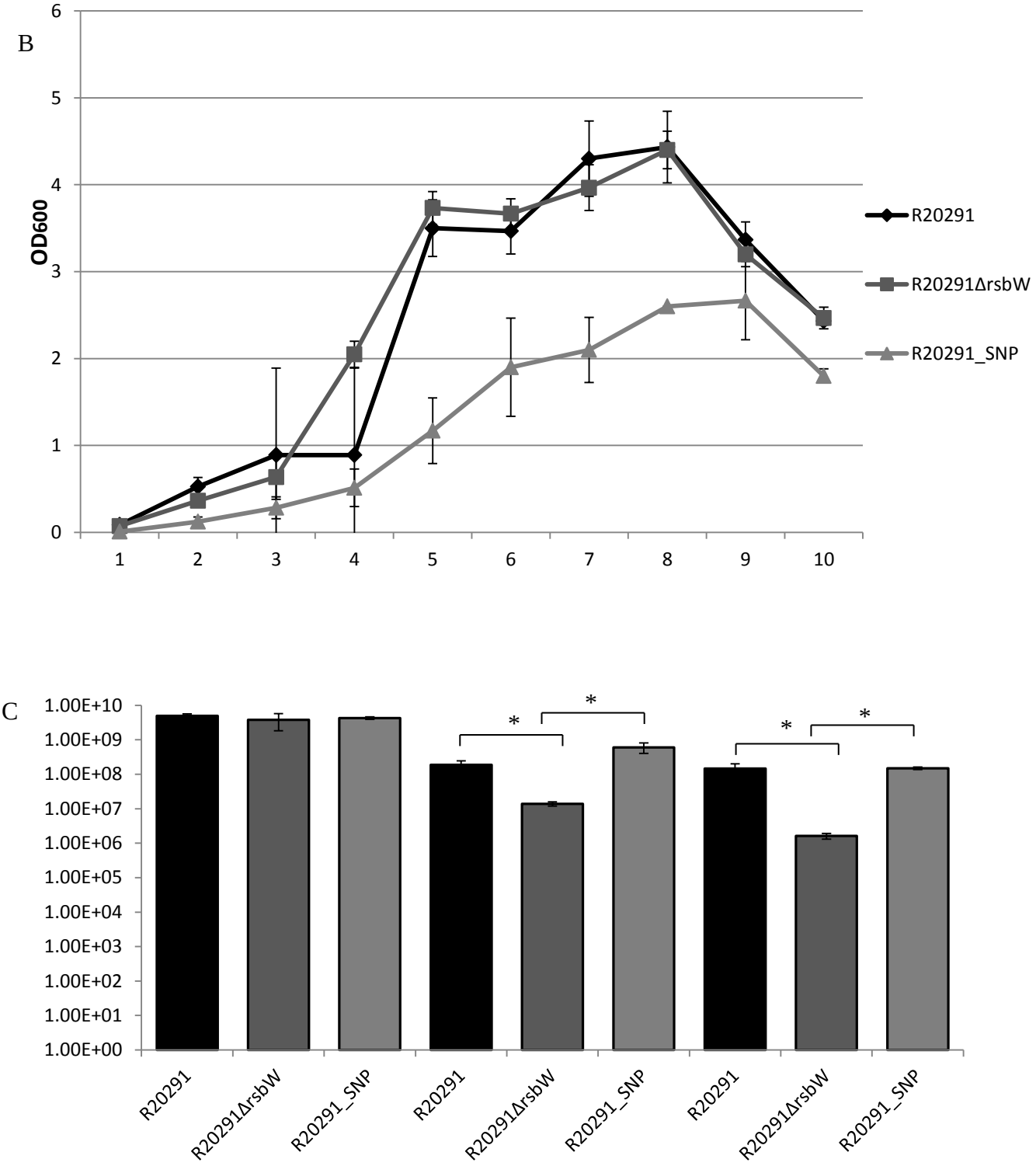


Figure 34 Role of the anti-sigma factor RsbW in biofilm formation and in bacterial growth. (**A**) Wild-type strain R20291, which has an active anti-sigma factor RsbW, forms significant less biofilm compared with strain with deleted *rsbW* strain R20291Δ*rsbW* and strain with a SNP in *rsbW*, R20291_SNP at days one, three and five. (**B**) Strain with the SNP in *rsbW*, R20291_SNP, has a defect in growth in a liquid medium, but not the strains R20291 or R20291Δ*rsbW*. (**C**) CFU counts showed decreased numbers of vital, colony forming bacteria, for the strain R20291Δ*rsbW* in the biofilm at the days three and five.

3.3.2.2.3 Confocal microscopy

To visually characterize biofilm, evaluate the bacterial viability, and biofilm thickness, we stained biofilm formed on culture slides with Live/Dead stain for all three strains, wild-type R20291 and two strains with a mutation in gene *rsbW*. Live/Dead stain is composed of Syto 9 dye, which stains live bacteria green, and propidium iodide dye, which stains dead bacteria red. Wild-type strain R20291 forms an ununiformed biofilm at day one, three and five (Figure 35, top panel). Biofilm thickness increases with time, and we can detect live (green) and dead (red) bacteria at all-time points. Mutant with deleted gene *rsbW* (R20291Δ*rsbW*) forms a thick and dense biofilm at day one, three and five (Figure 35, center panel). Unlike the biofilm formed by the wild-type R20291, biofilm by R20291Δ*rsbW* is composed of mostly dead bacteria at the day three and five, as majority of the bacteria is stained red by propidium iodide. This result confirms our data observed with CFU counts (Figure 34c), where we detected a drop in the number of vital colony forming bacteria in R20291Δ*rsbW* biofilms. Strain with a SNP in the gene *rsbW*, R20291_SNP, forms a similar structured biofilm as a mutant R20291Δ*rsbW*. Biofilm formed by R20291_SNP is thick and dense at all-time points, and forms 3D structures from day one, with well-structured 3D biofilm at the day five (Figure 35, bottom panel). The main difference in the biofilms between R20291Δ*rsbW* and R20291_SNP is that the strain with a SNP, but not the strain with a deleted *rsbW*, is composed of mostly live bacteria even at day five (majority of bacteria is stained green). These results suggest that anti-sigma factor RsbW has a role in biofilm formation.

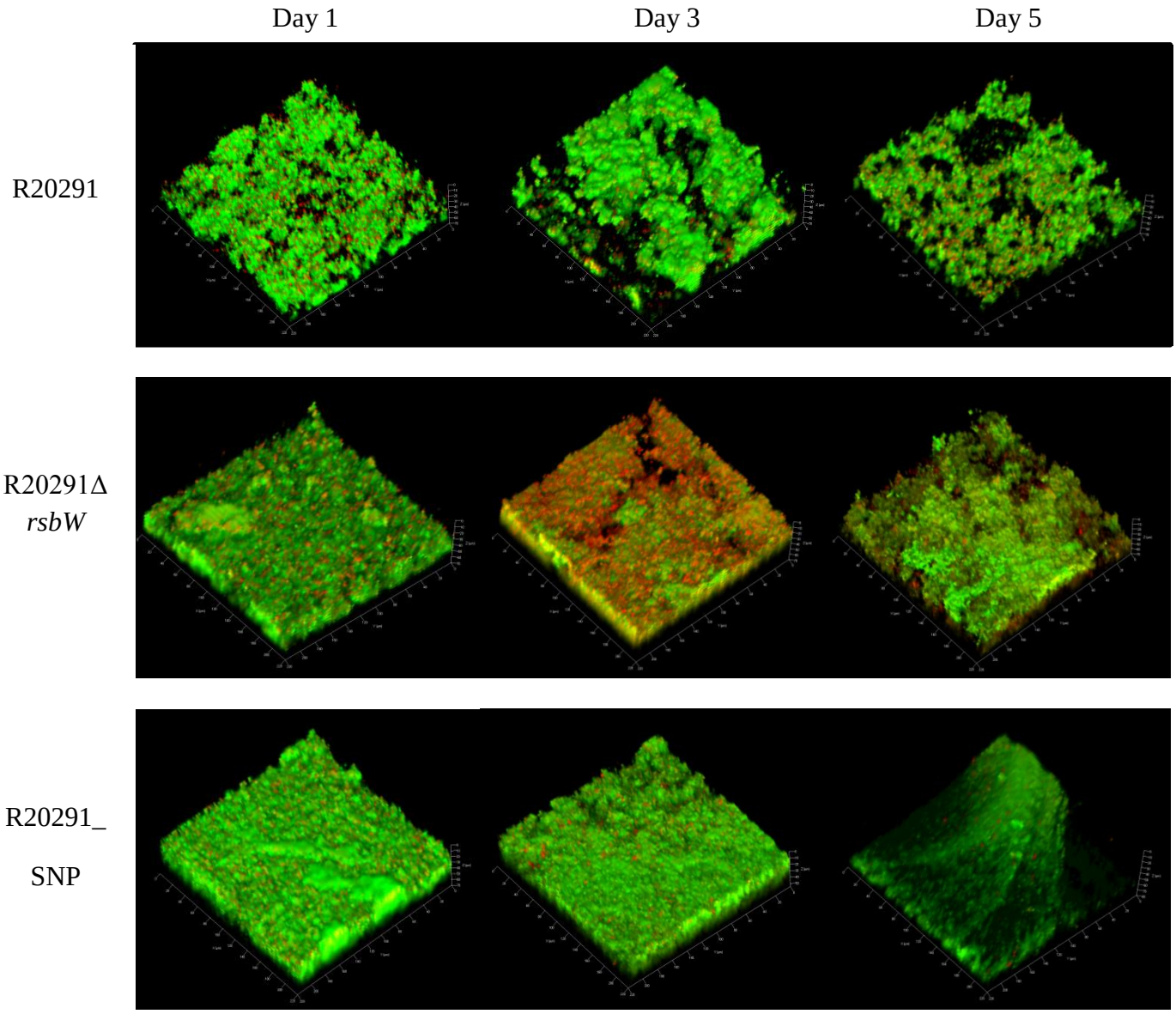


Figure 35 Confocal microscopy analysis biofilms formed by wild-type and rsbW mutant . Biofilms formed by wild-type strain R20291 (top panel), strain with a deletion in gene *rsbW* (middle panel) and strain with a SNP in *rsbW* gene, R20291_SNP (bottom panel) were evaluated for bacterial viability and biofilm thickness. Wild-type strain R20291 (top panel) forms an non uniform biofilm at all days, with the biofilm thickness increasing with the time. We can detect live (green) and dead (red) bacteria at all-time points. Mutant R20291Δ*rsbW* (middle panel) forms a thick and dense biofilm at day one, three and five, but majority of bacteria is dead at the day three and five. R20291_SNP (bottom panel) forms a structured, thick and dense biofilm at all-time points. 3D structures begin forming from day one.

3.3.2.2.4 Sporulation

We measured the germination of heat treated cultures in liquid culture and from the biofilms. We examined cultures over a period of 5 days (presented data for day one old culture, Figure 36). Initial CFU numbers for all three strains were comparable before the heat treatment (for the biofilm culture and culture in planktonic growth). After the heat treatment the wild-type strain R20291 formed significantly more colonies on BHIS supplemented with taurocholate in the liquid culture and in the biofilms over a period of 5 days compared with strains R20291Δ*rsbW* and R20291_SNP. Strains with the mutation in the gene *rsbW* formed significantly less colonies after the heat treatment. Our data are showing that *rsbW* mutants form significantly more biofilm *in vitro* compared to the wild-type, but are unable to form spores. This could additionally support our hypothesis that the master regulator Spo0A has a role in biofilm formation. Anti-sigma factor could indirectly regulate the concentration of Spo0A~P present in the cell, likely affecting the kinases responsible for the phosporylation of Spo0A.

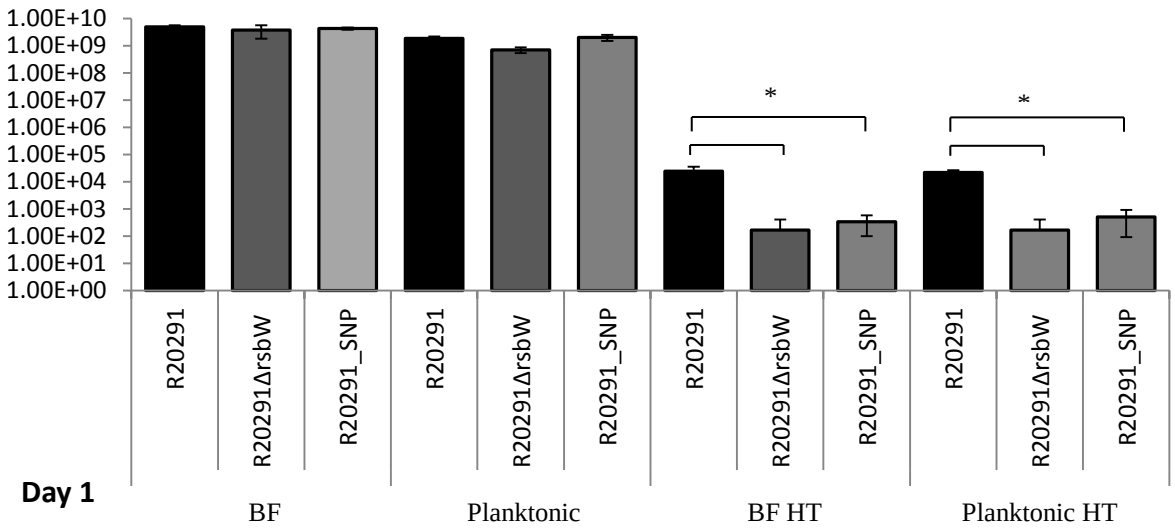


Figure 36 Sporulation in *C. difficile rsbW* mutants. Quantitation of the number of spores present in the biofilms (BF) and in planktonic tube culture (Planktonic). Heat treated cultures (BF HT and Planktonic HT) were plated on brain heart infusion plates (BHIS), supplemented with sodium taurocholate. Results are presented in log scale and the error bars represent standard deviations (P<0.05). Experiments were performed in triplicates, and data shown are representative of at least 3 independent experiments.

3.3.2.2.5 Motility

Recently two different groups in United Kingdom published studies where they examined wild-type R20291 with transmission electron microscope (TEM). In work described by Martin et al. (Martin et al., 2013) on the surface of the strain R20291 many flagella structures were present. When the same strain was exanimated with TEM by Baban and co-authors (Baban et al., 2013), just one flagella was present on the bacterial surface.

We performed a motility assay in low-medium agar with strains R20291, R20291Δ*rsbW* and R20291_SNP. Indeed, wild-type strain R20291 was more motile compared to R20291Δ*rsbW* and R20291_SNP in a low-agar medium. Strains R20291Δ*rsbW* and R20291_SNP demonstrated a defect in motility *in vitro* (Figure 37). This could suggest presence of different number of flagella present on the bacterial surface. Sigma factor B, and hence *rsbW*, could be involved in regulation of assembly of flagella.

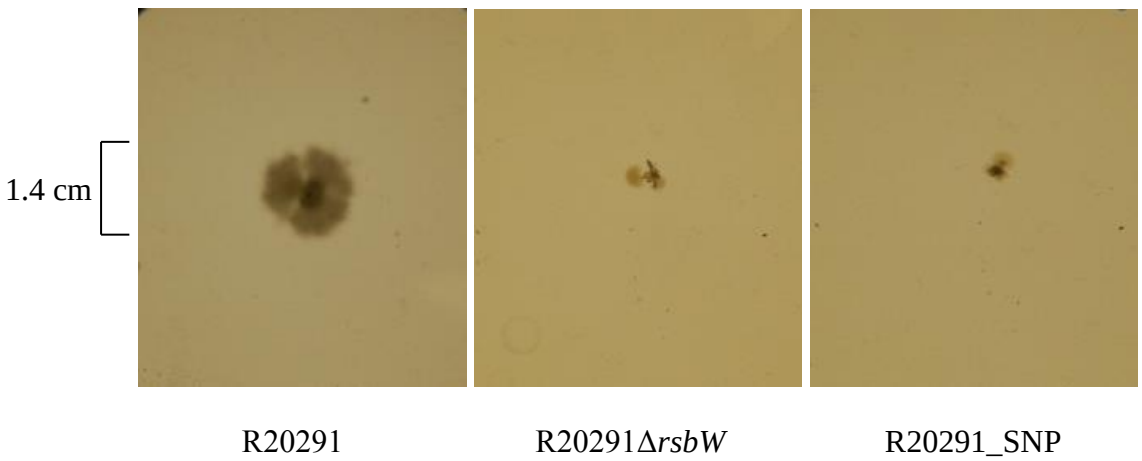


Figure 37 Motility assay *C. difficile rsbW* mutants. Strains with a mutation in a gene *rsbW* showed to be less motile compared to the wild-type R20291.

3.3.2.2.6 Complementation of *rsbW* mutants

Our results indicate that *rsbW* is a key regulator for biofilm formation and sporulation, and may suggest the SNP in *rsbW* gene could result in loss of function of anti-sigma factor RsbW. To confirm out results we tried to complement the strains R20291Δ*rsbW* and R20291_SNP with an episomal plasmid overexpressing gene *rsbW* without the SNP, and in parallel with an episomal plasmid overexpressing gene *rsbW* with the SNP. We were able to introduce both forms of plasmid in the wild-type strain R20291, but we were unsuccessful in introducing the plasmid with wild-type gene *rsbW* in both mutants. Although our attempts to episomally complement the *rsbW* mutant failed, we were able to introduce plasmid carrying gene *rsbW* with the SNP, which could suggest that unsuccessful complementation is due to potential toxicity of the overexpressed regulator in *rsbW* mutants.

4 DISCUSSION

Clostridium difficile is a spore-forming, Gram-positive anaerobic bacillus which can cause severe gastrointestinal infections in humans (Rupnik et al., 2009). *C. difficile* infection (CDI), one of the predominant nosocomial infections worldwide, usually occurs when the normal intestinal flora is damaged or absent, commonly after the use of antibiotics. The clinical symptoms can range from mild or severe diarrhea to serious inflammatory conditions, including pseudomembranous colitis (Rupnik et al., 2009). Recently it was demonstrated that transmission of clostridial disease occurs through spores (Lawley et al., 2009); (Deakin et al., 2012). The best-characterized virulence factors of *C. difficile* are two large clostridial toxins, toxin A (TcdA) and B (TcdB) (Kuehne et al., 2010); (Lyras et al., 2009); (Voth and Ballard, 2005). *C. difficile* toxins cause disorganization of the cell actin cytoskeleton and tight junctions, induction of apoptosis, fluid accumulation, and destruction of the epithelium (Voth and Ballard, 2005). Although the toxins are crucial for virulence, in recent years, attention has been focused on bacterial colonization of the gut, especially due to increased instances of recurrent CDI.

For several pathogens recurrent infections have been associated with the ability to form sessile surface-associated microbial communities or 'biofilms' (Romling and Balsalobre, 2012). Bacteria within biofilms are protected and more resistant to different environmental stresses, like antibiotic or oxygen stress (Davey and O'Toole G, 2000). Common diseases as dental caries and periodontitis are caused by bacteria in biofilms, and biofilm formation has been connected with persistent tissue infections such as chronic otitis media, chronic rhinosinositis, recurrent urinary tract infections, endocarditis and cystic fibrosis-associated lung infections (Costerton, 1999). Recently, biofilms were also associated with chronic inflammatory diseases as Crohn's disease (Claret et al., 2007) and acute infections (Hannan et

al., 2012); (Kumagai et al., 2011). Moreover, biofilms represent a big problem when formed on artificial devices used in medicine, as catheters, stents, orthopaedic implants, contact lenses and implantable electronic devices (Costerton, 1999); (Probert and Gibson, 2002). Resistance of bacteria within biofilms to antimicrobials makes treatment of the disease difficult and unsuccessful. Furthermore, mature biofilms are highly resilient to the action of the innate and adaptive immune defense systems (Hannan et al., 2012). Biofilms are the most representative form of growth of bacteria in the large intestine (Macfarlane and Dillon, 2007), but the biofilm formation by individual gut species, particularly anaerobic species, has not been well characterized. Ability to form biofilms is known to influence virulence and also transmission of intestinal pathogenic bacteria such as *Vibrio cholera* (Faruque et al., 2006); (Watnick et al., 2001); (Zhu and Mekalanos, 2003). In addition to spore formation, a known means of adaptation to stress, it is likely that *C. difficile* forms microcolonies *in vivo* to survive the unfavorable environment of the human gut. As biofilm formation by *C. difficile*, especially clinically important strains, was not previously characterized, we sought to develop *in vitro* assays to study *C. difficile* biofilm formation.

Our study was a first report of the ability of two clinically relevant *C. difficile* strains, laboratory strain 630 and strain isolated from Stoke Mandeville outbreak in 2004 and 2005, R20291, to form structured biofilms *in vitro* (Đapa et al., 2013). The ability to adhere and form biofilms influences the ability of pathogens to colonize and establish an infection (Allsopp et al., 2010); (Nobbs et al., 2009). We employed multiple techniques (confocal microscopy, crystal violet (CV) assay and CFU counts) to demonstrate and quantitate biofilms. Our data show that both the R20291 strains we studied form more biofilm in all tested conditions *in vitro*. It has been reported that strain R20291, a strain isolated in an outbreak, produces higher levels of toxin *in vitro* compared to 630 (Warny et al., 2005). Although

colonization of these strains has yet to be examined carefully *in vivo*, higher biofilm formation by this strain could indicate better colonization *in vivo*.

While for the strain R20291 addition of glucose does not increase the levels of biofilm formation, it is an important factor for biofilm formation for strain 630. It is well known that different carbohydrates can modulate biofilm formation. Carbohydrates induce biofilm formation in *Streptococcus gordonii* (Gilmore et al., 2003) while in *Bacillus subtilis* the CcpA protein represses formation of biofilm in medium with high levels of glucose (Stanley et al., 2003). It is possible that *in vivo* the nutritional environment in the gut modulates colonization of *C. difficile*.

While the strain R20291 forms maximum biofilm after incubation for 1 day by CV assays and CFU counts, strain 630 has the highest number of CFU on day 1, but when stained with CV shows the maximum biofilm on day 5. This suggests that strain 630 behaves differently to R20291, and may accumulate more biofilm matrix upon longer incubation. These *in vitro* data indicate that the strains may have different behaviors *in vivo* in terms of biofilm or microcolony formation, which may in turn influence their abilities to persist in the gut.

The self-produced biofilm matrix is known to protect bacteria during infections by providing an enclosed environment to escape immune responses (Beloin et al., 2008); (Lawley et al., 2009). Biofilm matrices are typically made up of extracellular polymeric substance (EPS), which comprises primarily of proteins, DNA and polysaccharide (Colvin et al., 2011); (Mann et al., 2009); (Mann and Wozniak, 2012). Similar to many clinically relevant biofilm formers bacteria, such as *Pseudomonas aeruginosa* and *S. aureus*, *C. difficile* also appears to form a complex matrix comprising of proteins, DNA and polysaccharide.

When *C. difficile* R20291 was stained with antibody against total bacteria we observed a complex biofilm matrix with just few stained individual bacteria. This suggested that the compact biofilm may comprise surface-associated or secreted bacterial components and may

be impenetrable to antibodies. Proteinase K and DNase I treatments inhibited the formation of biofilms and caused disassembly of pre-formed biofilms. This further showed that proteins as well as external DNA are part of the biofilm matrix. Furthermore, it is interesting that we observe biofilm matrix staining for a synthetic derivative of the *C. difficile* surface PSII polysaccharide. Staining with synthetic polysaccharide derivative, which was recently reported to be immunogenic (Adamo et al., 2012), revealed the presence of polysaccharide components in the biofilm matrix.

Presence of a complex and perhaps impermeable matrix may protect *C. difficile* from unfavorable agents *in vivo* in the gut. In all, the data clearly show that *C. difficile* biofilms are composed of a thick multi-component biofilm matrix. Biofilm impenetrability has been proposed as a feature of bacterial biofilms that contributes to escape of immune responses during the infection, as well as antibiotic resistance *in vivo* (Beloin et al., 2008); (Watnick et al., 2001).

The relevance of biofilm formation in the context of infection and treatment has been widely studied. One of the main functions of a biofilm is to protect bacteria within from unfavourable conditions such as antibiotics, particularly during infection. The rising incidence of resistance to antibiotics treatments for nosocomial pathogens like *S. aureus* and *C. difficile* has been well documented in recent years (French, 2010), and the role the bacterial biofilms play in resisting resistance to the antibiotics has been well demonstrated for many pathogenic species e.g., methicillin-resistant *S. aureus* (Olson et al., 2011). Resistance to antibiotics in biofilm can increase from 10- to 1,000-fold more compared to planktonic bacteria (Mann et al., 2009). Moreover, highly antibiotic-resistant *C. difficile* strains and treatment of recurring clostridial infections have been the major challenges for managing CDI (Surawicz and Alexander, 2011). On the other hand, antibiotics at minimal concentrations could also act as stress signals and biofilm formation can be a defensive reaction to the presence of antibiotics, as shown for

Gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* (Boehm et al., 2009); (Hoffman et al., 2005). Induction of biofilms in presence of sub-inhibitory concentrations of antibiotics has been previously attributed to alterations in the level of c-di-GMP (Hoffman et al., 2005). Such induction *in vivo* could be clinically relevant when there is exposure to low doses of antibiotics, like at the beginning or end of antibiotic therapy, which could perhaps explain ineffective treatment (Boehm et al., 2009). In CDI, the establishment of persistent biofilms *in vivo*, in addition to the formation of spores, could potentially explain the occurrence of recurrent infections.

We examined effects of a range of concentration of vancomycin, an antibiotic used for treatment of *C. difficile* infections. Bacteria within biofilms were found to be more resistant to high concentrations of vancomycin and *C. difficile* biofilm formation was induced when bacteria were exposed to sub-inhibitory and inhibitory concentrations of vancomycin. Thus, in the case of *C. difficile*, biofilms may have multiple roles depending on the antibiotic concentrations in the environment.

Several mechanisms are known to mediate antibiotic resistance of bacteria within biofilms. The biofilm matrix, a main player, can act as an initial physical barrier that can affect penetration of the antimicrobial agents (Mah and O'Toole, 2001), while other more complex attributes such as the physiological state of the bacterium, like the presence of persistent cells in population, can also contribute to resistance (Shah et al., 2006). Biofilm matrix has the role of a protective barrier within which bacteria are more tolerant to antibiotics. Concentration gradients are formed across the biofilm matrix decreasing the effective concentration of antibiotics reaching the bacteria within (Mah and O'Toole, 2001). Persistent cells are phenotypic variants of the wild-type in a dormant state and are more resistant to the antibiotics. Upon re-inoculation, the persistent bacteria display similar level of tolerance to antibiotics (Keren et al., 2004). Indeed, genetic mutations could also occur at low frequencies within bacteria in biofilm environments, contributing to antibiotic resistance (Tyerman et al.,

2013). Genetic changes mediated by systems such as stress responses and slow growth could be responsible for the increased tolerance to the antibiotics (Mah and O'Toole, 2001). In our initial studies on the mechanism involved in vancomycin resistance, we disrupted biofilms and treated with antibiotics. These bacteria were not resistant to vancomycin anymore, indicating that vancomycin resistance does not involve genetic or inherited changes in the bacteria. As seen in other bacterial species, our data indicate that antibiotic resistance of clostridial biofilms may be mediated by the thick biofilm matrix and/or the physiological state of bacteria within biofilms (Lewis, 2008). Clearly, much remains to be understood with regard to how clostridial biofilms may resistant antibiotics.

Regulation of biofilm formation in Gram-positive bacteria involves multiple factors including adhesins, surface structures e.g. flagella and pili (Abee et al., 2011). The *C. difficile* S-layer is composed of S-layer proteins (SLPs) that are present as heterodimeric complex (Fagan et al., 2009). The signal peptide of protein precursor SlpA is removed by proteolytic cleavage. Additional cleavage is essential for maturation of SLPs, which is mediated by the cysteine protease Cwp84 (Calabi et al., 2001); (Kirby et al., 2009). A mutant in *cwp84* has previously been shown to be defective in the S-layer synthesis (Kirby et al., 2009). This protease is also important for degradation of extracellular matrix proteins like fibronectin, laminin and vitronectin (Janoir et al., 2007). A mutant in *cwp84*, which shows similar growth rates as wild-type in broth culture, is unable to form biofilm, indicating that a mature S-layer, which also hosts several other CWPs, is needed for *C. difficile* biofilm formation. Our data prove that a mature S-layer is essential for *C. difficile* biofilm formation, perhaps due to the fact that this layer may be essential for anchoring various cell wall associated proteins, which may be required for the early steps such as adhesion during biofilm formation. A role for specific CWPs in biofilms remains to be investigated. Furthermore, the observation that antibodies

raised against fixed whole bacteria recognize complex structures on biofilms may indicate that surface components such as CWPs may compose the biofilm matrix.

Bacterial flagella are known to modulate attachment, the first step in biofilm formation in motile bacteria, however the role of flagella in biofilm development by motile bacteria varies between species (Kobayashi, 2007b); (Lemon et al., 2010). For Gram-positive bacteria like *B. subtilis* the presence of flagella is important, but not essential for formation of biofilms (Kobayashi, 2007b), while for *Listeria monocytogenes*, motility is essential for mature biofilm formation (Lemon et al., 2010). In our experiments a mutant in flagellin, a principal component of flagella, clearly affects biofilms *in vitro*, indicating that motility of *C. difficile* is key in formation of biofilms. Recently strain 630 *C. difficile* flagellar mutants were reported to have better adherence in an *in vitro* model (Dingle et al., 2011). Our data further suggest that flagella are important at later biofilm stages, as the mutant does not display defects in *in vitro* assays at earlier time points. Flagella may be involved in the maturation of *C. difficile* biofilms, as seen for other bacteria such as *Pseudomonas* sp., where flagella contribute to the architecture of mature biofilms (Barken et al., 2008). Studies on the functions of clostridial flagella in biofilms need to be performed under conditions of flow, where development and maturation of biofilms can be monitored.

Cell-cell communication is crucial in a complex structure like biofilms where bacteria are in strict contact with one another. Quorum sensing (QS) has an important role in bacterial biofilm formation (Hammer and Bassler, 2003); (Vendeville et al., 2005). The enzyme LuxS, that synthesizes autoinducer-2 (AI-2), is one of the major modulators of QS, and is largely conserved across bacterial species (Hardie and Heurlier, 2008). It was demonstrated that *C. difficile* genome carries a 453-bp gene that encodes a protein which shares 40% identity to the *V. harveyi* LuxS protein, and is responsible for autoinducer-2 (AI-2) production (Carter et al., 2005). Al-2 is one of the major modulators of quorum sensing and has an important role in bacterial biofilm formation (Vendeville et al., 2005); (Hammer and Bassler, 2003). The role

of *luxS* in toxin production is not clear as there are conflicting reports in the literature (Carter et al., 2005); (Lee and Song, 2005). While precise mechanisms by which *luxS* functions in *C. difficile* is unclear at present, our data suggest a role for putative *luxS*-encoded molecules in formation of biofilms *in vitro*, and may indicate that a *luxS*-mediated quorum sensing system exists in *C. difficile*. Further work needs to be done to clarify the mechanisms involved in the *luxS* mediated control of biofilm formation.

Interestingly, we find that a mutant lacking SleC, a protein recently reported to be specifically involved in germination of *C. difficile* spores (Burns et al., 2010), is defective for biofilm formation. A role for spore germination in biofilms has not been well studied, however in our conditions given the lack of spores, it is unlikely that SleC is involved in germination of spores. Given that the *sleC* mutant biofilms shows strikingly different cellular morphologies, it is possible that SleC has other functions such as in hydrolysis of vegetative cell peptidoglycans.

All the mutants were genetically complemented either episomally or chromosomally, and biofilm formation was completely or partly restored.

Biofilm formation is a complex process, evolving many different pathways and controlled by several regulators. In the model organism in Gram positive bacteria, *Bacillus subtilis*, four different subnetworks are described (Figure 11) (Vlamakis et al., 2013). One of main and best described pathways is regulated by master regulator Spo0A, regulator which controls the process of sporulation, a critical pathway in bacterial responses to environmental stresses. The main regulator that controls entry into sporulation, Spo0A, is well conserved in *Bacillus* and *Clostridium* species (Lopez and Kolter, 2010); (Underwood et al., 2009). In addition to control of entry into sporulation, Spo0A controls a range of other regulatory factors, including pathways unrelated to sporulation (Molle et al., 2003). Sporulation and biofilm formation have been linked in other bacteria, particularly in *Bacillus subtilis*. Among more than 100

genes that are regulated by Spo0A are genes involved in biofilm matrix expression (Vlamakis et al., 2013). Biofilm environments have been shown to be optimal for spore formation and spores are part of biofilms for many spore-forming bacteria under nutrient-starved conditions (Abee et al., 2011). However, we find extremely low numbers of spores in biofilms (adherent and planktonic phases of biofilms) under our conditions. Previously was demonstrated that a *B. subtilis spo0A* mutant was defective for biofilm formation (Hamon and Lazazzera, 2001), and similar to our observation, they did not detect spores in *B. subtilis* biofilms *in vitro* (Hamon and Lazazzera, 2001). In the case of *B. subtilis* the switch between biofilm formation and sporulation depends on the concentration of phosphorylated Spo0A (Spo0A~P). Intermediate levels of Spo0A~P induce expression of genes for biofilm matrix formation, while high levels induce sporulation in the cell. Initial phosphorylation induces biofilm formation as result of matrix gene expression; once biofilm matures Spo0A~P accumulates in some cells and activates sporulation (Vlamakis et al., 2013).

Our data, and recent data reported by Dawson et al., demonstrate the importance of *spo0A* for biofilm formation by *C. difficile* (Đapa et al., 2013); (Dawson et al., 2012). Under our *in vitro* conditions, where biofilms are not mature, we detected very low number of spores. Yet, we have defective biofilm formation for a *spo0A* mutant. Our data indicate that the lack of *spo0A* may result in decreased adhesion to surfaces in early stages of biofilm formation. Spo0A has also been previously implicated in toxin production in *C. difficile*, although the exact relationship between the two is currently unclear (Underwood et al., 2009); (Deakin et al., 2012). Thus, *spo0A* appears to control multiple stress-induced pathways in *C. difficile*. We hypothesize that this regulator may act as a switch between different pathways such as sporulation, biofilm formation, and toxin production, selectively inducing one or more of these pathways, depending on the local environment. Our observation that bacteria sporulate during tube culture but not in biofilms, when incubated under same conditions, may support this hypothesis. Recently *spo0A* mutants were shown to be defective in persistence and

transmission in a murine infection model, primarily due to the inability of the *spo0A* mutant strain to form spores (Deakin et al., 2012). In addition to production of spores, the formation of biofilms *in vivo* may also account for the persistence defects observed for the *spo0A* strain. Indeed, during infection, *C. difficile* spores may be a major part of mature biofilms, where spores form as a response to nutrient starvation, as seen in other bacteria (Piggot, 1996). Examining the *spo0A* regulon in *C. difficile* under different stress conditions could provide valuable information about the role/s of this regulator in *C. difficile* physiology. Moreover, how the switch between the biofilm formation and sporulation works in case of *C. difficile* would be interesting to study, preferably by employing mutants in kinases, which are responsible for phosphorylation of Spo0A, or by study potential targets, that are affected by the concentration of Spo0A~P present in the cell. Employing isogenic mutants, that are defective in sporulation, but not in biofilm formation *in vivo*, and vice versa, would clarify the role and the importance two different pathways have for the persistence of the CDI.

The biofilm defect of the *spo0A* mutant was partly restored by low inhibitory concentrations of antibiotic vancomycin. This suggests that, as it is in *B. subtilis*, Spo0A-independent pathways, which regulate biofilm formation, may exist in *C. difficile*. In case of *B. subtilis* different enviromental stimuli, e.g. cell wall stress and specific antibiotics stresses, can activate several extracytoplasmic function (ECF) RNA polymerase σ-factors, including σ^M, σ^W and σ^X (Helmann, 2002). Transcription of Abh, which can indirectly repress the repressor SinR, is under the control of σ^M, σ^W and σ^X. Repression of SinR leads to the transcription of biofilm matrix genes. As there are no known orthologs for any of this genes in *C. difficile*, a possible way to study the Spo0A-independent biofilm pathway, and the regulation of biofilm formation in general, would be to preform a transcriptome analysis on mutant *spo0A* induced with inhibitory concentrations of antibiotic vancomycin. This would give as new insights in the regulation of biofilm formation in *C. difficile*.

A Single Nucleotide Polymorphism (SNP) is a DNA sequence variation, which occurs when a single nucleotide in the genome is different between members of same biological species. SNPs are the most common type of genetic variations. SNPs, insertion and deletion of a single nucleotide are called point mutations. SNPs occurring in a DNA sequence, which encodes a protein, might result either in a synonymous (silent) codon mutation (no change in the encoded amino acid), or in a nonsynonymous mutation (an amino acid change, and has an impact on the function of the encoded protein). Point mutations are referred to as the raw material of evolution. Presence of a point mutation may provide selective advantage, or disadvantage, against other individuals of the same biological species.

Whole-genome sequencing offers us an efficient approach to associate genotype with phenotype. Renzoni et al. used whole-genome sequencing to identify SNPs in two isogenic strains of *Staphylococcus aureus* (Renzoni et al., 2011). A parental strain and a derivative strain, which had undergone an *in vitro* selection for resistance to antibiotic teicoplanin. Whole genome sequences showed presence of only three SNPs (in the genes *stp1*, *yjbH* and *vraS*). All three SNPs were shown to be required for the highest level of glyopeptide resistance (Renzoni et al., 2011). In another study SNPs were connected with rifampicin resistance in *Mycobacterium tuberculosis* (Comas et al., 2012).

Many contradictions are known in *Clostridium difficile* literature. The most apparent are two studies, published by Nature, which attribute different roles to two main *C. difficile* toxins, ToxA and ToxB. Lyras and co-authors claim that only toxin B is essential for the pathogenesis of *C. difficile* (Lyras et al., 2009), while Kuehne and co-authors found that toxin A and toxin B are both needed for the pathogenesis of *C. difficile* (Kuehne et al., 2010). Moreover, the role of Spo0A (Underwood et al., 2009); (Deakin et al., 2012) and *luxS* (Carter et al., 2005); (Lee and Song, 2005) in toxin production remains to be understood, as there are conflicting reports in the literature.

In our studies we found that clinical *C. difficile* strains, originally coming from the same outbreak, but stored in different laboratories around United Kingdom, showed differences in biofilm formation, sporulation, minimal inhibitory concentration to the antibiotic vancomycin, and motility. In collaboration with the Minton laboratory, Clostridia research group (CRG), University of Nottingham, whole genome sequencing was performed for two strains of R20291, originally from different laboratories. Sequences of these strains showed the presence of single nucleotide polymorphisms (SNPs) in different regulatory genes, as compared to the published genome sequence. Further to this study, genome sequencing of alternate R20291 strains from other laboratories in the Minton Laboratory has also revealed SNPs in other genes (unpublished data). The strain, in which we performed our previous studies, carries a SNP in anti-sigma factor RsbW and in the gene *vncR*. Nucleotide in the position 245 in gene *rsbW* changed from a nucleotide 'G' to a nucleotide 'T', compared to a reference genome R20291. The SNP in the genome caused a change in amino acid sequence, changing a glycine to valine.

RsbW is an anti-sigma factor which regulates the stress response induced by alternative sigma factor (σ^B), and its role in biofilm formation was previously shown in other bacteria, e.g. *Staphylococcus epidermidis* (Knobloch et al., 2004). σ-factor B initiates the transcription in bacteria by associating with the RNA polymerase core enzyme. Binding of σ-factors to the promotors results in a change in the pattern of gene expression. σ-factor B in *B. subtilis* is stimulated by numerous environmental stresses, e.g. signals of energy stress like carbon, phosphate or oxygen starvation, and salt, heat, acid, or ethanol shock stresses (Hecker et al., 1996). The activity of σ-factor B is controlled by a RsbVW partner-switching. RsbVW partner-switching is highly conserved among gram positive bacteria that contain σ-factor B operon (de Been et al., 2011). In normal conditions σ-factor B is in an inactive state, forming a complex with the anti-sigma factor RsbW. When stress conditions occurs, the release of σ-factor B from RsbW is accomplished by anti-anti σ-factor RsbV, which upon

dephosphorylation, sequesters RsbW. Anti σ-factor RsbW in addition acts as a kinase of RsbV, providing a negative feedback on activation of σ-factor B. RsbV is dephosphorylated during stress conditions, sequestring in this way RsbW, and activating σ-factor B (Hecker et al., 2007). In case of *S. epidermidis,* σ-factor B controls the transcription of genes responsible for biofilm formation, and the lack of active anti-sigma factor RsbW results in constantly activated σ-factor B and in increase of biofilm production.

In our studies, a strain with a deleted *rsbW*, R20291Δ*rsbW*, and the gene with a SNP in *rsbW*, R20291_SNP, form significantly more biofilm comparing to the wild-type strain R20291. Furthermore, mutants in *rsbW* form significantly lesser spores (counted by germination of heat treated cultures) in biofilm culture and in planktonic growth. Similar results are expected when low concentration of Spo0A~P are present in the bacterial cells in case of *B. subtilis*, biofilm formation is induced, and sporulation is repressed. Indeed while we do not know the mechanism of action of Spo0A in *C. difficile*, nevertheless, given the similarities to the *Bacillus* sporulation pathways, we suppose that Spo0A activity is modulated by phosphorylation. We hypothesise that σ-factor B indirectly controls biofilm formation by repressing transcription of kinases, which would result in low concentrations of Spo0A~P present in the cell, or directly, by stimulating the transcription of still unknown genes involved in biofilm matrix production.

Mutants in the gene *rsbW*, strains R20291Δ*rsbW* and R20291_SNP, showed few differences in growth, and many similarities, e.g. biofilm formation, sporulation and motility. Although our attempts to episomally complement the *rsbW* mutants failed due to potential toxicity of the overexpressed regulator in the mutant strains, our results indicate that *rsbW* is a key regulator for biofilm formation and sporulation, and suggest the 245bp-SNP in *rsbW* gene could result in loss of function of *rsbW*.

The human large intestine represents a huge variety of bacterial species, constituting an extremely complex and metabolically active site (Probert and Gibson, 2002). Most studies on the colonic microbiota have been done on planktonic bacteria found in faeces, but the sessile bacteria that form biofilms in the mucus layer of the gut, are likely to play a fundamental role in gut health and disease (Croucher et al., 1983). Biofilms formed by anaerobic bacteria from the human intestinal tract have been poorly characterized. One of the reasons for this is perhaps the difficulties associated with cultivation and standardizing conditions for biofilm formation *in vitro*. Recently the ability to form mono- and duo- species biofilms for several gut bacteria such as *Bacteroides oralis*, various *Clostridium* species including *C. difficile*, *Finegoldia magna*, and *Fusobacterium necrophorum* was shown *in vitro* (Donelli et al., 2012). *C. difficile* colonization of the gut and mechanisms involved in this process *in vivo* are poorly understood, but it is likely that formation of large microcolonies or biofilm communities have a key role in gut colonization and bacterial survival. It is also possible that microcolony formation precedes toxin production and modulates spore formation in the colon. Formation of bacterial mats has been reported previously in murine *C. difficile* infections (Lawley et al., 2009). Such biofilm or multicellular structures could potentially protect bacterium from cellular immune responses, invoked by toxin production, and from antibiotics used for the treatment of CDI. In addition to spores, which have been linked with persistence of clostridial disease (Lawley et al., 2009); (Deakin et al., 2012), biofilm formation *in vivo* could be another factor contributing to the complexities of treatment of CDI, and could explain recurrence of clostridial infections. Investigating biofilm development by *C. difficile*, particularly during infection *in vivo*, is therefore essential for a better understanding of clostridial pathogenesis. Screening for inhibitors or designing vaccines against proteins crucial for biofilm development could provide effective solutions for treatment or prophylaxis of CDI.

Based on our results, we have summarized the roles of *C. difficile* factors in a hypothetical model of biofilm development (Figure 38) (Dapa and Unnikrishnan, 2013). Mechanistic studies on how these different proteins mediate biofilm formation, and how their functions are coordinated, will shed more light on this complex process.

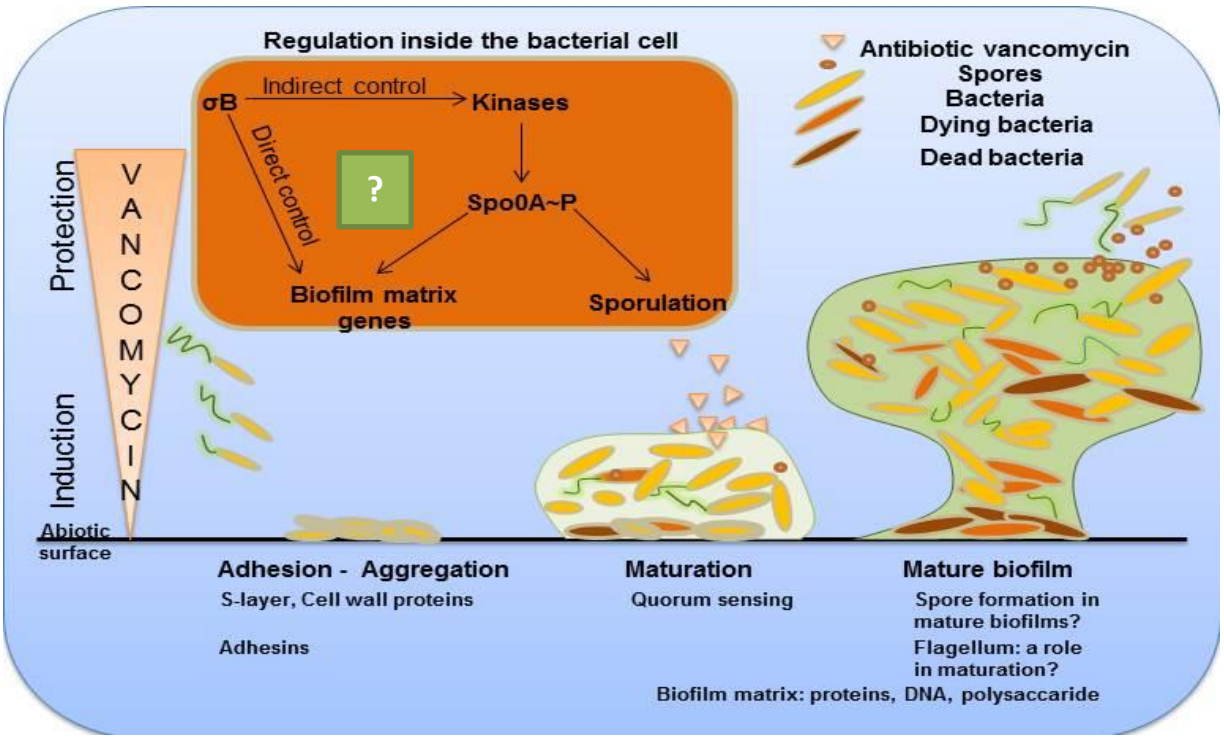


Figure 38 A hypothetical model for *C. difficile* biofilm development. Bacteria first recognize specific or nonspecific attachment sites on an appropriate surface and adhere to it. An intact S-layer and/or adhesins are important for this initial step of biofilm formation. Quorum sensing mediated by *luxS* is crucial during the early and late maturation phases, when the cells start to produce a biofilm matrix composed of proteins, extracellular DNA and polysaccharides. *C. difficile* flagella have a role in maturation of biofilms and perhaps in the early steps, in directing bacteria to the right attachment sites. Spores are part of *C. difficile* biofilms, although numbers of spores may be maximal in mature biofilms where conditions of nutrient stress are likely. *C. difficile* biofilms can protect bacteria within from the antibiotic vancomycin, although at low concentrations (sub-inhibitory and inhibitory concentrations) biofilm formation is induced. The regulator of sporulation, Spo0A controls the step of adhesion-aggregation, likely by upregulating expression of adhesins or biofilm matrix genes. Sigma factor B, which is controlled by the anti sigma factor B, rsbW, could control biofilm formation indirectly, by regulating activity of kinases, or directly, controlling expression of biofilm matrix genes (Modified from Dapa and Unnikrishnan, 2013).

5 CONCLUSIONS

In conclusion, we demonstrate that clinically relevant strains of the anaerobic gut pathogen *Clostridium difficile* are able to form complex biofilms *in vitro*. *C. difficile* biofilm formation appears to be a multifactorial process with a role for proteins that are important in different aspects of bacterial physiology. Indeed, the details of the precise roles of each of these proteins/pathways and their regulation remain to be studied. A possible model of infection is that *C. difficile* colonizes the colon via formation of microcolonies or biofilms, followed by toxin production. Formation of biofilms *in vivo* perhaps provides the bacterium with a mechanism to protect itself from the cellular immune responses invoked by the toxins, in addition to a mechanism of persistence in presence of antibiotics. Investigation of *C. difficile* biofilm development during infection and factors controlling it could give us a better insight into their role in *C. difficile* pathogenesis.

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APPENDIX

APPENDIX 1 – Growth media

BHIS

BHI	37 g/l
Yeast extract	5 g/l
L-cysteine	1 g/l
ddH2O	

15 g/l agar is added to have a solid medium. The medium is sterilized in autoclave and stored at 4°C.

LB

Tryptone peptone	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l
dH2O	

The pH is adjusted to 7.6, then the medium is sterilized in autoclave and stored at 4°C.
15 g/l agar are added to have a solid medium.

TYM

Tryptone peptone (Difco)	24 g/l
Yeast extract	12 g/l
Mannitol	10 g/l
Glycerol	1 g/l
ddH2O	

The pH is adjusted to 6.8. The medium is sterilized in autoclave and stored at 4°C.

CDMM-*Clostridium difficile* Minimal medium

Aminoacids:

L-cysteine	0.5 g/l
L-isoleucine	0.1 g/l
L-leucine	1.0 g/l
L-proline	0.8 g/l
L-tryptophan	0.1 g/l
L-valine	0.1 g/l
L-arginine	0.1 g/l
Glycine	0.1 g/l
L-histidine	0.1 g/l
L-methionine	0.1 g/l
L-threonine	0.1 g/l

Salts:

KH2PO4	0.3 g/l
Na2HPO4	1.5 g/l
NaCl	0.9 g/l
CaCl2·2H2O	26 mg/l
MgCl2·6H2O	20 mg/l
MnCl2·4H2O	10 mg/l
(NH4)2SO4	0.44 g/l
FeSO4·7H2O	4 mg/l
NaHCO3	5 g/l

Vitamins:

Biotin	10 μg/l
Calcium pantothenate	1 mg/l
Pyridoxine hydrochloride	100 μg/l

Powders are dissolved in ddH2O. The pH is adjusted to 7.4. The medium is filter sterilized and stored at 4°C.

CDM - MINIMAL MEDIUM FOR SELECTION OF DOUBLE CROSS-OVER CLONES

To make 100 ml Minimal medium:

Sterile anaerobic ddH2O	61 ml
Amino acid solution (5X)	20 ml
Salt solution (10X)	10 ml
20% glucose solution	5 ml
Trace salt solution (50X)	2 ml
FeSO4·7H2O solution (100X)	1 ml
Vitamin solution (100X)	1 ml

Amino acid solution (5X):
Dissolve the following quantities in 200 ml of ddH2O:

Cas-amino acids	10.0 g
L-Tryptophan	0.5 g
L-Cysteine	0.5 g

Salt solution (10X):
Dissolve the following quantities in 200 ml of ddH2O:

KH2PO4	1.8 g
NaCl	1.8 g
Na2HPO4	10.0 g
NaHCO3	10.0 g
dH2O	

Glucose solution:
20% w/v glucose

Trace salt solution (50X):
Dissolve the following quantities in 200 ml of ddH2O:

CaCl2·2H2O	260 mg
MnCl2·4H2O	100 mg
MgCl2·6H2O	200 mg
(NH4)2SO4	400 mg

FeSO4·7H2O solution (100X):
Dissolve 20 mg FeSO4·7H2O in 50 ml anaerobic water

Vitamin solution (100X):
Dissolve each of the following vitamins in 200 ml of water:

Ca-D-panthotenate	20 mg
Pyridoxine	20 mg
d-biotin	20 mg

The medium is filter sterilized and stored at 4°C.

APPENDIX 2 – Bacterial strains and plasmids

Strain or plasmid	**Relevant properties**	**Source or reference**
E. coli CA434	Conjugation donor	(Purdy et al., 2002)
CdiR20291Δ*cwp84* (Δ*cwp84*)	*C. difficile* R20291 strain with an in frame deletion of *cwp84* from 642-bp to 1887-bp	(Ng et al., 2013)
CRG3351(*fliC*)	CdiR20291-*fliC*430s::CT	(Baban et al., 2013)
CRG1183 (*luxS*)	CdiR20291-*luxS*161a::CT	(Đapa et al., 2013)
CRG1166 (*sleC*)	CdiR20291-*sle*C128a::CT	(Burns et al., 2010)
CRG1375 (*spo0A*)	CdiR20291-*spo0A*178a	(Heap et al., 2010)
CdiR20291Δ*cwp84*-C (Δ*cwp84*-C)	*C. difficile* R20291 Δ*cwp84* complemented with *cwp84* on the chromosome	(Ng et al., 2013)
CRG1183-C (*luxS*-C)	CRG1183 containing pMTL-TD1	(Đapa et al., 2013)
CRG1634 (*sleC*-C)	CRG1166 containing pMTL-DB1	(Burns et al., 2010)
CRG3359 (*fliC*-C)	CRG3351 mutant containing pMTLSB1	(Baban et al., 2013)
CRG1375-C (*spo0A*-C)	CRG1375 containing pMTL960:*spo0A*(Cdi)	(Đapa et al., 2013)

CdiR20291Δ*rsbW* (Δ*rsbW*)	*C. difficile* R20291 strain with an in frame deletion of *rsbW* from 10-bp to 401-bp	This study
Plasmids	**Relevant properties**	**Source or reference**
pMTL84151	Clostridium modular plasmid containing *catP*	(Heap et al., 2009)
pMTL-DB1	pMTL84151 containing 1,272-bp *sleC* coding region and 244-bp upstream promoter region	(Burns et al., 2010)
pMTL-TD1	pMTL84151 containing 456-bp *luxS* coding region and 124-bp upstream promoter region	(Đapa et al., 2013)
pMTLSB1	pMTL84151 containing 873-bp *fliC* coding region and 100-bp upstream *fliC* promotor region	(Baban et al., 2013)
pMTL960:s*po0A*(Cdi)	pMTL-960 containing *spo0A* open reading frame with 174-bp of upstream sequence and 205-bp of downstream sequence	(Đapa et al., 2013)

APPENDIX 3 – Primers

F1_*rsbW*	TTTTTTGTTTAAACTAAACAGCATAAATAAGGTTGT
	*Pme*I
R1_*rsbW*	GGGCTAGAATTCGTAATTTCCATCTTTATAGTCT
	*Eco*RI
F2_*rsbW*	GGGCTAGAATTCAATGACTAAATATTTAGGAGTTGA
	*Eco*RI
R2_*rsbW*	TTTTTTGTTTAAACTTGATATCCATAAGAAGCCTCC
	*Pme*I
SC7_F	GACGGATTTCACATTTGCCGTTTTGTAAACGAATTGCAGG
SC7_R	AGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGG
Rsb_contr_F	TAGTAGGAAGCTCTGCTCTTATAGTAGC
Rsb_contr_R	AATCTTCATACTCTATACTTCCAAAGTTACC
*luxS*_95_F	AGTAACTAAATTTGACTTGAGATTTTTACAGCC
*luxS*_262_R	AACCAATCTTAACTGTTTTGGCATCTACATCTCCCC
F1_*luxS*	TAAAGAATGCGGCCGCGTACGATTATGTGATATAAATATTATAAC
	*Not*I

R1_*luxS*	TAAAGAATCTCGAGTTATTCTCCATATATATTTAAAGAAAATCC
	*Xho*I
Cdi-*spo0A*-F1	AACTAGTGGTATTTTTATAGATGAAATGATAAAATTGTAGGTGAG
	*Spe*I
Cdi-*spo0A*-R1	GGATCCTCAGTTTACAACTTGTAAAGACA CATACTATATCC
	*Bam*HI
*rsbW*_F	TTTGGAATGTTTGTCTTGATGG
*rsbW*_R	GCTAACAATCTTGCTAAATACAGATG