Sede Amministrativa: Università degli Studi di Padova

Dipartimento di
SCIENZE BIOMEDICHE

CORSO DI DOTTORATO DI RICERCA IN SCIENZE BIOMEDICHE
CICLO XXXI

DUAL ROLE OF THE COLONIZATION FACTOR CD2831 IN CLOSTRIDIUM DIFFICILE PATHOGENESIS

Tesi redatta con il contributo finanziario di GSK Vaccines S.r.l.

Coordinatore: Ch.mo Prof. Paolo Bernardi
Supervisore: Ch.mo Prof. Cesare Montecucco
Supervisore: Dott.ssa Rosanna Leuzzi

Dottoranda
Vanessa Arato

2018

This work was sponsored by Novartis Vaccines and Diagnostics Srl; in March 2015 the Novartis non-influenza Vaccines business was acquired by the GSK group of companies.

Vanessa Arato is a student from The University of Padua, involved in a PhD studentship program at GSK.

TABLE OF CONTENTS

1 ABSTRACT

Clostridium difficile is a Gram-positive, anaerobic bacterium and the leading cause of antibiotic-associated diarrhea and pseudomembranous colitis. *C. difficile* modulates the transition from a motile to a sessile lifestyle through a mechanism of riboswitches regulated by cyclic diguanosine monophosphate (c-di-GMP). Previously described as positively regulated by c-di-GMP, CD2831 was annotated as putative collagen-binding protein and thus potentially involved in sessility. CD2831 is a Sortase substrate and it belongs to the Microbial Surface Components Recognizing Adhesive Matrix Molecule (MSCRAMMs) family, a class of virulence factors that has been recently reported to play a role also in host immune evasion by binding to human complement components. With the overexpression of CD2831 in *C. difficile* and the heterologous expression on *Lactococcus lactis* surface, here we show that CD2831 is a collagen-binding protein, able to bind to immobilized collagen types I, III and V and to the collagen produced by human fibroblasts. We also observed that the overexpression of CD2831 increases the ability to form biofilm on abiotic surface in both *C. difficile* and *L. lactis*. Notably, similarly to other MSCRAMMs, we showed that CD2831 binds to the collagen-like domain of the human complement C1q, preventing the C1 complex formation and the activation of the complement cascade via classical pathway.

2 INTRODUCTION

2.1 *Clostridium difficile* infection

2.1.1 Epidemiology of *Clostridium difficile* Infection

Clostridium difficile is a Gram-positive, strictly anaerobic, spore-forming bacterium that colonizes the human intestine causing *Clostridium difficile* infection (CDI). Originally named *Bacillus difficilis* due to its morphology and the issues in finding the appropriate growth conditions, it was isolated in 1935 from feces of newborn infants with the description of a new commensal anaerobe until the end of 1970s, when it was recognized as the etiologic agent of pseudomembranous colitis (Khan and Elzouki 2014). CDI is now well recognized as a cause of significant patient morbidity and mortality and as a major burden to the health care costs with an attributable mortality ranging from 6.9% to 16.7% in endemic periods (Kwon, Olsen, and Dubberke 2015).

Clinical symptoms of CDI include diarrhea (Bristol stool chart types ranging from 5 to 7), pseudomembranous colitis and toxic megacolon (defined as a distention of the colon, usually to ≥10 cm in diameter, and signs of a severe systemic inflammatory response) (Debast, Bauer, and Kuijper 2014).

CDI is associated to long-term antimicrobial treatments and the 10-35% of antibiotic-associated diarrhea is caused by *Clostridium difficile*. Recent statistical data from Canada, USA and Europe indicate an increased incidence and severity of the disease (Khan and Elzouki 2014). This has been explained as result of changes in demographic situation, increased use of broad-spectrum antibiotics and emergence of hypervirulent *C. difficile* strains (Freeman et al. 2010).

Notably, it is important to discriminate between *C. difficile* colonization and infection. With the term “colonization” we refer to the detection of the organism in the absence of CDI symptoms

whereas “infection” indicates the presence of *C. difficile* toxin or a toxigenic strain type and clinical manifestations of CDI (Crobach et al. 2018). *Clostridium difficile* transmission occurs via spores but the incubation time between spore ingestion and the occurrence of clinical symptoms of the disease is yet to be determined. Asymptomatic carriers, that represent the reservoir for environmental contamination, vary from 2% in the community to more than 30% in hospitals and long-term care facilities (McFarland, Surawicz, and Stamm 1990).

2.1.2 Risks factors and clinical features

As mentioned above, the major risk factor for CDI is a long exposure to antimicrobials, particularly within the hospital setting. The incidence of CDI in some community hospitals is now greater than methicillin-resistant *Staphylococcus aureus* (MRSA) infections (Miller et al. 2011). Almost all antimicrobials, with a particular relevance for the commonly used β-lactam agents, have been reported to be associated with CDI and the reason is attributable to their ability to alter the commensal intestinal flora (Bartlett 2002). The risk to develop the antimicrobials-associated CDI is variable and depends on host factors such as the age and the immune system function but also on the type and dose of antibiotic, and the duration of the treatment.

Other risk factors can be recent surgery (transplant, gastrointestinal procedures), treatment with proton pump inhibitors and immunosuppressant, underlying debilitating conditions, inflammatory bowel disease, prolonged hospitalization (>15 days), and nasogastric tube feeding. Elderly patients are at noticeably higher risk, with disease rates for patients over 65 years of age as much as 20-fold higher than those for younger patients (Surowiec et al. 2006; Schroeder 2005).

The most common clinical presentation of CDI is diarrhea associated with antimicrobials therapy. Diarrhea typically occurs from a few days after the initiation of antibiotic treatment to as long as 8 weeks after the end of the therapy (Bartlett 2002). For mild-to-moderate disease, diarrhea is usually the only symptom. It can be difficult to discriminate between CDI-associated diarrhea and other causes of antibiotic-associated diarrhea (AAD) so definitive diagnosis of CDI is generally accomplished through laboratory confirmation of the presence of *C. difficile* toxins. Other clinical manifestations of CDI include fever that occurs in ∼28% of cases, leukocytosis

(~50%), and abdominal cramps (~22%) (Bartlett et al. 1980). Severe forms of the disease are characterized of paralytic ileus, which can evolve into toxic megacolon. In this scenario also other symptoms co-exist: vomiting, dehydration, lethargy, or tachycardia, in addition to fever and abdominal pain. Importantly, CDI can result in pseudomembranous colitis and life-threatening fulminant colitis, which may lead even to death (Bartlett 2002).

2.2 *Clostridium difficile* Pathogenesis

2.2.1 Spores

The anaerobic nature of *C. difficile* does not allow the vegetative form to survive in aerobic environments so the transmission of CDI relies on the production of dormant spores. After ingestion of *C. difficile* spores, these can pass through the stomach and, upon exposure to bile acids, spores germinate into vegetative cells in the small bowel (Poutanen and Simor 2004) (**Fig. 2.1**). Spores are not only resistant to oxygen but also to many physical and chemical treatments such as heat and the bleach-free disinfectants commonly used in the hospital settings (Nakamura et al. 1985; Lawley, Croucher, et al. 2009; Ali, Moore, and Wilson 2011). Being metabolically dormant, spores are also intrinsically resistant to antibiotics and attacks from the human immune system (Baines et al. 2009; Paredes-Sabja et al. 2012). During the course of the disease, *C. difficile* initiates a sporulation pathway that culminates in the production of dormant spores that are responsible of CDI persistence in the patients and dissemination through patient-to-patient contact. Indeed, Deakin and its group demonstrated in 2012 that non-sporulating strains of *C. difficile* were unable to persist in the colon of the host and be horizontally transmitted (Deakin et al. 2012).

The signals that induce sporulation *in vitro* and *in vivo* have not been yet identified. As demonstrated for *Bacillus subtilis*, external stimuli such as nutrients availability, quorum sensing or stress factors could be related. In many Bacillus and Clostridium species, the switch to enter sporulation is regulated by several orphan histidine kinases that can phosphorylate the master transcriptional regulator Spo0A (Higgins and Dworkin 2012). Consistently, SpoOA mutants of *C. difficile* are defective in sporulation (Underwood et al. 2009) and unable to persist and cause transmission between mice, suggesting for this factor a pivotal role in sporulation and

transmission of CDI (Deakin et al. 2012). Nevertheless, if sporulation is related to hypervirulence is still under question (Vohra and Poxton 2011).

Germination of *C. difficile* spores is an important step for initiating CDI. Bacterial spore germination is induced when bacteria sense the presence of species-specific small molecules (germinants). Sodium taurocholate, the main component of bile, chlorine derivatives and the amino acid glycine enhance germination of spores into vegetative cells (Wilson, Kennedy, and Fekety 1982). In *in vivo* conditions germination presumably occurs in a neutralized region of the intestine as the optimal pH for germination has been shown to be around 6.5-7.5 (Wheeldon et al. 2008) and this process may also require the adhesion to semi-solid surfaces since experiments demonstrated that enhanced germination is observable on agar rather than in liquid broth (Sorg and Sonenshein 2008).

2.2.2 Toxins

There are more than 400 of *Clostridium difficile* strains but only the toxigenic ones are able to produce disease (Samore et al. 1994). The major virulence factors are in fact the two toxins of *Clostridium difficile*, Toxin A and Toxin B (TcdA and TcdB), which have been extensively studied since their first recognition and represent the key factors in *C. difficile* pathogenesis. TcdA (308 kDa) and TcdB (270 kDa) are glucosyltransferases, targeting the Ras superfamily of small GTPases via glycosylation. These small regulatory proteins are therefore inactivated by the irreversible modification leading to disruption of vital signaling pathways in the host cell (Voth and Ballard 2005).

TcdA and *tcdB*, together with three other genes *tcdR*, *tcdC* and *tcdE* are found in a 19,6 kb pathogenicity locus (PaLoc) (Braun et al. 1996). TcdR is homologous to proteins annotated as DNA-binding proteins and it is predicted to positively regulate toxins as TcdR expression has been shown to enhance toxin promoter activity in *E. coli* (Moncrief, Barroso, and Wilkins 1997). TcdE is thought to be a holin-like protein involved in toxin release (Govind and Dupuy 2012) whereas TcdC, whose regulation is inverse to the other PaLoc genes is suggested to act as a negative regulator of toxins expression (Hundsberger et al. 1997). The TcdC role is still controversial: mutation in the strain R20291 shows increased toxins expression bringing to a

hypervirulent strain (Curry et al. 2007), however Cartman et al. demonstrated no change in toxins expression upon genetic mutation of TcdC in the R20291 strain (Cartman et al. 2012). This may be explained as a possible gene duplication event occurred in *tcdA* and *tcdB* genes.

Notably, in addition to TcdA and TcdB, some strains have shown to present a third toxin, called binary toxin (CDT), homologous to other actin-specific ADP-ribosyltransferase of other Clostridia (Stubbs et al. 2000; Popoff and Boquet 1988). The genes encoding the binary toxin, *ctdA* and *ctdB* are not present in the PaLoc region of the chromosome as well as the negative regulator gene, *ctdR* (Carter et al. 2007). In binary toxin, *ctdA* gene encodes for the CDTa, the enzymatic ADP-ribosyltransferase that modifies actin, whereas *ctdB* encodes for CDTb, which binds to host cells and translocates CDTa into the cytosol. CDTb is activated by serine proteases and binds to lipoprotein receptors. ADP-ribosylation induces depolymerization of the actin cytoskeleton and microtubule-based membrane protrusions which form a network on epithelial cells and increase bacterial adherence and colonization (Gerding et al. 2014).

TcdA and TcdB are internalized into the host cell cytosol via endocytosis through the formation of acidic endosomes (Florin and Thelestam 1983). Receptor binding is the first and essential step of the cell entry mechanism and TcdA has been shown to recognize the disaccharide Galβ1-4GlcNac which is found in the I, X and Y blood antigens present in many cells (Tucker and Wilkins 1991). Recently, an extensive genome-wide screening on HeLa cells using the CRISPR-Cas9 approach led to the identification of Frizzled proteins as TcdB receptors (Tao et al. 2016).

Upon receptors binding, toxins require acidified endosomes to translocate. Lysosomotropic inhibitors, such as bafilomycin A and ammonium chloride have been shown to inhibit the cytotoxicity of several clostridial toxins and TcdB activity has been abolished by using conditions that prevent the fusion between lysosomes and endosomes (Florin and Thelestam 1986). The low pH is a required condition for the correct folding of the toxins leading to the exposure of the hydrophobic domain (Qa'Dan, Spyres, and Ballard 2000). Once internalized, the prominent toxins effect is the profound change in cellular cytoskeleton organization (Mitchell, Laughon, and Lin 1987; Thelestam and Bronnegard 1980). Several studies from Just and colleagues demonstrated that TcdB was able to glucosylate the GTPases RhoA, Rac and Cdc42 using UDP-glucose as cosubstrate and that TcdA could modify RhoA with the same mechanism, concluding

that both the toxins are glucosyltransferases capable of inactivating small GTPases within the cell (Just, Selzer, et al. 1995; Just, Wilm, et al. 1995). The phenotype in infected cells results in cellular rounding due to actin condensation, membrane blebbing and eventually in apoptosis. Importantly, the decline in F-actin results also in tight junction disruption between intestinal epithelial cells (Nusrat et al. 2001). Both TcdA and TcdB induce the influx of neutrophils that causes inflammation typical of pseudomembranous colitis. This may be due to tight junctions disruption but also to stimulation of cytokine expression and macrophage derived TNF-α (Kim et al. 2002; Kelly et al. 1994) (**Fig 2.1**).

A study from our group showed the importance of toxins not only as effectors of toxicity but also in facilitating the bacterial colonization. Indeed, TcdA-mediated subversion of cell polarity facilitates *C. difficile* adhesion and translocation of Caco-2 monolayer (Kasendra et al. 2014).

Other studies demonstrated that when Binary Toxin is present, strains can develop more severe diarrhea and that Toxin A^-/B^- Binary Toxin positive strains have been isolated from symptomatic patients (Geric et al. 2003; Barbut et al. 2005). Despite this, one study shows that Toxin A^-/B^- strains which express binary toxin are non-pathogenic in hamsters and do not cause the same level of mucosal damage in the rabbit as toxin positive strains do (Geric et al. 2006).

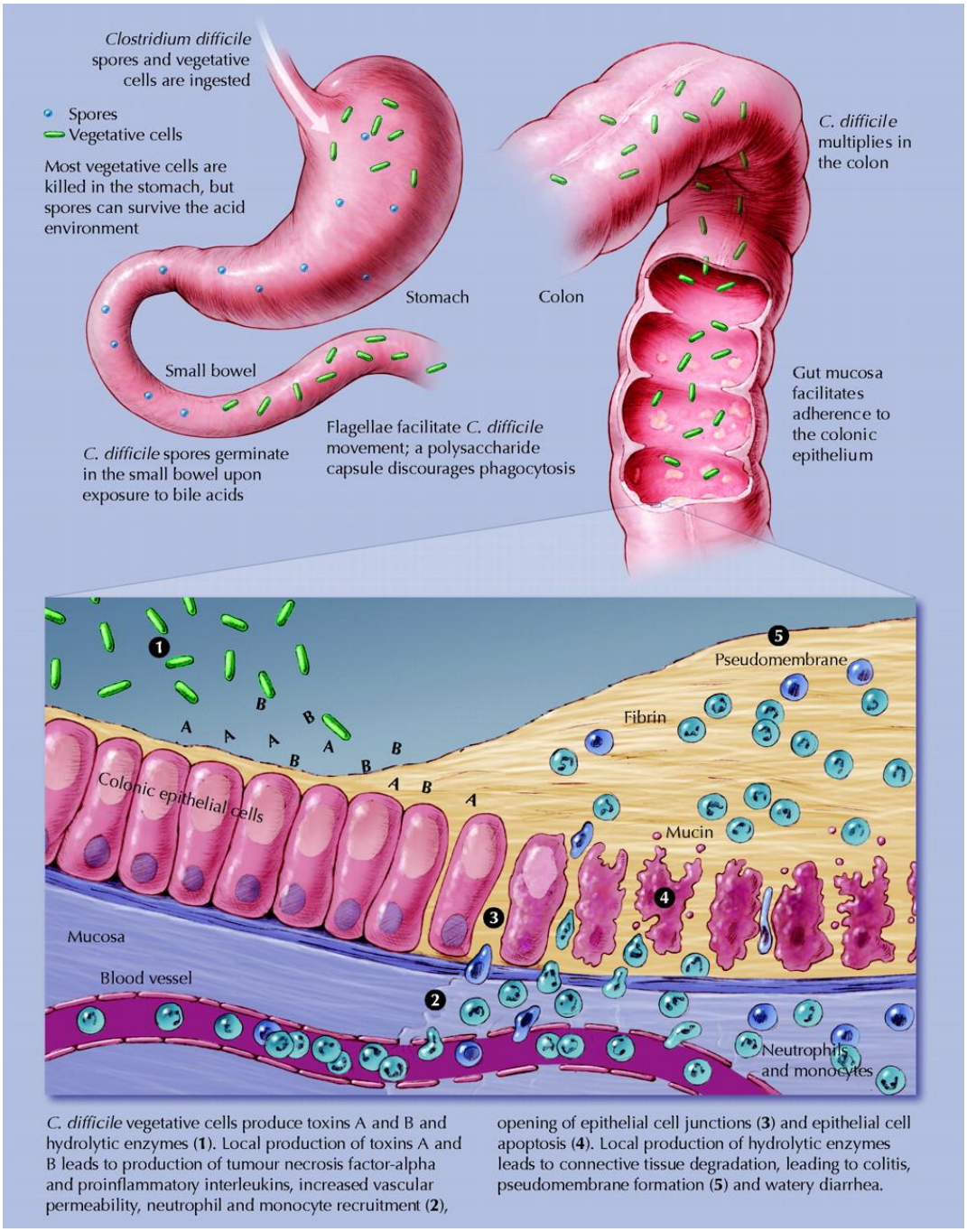

Fig. 2.1 | *Clostridium difficile* infection of the human gut

Once ingested, spores can survive in the stomach environment and germinate into the small bowel upon exposure to bile acids. *C. difficile* vegetative cells arrive in the colon, adhere to the gut mucosa and proliferate. Toxins production leads to epithelial integrity perturbation (1) and to production of tumour necrosis factor-alpha and proinflammatory interleukins. This results in increased vascular permeability, neutrophil and monocyte recruitment (2), opening of epithelial cell junctions (3) and epithelial cell apoptosis (4). Local production of hydrolytic enzymes leads to connective tissue degradation, conducting to colitis, pseudomembrane formation (5) and diarrhea (Poutanen and Simor, 2004).

2.2.3 Colonization and adhesion to the host

2.2.3.1 S-layer and adhesins

In order to induce its pathogenicity, vegetative *C. difficile* cells need to be implanted in the gut epithelial cells, which are protected by a layer of dense mucus. Once adhered to the mucus layer, *C. difficile* can reach the enterocytes present in the small bowel and colon by means of its multiple adhesins (Deneve et al. 2009). The hydrophobic nature of *C. difficile* cell surface as well as the positive charges distributed within the cell wall, may play a role during the gut colonization via interaction with negatively charged intestinal cells (Borriello and Wilcox 1998; Borriello 1998).

The crossing of the first barrier represented by the mucus layer involves flagellar components such as cap protein (FliD) and flagellin (FliC) (Deneve et al. 2008). Important is also the presence of proteolytic enzymes such as hyaluronidase and collagenase that can contribute to the release of essential nutrients facilitating *C. difficile* establishment in the gut (Seddon, Hemingway, and Borriello 1990).

Pivotal is the contribution given by Surface Layer proteins (SLPs), which mediate the adherence to the mucus and to epithelial cells of the gut. SLPs are part of the S-layer that completely cover the outer cell surface of the bacterium. Specifically, the S-layer consists of two proteins, the high-molecular weight P47 (HMW) and the low-molecular weight P36 (LMW) that derive from SlpA precursor clevage and interact to each other in a heteromeric complex (Emerson et al. 2009). HMW SlpA can strongly associate to human and murin gastrointestinal tissues and to some extracellular matrix components such as collagen, thrombospondin and vitronectin (Emerson et al. 2009; Janoir et al. 2007).

Other surface proteins likely involved in the host colonization include the heat shock protein GroEL (Hennequin, Collignon, and Karjalainen 2001; Hennequin et al. 2001) the adhesin Cwp66 (Clostridial Wall Protein, 66 kDa) that mediates *in vitro* adherence to Vero cells (Pechine, Deneve-Larrazet, and Collignon 2016; Wright et al. 2005) and the lipoprotein CD0872 (Kovacs-Simon et al. 2014).

2.2.3.2 MSCRAMM family proteins

As other pathogens, *C. difficile* targets a wide variety of host molecules via multiple mechanisms, including binding to components of the host extracellular matrix (ECM). The surface proteins known to mediate adhesion to ECM are designated as "Microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs). Their role could be crucial in *C. difficile* pathogenesis as the disruption of epithelial barrier caused by the toxins determines the accessibility to the underlying matrix and therefore favors bacterial colonization (**Fig 2.1**). Fibronectin-binding protein A (FbpA) and Collagen-binding protein A (CbpA) are examples of *C. difficile* MSCRAMMs playing a role in this mechanism (Hennequin et al. 2003; Tulli et al. 2013).

The most characterized collagen-binding MSCRAMM is the *Staphylococcus aureus* Cna protein. CNA-like collagen-binding proteins have been identified in other bacteria, among which are ACE (adhesin of collagen from *Enterococcus faecalis*) (Rich et al. 1999), Acm (adhesin of collagen from *Enterococcus faecium*) (Nallapareddy, Weinstock, and Murray 2003) and CNE (collagen-binding protein from *Streptococcus equi*) (Lannergard, Frykberg, and Guss 2003). These proteins share the Cna domain organization, however the specific binding mechanisms for each collagen adhesin may be somewhat different.

A second role for MSCRAMMs has been identified in the host immune system evasion with the binding to the complement component C1q. The human complement C1q presents a shape resembling a bunch of flowers, with six peripheral globular heads, each connected by fibrillar strands to a central bundle of collagen-like fibers (Reid 1979; Reid, Gagnon, and Frampton 1982). Globular regions of C1q recognize the Fc region of antibodies on the bacterial surface (Hughes-Jones and Gardner 1979) and the central collagen-like structure activates the complement cascade via the classical pathway through the interaction with the other two components of the C1 complex, C1s and C1r. Besides complement activation, C1q is also able to enhance phagocytosis independently from C1r and C1s (Bobak et al. 1987) and this activity requires the collagen-like tail of C1q (Bobak, Frank, and Tenner 1988; Thielens et al. 2017).

The collagen-binding protein Cna from *Staphylococcus aureus,* and other similar proteins of Gram-positive bacteria, can bind to the "collagenous domain" of C1q. Interaction of Cna with

C1q has been shown to prevent the binding of C1r to C1q thus inhibiting the C1 complex formation and consequently the classical complement pathway activation (Kang et al. 2013).

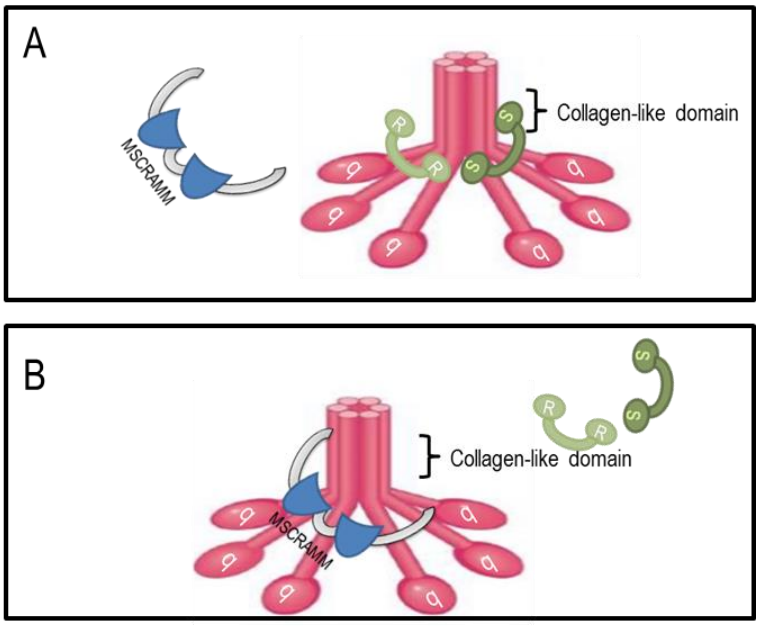

Fig. 2.2 | Schematic representation of MSCRAMMs interfering with C1r/C1s binding to C1q

Binding of dimers of C1s and C1r to the collagen-like domain of the C1q stalk is required for initiation of the human complement cascade via classical pathway (A). When MSCRAMMs proteins such as Cna of *S. aureus* interact with antibodies-associated C1q, the binding of C1s and C1r to C1q as well as the activation of the complement cascade is prevented (B).

2.2.4 C-di-GMP regulation

An important role in regulating *C. difficile* colonization is played by the second messenger c-di-GMP whose levels can control the expression of proteins involved in bacterial motility and sessility. C-di-GMP acts by binding riboswitches, tridimensional structures present at the mRNA level located upstream of the target genes (Peltier and Soutourina 2017). *C. difficile* encodes a large number of diguanylate cyclases and phosphodiesterases that are responsible for the synthesis and degradation of c-di-GMP, respectively, and 16 predicted c-di-GMP-responsive riboswitches have been identified in *C. difficile* genome (Bordeleau et al. 2011; Sudarsan et al. 2008; Soutourina et al. 2013). C-di-GMP controls bacterial physiology and virulence by modulating the expression levels of *tcdA* and *tcdB*, as well as that of *tcdR*, which encodes an alternative sigma factor that activates *tcdA* and *tcdB* expression (McKee et al. 2013; Martin-Verstraete, Peltier, and Dupuy 2016). In many bacterial species, including *C. difficile*, c-di-GMP controls lifestyle switch from free-living motile state to biofilm communities (Romling, Galperin, and Gomelsky 2013; Purcell and Tamayo 2016). In particular, c-di-GMP negatively regulates flagellar swimming motility and positively regulates expression of putative adhesins and type IV pili (TFP) biosynthesis. Indeed, TFP have been recently shown to be fundamental for adherence and persistence of *C. difficile* in a mouse model of infection (Purcell et al. 2016; Bordeleau et al. 2015; Purcell et al. 2012; McKee et al. 2018) (**Fig. 2.3 A**).

Interestingly, a type I riboswitch that is activated at low levels of c-di-GMP, was found upstream of the *C. difficile* protease *ZmpI* (CD2830), whereas the close gene coding for the putative collagen-binding adhesin CD2831 (Sebaihia et al. 2006) presents a type II c-di-GMP riboswitch, activated at high levels of cellular c-di-GMP. CD2831 and another c-di-GMP controlled putative adhesin, CD3246, are recognized and cleaved by ZmpI. This mechanism suggests that expression of ZmpI could be upregulated in the presence of low concentration of c-di-GMP, whereas the expression of CD2831 may be upregulated in presence of high levels of intracellular c-di-GMP (Soutourina et al. 2013). Consistently, it was demonstrated that at low c-di-GMP levels, ZmpI is produced and it cleaves the low amount of CD2831 and CD3246, thus limiting bacterial adhesion to the host (Hensbergen et al. 2015; Peltier et al. 2015; Corver et al. 2017) (**Fig. 2.3 B**).

C-di-GMP positively regulates *C. difficile* biofilm formation, and high level of intracellular c-di-GMP in the 630 strain results in a more robust biofilm (Soutourina et al. 2013; Purcell et al. 2017). There are many studies and evidences demonstrating the ability of *C. difficile* to form biofilm *in vitro*. *C. difficile* biofilm on abiotic surface varies between strains even though no correlation between biofilm robustness and virulence has been found (Ethapa et al. 2013; Pantaléon et al. 2018). In the biofilm asset, bacteria have been shown to be embedded in a matrix rich of extracellular DNA, polysaccharides and proteins, including toxin A and B. Besides c-di-GMP, biofilm formation process is modulated by the master regulator Spo0A, as in R20291 strain a genetic inactivation of spo0A exhibits decreased biofilm formation (Dawson et al. 2012). Also Cwp84, flagella, TFP and the quorum-sensing regulator LuxS, are all required for maximal biofilm formation by *C. difficile* (Pantaleon et al. 2015; Maldarelli et al. 2016; Ethapa et al. 2013). Importantly, evidences support *C. difficile* biofilm formation *in vivo:* observations in a mouse model showed that *C. difficile* forms clumps associated with damaged tissue suggesting the formation of microcolonies (Lawley, Clare, et al. 2009). Also, infected hamsters and mice have been reported to present aggregation or clusters of *C. difficile* (Buckley et al. 2016; Soavelomandroso et al. 2017) and recent studies show the presence of *C. difficile* biofilm communities in association with the gut mucus layer (Semenyuk et al. 2015; Soavelomandroso et al. 2017).

Although many advances have occurred in understanding c-di-GMP effects on bacterial physiology, the stimuli and the mechanisms present upstream the modulation of c-di-GMP in *C. difficile* are still unknown.

In this study we establish that the c-di-GMP regulated CD2831 is a collagen-binding protein contributing to *C. difficile* adhesion to the host extracellular matrix. Gene overexpression of CD2831 significantly increased biofilm formation *in vitro*, suggesting a role for CD2831 in the process of biofilm formation. Moreover, as other MSCRAMMs, CD2831 can bind to human complement C1q and thus potentially be involved in host immune evasion mechanisms.

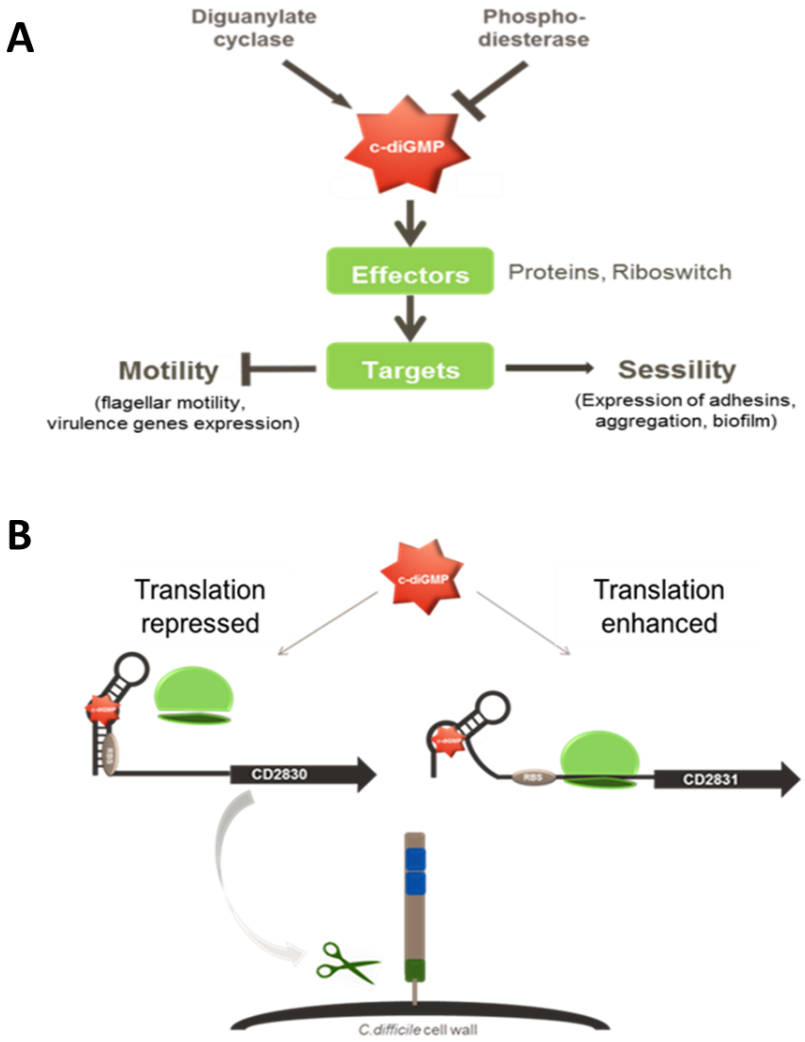

Fig. 2.3 c-di-GMP regulates *C. difficile* lifestyle

(A) Second messenger c-di-GMP is synthesized by proteins with diguanylate cyclase activity and degraded by phosphodiesterases. C-di-GMP can act on effectors involved in flagellar motility, adhesion and virulence factors expression. In particular, increased c-di-GMP levels downregulate flagellar motility whereas upregulate expression of adhesines and other molecules involved in sessility and biofilm formation. (B) c-di-GMP inversely modulates CD2831 and the protease CD2830 (ZmpI) by targeting riboswitches located upstream of the target mRNA. Binding of c-di-GMP allosterically modify the riboswitch, changing the accessibility of ribosomes to the ribosome-binding site. CD2831 translation is enhanced after c-di-GMP binding, whereas ZmpI translation is repressed upon c-di-GMP binding.

3 EXPERIMENTAL PROCEDURES

3.1 Bacterial strains and growth conditions

C. difficile 630Δ*erm* strain (an erythromycin-sensitive derivative of *C. difficile* strain 630) was grown anaerobically (10 % H_2 , 10 % CO_2 and 80 % N_2) at 37 °C in Brain-Heart Infusion (BHI) (ThermoFisher, Massachussetts, USA) broth or agar supplemented with 0.05% L-cysteine and 0.5% yeast extract (BHIS). When necessary, *C. difficile* cultures were supplemented with D-cycloserine (25 µg/ml) and/or thiamphenicol (20 µg/ml) (Sigma, Missouri, USA). *E. coli* HK100 cells and BL21(DE3)T1R strains, used to clone and express CD2831, were cultured at 37°C in Luria-Bertani (LB) broth supplemented with 100 µg/ml ampicillin. *L. lactis* MG1363 strain was cultured at 30 °C in M17 growth medium supplemented with 0.5% glucose and, when required, with 20 µg/ml chloramphenicol. Anhydrotetracycline (aTC) was used from 10 to 100 ng/ml for induction of the pTet promoter in *C. difficile*.

3.2 Cloning, expression and purification of CD2831

CD2831 domain prediction was assessed with a blast search against the CDD database available at NCBI. Localization of collagen-binding domains was further investigated with Pfam (https://pfam.xfam.org/). For the expression of recombinant CD2831 full length and subdomain 1 (157-432), 2 (432-840), 3 (311-940), 4 (157-830), 5 (32-830) and 6 (594-940), the corresponding sequences were amplified by PCR using chromosomal DNA from *C. difficile* 630 strain as template. The PCR products were cloned in pET21 vector (Novagen, Wisconsis, USA) using the PIPE method (Klock et al. 2008). BL21(DE3)T1^R chemically competent cells were used for expression of the N-terminal His-tag proteins. A single colony of *E. coli* BL21(DE3)T1^R strain expressing CD2831 was inoculated in LB containing 100 µg/ml ampicillin and grown overnight at 37°C. Bacterial suspension was diluted in fresh HTMC medium supplemented with 100 µg/ml ampicillin and grown for 30 hours at 25 °C. Proteins expression was induced by adding 0.5 mM Isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma, Missouri, USA). Recombinant CD2831 His-

tagged proteins were purified by affinity chromatography on Ni2+-NTA Superflow (Qiagen, Italy). The purity of the proteins was checked by SDS-PAGE followed by Coomassie Blue staining and the proteins concentration was determined by BCA reagents (ThermoFisher, Massachussetts, USA).

3.3 Construction of CD2831 overexpressing strains of *C. difficile*

The CD2831 gene was amplified from *C. difficile* strain 630Δ*erm* genomic DNA using Phusion® High-Fidelity DNA Polymerase (NEB, England, UK). Region containingribosome-binding site (RBS) of SlpA protein of *C. difficile* was included in the forward primer before the ATG start codon (primer Fw: CGAGCTCAATATAATGTTGGGAGGAATTTAAGAAATGAAGAAAGGAAATAGAAAGGCA and primer Rv GACTAGTCTAATTTGTATTTTTATTTCTTCT). PCR product was digested with SacI-SpeI and cloned into pMTL960 derivative vectors (Fagan and Fairweather 2011) containing either cwp2 constitutive promoter (producing pCwp2-2831) or pTet inducible promoter (producing pTet-2831).

3.4 *E. coli-C. difficile* conjugation

Overnight cultures of *E. coli* SM10λpir carrying CD2831 expression vector were harvested and pellets were resuspended in 200 µl of *C. difficile* overnight culture in the anaerobic cabinet. The mixed bacterial suspension was then spotted onto BHIS agar plates and conjugation proceeded for 8 hours at 37 °C. 1:5 dilutions were plated onto BHIS agar containing 20 µg/ml thiamphenicol for plasmid selection and 250 µg/ml cycloserine for *E. coli* growth inhibition. Plates were grown overnight at 37 °C in the anaerobic cabinet. Transconjugants were picked and streaked onto fresh selective plates twice to purify *C. difficile* containing CD2831 overexpressing plasmids from *E. coli*.

3.5 Generation of *Lactococcus lactis* expressing CD2831 strain

The construction of *L. lactis* strain expressing the *C. difficile* protein CD2831 was performed as previously described (Tulli et al. 2013). Briefly, the CD2831 gene was amplified by PCR using chromosomal DNA from *C. difficile* 630Δ*erm* strain as template (primer1: 5'-

GAGGTTAAGGCTAACGGTTCAGAATTAGGAGAGAATAGTCAGATTCAAAG -3' and primer2: 5'-ACCTGTTGATGGTAATACTGGTGGATTTACCAAAGTATCATCTTTAAC -3') and cloned in pAM401 expression vector (Buccato et al. 2006). The coding sequence for the leader peptide (1-93 bp) and the PPKTG-motif at C-terminal (2817-2829 bp) of CD2831 were substituted with the same regions of the GAS *emm1* gene encoding the streptococcal M1 protein to ensure the surface exposure in *L. lactis* as described by Edwards et al. (Edwards et al. 2008). pAM401-M1 was used as template to amplify the expression vector containing the leader peptide and the C-terminal of M1 protein (Primer3: 5'-GATAGTAATGTAGGTCAATTACCATCAACAGGTGAAACAGCTAACCCA-3'; primer4: 5'-GTATCTGCGAAAGTATTTGAACCGTTAGCCTTAACCTCTGTTTGATTCGC-3'). The two products were assembled by PIPE method in *E. coli* HK100 cells and the resulting vector was introduced in *L. lactis* subspecies cremoris MG1363 strain by electroporation.

3.6 Production of polyclonal antibody against CD2831

Rabbits were immunized three times, 2 weeks apart, with 10 µg of recombinant CD2831 in the presence of aluminum hydroxide. The immune serum was obtained 14 days after the last immunization. All animal experiments were performed in accordance with relevant guidelines and regulations established by the Italian law, including the approval of the local Animal Welfare Body followed by authorization of Italian Ministry of Health.

3.7 Cell fractionation and protein analysis

To obtain cell wall-associated proteins of *C. difficile* the following protocol was applied. Bacteria were grown until exponential phase, harvested by centrifugation at 3000 x g for 10 minutes and washed in Tris-sucrose buffer (TS: 10 mM Tris-HCl pH 6.9, 10 mM MgCl2, 0.5 M sucrose). Cells were resuspended in TS buffer, protease inhibitors cocktail (Complete Mini EDTA-free, Roche) and 250 µg/ml of mutanolysin (Sigma, Missouri, USA) and digestion was allowed to proceed for 2 hours at 37 °C with gentle agitation. The majority of intact protoplasts were removed by centrifugation at 3000 x g for 30 minutes. The supernatant was then subjected to microcentrifugation at 17000 x g for 30 minutes to remove cell debris and any remaining protoplasts. The supernatant containing the solubilized cell surface proteins was recovered.

Proteins were separated by SDS-PAGE electrophoresis using NuPage Gel System (ThermoFisher, Massachussetts, USA), according to the manufacturer's instructions. Samples were transferred to nitrocellulose membranes for Western blot analysis. After blocking with PBS containing 0.05% Tween 20 (PBST) and 10% w/v skim milk powder (Sigma, Missouri, USA), proteins were detected with anti-CD2831 serum followed by the HRP-conjugated secondary antibodies (DAKO, Glostrup, Denmark) diluted in PBST and 3% w/v skim milk powder. Bands were visualized with Super Signal® West Pico Chemiluminescent Substrate (Thermo Scientific, Massachussetts, USA).

3.8 Flow cytometry analysis

C. difficile and *L. lactis* cultures were harvested at 6000 x g for 1 minute and washed twice with PBS. The pellet was resuspended in 4% (v/v) formaldehyde (Carlo Erba Reagents, Italy) in PBS and incubated for 30 minutes at room temperature. Pellets were washed twice and incubated with rabbit anti-CD2831 serum diluted 1:100 for 1 hour at room temperature. After two washes with PBS, bacterial pellets were incubated with Alexa Fluor 647-conjugated secondary anti-rabbit antibody (Thermo Scientific, Massachussetts, USA) diluted 1:500 for 30 minutes at room temperature in the dark. All washing steps and antibodies dilutions were performed using 1% (w/v) bovine serum albumin (BSA) in PBS. Bacterial samples were analyzed by BD FACS Canto II system (BD Bioscience, USA).

3.9 Proteins ELISA

96-well MaxiSorp PS Immuno F96 plates (Thermo Scientific, Massachussetts, USA) were coated overnight at 4 °C with 100 µl collagen I, III , V or human C1q (Sigma, Missouri, USA) at the final concentration of 10 µg/ml. Plates were washed three times with 0.9% NaCl + 0.05% Tween-20 (NaCl-T) and blocked with 3% (w/v) BSA + 0.05% Tween + in PBS for 2 hours at 37 °C. Plates were washed three times with NaCl-T. Recombinant *C. difficile* proteins were two-fold serially diluted in PBS, then added to the collagen/C1q-coated wells and incubated for 1 hour at 37 °C. Following incubation, the wells were gently washed three times with NaCl-T. Anti-CD2831 antibodies diluted 1:500 in 1% BSA/PBS were added to each well and incubated for 1 hour. Wells were washed three times with NaCl-T before incubation with anti-rabbit HRP-conjugated

secondary antibodies (DAKO, Glostrup, Denmark) diluted 1:2.000 in 3% BSA/PBS w/v for 1 hour. Wells were washed three times in NaCl-T and 50 µl OPD substrate (5 ml 0.1 M citric acid, 5 ml 0.2 M Na_2HPO_4, 10 ml H_2O, 1 OPD tablet (Sigma, Missouri, USA), 8 µl H_2O_2) was added to each well. Plates were incubated at room temperature for 15 minutes and the reaction was stopped with 50 µl of 3M H_2SO_4 per well. The optical density at 450 nm was determined using a microplate reader.

The competition assay was performed by the procedure described above, with the following alterations: two-fold diluted CD2831 protein was pre-incubated with 1% Normal Human Serum (NHS) (Sigma, Missouri, USA) at RT for 1 hour. The mixture was subsequently added to microtiter plates previously coated overnight at 4 °C with purified IgM (1 µg/well). Rabbit antibodies against C1q, C1r and C1s (Sigma, Missouri, USA) diluted 1:250 in 1% BSA/PBS were used to detect proteins deposition. C1q-depleted serum (Sigma, Missouri, USA) was used as negative control. Detection was performed as described above.

3.10 Protein binding and bacterial adhesion assay to IMR-90 cells

IMR-90 human fibroblasts (ATCC, Virginia, USA) were cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC, Virginia, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Scientific, Massachussetts, USA) and antibiotics at 37 °C with 5% CO_2. Cells were seeded onto 8-well chamber slides or 96-well plates coated with collagen I (BD BioCoat, England, UK) and cultured for 3 days to induce ECM protein production.

Cells were then incubated with serially diluted recombinant CD2831 proteins (0.08-10 µM). After washing to remove the unbound proteins, cells were fixed with 4% (v/v) formaldehyde in H_2O for 30 minutes at RT. Samples were washed and incubated with rabbit anti-CD2831 sera (1:500 in PBS/BSA) for 1 hour at RT. After multiple washes, samples were incubated with FITC-conjugated goat anti-rabbit IgG (1:500 in PBS/BSA) for 1 hour at RT. Binding of the proteins was quantified with a microplate fluorescence reader.

Similarly, for bacterial adhesion assay, *L. lactis* or *C. difficile* strains were grown until exponential phase, resuspended in DMEM medium and added to the microtiter wells. After 2 hours of incubation at 37 °C in anaerobic conditions, plates were washed with PBS and fixed with 4%

(v/v) formaldehyde in H_2O for 30 minutes at room temperature. After three washes with PBS, samples where incubated with 3% (w/v) BSA in PBS for 30 minutes and immunofluorescence staining was performed as follows. To detect collagen-adhering bacteria, an anti-*C. difficile* 630 polyclonal serum diluted 1:500 and a mix of anti- Collagen I, III and V (Santa Cruz, Texas, USA) diluted 1:250 in 1% (w/v) BSA in PBS were added for 1 hour, followed by washes and incubation with Alexa Fluor-conjugated secondary antibodies 1:500 in 1% (w/v) BSA in PBS for 30 minutes. Adhering bacteria were measured by using a fluorescence plate reader. For microscopy visualization, wells were incubated with Alexa-Fluor-conjugated phalloidin (Thermo Scientific, Massachussetts, USA) and DAPI (Sigma, Missouri, USA) and glass coverslips were mounted with ProLong® Gold antifade reagent and analysed with Zeiss LSM710 confocal microscope.

3.11 Biofilm formation assay

Overnight cultures of *C. difficile* or *L. lactis* were diluted 1:100 into fresh medium containing 0.1 M glucose and 1 ml was seeded on 24-well or 48-well polystyrene plates (Sigma, Missouri, USA) for the desired time. To prevent evaporation of liquid, plates were wrapped with parafilm. Measurement of biofilm biomass was assessed using crystal violet as previously described (Varga, Therit, and Melville 2008; Ethapa et al. 2013). After the required incubation, wells were gently washed with sterile PBS and then allowed to dry for 10 minutes. The biofilm was stained with filter-sterilized 0.2% crystal violet (Sigma, Missouri, USA) and incubated for 30 minutes at 37 °C. Crystal violet was removed from the wells, followed by two washes with sterile PBS. The dye was extracted by adding 1 ml methanol to each well and incubation for 30 minutes at room temperature in aerobic conditions. The methanol-extracted dye was diluted 1:2 or 1:10 and absorbance at 570 nm was measured using a microplate reader.

3.12 Confocal microscopy analysis of biofilm formation

For microscopy visualization of microbial biofilm, bacteria were grown in 8-well glass chamber slides (BD Biosciences, New Jersey, USA) in BHIS supplemented with 0.1 M glucose, at 37 °C in anaerobic conditions for 72 hours. The wells were washed twice, and before removing from the anaerobic chamber, bacteria were fixed with 4% (v/v) formaldehyde in H_2O for 30 minutes.

Extracellular DNA and nuclei were stained by using DAPI (ThermoFisher, Massachusetts, USA) diluted 1:5000 in PBS for 30 minutes. Chamber slides were mounted with ProLong Gold Antifade Reagent (ThermoFisher, Massachusetts, USA) and analyzed with a Zeiss Observer LSM710 confocal microscope.

3.13 Electron Microscopy

A 5 µl aliquot of purified proteins CD2831 and/or C1q (10 ng/µl) were charged on a 300-mesh copper TEM grid (Agar Scientific, Stansted, UK). After 1 minute of adsorption the excess was blotted with Whatman filter paper and the samples were counterstained with Nano-W (Nanoprobes, Yaphank, NY, USA) for 30 seconds. The images were acquired at 120000x magnification using a TEM FEI Tecnai G2 spirit microscope operating at 100kV and equipped with an 2K × 2K CCD Veleta (Emsis, Germany).

3.14 Statistical analysis

At least two independent experiments, run under the same conditions, were performed for all studies. Results were analyzed with GraphPad Software (La Jolla California, USA) by applying the Student *t* test for unpaired data. $P \leq 0.05$ was considered statistically significant (*,$P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$, ****, $P \leq 0.0001$).

4 RESULTS

4.1 CD2831 structure and domains prediction

CD2831 is a protein codified by the homonymous gene and consists of 972 amino acid residues. The protein was first identified when the complete genome sequence of *C. difficile* 630 strain was determined and it was annotated as a putative collagen-binding adhesin (Sebaihia et al. 2006). CD2831 protein presents an N-terminal putative leader peptide of 31 amino acids followed by a short repeat region (AA 56-157). Despite a previous *in silico* analysis of CD2831 identified the presence of a central "Collagen-Hug" motif comprised between amino acids L173 and G459 (Hensbergen et al. 2015), our study revealed the presence of a collagen-binding superfamily region spanning from residues 225 to 797. Weak sequence similarities (20% amino acid identity) to the *S. epidermidis* Adhesin Sdrg and the *S. pyogenes* T6 Backbone Pilin were detectable in the 165-460 and 461-820 regions respectively, suggesting the typical MSCRAMM structural organization in IgG-like sub-domains. Localization of putative collagen-binding domains was further investigated and predicted the location of two collagen-binding domains (CBDs), from here on indicated as CBD 1 and 2. The first extends from amino acids N334 to D431 whereas CBD2 corresponds to the region from G467 to E592. The CBDs are followed by a region of 110 amino acids rich in Proline that has been described to be recognized and cleaved by the endogenous metalloprotease ZMP1/CD2830 (Hensbergen et al. 2015; Peltier et al. 2015). Interestingly, as described above, CD2831 and ZmpI are inversely regulated by c-di-GMP through a riboswitches mechanism. The C-terminal region of CD2831 encompasses an LPXTG-like motif (PPKTG) recognized by the Sortase B enzyme, which is responsible for the cell-wall proteins attachment to the peptidoglycan (Peltier et al. 2015). The sortase motif is followed by a hydrophobic region, most probably representing the transmembrane domain (**Fig. 4.1**).

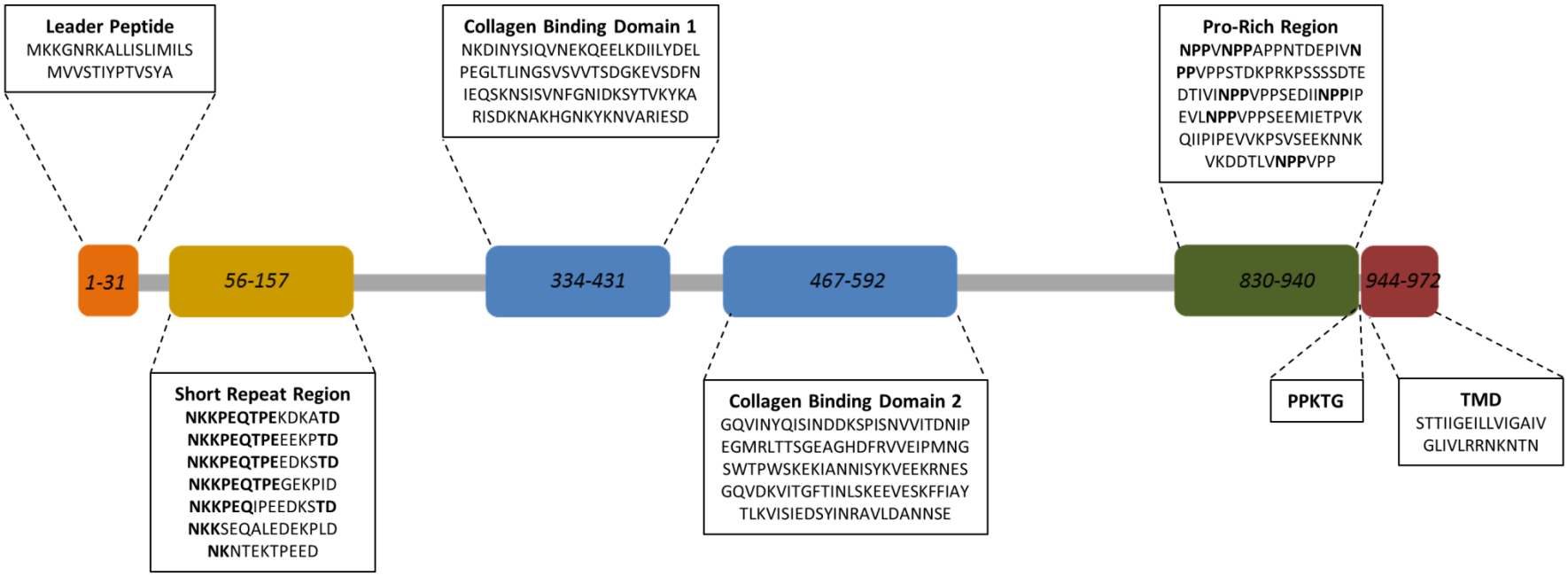

Fig. 4.1 | Domain organization of CD2831.

Schematic representation of CD2831 domains organization displays the Leader Peptide at the N-terminus (orange), necessary for the export of the full-length precursor across the cytoplasm. CD2831 also contains a Short Repeat Region (yellow) in which repeated amino acids sequences are marked in bold, two Collagen-binding domains (blue) and a Proline-rich region (green) recognized by the protease ZmpI, which cleaves within the seven NPP amino acids sequences of the domain. The protein is cleaved between the threonyl and glycil residues of the PPKTG motif and transferred to the cell-wall peptidoglycan by Sortase B enzyme. The charged tail and the hydrophobic domain (TMD) at the C-terminus (red) prevent the protein from release into the extracellular milieu.

4.2 Recombinant CD2831 and subdomains containing CBD1 and CBD2 bind to native collagen produced by cells and to collagen types I, III and V

To investigate the predicted collagen-binding activity of CD2831, the sequences coding for the full-length CD2831 and six different portions of the protein were cloned and expressed in *E. coli*. Subdomains 1 (157-432) and 2 (432-830) include the N-terminal region containing only the CBD1 and the C-terminal region presenting CBD2, respectively. CD2831 subdomains 3 (311-940), 4 (157-830) and 5 (32-830) encompass both the CBDs with subdomain 3 including also the Pro-rich and subdomain 5 the short repeat region. Subdomain 6 (594-940) represents the C-terminus of CD2831 and, lacking any CBD, was used as negative control in the binding assay (**Fig. 4.2 A**).

After purification, full length CD2831 and subdomains were tested in their binding activity toward native collagen produced by human cells. For this, confluent IMR90 human fibroblasts were cultured on 96-well plates for three days. In these conditions IMR90 cells are known to produce a considerable amount of extracellular matrix components (ECM), with a relevant presence of collagen.

Experiments using IMR90 showed that CD2831 displays a dose-dependent binding activity to native collagen. Subdomains 3 (311-940), 4 (157-830) and 5 (32-830), encompassing the CBD1 and CBD2 portions, showed a binding kinetic similar to the full-length protein, confirming the predicted collagen binding activity. Addition of the short repeat region at N-terminal (subdomain 5) or the Proline-rich region (subdomain 3) at C-terminal showed no impact on binding activity, further confirming that the minimal domain mediating the interaction to collagen resides in the CBD1 and CBD2 portions of the protein. When expressed individually, CBD1 and CBD2 (subdomains 1 and 2) showed higher binding activity compared to the full-length protein. portions of the protein. Unexpectedly, when expressed individually, CBD1 and CBD2 (subdomains 1 and 2) showed a higher binding activity compared to the full-length protein (**Fig. 4.2 B**). Equal recognition of each domain from CD2831 anti-sera was confirmed by dot blot (**Fig. 4.2 C**).

Full length recombinant CD2831 binding to native collagen was also visualized by immunofluorescence showed in **Fig. 4.2 D**, which displays a spatial co-localization between CD2831 (green) and native collagen (red) produced by human fibroblasts IMR90.

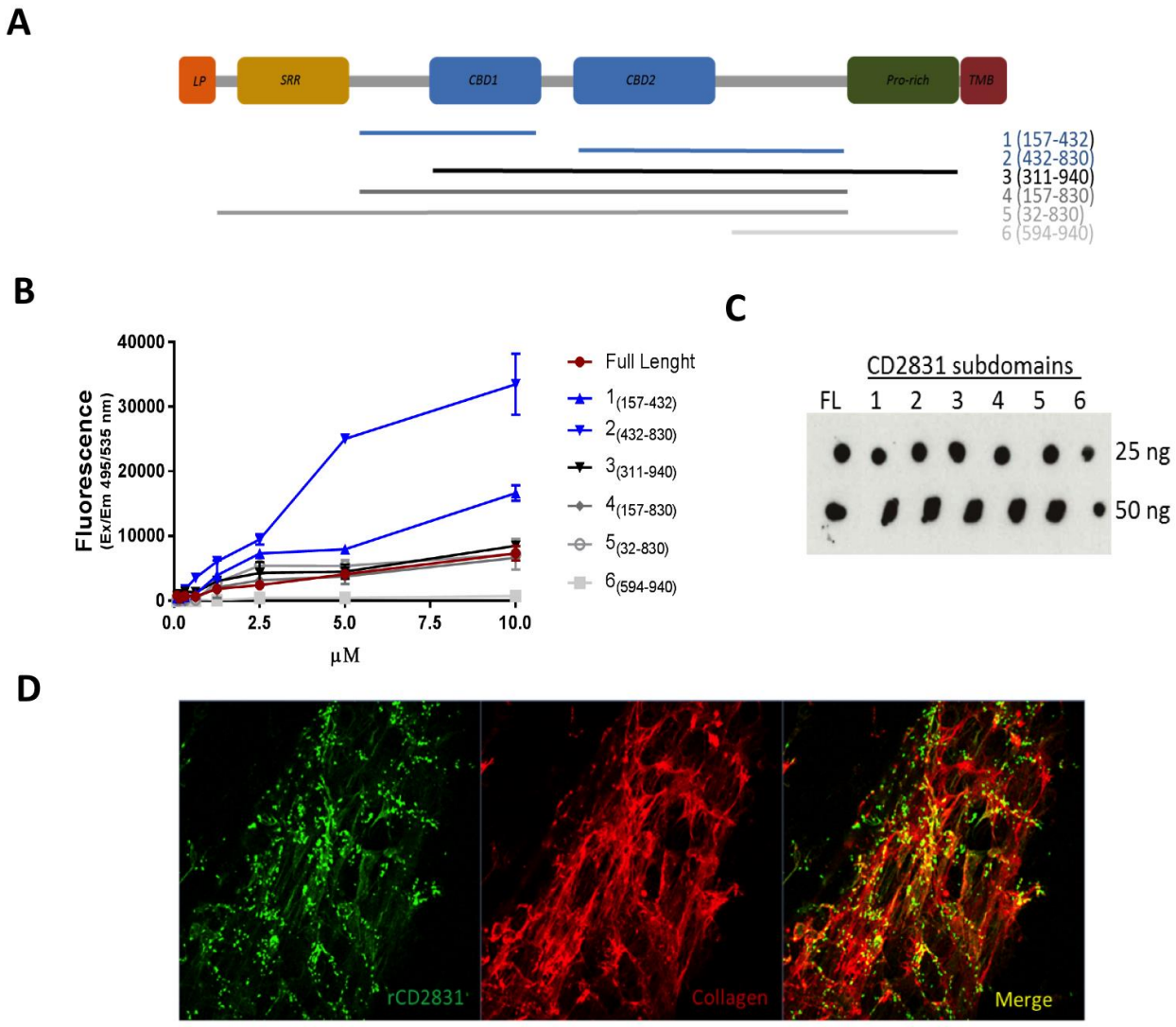

Fig. 4.2 | Binding of recombinant CD2831 full length protein and subdomains to IMR90 cells.

A. Schematic representation of CD2831; Blue, black and grey lines indicate length of recombinant CD2831 subdomains designed for the study. **B.** Human fibroblasts IMR90 were cultured into collagen I-coated 96 well plates for three days after seeding. Cells were then incubated with serially diluted recombinant CD2831 proteins ranging from 0.08 to 10 µM. Binding of the proteins was detected by using anti-CD2831 and secondary Alexa Fluor antibodies and quantified by a multi-well fluorescent reader. **C.** Dot Blot on 25 and 50 ng of recombinant proteins spotted on nitrocellulose membrane. Proteins were revealed by using rabbit anti-sera raised against the full length recombinant CD2831 diluted 1:500. **D.** Immunofluorescence of 1 µM of CD2831 full-length (rCD2831) (green) binding to collagen (red) produced by IMR90 cells. Collagen staining results from a mix of antibodies against Collagen types I, III and V in a 1:1:1 ratio.

To further define the affinity of CD2831 to collagens types known to be more represented in the gut tissue (Graham et al. 1988), collagen-binding activity of CD2831 full-length and subdomains 1 and 2 (containing single CBD) was further investigated through ELISA test on immobilized collagen type I, III and V. Recombinant proteins in a range of concentrations between 0.15 and 2 µM showed a dose-depending binding activity to Collagens types I, III and V. All the recombinant proteins showed a higher binding affinity to collagen type V, whereas comparable was the affinity to collagens types I and III. Consistent with what observed in the experiments described above with human cells, binding of single CBD (subdomains 1 and 2) revealed to be higher compared to the full-length CD2831 **(Fig. 4.3)**.

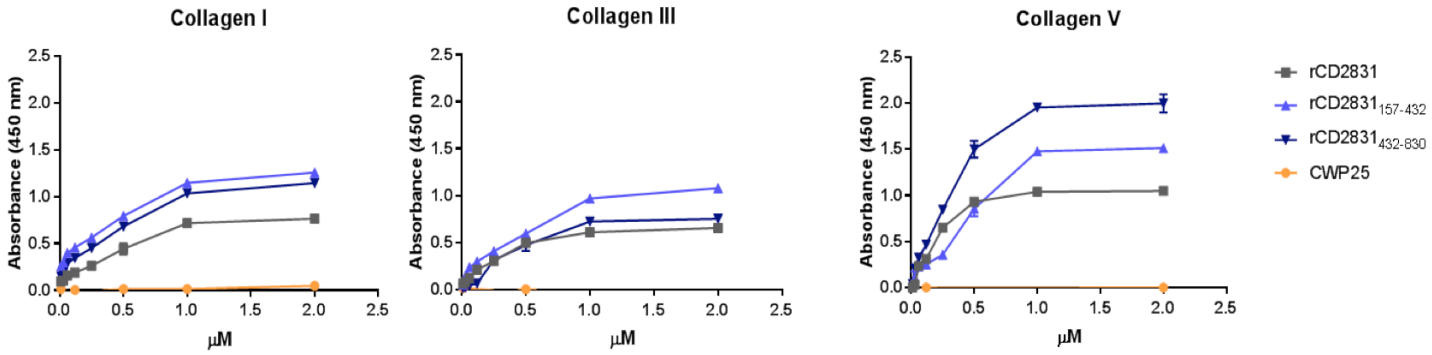

Fig. 4.3 | Binding of recombinant CD2831 full-length protein and subdomains to Collagen.

ELISA plates were coated with 10 µg/ml of purified collagens and incubated with serially diluted recombinant CD2831 proteins ranging from 15 nM to 2 µM. Binding of the proteins was detected by anti-CD2831 polyclonal serum diluted 1:500, followed by HRP-conjugated-secondary antibody. CWP25 protein was used as negative control. CD2831 CBD1 and CD2831 CBD2 represent subdomains 1 and 2 previously described.

4.3 Expression and cleavage of CD2831 in CD630*Δerm*

As previously mentioned, CD2831 presents at its C-terminal a 111 amino acids sequence rich in prolines that is recognized and efficiently cleaved by the endogenous secreted metalloprotease ZmpI which eventually brings to secretion of the protein (Schacherl et al. 2015). In *in vitro* conditions, c-di-GMP levels that inversely regulate CD2831 and ZmpI are almost undetectable and this results in a large protease production with a subsequent low presence of CD2831 in the cell wall (Peltier et al. 2015).

Cell-associated CD2831 levels in a wild type condition were monitored by western blot in a time-course experiment. *C. difficile* 630Δ*erm* was grown overnight in BHIS, bacterial growth was then centrifuged, washed once with PBS to remove all secreted proteins and resuspended in fresh medium. Bacterial samples were collected every 15 minutes and presence of CD2831 in the cell wall was investigated through western blot. As shown in **Fig. 4.4**, after 6 hours of growth CD2831 was barely detectable in the cell wall due to an accumulation of ZmpI in the liquid culture. Upon removal of supernatant with ZmpI (medium refresh), cell-associated CD2831 promptly increased in the first hour to diminishing again as soon as the protease ZmpI accumulates into the supernatant.

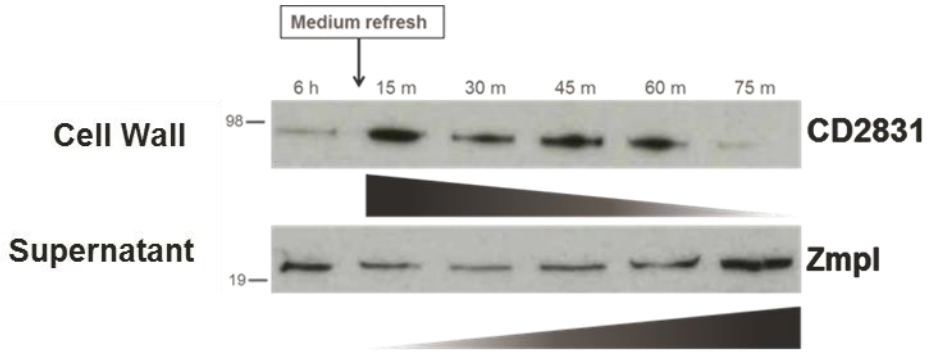

Fig. 4.4 | CD2831 is efficiently cleaved from the *C. difficile* cell wall from the protease ZmpI.

Western blot (WB) on *C. difficile* 630*Δerm* cell wall lysate and culture supernatant (SN). First lane shows presence of CD2831 on the cell wall and ZmpI in the SN after 6 hours of growth in BHIS. Bacteria were then centrifuged and resuspended in fresh BHIS and samples were taken every 15 minutes for 75 minutes and processed for WB to monitoring presence of CD2831 in the cell wall and of ZmpI in the SN. Rabbit CD2831 and mouse ZmpI anti-sera diluted 1:1000 were used to detect proteins of interest followed by secondary HRP-conjugated antibodies diluted 1:2000.

4.4 Generation of CD2831 overexpressing *C. difficile* strain

CD2831 is poorly detected in both the supernatant and in the whole cell lysate of strain 630Δ*erm* due to the very low levels of c-di-GMP in *in vitro* conditions. Stimuli inducing high levels of c-di-GMP *in vitro* are unknown; therefore, in order to investigate the activity of CD2831 in the context of the bacterial cell wall, the gene was constitutively overexpressed in *C. difficile*. Genomic DNA was extracted from wild type 630Δ*erm* and primers designed to amplify the entire 2.9 kb sequence of *CD2831* gene, with the forward primer containing a 28 bp region corresponding to the protein SlpA Ribosome-binding site (RBS). SlpARBS-*CD2831* was then ligated between SacI and SpeI sites of pRPF144 shuttle vector (under the regulation of the constitutive cwp2 promoter) generating pCwp2-2831 (**Fig. 4.5 A**). Plasmids pCwp2-empty and pCwp2-2831 were conjugated from *E. coli* SM10-lambda pir donor strain into 630Δ*erm.* CD2831 expression in cytosolic, supernatant and cell wall fractions was investigated by western blot, flow-cytometry and immunofluorescence. The wild type strain containing only the empty vector presented CD2831 exclusively in the supernatant (Sn), whereas in the overexpressing strain (pCwp2-2831), CD2831 was also detected in the fraction containing cytosol and membranes (CM) and was associated with the cell wall (CW) **(Fig. 4.5 B)**. Cell wall expression of CD2831 was also investigated and confirmed by flow-cytometry and immunofluorescence analysis (**Fig. 4.5 C D**).

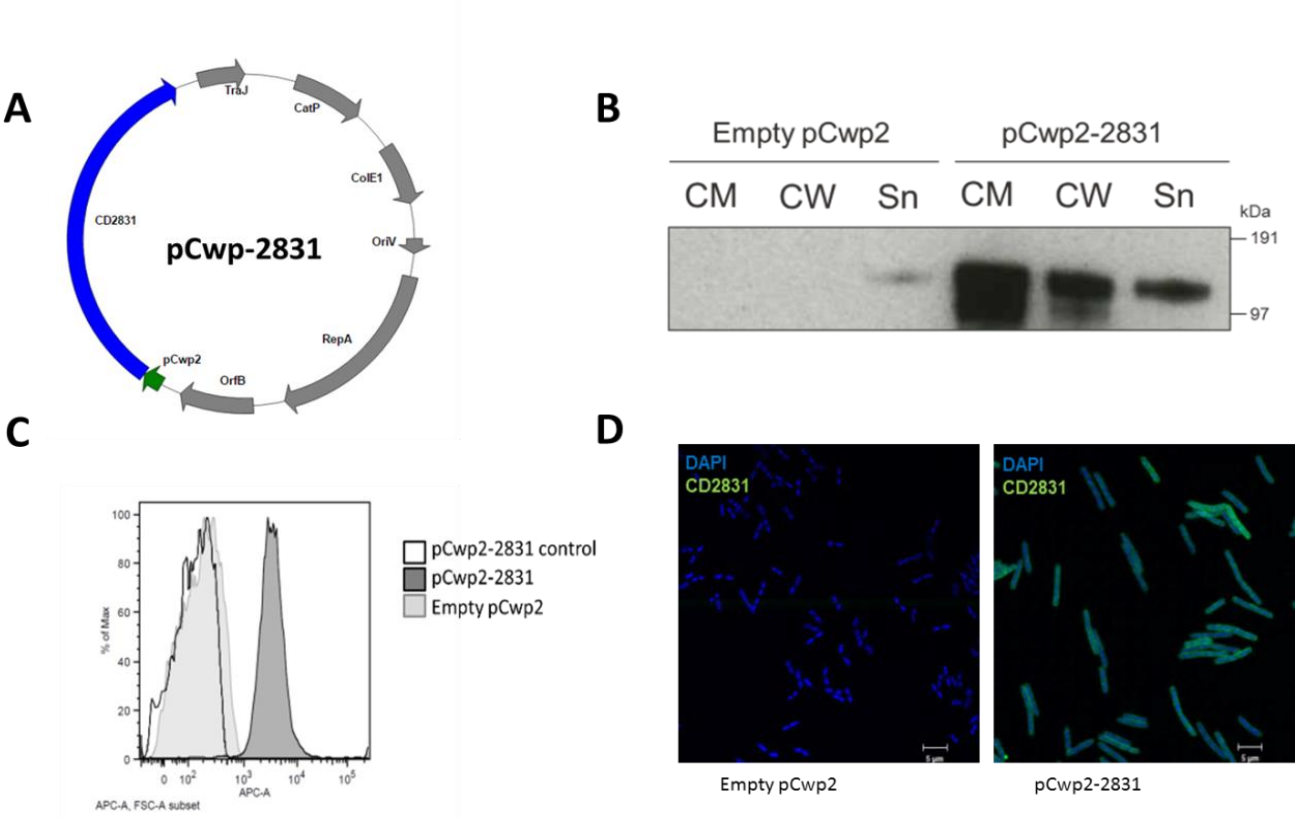

Fig. 4.5| Overexpression of *CD2831* in *C. difficile* 630*Δerm*.

A. Schematic representation of pRPF144 vector used to overexpress CD2831 in *C. difficile*. **B.** *C. difficile* overnight cultures were centrifuged and proteins released in the supernatant (Sn) were TCA precipitated. The Cell wall (CW) was isolated from cytoplasm and membranes (CM) and the three fractions were analyzed by western blot using sera against CD2831. **C.** Flow-cytometry analysis of CD2831 exposure: in light gray is showed the wild type condition (empty pCwp2), in dark gray is the overexpressing condition (pCwp2-2831). The negative black line peak represents the overexpressing strain stained with the only secondary antibody as negative control. **D.** The same bacteria were visualized by immunofluorescence microscopy, where nuclei are stained in blue (DAPI) and CD2831 is stained in green.

4.5 CD2831 overexpression enhances *C. difficile* binding to immobilized collagen and to native collagen produced by human IMR90 cells

The adhesive properties of CD2831 observed in the experiments conducted by using the recombinant proteins were further explored by analyzing binding of 630Δ*erm* constitutively overexpressing CD2831 (pCwp2-2831) to collagen type I, III and V. Therefore, *C. difficile* growth cultures at late-exponential phase (OD_{600} 0.8-1) were diluted and inoculated into collagen-coated plates. Bacteria were allowed to adhere to collagens for one hour in anaerobic conditions. After removal of non-adhering bacteria, wells were fixed and adhesion of *C. difficile* was detected by immunofluorescence, using anti-sera raised against *C. difficile* 630. With these experiments we observed that overexpression of CD2831 confers significantly increased adhesiveness of *C. difficile* to immobilized collagens, compared to the wild type (containing empty pCwp2 vector) (**Fig. 4.6 A**). With similar experimental procedures we also measured CD2831 contribution to adhesion to collagen-producing cells IMR90. Cells were grown on 96 well-plates or chamber-slides for three days and were incubated with 100 µL of a *C. difficile* suspension at OD_{600} 0.2 for 1 hour in anaerobic conditions. Samples were washed and fixed before staining with antibodies raised against *C. difficile* 630. Presence of fluorescent adhering bacteria was evaluated either by confocal microscopy or for quantitative analysis through the use of a fluorescence plate reader. As expected, CD2831 revealed to contribute to *C. difficile* adhesion to native collagen-producing cells as the overexpressing strain showed increased binding to IMR90 compared to the empty vector control (**Fig. 4.6 B**).

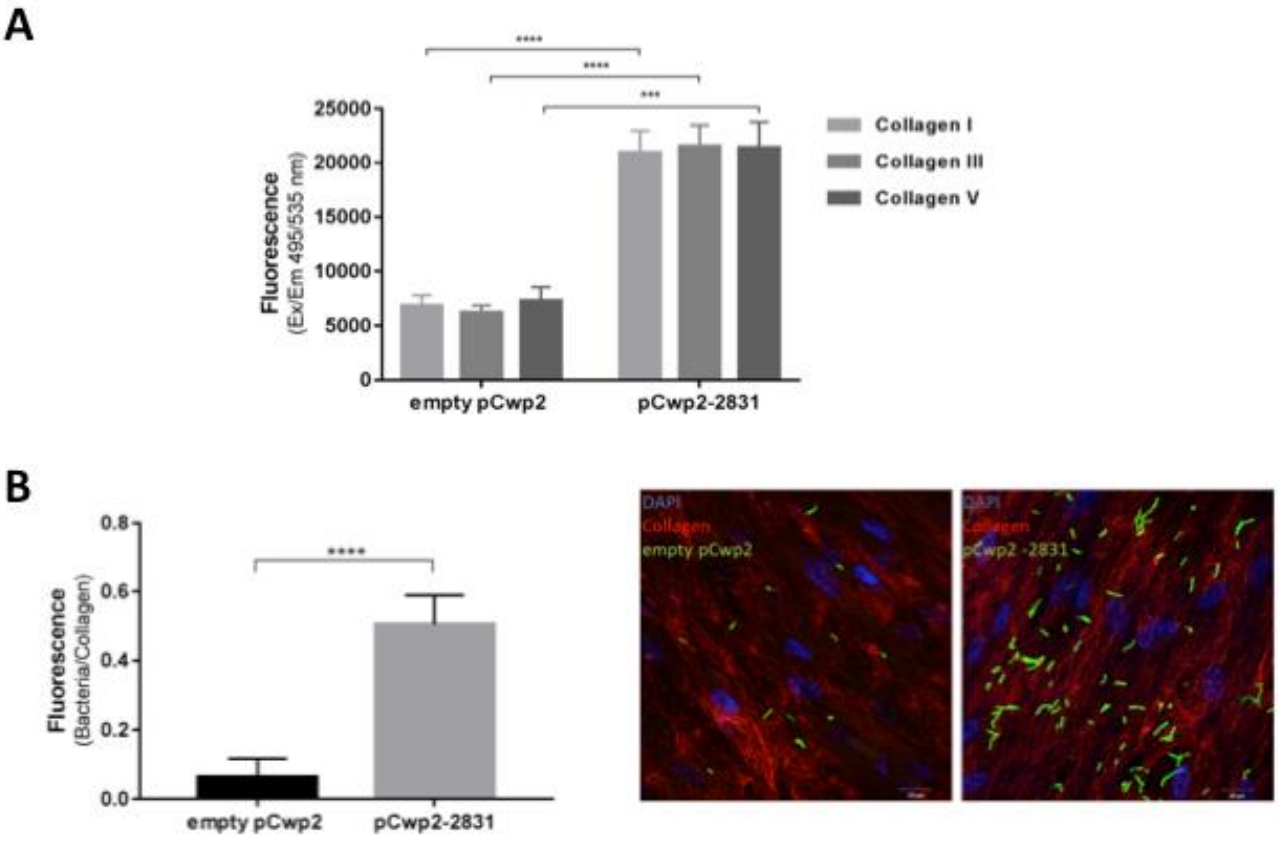

Fig. 4.6| Interaction of CD2831-overexpressing *C. difficile* with collagens and human fibroblasts.

A. Adhesion of *C. difficile* overexpressing CD2831 (pCwp2-2831) and control (empty pCwp2 vector) strains to immobilized collagens type I, III and V after one hour of incubation in anaerobic condition. **B.** (Left panel) Adhesion assay in which *C. difficile* 630Δ*erm* control and CD2831-overexpressing strain were allowed to adhere to IMR90 cells for 1 hour at 37 °C in anaerobic conditions. Bacteria were stained by using an anti-CD630 serum followed by Alexa-fluor conjugated secondary antibody. Levels of collagens and cells-associated bacteria were detected by using a fluorescence plate reader. (Right panel) Confocal images of *C. difficile* 630Δ*erm* control and CD2831-overexpressing strains adhering to the collagen-producing IMR90 cells, after 1 hour incubation at 37 °C in anaerobic conditions.

4.6 Contribution of CD2831 in biofilm formation

As mentioned before, second messenger c-di-GMP has been shown to be involved in controlling *C. difficile* lifestyle switch from motility to sessility. Specifically, this results in the down-regulation of flagella and toxins in favor of an increased expression of adhesins or biofilm-related proteins. As a c-di-GMP-regulated adhesin, we also investigated CD2831 for playing a role in biofilm formation. *C. difficile* constitutively overexpressing CD2831 was compared to a wild type control strain biofilm formation on both collagen-coated plates and abiotic surface in BHIS medium supplemented with 2% Glucose. Biofilm measurement through crystal violet assay showed a two-fold increase in biofilm for the CD2831-overexpressing strain compared to the control, after 24 hours (**Fig. 4.7 A)**. In order to measure biofilm thickness through confocal microscopy, an increased bacterial biomass was required so pCwp2-2831 *C. difficile* and control strains were incubated for three days on glass chamber slides. Nuclei as well as extracellular DNA present in the matrix were stained with DAPI and Z-stack confocal microscopy images confirmed a two-fold increase in thickness of bacterial biofilm (**Fig. 4.7 B**).

To modulate CD2831 expression levels more precisely, plasmid pTet-2831, carrying CD2831 under the control of the pTet-inducible promoter, was constructed and introduced into the wild-type 630*Δerm* strain. Expression was then induced by a range of anhydrotetracycline (ATc) concentrations, and CD2831 levels were determined by immunoblotting. pTet promoter controlling CD2831 was induced with ATc (10, 20, 50 and 100 ng/ml) and biofilm formation was measured after 24-hour of growth on a plastic surface. The biofilm was then measured by Crystal Violet assay, which showed that *C. difficile* biofilm increased proportionally to the expression of CD2831 (**Fig. 4.7 C**).

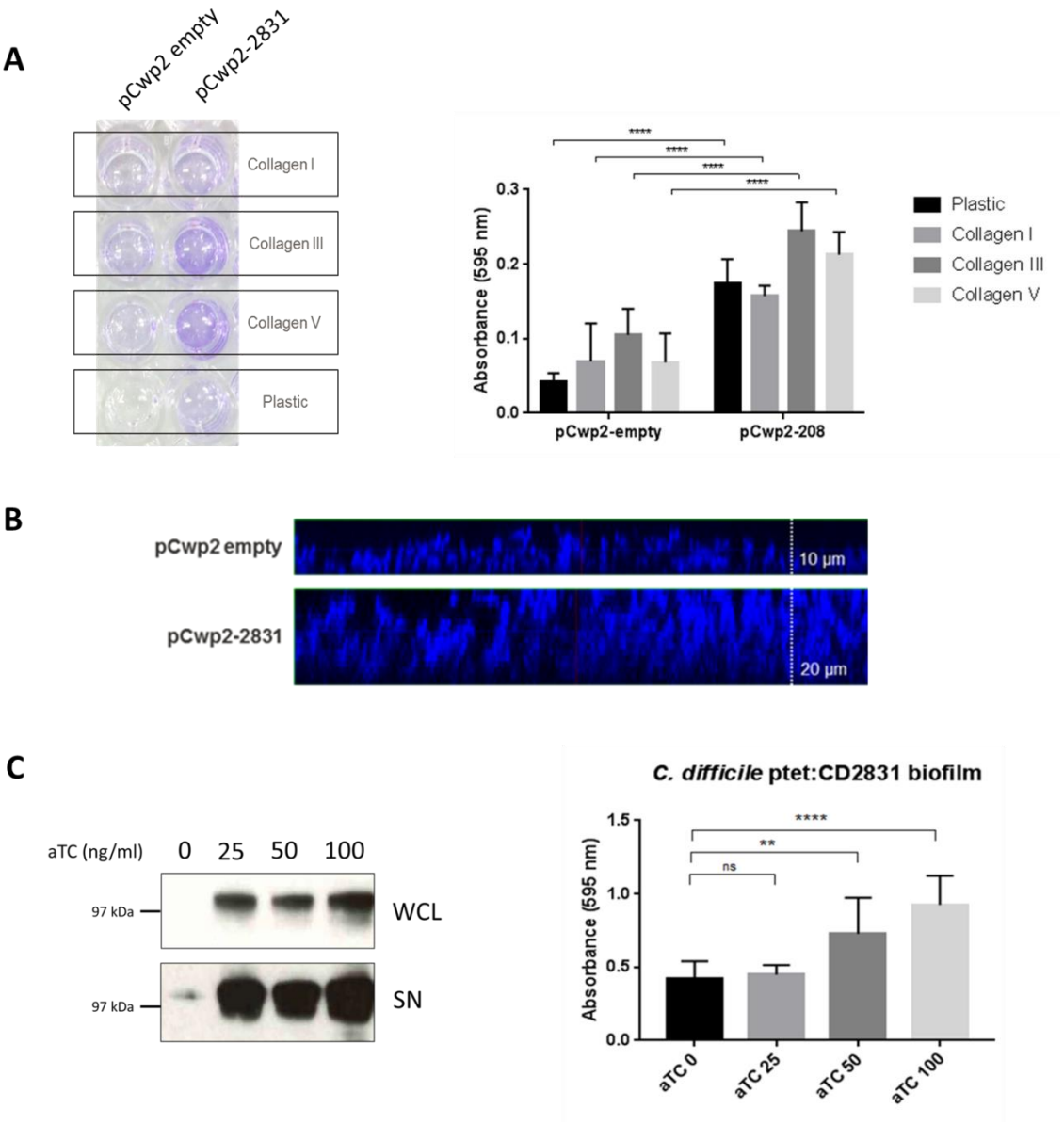

Fig. 4.7 | CD2831 contribution to biofilm formation.

A. Crystal violet assay on *C. difficile* 24 hours biofilm on plastic and collagen-coated plates. Plates were coated overnight at 4°C with 10 µg/ml of collagens in PBS. Bacteria were allowed to grow overnight and cultures were diluted at OD_{600} 0,1 in fresh BHIS + 2% Glucose in plastic and collagens well and biofilm formation was allowed for 24 hours. Absorbance corresponding to crystal violet extracted dye from biomass was measured with a plate reader. **B.** 3 days *C. difficile* biofilm on chamber slides was stained with DAPI and thickness of biofilm Z-stack was measured through confocal microscopy. **C.** WB showing CD2831 expression in bacterial lysates and supernatants of *C. difficile* pTet-2831 after induction with 0, 25, 50 and 100 ng/mL of anhydrotetracycline (aTC) for 24 hours. The right graph shows *C. difficile* pTet-CD2831 biofilm measured by crystal violet assay for 24 hours at the tested aTC concentrations.

4.7 Heterologous expression of CD2831 in *Lactococcus lactis* surface results in an increased adherence to collagen and biofilm formation

A useful tool to demonstrate putative adhesive properties of proteins of interest is the heterologous expression on the surface of *Lactococcus lactis* (Tulli et al. 2013; Buccato et al. 2006; Sinha et al. 2000; Arrecubieta et al. 2007). We therefore constitutively expressed CD2831 on *L. lactis* surface using vector pAM401 and we evaluated the heterologous protein contribution to the adhesion. Protein anchoring to *L. lactis* cells was achieved by substituting the original cell wall anchoring motif of CD2831 with a previously described motif (Edwards et al. 2008) (**Fig. 4.8 A**).

We applied immunofluorescence microscopy and flow-cytometry to confirm that export of CD2831 on *L. lactis* surface was successfully obtained. This allowed us to generate a strain in which CD2831 was exclusively cell wall-associated. Moreover, we were able to observe during bacterial liquid culture an aggregation phenotype in bacteria expressing CD2831 that was observable also by fluorescence microscopy (**Fig. 4.8 B**).

L. lactis expressing CD2831 was investigated for its binding activity to collagen-producing cells and, as observed for *C. difficile*, expression of CD2831 conferred an advantage in binding to IMR90 human fibroblasts. Moreover, a pre-incubation of *L. lactis* with 0,1% CD2831 anti-sera was sufficient to reduce CD2831-expressing *L. lactis*- adhesion to IMR90 reaching the same level as wild type *L. lactis* (**Fig. 4.8 C**).

The *L. lactis* heterologous system was also employed to study the contribution of CD2831 in bacterial biofilm formation. As for *C. difficile, L. lactis* expressing CD2831 and control strains were allowed to grow in biofilm on abiotic surface for 24 hours. Crystal violet assay showed that also in *L. lactis* bacterial background the overexpression of CD2831 contributes to increase biofilm formation (**Fig.** 4.**8 D**).

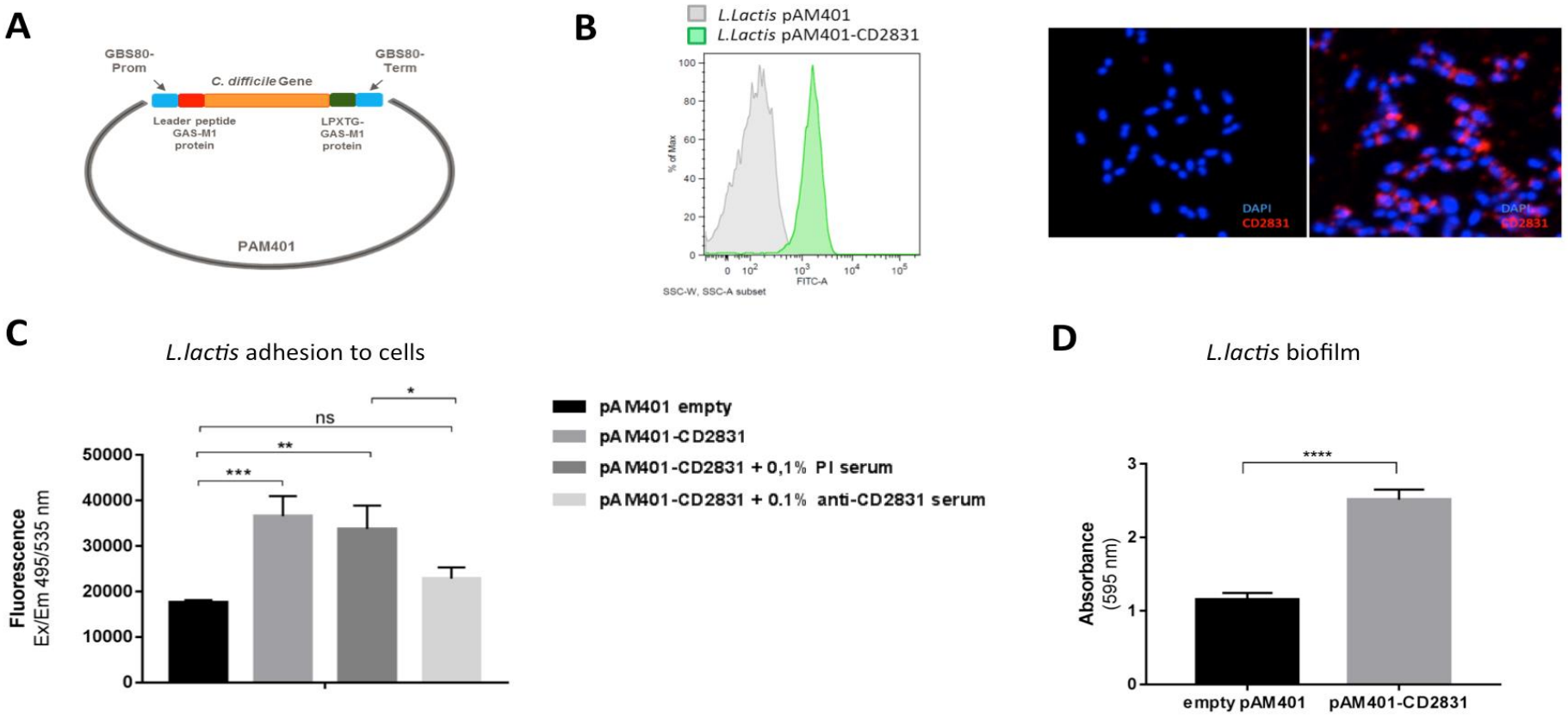

Fig. 4.8| *L. lactis* as heterologous system to study CD2831 adhesive properties.

A. Schematic representation of the vector pAM401 used to express CD2831 on *L. lactis* surface. The sequence coding for the leader peptide and the LPXTG-motif at C-terminal of the *C. difficile* gene are substituted with the same regions of the GAS M1 gene (1–129 bp and 1348–1455 bp respectively), to ensure the surface exposure in *L. lactis*. **B.** Flow-cytometry (left) and fluorescence microscopy (right) of *L. lactis* showing surface exposure of CD2831. **C.** *L. lactis* strains were allowed to adhere to matrix-producing human IMR90 fibroblasts for 1 hour at 37 °C. Data show the contribution of CD2831 in adhesion of *L. lactis* to cells and inhibition of *L. lactis* expressing CD2831 adhesion after pre-incubation of bacteria with 0.1 % CD2831 anti-serum for 1h. **D.** *L. lactis* strains were allowed to grow on biofilm on abiotic surface for 24 hours, bacterial biomass was measured by crystal violet and absorbance at 595 nm was measured.

4.8 CD2831 binds to human complement C1q preventing C1 complex formation

In addition to binding to matrix components, members of MSCRAMMs family from Gram-positive bacteria are also known to bind to human complement C1q and act as inhibitors of the classical complement activation pathway (Kang et al. 2013). As CD2831 can be classified within this family of proteins, we tested recombinant CD2831 full-length protein binding to human C1q. ELISA test on immobilized C1q were performed and a dose-range binding activity of CD2831 towards C1q was registered as shown in figure 4.**9 A**. CD2831/C1q interaction was investigated also through Transmission Electron Microscopy (TEM). CD2831 displays a "comma-like" shape and notably, when incubated in a 1:1 ratio with human C1q, binding of CD2831 to the C1q stalk (also known as "collagen-like" region) was detected (**Fig. 4.9 B**).

Host adaptive immune response against bacteria starts with C1q binding to the Fc region of antibodies surrounding the bacterial surface. This initiates the complement cascade via classical pathway that proceeds with the C3b deposition on the bacterium, allowing its recognition from macrophages. To initiate this immune defense mechanism, C1q stalk must be recognized by two other molecules called C1s and C1r; all together these proteins interact and form the C1 complex, which activates the classical pathway of the complement cascade (**Fig. 4.10 A**). To investigate a possible interference of CD2831 in the C1 complex formation, we performed ELISA tests on immobilized IgM in which we measured C1q, C1s and C1r deposition in presence of different concentration of recombinant CD2831. ELISA tests showed that recombinant CD2831 binding to C1q stalk reduces C1s and C1r deposition on IgM-associated C1q and therefore C1 complex formation is compromised (**Fig. 4.10 B**).

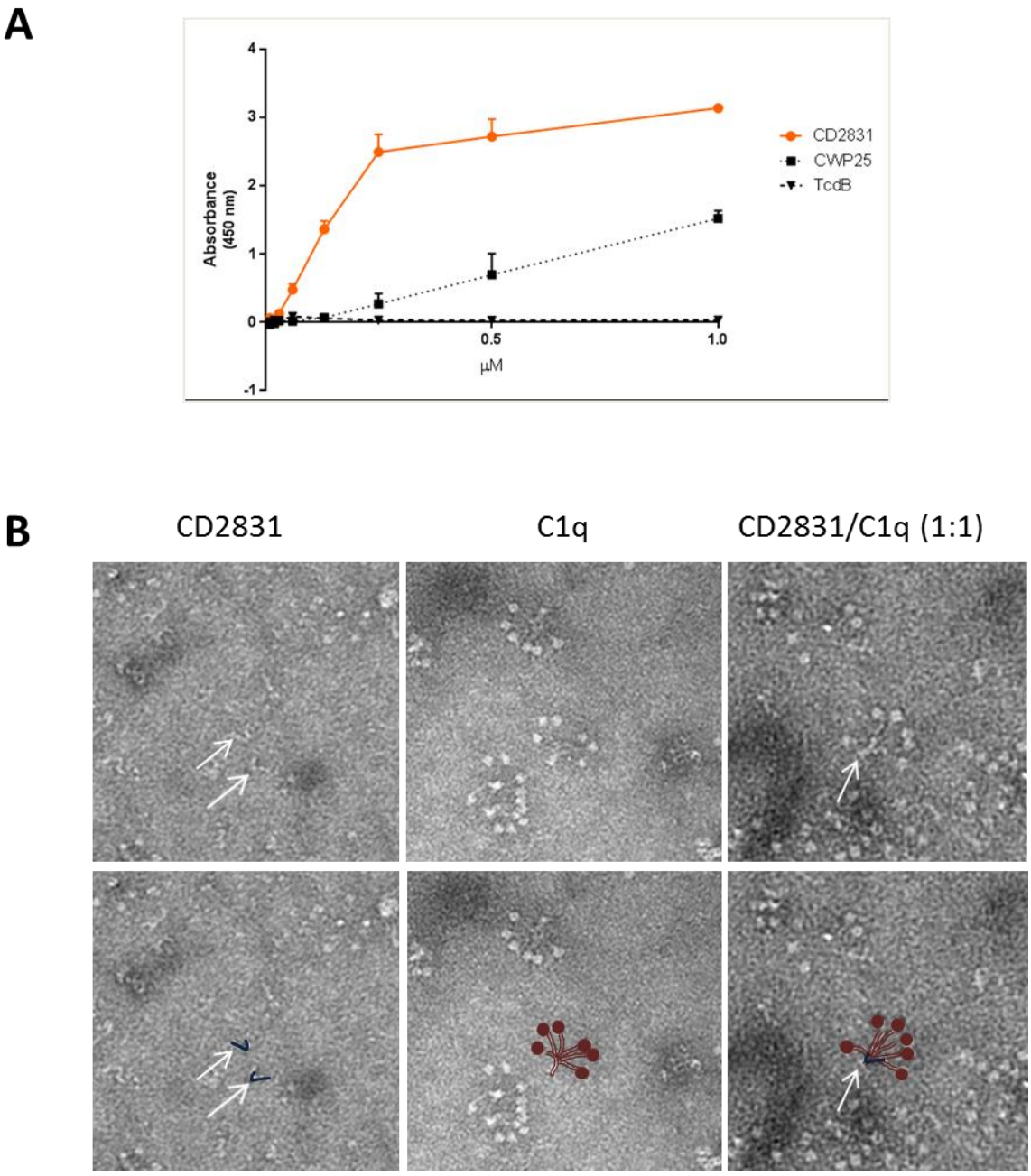

Fig. 9| Interaction of CD2831 with human complement C1q.

A. ELISA plates coated with 10 µg/ml of purified human complement component C1q were incubated with several dilutions of recombinant CD2831 (15 nM-1 µM) for 1 hour. The experiment shows a dose-dependent binding activity of CD2831 to human C1q. *C. difficile* recombinant toxin B and Cwp25 proteins were used as controls. **B.** Transmission Electron Microscopy images showing CD2831 (white arrows), human C1q and CD2831/C1q in 1:1 ratio. The lower panels show drawings of CD2831 (blue) interacting with the stalk region of C1q (red).

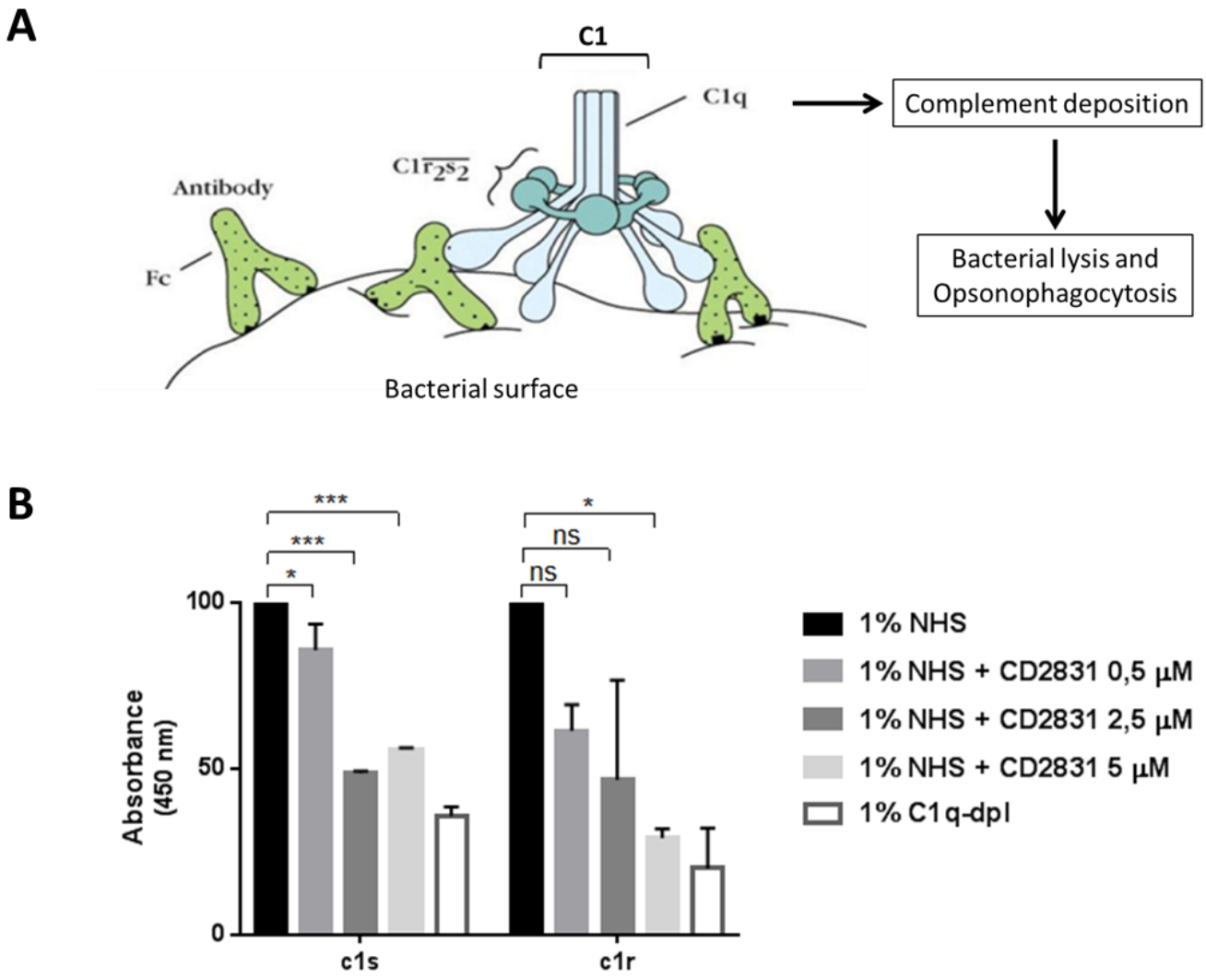

Fig. 4.10| C1 complex formation is abolished in presence of CD2831

A. Drawing reassuming complement activation via classical pathway: C1 complex, consisting of C1q interacting with two molecules of C1s and C1r, initiates the complement cascade. C1q recognizes IgM or certain subclasses of IgG complexed with antigens on the bacterial surface. Such binding of C1q leads to conformational changes in the C1q molecule, which activates the associated C1r molecules. Active C1r cleaves the C1s molecules, activating them. Active C1s splits other components of the cascade, which eventually leads to bacterial opsonization and lysis. **B.** ELISA plates coated with 10 µg/ml purified human IgM were incubated with 1% Normal Human Serum (NHS) and different concentrations of recombinant CD2831. After 1 hour, deposition of human C1s and C1r was measured. 1% C1q-depleted serum (C1q-dpl) was used as negative control.

5 DISCUSSION

Bacterial infections involve binding of pathogens to ligands located on the surface of host cells and in the extracellular matrix (Cue et al. 1998; Talay et al. 2000; Margarit et al. 2009). In an intact healthy tissue, ECM is not exposed to the environment and may not be accessible for interaction with microbial MSCRAMMs. Characteristic of *C. difficile* pathogenesis is the disruption of intestinal epithelial tight junctions caused by the glycosylating toxins TcdA and TcdB with the consequent exposure of the underlying ECM (Pothoulakis 2000). In these conditions, MSCRAMMs can bind to ECM ligands, such as fibronectin or collagen and thus operate as important virulence factors. To date, high-molecular weight S-layer protein (HMW-SLP) and Collagen-binding protein A (CbpA) of *C. difficile* have been described as surface proteins that are able to bind to collagen and that could be implicated in this mechanism (Calabi et al. 2002; Tulli et al. 2013). CD2831 is another collagen-binding protein of *C. difficile* whose presence on the bacterial surface is tightly regulated by the endogenous protease ZmpI that is abundant in low intracellular c-di-GMP levels conditions and efficiently cleaves CD2831, allowing its release in the extracellular milieu.

First, we analyzed CD2831 protein sequence and we located the two predicted collagen-binding domains. Then we cloned and expressed the full-length CD2831, two subdomains containing individual Collagen-binding domain (CBD) and three other CD2831 subdomains containing both CBDs in combination with additional portions of the protein. Therefore we tested the recombinant proteins in collagen-binding assays. Our experiments confirmed CD2831 as a protein able to bind to Collagen type I, III and V and to native collagen produced by human cells and similar results were obtained for subdomains containing both CBDs confirming that the minimal domain mediating the interaction to collagen resides in the two predicted collagen-binding domains. Interestingly, in these experiments, the recombinant proteins presenting the single collagen-binding domain, showed higher affinity for collagen compared to the full length CD2831. Our hypothesys is that this result could be due to a better exposure to the ligand of the collagen-binding domain when the protein is halved.

When grown *in vitro, C. difficile* c-di-GMP levels have been shown to be almost undetectable and so are CD2831 levels on the bacterial cell wall (Hensbergen et al. 2015; Peltier et al. 2015). Physiological stimuli increasing c-di-GMP levels are still unknown but it is clear that it coincides with the switch of *C. difficile* to a sessile lifestyle, as c-di-GMP positively regulates adhesins such as TFP and negatively controls flagellar motility. Presence of bile salts as a signal upstream c-di-GMP regulations has been described for *Vibrio Cholerae* (Koestler and Waters 2014). Nevertheless, incubation of *C. difficile* with bile acids mixtures did not drive c-di-GMP increase in our experimental conditions (data not shown). Overexpression of diguanylate cyclases and genetic depletion of phosphodiesterases are the approaches followed in several studies for increasing c-di-GMP levels *in vitro* in *C. difficile*. However, in this condition the bacterial cell wall will be dramatically changed by the effects of c-di-GMP on many proteins and this could not be the optimal asset for studying single protein functionality. Our approach was to overexpress CD2831 in *C. difficile* and although a considerable amount of the protein was still cleaved by ZmpI and released from the cell wall, collagen-binding activity toward immobilized collagens and native collagen produced by human cells was confirmed by comparing the overexpressing strain with the wild type.

In order to overcome protease cleavage and release of CD2831 by the ZmpI protease, which targets also the putative adhesin CD3246, we employed the heterologous expression of CD2831 in *L. lactis* cell wall and confirmed collagen binding activity of CD2831 also in this strain. The increase of *L. lactis*-CD2831 adhesion to collagen-producing cells was reversed upon addiction of CD2831 anti-sera. Results obtained using the Lactococcus system suggest that also in *C. difficile*, bacterial adhesion to collagen could be mediated by the cell wall-anchored form of CD2831.

Other MSCRAMMs of Gram-positive bacteria, such as the fibronectin-binding protein FbsC of *Group B streptococcus*, have been shown to be involved in host epithelium invasion and in *in vitro* biofilm formation (Buscetta et al. 2014). This, together with positive regulation of CD2831 from c-di-GMP, which also positively modulates *C. difficile* biofilm, drove us to investigate CD2831 as possibly implicated in biofilm formation. We addressed this hypothesis by comparing CD2831-overexpressing strain with *C. difficile* wild type in their ability to form biofilm *in vitro* and we showed that increasing expression of CD2831 raised bacterial biomass formation. Also, we were

able to observe this phenotype in the *L. lactis* expressing CD2831. This could be reasonably explained as result of an increased aggregative phenotype in bacteria overexpressing CD2831. Interestingly, biofilm assay using *C. difficile* overexpressing CD2831 resulted in increased bacterial biomass not only on collagen, but even in not-coated plastic wells. This suggests that CD2831 contribution to biofilm formation is presumably not limited to increase the first attachment to the surface and that this protein plays an active role during the bacterial biofilm construction.

Kang and co-authors recently showed that the MSCRAMM Cna protein from *Staphylococcus aureus* and other similar Gram-positive adhesins, can also interfere with the complement cascade activation by interacting with human complement C1q (Kang et al. 2013). Host adaptive immune response against bacteria starts with C1q binding to the Fc region of antibodies surrounding the bacterial surface. This initiates the complement cascade via classical pathway that proceeds with the C3b opsonization of the pathogen and the associated enhanced phagocytosis. Complement cascade also brings to the generation of chemotaxis factors such as C3a and C5a involved in phagocytes recruitment as well as lysis of the pathogen by formation of the membrane attack complex on the pathogen surface. To initiate this immune defense mechanism, C1q stalk must be recognized by two other molecules called C1r and C1s. Association of these proteins leads to the formation of the C1 complex, which activates the classical pathway of the complement cascade (Wallis et al. 2010). Cna/C1q interaction was shown to inhibit C1r recognition of C1q and complement activation via classical pathway (Kang et al. 2013). In light of the similarity of Cna with our protein of interest, we investigated CD2831 binding to C1q. Besides confirming a dose-range affinity of CD2831 to human C1q, we also visualized the complex by transmission electron microscopy (TEM). Notably, TEM analysis showed CD2831 interacting with the collagen-like domain of the C1q protein stalk, which is the region where the C1r and C1s associate to form the heterotetramer ($C1r_2C1s_2$). We performed ELISA tests using human serum on immobilized IgM, in which we measured C1q, C1s and C1r deposition in presence of different concentration of recombinant CD2831. Whereas C1q recognition of IgM is not altered by the presence of CD2831 (data not shown), we were able to demonstrate that recombinant CD2831 binding to C1q stalk reduced C1s and C1r deposition on IgM-associated C1q. In presence of CD2831, C1 complex

formation is therefore abolished and complement cascade via classical pathway can not be activated.

Importantly, in the late 1980s, a role for C1q independent from complement activation emerged with the observation that immobilized C1q, in the absence of C1r and C1s, triggered an enhancement of FcγR-mediated phagocytosis when targets were coated with a sub-optimal concentration of antibody (Bobak et al. 1987). Moreover, this activity required the collagen-like tail of C1q and extended to enhancement of CR1-mediated phagocytosis (Bobak, Frank, and Tenner 1988). These observations suggest that microbial MSCRAMMs binding to C1q is beneficial even during early stages of infection when production of anti-microbial antibodies is still not efficient and that employment of this mechanism could be crucial for bacteria to evade host immune response.

In conclusion we have characterized the novel collagen-binding protein CD2831 that contributes to bacterial adhesion to the host and to biofilm formation. On the other hand CD2831, with its complement inhibition activity, represents for *C. difficile* a defense weapon from the immune system.

6 REFERENCES

Ali, S., G. Moore, and A. P. Wilson. 2011. 'Spread and persistence of Clostridium difficile spores during and after cleaning with sporicidal disinfectants', *J Hosp Infect*, 79: 97-8.

Arrecubieta, C., M. H. Lee, A. Macey, T. J. Foster, and F. D. Lowy. 2007. 'SdrF, a Staphylococcus epidermidis surface protein, binds type I collagen', *J Biol Chem*, 282: 18767-76.

Baines, S. D., R. O'Connor, K. Saxton, J. Freeman, and M. H. Wilcox. 2009. 'Activity of vancomycin against epidemic Clostridium difficile strains in a human gut model', *J Antimicrob Chemother*, 63: 520-5.

Barbut, F., D. Decre, V. Lalande, B. Burghoffer, L. Noussair, A. Gigandon, F. Espinasse, L. Raskine, J. Robert, A. Mangeol, C. Branger, and J. C. Petit. 2005. 'Clinical features of Clostridium difficile-associated diarrhoea due to binary toxin (actin-specific ADP-ribosyltransferase)-producing strains', *J Med Microbiol*, 54: 181-5.

Bartlett, J. G. 2002. 'Clinical practice. Antibiotic-associated diarrhea', *N Engl J Med*, 346: 334-9.

Bartlett, J. G., N. S. Taylor, T. Chang, and J. Dzink. 1980. 'Clinical and laboratory observations in Clostridium difficile colitis', *Am J Clin Nutr*, 33: 2521-6.

Bobak, D. A., M. M. Frank, and A. J. Tenner. 1988. 'C1q acts synergistically with phorbol dibutyrate to activate CR1-mediated phagocytosis by human mononuclear phagocytes', *Eur J Immunol*, 18: 2001-7.

Bobak, D. A., T. A. Gaither, M. M. Frank, and A. J. Tenner. 1987. 'Modulation of FcR function by complement: subcomponent C1q enhances the phagocytosis of IgG-opsonized targets by human monocytes and culture-derived macrophages', *J Immunol*, 138: 1150-6.

Bordeleau, E., L. C. Fortier, F. Malouin, and V. Burrus. 2011. 'c-di-GMP turn-over in Clostridium difficile is controlled by a plethora of diguanylate cyclases and phosphodiesterases', *PLoS Genet*, 7: e1002039.

Bordeleau, E., E. B. Purcell, D. A. Lafontaine, L. C. Fortier, R. Tamayo, and V. Burrus. 2015. 'Cyclic di-GMP riboswitch-regulated type IV pili contribute to aggregation of Clostridium difficile', *J Bacteriol*, 197: 819-32.

Borriello, S. P. 1998. 'Pathogenesis of Clostridium difficile infection', *J Antimicrob Chemother*, 41 Suppl C: 13-9.

Borriello, S. P., and M. H. Wilcox. 1998. 'Clostridium difficile infections of the gut: the unanswered questions', *J Antimicrob Chemother*, 41 Suppl C: 67-9.

Braun, V., T. Hundsberger, P. Leukel, M. Sauerborn, and C. von Eichel-Streiber. 1996. 'Definition of the single integration site of the pathogenicity locus in Clostridium difficile', *Gene*, 181: 29-38.

Buccato, S., D. Maione, C. D. Rinaudo, G. Volpini, A. R. Taddei, R. Rosini, J. L. Telford, G. Grandi, and I. Margarit. 2006. 'Use of Lactococcus lactis expressing pili from group B Streptococcus as a broad-coverage vaccine against streptococcal disease', *J Infect Dis*, 194: 331-40.

Buckley, A. M., C. Jukes, D. Candlish, J. J. Irvine, J. Spencer, R. P. Fagan, A. J. Roe, J. M. Christie, N. F. Fairweather, and G. R. Douce. 2016. 'Lighting Up Clostridium Difficile: Reporting Gene Expression Using Fluorescent Lov Domains', *Sci Rep*, 6: 23463.

Buscetta, M., S. Papasergi, A. Firon, G. Pietrocola, C. Biondo, G. Mancuso, A. Midiri, L. Romeo, G. Teti, P. Speziale, P. Trieu-Cuot, and C. Beninati. 2014. 'FbsC, a novel fibrinogen-binding protein, promotes Streptococcus agalactiae-host cell interactions', *J Biol Chem*, 289: 21003-15.

Calabi, E., F. Calabi, A. D. Phillips, and N. F. Fairweather. 2002. 'Binding of Clostridium difficile surface layer proteins to gastrointestinal tissues', *Infect Immun*, 70: 5770-8.

Carter, G. P., D. Lyras, D. L. Allen, K. E. Mackin, P. M. Howarth, J. R. O'Connor, and J. I. Rood. 2007. 'Binary toxin production in Clostridium difficile is regulated by CdtR, a LytTR family response regulator', *J Bacteriol*, 189: 7290-301.

Cartman, S. T., M. L. Kelly, D. Heeg, J. T. Heap, and N. P. Minton. 2012. 'Precise manipulation of the Clostridium difficile chromosome reveals a lack of association between the tcdC genotype and toxin production', *Appl Environ Microbiol*, 78: 4683-90.

Corver, J., V. Cordo, H. C. van Leeuwen, O. I. Klychnikov, and P. J. Hensbergen. 2017. 'Covalent attachment and Pro-Pro endopeptidase (PPEP-1)-mediated release of Clostridium difficile cell surface proteins involved in adhesion', *Mol Microbiol*, 105: 663-73.

Crobach, M. J. T., J. J. Vernon, V. G. Loo, L. Y. Kong, S. Pechine, M. H. Wilcox, and E. J. Kuijper. 2018. 'Understanding Clostridium difficile Colonization', *Clin Microbiol Rev*, 31.

Cue, D., P. E. Dombek, H. Lam, and P. P. Cleary. 1998. 'Streptococcus pyogenes serotype M1 encodes multiple pathways for entry into human epithelial cells', *Infect Immun*, 66: 4593-601.

Curry, S. R., J. W. Marsh, C. A. Muto, M. M. O'Leary, A. W. Pasculle, and L. H. Harrison. 2007. 'tcdC genotypes associated with severe TcdC truncation in an epidemic clone and other strains of Clostridium difficile', *J Clin Microbiol*, 45: 215-21.

Dawson, L. F., E. Valiente, A. Faulds-Pain, E. H. Donahue, and B. W. Wren. 2012. 'Characterisation of Clostridium difficile biofilm formation, a role for Spo0A', *PLoS One*, 7: e50527.

Deakin, L. J., S. Clare, R. P. Fagan, L. F. Dawson, D. J. Pickard, M. R. West, B. W. Wren, N. F. Fairweather, G. Dougan, and T. D. Lawley. 2012. 'The Clostridium difficile spo0A gene is a persistence and transmission factor', *Infect Immun*, 80: 2704-11.

Debast, S. B., M. P. Bauer, and E. J. Kuijper. 2014. 'European Society of Clinical Microbiology and Infectious Diseases: Update of the Treatment Guidance Document for Clostridium difficile Infection', *Clinical Microbiology and Infection*, 20: 1-26.

Deneve, C., C. Delomenie, M. C. Barc, A. Collignon, and C. Janoir. 2008. 'Antibiotics involved in Clostridium difficile-associated disease increase colonization factor gene expression', *J Med Microbiol*, 57: 732-8.

Deneve, C., C. Janoir, I. Poilane, C. Fantinato, and A. Collignon. 2009. 'New trends in Clostridium difficile virulence and pathogenesis', *Int J Antimicrob Agents*, 33 Suppl 1: S24-8.

Edwards, A. M., A. G. Manetti, F. Falugi, C. Zingaretti, S. Capo, S. Buccato, G. Bensi, J. L. Telford, I. Margarit, and G. Grandi. 2008. 'Scavenger receptor gp340 aggregates group A streptococci by binding pili', *Mol Microbiol*, 68: 1378-94.

Emerson, J. E., C. B. Reynolds, R. P. Fagan, H. A. Shaw, D. Goulding, and N. F. Fairweather. 2009. 'A novel genetic switch controls phase variable expression of CwpV, a Clostridium difficile cell wall protein', *Mol Microbiol*, 74: 541-56.

Ethapa, T., R. Leuzzi, Y. K. Ng, S. T. Baban, R. Adamo, S. A. Kuehne, M. Scarselli, N. P. Minton, D. Serruto, and M. Unnikrishnan. 2013. 'Multiple factors modulate biofilm formation by the anaerobic pathogen Clostridium difficile', *J Bacteriol*, 195: 545-55.

Fagan, R. P., and N. F. Fairweather. 2011. 'Clostridium difficile has two parallel and essential Sec secretion systems', *J Biol Chem*, 286: 27483-93.

Florin, I., and M. Thelestam. 1983. 'Internalization of Clostridium difficile cytotoxin into cultured human lung fibroblasts', *Biochim Biophys Acta*, 763: 383-92.

———. 1986. 'Lysosomal involvement in cellular intoxication with Clostridium difficile toxin B', *Microb Pathog*, 1: 373-85.

Freeman, J., M. P. Bauer, S. D. Baines, J. Corver, W. N. Fawley, B. Goorhuis, E. J. Kuijper, and M. H. Wilcox. 2010. 'The changing epidemiology of Clostridium difficile infections', *Clin Microbiol Rev*, 23: 529-49.

Gerding, D. N., S. Johnson, M. Rupnik, and K. Aktories. 2014. 'Clostridium difficile binary toxin CDT: Mechanism, epidemiology, and potential clinical importance', *Gut Microbes*, 5: 15-27.

Geric, B., R. J. Carman, M. Rupnik, C. W. Genheimer, S. P. Sambol, D. M. Lyerly, D. N. Gerding, and S. Johnson. 2006. 'Binary toxin-producing, large clostridial toxin-negative Clostridium difficile strains are enterotoxic but do not cause disease in hamsters', *J Infect Dis*, 193: 1143-50.

Geric, B., S. Johnson, D. N. Gerding, M. Grabnar, and M. Rupnik. 2003. 'Frequency of binary toxin genes among Clostridium difficile strains that do not produce large clostridial toxins', *J Clin Microbiol*, 41: 5227-32.

Govind, R., and B. Dupuy. 2012. 'Secretion of Clostridium difficile toxins A and B requires the holin-like protein TcdE', *PLoS Pathog*, 8: e1002727.

Graham, M. F., R. F. Diegelmann, C. O. Elson, W. J. Lindblad, N. Gotschalk, S. Gay, and R. Gay. 1988. 'Collagen content and types in the intestinal strictures of Crohn's disease', *Gastroenterology*, 94: 257-65.

Hennequin, C., A. Collignon, and T. Karjalainen. 2001. 'Analysis of expression of GroEL (Hsp60) of Clostridium difficile in response to stress', *Microb Pathog*, 31: 255-60.

Hennequin, C., C. Janoir, M. C. Barc, A. Collignon, and T. Karjalainen. 2003. 'Identification and characterization of a fibronectin-binding protein from Clostridium difficile', *Microbiology*, 149: 2779-87.

Hennequin, C., F. Porcheray, A. Waligora-Dupriet, A. Collignon, M. Barc, P. Bourlioux, and T. Karjalainen. 2001. 'GroEL (Hsp60) of Clostridium difficile is involved in cell adherence', *Microbiology*, 147: 87-96.

Hensbergen, P. J., O. I. Klychnikov, D. Bakker, I. Dragan, M. L. Kelly, N. P. Minton, J. Corver, E. J. Kuijper, J. W. Drijfhout, and H. C. van Leeuwen. 2015. 'Clostridium difficile secreted Pro-Pro endopeptidase PPEP-1 (ZMP1/CD2830) modulates adhesion through cleavage of the collagen binding protein CD2831', *FEBS Lett*, 589: 3952-8.

Higgins, D., and J. Dworkin. 2012. 'Recent progress in Bacillus subtilis sporulation', *FEMS Microbiol Rev*, 36: 131-48.

Hughes-Jones, N. C., and B. Gardner. 1979. 'Reaction between the isolated globular sub-units of the complement component C1q and IgG-complexes', *Mol Immunol*, 16: 697-701.

Hundsberger, T., V. Braun, M. Weidmann, P. Leukel, M. Sauerborn, and C. von Eichel-Streiber. 1997. 'Transcription analysis of the genes tcdA-E of the pathogenicity locus of Clostridium difficile', *Eur J Biochem*, 244: 735-42.

Janoir, C., S. Pechine, C. Grosdidier, and A. Collignon. 2007. 'Cwp84, a surface-associated protein of Clostridium difficile, is a cysteine protease with degrading activity on extracellular matrix proteins', *J Bacteriol*, 189: 7174-80.

Just, I., J. Selzer, M. Wilm, C. von Eichel-Streiber, M. Mann, and K. Aktories. 1995. 'Glucosylation of Rho proteins by Clostridium difficile toxin B', *Nature*, 375: 500-3.

Just, I., M. Wilm, J. Selzer, G. Rex, C. von Eichel-Streiber, M. Mann, and K. Aktories. 1995. 'The enterotoxin from Clostridium difficile (ToxA) monoglucosylates the Rho proteins', *J Biol Chem*, 270: 13932-6.

Kang, M., Y. P. Ko, X. Liang, C. L. Ross, Q. Liu, B. E. Murray, and M. Hook. 2013. 'Collagen-binding microbial surface components recognizing adhesive matrix molecule (MSCRAMM) of Gram-positive bacteria inhibit complement activation via the classical pathway', *J Biol Chem*, 288: 20520-31.

Kasendra, M., R. Barrile, R. Leuzzi, and M. Soriani. 2014. 'Clostridium difficile toxins facilitate bacterial colonization by modulating the fence and gate function of colonic epithelium', *J Infect Dis*, 209: 1095-104.

Kelly, C. P., S. Becker, J. K. Linevsky, M. A. Joshi, J. C. O'Keane, B. F. Dickey, J. T. LaMont, and C. Pothoulakis. 1994. 'Neutrophil recruitment in Clostridium difficile toxin A enteritis in the rabbit', *J Clin Invest*, 93: 1257-65.

Khan, Fahmi Yousef, and Abdul-Naser Elzouki. 2014. 'Clostridium difficile infection: a review of the literature', *Asian Pacific Journal of Tropical Medicine*, 7: S6-S13.

Kim, J. M., J. S. Kim, H. C. Jun, Y. K. Oh, I. S. Song, and C. Y. Kim. 2002. 'Differential expression and polarized secretion of CXC and CC chemokines by human intestinal epithelial cancer cell lines in response to Clostridium difficile toxin A', *Microbiol Immunol*, 46: 333-42.

Klock, H. E., E. J. Koesema, M. W. Knuth, and S. A. Lesley. 2008. 'Combining the polymerase incomplete primer extension method for cloning and mutagenesis with microscreening to accelerate structural genomics efforts', *Proteins*, 71: 982-94.

Koestler, B. J., and C. M. Waters. 2014. 'Bile acids and bicarbonate inversely regulate intracellular cyclic di-GMP in Vibrio cholerae', *Infect Immun*, 82: 3002-14.

Kovacs-Simon, A., R. Leuzzi, M. Kasendra, N. Minton, R. W. Titball, and S. L. Michell. 2014. 'Lipoprotein CD0873 is a novel adhesin of Clostridium difficile', *J Infect Dis*, 210: 274-84.

Kwon, Jennie H., Margaret A. Olsen, and Erik R. Dubberke. 2015. 'The Morbidity, Mortality, and Costs Associated with Clostridium difficile Infection', *Infectious Disease Clinics of North America*, 29: 123-34.

Lannergard, J., L. Frykberg, and B. Guss. 2003. 'CNE, a collagen-binding protein of Streptococcus equi', *FEMS Microbiol Lett*, 222: 69-74.

Lawley, T. D., S. Clare, A. W. Walker, D. Goulding, R. A. Stabler, N. Croucher, P. Mastroeni, P. Scott, C. Raisen, L. Mottram, N. F. Fairweather, B. W. Wren, J. Parkhill, and G. Dougan. 2009. 'Antibiotic treatment of clostridium difficile carrier mice triggers a supershedder state, spore-mediated transmission, and severe disease in immunocompromised hosts', *Infect Immun*, 77: 3661-9.

Lawley, T. D., N. J. Croucher, L. Yu, S. Clare, M. Sebaihia, D. Goulding, D. J. Pickard, J. Parkhill, J. Choudhary, and G. Dougan. 2009. 'Proteomic and genomic characterization of highly infectious Clostridium difficile 630 spores', *J Bacteriol*, 191: 5377-86.

Maldarelli, G. A., K. H. Piepenbrink, A. J. Scott, J. A. Freiberg, Y. Song, Y. Achermann, R. K. Ernst, M. E. Shirtliff, E. J. Sundberg, M. S. Donnenberg, and E. C. von Rosenvinge. 2016. 'Type IV pili promote early biofilm formation by Clostridium difficile', *Pathog Dis*, 74.

Margarit, I., S. Bonacci, G. Pietrocola, S. Rindi, C. Ghezzo, M. Bombaci, V. Nardi-Dei, R. Grifantini, P. Speziale, and G. Grandi. 2009. 'Capturing host-pathogen interactions by protein microarrays: identification of novel streptococcal proteins binding to human fibronectin, fibrinogen, and C4BP', *FASEB J*, 23: 3100-12.

Martin-Verstraete, I., J. Peltier, and B. Dupuy. 2016. 'The Regulatory Networks That Control Clostridium difficile Toxin Synthesis', *Toxins (Basel)*, 8.

McFarland, L. V., C. M. Surawicz, and W. E. Stamm. 1990. 'Risk factors for Clostridium difficile carriage and C. difficile-associated diarrhea in a cohort of hospitalized patients', *J Infect Dis*, 162: 678-84.

McKee, R. W., N. Aleksanyan, E. M. Garrett, and R. Tamayo. 2018. 'Type IV Pili Promote Clostridium difficile Adherence and Persistence in a Mouse Model of Infection', *Infect Immun*, 86.

McKee, R. W., M. R. Mangalea, E. B. Purcell, E. K. Borchardt, and R. Tamayo. 2013. 'The second messenger cyclic Di-GMP regulates Clostridium difficile toxin production by controlling expression of sigD', *J Bacteriol*, 195: 5174-85.

Miller, B. A., L. F. Chen, D. J. Sexton, and D. J. Anderson. 2011. 'Comparison of the burdens of hospital-onset, healthcare facility-associated Clostridium difficile Infection and of healthcare-associated infection due to methicillin-resistant Staphylococcus aureus in community hospitals', *Infect Control Hosp Epidemiol*, 32: 387-90.

Mitchell, M. J., B. E. Laughon, and S. Lin. 1987. 'Biochemical studies on the effect of Clostridium difficile toxin B on actin in vivo and in vitro', *Infect Immun*, 55: 1610-5.

Moncrief, J. S., L. A. Barroso, and T. D. Wilkins. 1997. 'Positive regulation of Clostridium difficile toxins', *Infect Immun*, 65: 1105-8.

Nakamura, S., K. Yamakawa, J. Izumi, S. Nakashio, and S. Nishida. 1985. 'Germinability and heat resistance of spores of Clostridium difficile strains', *Microbiol Immunol*, 29: 113-8.

Nallapareddy, S. R., G. M. Weinstock, and B. E. Murray. 2003. 'Clinical isolates of Enterococcus faecium exhibit strain-specific collagen binding mediated by Acm, a new member of the MSCRAMM family', *Mol Microbiol*, 47: 1733-47.

Nusrat, A., C. von Eichel-Streiber, J. R. Turner, P. Verkade, J. L. Madara, and C. A. Parkos. 2001. 'Clostridium difficile toxins disrupt epithelial barrier function by altering membrane microdomain localization of tight junction proteins', *Infect Immun*, 69: 1329-36.

Pantaléon, V., M. Monot, C. Eckert, S. Hoys, A. Collignon, C. Janoir, and T. Candela. 2018. 'Clostridium difficile forms variable biofilms on abiotic surface', *Anaerobe*.

Pantaleon, V., A. P. Soavelomandroso, S. Bouttier, R. Briandet, B. Roxas, M. Chu, A. Collignon, C. Janoir, G. Vedantam, and T. Candela. 2015. 'The Clostridium difficile Protease Cwp84 Modulates both Biofilm Formation and Cell-Surface Properties', *PLoS One*, 10: e0124971.

Paredes-Sabja, D., G. Cofre-Araneda, C. Brito-Silva, M. Pizarro-Guajardo, and M. R. Sarker. 2012. 'Clostridium difficile spore-macrophage interactions: spore survival', *PLoS One*, 7: e43635.

Pechine, S., C. Deneve-Larrazet, and A. Collignon. 2016. 'Clostridium difficile Adhesins', *Methods Mol Biol*, 1476: 91-101.

Peltier, J., H. A. Shaw, E. C. Couchman, L. F. Dawson, L. Yu, J. S. Choudhary, V. Kaever, B. W. Wren, and N. F. Fairweather. 2015. 'Cyclic diGMP regulates production of sortase substrates of Clostridium difficile and their surface exposure through ZmpI protease-mediated cleavage', *J Biol Chem*, 290: 24453-69.

Peltier, J., and O. Soutourina. 2017. 'Identification of c-di-GMP-Responsive Riboswitches', *Methods Mol Biol*, 1657: 377-402.

Popoff, M. R., and P. Boquet. 1988. 'Clostridium spiroforme toxin is a binary toxin which ADP-ribosylates cellular actin', *Biochem Biophys Res Commun*, 152: 1361-8.

Pothoulakis, C. 2000. 'Effects of Clostridium difficile toxins on epithelial cell barrier', *Ann N Y Acad Sci*, 915: 347-56.

Poutanen, S. M., and A. E. Simor. 2004. 'Clostridium difficile-associated diarrhea in adults', *CMAJ*, 171: 51-8.

Purcell, E. B., R. W. McKee, E. Bordeleau, V. Burrus, and R. Tamayo. 2016. 'Regulation of Type IV Pili Contributes to Surface Behaviors of Historical and Epidemic Strains of Clostridium difficile', *J Bacteriol*, 198: 565-77.

Purcell, E. B., R. W. McKee, D. S. Courson, E. M. Garrett, S. M. McBride, R. E. Cheney, and R. Tamayo. 2017. 'A Nutrient-Regulated Cyclic Diguanylate Phosphodiesterase Controls Clostridium difficile Biofilm and Toxin Production during Stationary Phase', *Infect Immun*, 85.

Purcell, E. B., R. W. McKee, S. M. McBride, C. M. Waters, and R. Tamayo. 2012. 'Cyclic diguanylate inversely regulates motility and aggregation in Clostridium difficile', *J Bacteriol*, 194: 3307-16.

Purcell, E. B., and R. Tamayo. 2016. 'Cyclic diguanylate signaling in Gram-positive bacteria', *FEMS Microbiol Rev*, 40: 753-73.

Qa'Dan, M., L. M. Spyres, and J. D. Ballard. 2000. 'pH-induced conformational changes in Clostridium difficile toxin B', *Infect Immun*, 68: 2470-4.

Reid, K. B. 1979. 'Complete amino acid sequences of the three collagen-like regions present in subcomponent C1q of the first component of human complement', *Biochem J*, 179: 367-71.

Reid, K. B., J. Gagnon, and J. Frampton. 1982. 'Completion of the amino acid sequences of the A and B chains of subcomponent C1q of the first component of human complement', *Biochem J*, 203: 559-69.

Rich, R. L., B. Kreikemeyer, R. T. Owens, S. LaBrenz, S. V. Narayana, G. M. Weinstock, B. E. Murray, and M. Hook. 1999. 'Ace is a collagen-binding MSCRAMM from Enterococcus faecalis', *J Biol Chem*, 274: 26939-45.

Romling, U., M. Y. Galperin, and M. Gomelsky. 2013. 'Cyclic di-GMP: the first 25 years of a universal bacterial second messenger', *Microbiol Mol Biol Rev*, 77: 1-52.

Samore, M. H., K. M. Bettin, P. C. DeGirolami, C. R. Clabots, D. N. Gerding, and A. W. Karchmer. 1994. 'Wide diversity of Clostridium difficile types at a tertiary referral hospital', *J Infect Dis*, 170: 615-21.

Schacherl, M., C. Pichlo, I. Neundorf, and U. Baumann. 2015. 'Structural Basis of Proline-Proline Peptide Bond Specificity of the Metalloprotease Zmp1 Implicated in Motility of Clostridium difficile', *Structure*, 23: 1632-42.

Schroeder, M. S. 2005. 'Clostridium difficile--associated diarrhea', *Am Fam Physician*, 71: 921-8.

Sebaihia, M., B. W. Wren, P. Mullany, N. F. Fairweather, N. Minton, R. Stabler, N. R. Thomson, A. P. Roberts, A. M. Cerdeno-Tarraga, H. Wang, M. T. Holden, A. Wright, C. Churcher, M. A. Quail, S. Baker, N. Bason, K. Brooks, T. Chillingworth, A. Cronin, P. Davis, L. Dowd, A. Fraser, T. Feltwell, Z. Hance, S. Holroyd, K. Jagels, S. Moule, K. Mungall, C. Price, E. Rabbinowitsch, S. Sharp, M. Simmonds, K. Stevens, L. Unwin, S. Whithead, B. Dupuy, G. Dougan, B. Barrell, and J. Parkhill. 2006. 'The multidrug-resistant human pathogen Clostridium difficile has a highly mobile, mosaic genome', *Nat Genet*, 38: 779-86.

Seddon, S. V., I. Hemingway, and S. P. Borriello. 1990. 'Hydrolytic enzyme production by Clostridium difficile and its relationship to toxin production and virulence in the hamster model', *J Med Microbiol*, 31: 169-74.

Semenyuk, E. G., V. A. Poroyko, P. F. Johnston, S. E. Jones, K. L. Knight, D. N. Gerding, and A. Driks. 2015. 'Analysis of Bacterial Communities during Clostridium difficile Infection in the Mouse', *Infect Immun*, 83: 4383-91.

Sinha, B., P. Francois, Y. A. Que, M. Hussain, C. Heilmann, P. Moreillon, D. Lew, K. H. Krause, G. Peters, and M. Herrmann. 2000. 'Heterologously expressed Staphylococcus aureus fibronectin-binding proteins are sufficient for invasion of host cells', *Infect Immun*, 68: 6871-8.

Soavelomandroso, A. P., F. Gaudin, S. Hoys, V. Nicolas, G. Vedantam, C. Janoir, and S. Bouttier. 2017. 'Biofilm Structures in a Mono-Associated Mouse Model of Clostridium difficile Infection', *Front Microbiol*, 8: 2086.

Sorg, J. A., and A. L. Sonenshein. 2008. 'Bile salts and glycine as cogerminants for Clostridium difficile spores', *J Bacteriol*, 190: 2505-12.

Soutourina, O. A., M. Monot, P. Boudry, L. Saujet, C. Pichon, O. Sismeiro, E. Semenova, K. Severinov, C. Le Bouguenec, J. Y. Coppee, B. Dupuy, and I. Martin-Verstraete. 2013. 'Genome-wide identification of regulatory RNAs in the human pathogen Clostridium difficile', *PLoS Genet*, 9: e1003493.

Stubbs, S., M. Rupnik, M. Gibert, J. Brazier, B. Duerden, and M. Popoff. 2000. 'Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of Clostridium difficile', *FEMS Microbiol Lett*, 186: 307-12.

Sudarsan, N., E. R. Lee, Z. Weinberg, R. H. Moy, J. N. Kim, K. H. Link, and R. R. Breaker. 2008. 'Riboswitches in eubacteria sense the second messenger cyclic di-GMP', *Science*, 321: 411-3.

Surowiec, D., A. G. Kuyumjian, M. A. Wynd, and C. E. Cicogna. 2006. 'Past, present, and future therapies for Clostridium difficile-associated disease', *Ann Pharmacother*, 40: 2155-63.

Talay, S. R., A. Zock, M. Rohde, G. Molinari, M. Oggioni, G. Pozzi, C. A. Guzman, and G. S. Chhatwal. 2000. 'Co-operative binding of human fibronectin to Sfbl protein triggers streptococcal invasion into respiratory epithelial cells', *Cell Microbiol*, 2: 521-35.

Tao, L., J. Zhang, P. Meraner, A. Tovaglieri, X. Wu, R. Gerhard, X. Zhang, W. B. Stallcup, J. Miao, X. He, J. G. Hurdle, D. T. Breault, A. L. Brass, and M. Dong. 2016. 'Frizzled proteins are colonic epithelial receptors for C. difficile toxin B', *Nature*, 538: 350-55.

Thelestam, M., and M. Bronnegard. 1980. 'Interaction of cytopathogenic toxin from Clostridium difficile with cells in tissue culture', *Scand J Infect Dis Suppl*: 16-29.

Thielens, N. M., F. Tedesco, S. S. Bohlson, C. Gaboriaud, and A. J. Tenner. 2017. 'C1q: A fresh look upon an old molecule', *Mol Immunol*, 89: 73-83.

Tucker, K. D., and T. D. Wilkins. 1991. 'Toxin A of Clostridium difficile binds to the human carbohydrate antigens I, X, and Y', *Infect Immun*, 59: 73-8.

Tulli, L., S. Marchi, R. Petracca, H. A. Shaw, N. F. Fairweather, M. Scarselli, M. Soriani, and R. Leuzzi. 2013. 'CbpA: a novel surface exposed adhesin of Clostridium difficile targeting human collagen', *Cell Microbiol*, 15: 1674-87.

Underwood, S., S. Guan, V. Vijayasubhash, S. D. Baines, L. Graham, R. J. Lewis, M. H. Wilcox, and K. Stephenson. 2009. 'Characterization of the sporulation initiation pathway of Clostridium difficile and its role in toxin production', *J Bacteriol*, 191: 7296-305.

Varga, J. J., B. Therit, and S. B. Melville. 2008. 'Type IV pili and the CcpA protein are needed for maximal biofilm formation by the gram-positive anaerobic pathogen Clostridium perfringens', *Infect Immun*, 76: 4944-51.

Vohra, P., and I. R. Poxton. 2011. 'Comparison of toxin and spore production in clinically relevant strains of Clostridium difficile', *Microbiology*, 157: 1343-53.

Voth, D. E., and J. D. Ballard. 2005. 'Clostridium difficile toxins: mechanism of action and role in disease', *Clin Microbiol Rev*, 18: 247-63.

Wallis, R., D. A. Mitchell, R. Schmid, W. J. Schwaeble, and A. H. Keeble. 2010. 'Paths reunited: Initiation of the classical and lectin pathways of complement activation', *Immunobiology*, 215: 1-11.

Wheeldon, L. J., T. Worthington, A. C. Hilton, T. S. Elliott, and P. A. Lambert. 2008. 'Physical and chemical factors influencing the germination of Clostridium difficile spores', *J Appl Microbiol*, 105: 2223-30.

Wilson, K. H., M. J. Kennedy, and F. R. Fekety. 1982. 'Use of sodium taurocholate to enhance spore recovery on a medium selective for Clostridium difficile', *J Clin Microbiol*, 15: 443-6.

Wright, A., R. Wait, S. Begum, B. Crossett, J. Nagy, K. Brown, and N. Fairweather. 2005. 'Proteomic analysis of cell surface proteins from Clostridium difficile', *Proteomics*, 5: 2443-52.