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CbpA: a novel surface exposed adhesin of *Clostridium difficile* targeting human collagen

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1. Abstract

Clostridium difficile is the leading cause of antibiotic-associated diarrhea and pseudomembranous colitis. While toxins A and B are primarily responsible for the pathogenesis, determinants of bacterial adherence are also essential for intestinal colonization process. We focused our study on a novel member of the MSCRAMM family, named CbpA (Collagen binding protein A) for its adhesive properties towards these components of the extracellular matrix (ECM). We demonstrate that CbpA, which carries an LPXTG-like cell wall anchoring domain, is expressed on the bacterial surface of *C. difficile* and that the recombinant form binds at high affinity to collagen I and V (apparent K_D in the order of 10^{-8} and 10^{-9} M respectively). Such evidences were confirmed by confocal microscopy studies showing the association of the protein with Type I and V collagen fibers produced in human fibroblasts and mouse intestinal tissues. However, the collagen binding activity of the wild-type *C. difficile* 630 strain was indistinguishable to *cbpA* knock-out strain. To overcome this apparent clostridial adherence redundancy, we engineered a *Lactococcus lactis* strain for the heterologous expression of CbpA. By this approach, we were able to demonstrate that when exposed on the surface of *L. Lactis*, CbpA significantly enhances the ability of the bacterium to interact with collagen and to adhere to ECM-producing cells. To prove the specificity of the interaction, we showed that the binding activity of *L. lactis*-CbpA strain was prevented by pre-incubating bacteria with an antiserum raised against CpbA. The results reported in this study suggest a role for CpbA in the adherence to intestinal submucosa during *C. difficile* colonization of the gut.

2. Introduction

2.1 Clostridium difficile: emergence of a significant human pathogen

Clostridium difficile is a Gram-positive, spore-forming, obligate anaerobic, rod-shaped bacterium. Originally named *Bacillus difficilis*, it was originally isolated in 1935 by Hall and O'Toole, as part of neonatal intestinal flora. Later on, in 1978, *C. difficile* was identified as the agent of antibiotic-associated pseudomembranous colitis (PMC) (Larson *et al.*, 1978) and is now recognized as the major cause of hospital-acquired diarrhea. *C. difficile* infection (CDI) is primarily associated with the administration of antibiotics, most famously clindamycin, but particularly broad spectrum of penicillin, third generation cephalosporins and fluoroquinolones (Bartlett, 2006).

CDI symptoms range from mild self-limiting diarrhea without appreciable lesions through colitis, PMC and finally, if left untreated, CDI culminates with colonic perforation, toxin megacolon, prolonged ileus, ascites and death (Kelly and LaMont, 1998). *C. difficile* is responsible for 15-25% of the cases of Antibiotic-Associated Diarrhea (AAD) and for almost cases of antibiotic-associated pseudomembranous colitis (more than 95%) with a mortality rate of approximately 6% (Barbut *et al*., 2007; Bartlett, 2009).

CDI develops in patient after hospitalization and antibiotic treatment, as *C. difficile* can colonize the gut if the normal intestinal flora is disturbed or absent. Although elderly hospitalized patients receiving

antibiotics are still the main group at risk of infection (**fig 2.1**), an increase in CDI in younger populations with no previous contact with the hospital environment is emerging (Rupnik *et al*., 2009).

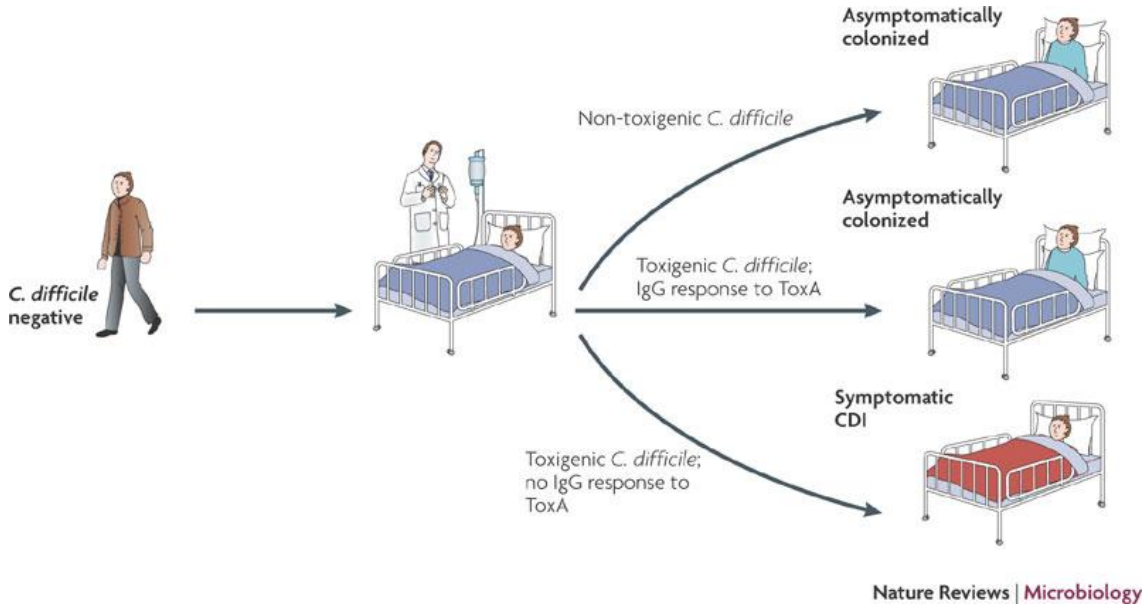


Fig. 2.1 | **Model for the acquisition of *Clostridium difficile* infection (CDI).** Patients are exposed to *C. difficile* spores through contact with the hospital environment or health care workers. After taking an antibiotic, they develop CDI if they acquire a toxigenic *C. difficile* strain and fail to mount an anamnestic serum immunoglobulin G (IgG) antibody response to toxin A (ToxA; also known as TcdA); if they can mount an antibody response they become asymptomatically colonized with *C. difficile*. If they acquire a non-toxigenic *C. difficile* strain, they also become asymptomatically colonized. Colonized patients have been shown to be protected from CDI (Rupnik *et al.*, 2009).

In late 2002 in Quebec, Canada, there was a marked increase noted in the incidence and severity of CDI from a baseline of 6 cases per 1000 admissions, with an attributable mortality of 1.5% reported in 1997, to 22.5 cases per 1000 admissions, with an attributable mortality of 6.9% in 2003 (Loo *et al.*, 2005). A similar increase in the incidence of CDI in the United Stated was also observed (McDonald *et al*., 2005). This increase was found to be associated with the emergence of a new strain of *C. difficile*, alternatively designated as BI, NAP1, or ribotype 027 toxinotype III. This hypervirulent strain

produced increased levels of toxins A and B, as well as an additional toxin, named binary toxin. This previously uncommon strain now has become epidemic, and has been reported in populations that previously has been thought to be at low risk, including peripartum women and healthy persons living in the community (Hookman and Barkin, 2009). Delay in diagnosing and treating CDAD are due to the fact that it can mimic the more common antibiotic associated diarrhea that is not caused by *C. difficile*. Thus diarrhea from C. difficile will be ascribed to other causes: food poisoning, viral infection, *Klebsiella pneumonia, Candida* species and *Staphylococcus aureus.* Patients can be infected with this microorganism and may have no symptoms of colitis. These asymptomatic carriers become vectors during outbreaks and can transmit the pathogen to other susceptible patients.

The standard assay for CDI is the detection of *C. difficile* toxin stool. However, the limitation of this test is the time required for results (24-48 hours), the work intensity and cost (Bartlett, 2006). Other tests include enzyme immunoassays to detect toxin A and B, that take less time to produce a result. Alternative methods include *C. difficile* detection by culture, by PCR testing, by analysis of the "common antigen" of *C. difficile* (glutamate dehydrogenase - GDH – detection) (Bartlett. 2006).

Patients with CDI are usually treated by stopping current antibiotic therapy (if possible) and then treating with metronidazole or oral vancomycin, as presented in 2012 SHEA/IDSA guidelines. Metronidazole is usually recommended as a first line treatment whereas vancomycin is reserved for severe CDI. No highly effective treatment has been found for severe complicated CDI, and if medical management with intravenous fluids, vasosoppressor, oral vancomycin, intravenous metronidazole and vancomycin enemas is not effective, surgical removal of the colon can be the only remaining life-saving measure (Lamontagne *et al*., 2007).

The major complication of CDI is relapses, which can occur in about 20% of patients treated with standard therapy (Bartlett, 2006). Usually patients with multiple recurrences of CDI typically respond to this treatment, but then diarrhea symptoms resume within days to week after therapy is stopped.

Between 20% and 50% of these recurrences are caused by new *C. difficile* organisms, indicating reinfection rather than a relapse of the original infection (Bartlett, 2006). No consensus exists regarding the treatment of relapses but several approaches have been proposed, including probiotics (Bartlett, 2009) in combination with standard therapy. Another alternative is a tapering and pulse dosing of vancomycin given every third day in the hope of keeping *C. difficile* from regrowing while the normal flora recovers. The most effective treatment for these patients has been replenishment of the normal bacterial flora with a faecal transplant delivered either by nasogastric tube or by enema (Aas *et al.*, 2003). Trasmission of *C. difficile* occours by the faecal-oral route after transient contamination of the hands of healthcare workers and patients. Vegetative cells die within minutes after exposure to air, however, the spores are extremely resistant to most disinfectants and can survive up to 70 dalys in a hospital room after a patien with CDI has been discharged (Rupnik *et al*., 2009). *C. difficile* spores are persistent (they last for months to years) and difficult to eradicate, so they are a challenge to hospital control of infections. Several methodologies have been shown to reduce their persistence (Cohen *et al.*, 2010). Hand hygiene is essential for controlling CDI infections. Spores are resistant to alcohol, so it is recommended that healthcare workers use soap and water in presence of high or increasing rates of CDI. Contact precautions and single rooms are recommended for infected patients. Appropriate cleaning of the environment and medical equipment is useful in preventing CDI. Cleaning with stronger chlorine solutions seems to more effectively rid an area of *C. difficile* spores that less-concentrated solutions.

2.2 *C. difficile* physiopathology

CDI typically occurs in hospitalized patients when the exposure to antibiotics treatment distrupted normal microbiota. Due to its anaerobic nature, *C. difficile* spores are recognized as the vector of

transmission, infection and persistence (Deneve *et al*., 2009). *C. difficile* spores are ubiquitously found in the environment but they are highly abundant in the surface of clinical setting, because patients with CDI infection enter in a contagious super-shedder state excreting high levels of infective spore (Fawley *et al*., 2007). After contamination by *C. difficile* spores, they pass through the stomach and, once exposed to bile acids germinate in vegetative cells in the small bowel (Poutanen and Simor, 2004) (**fig. 2.2**).

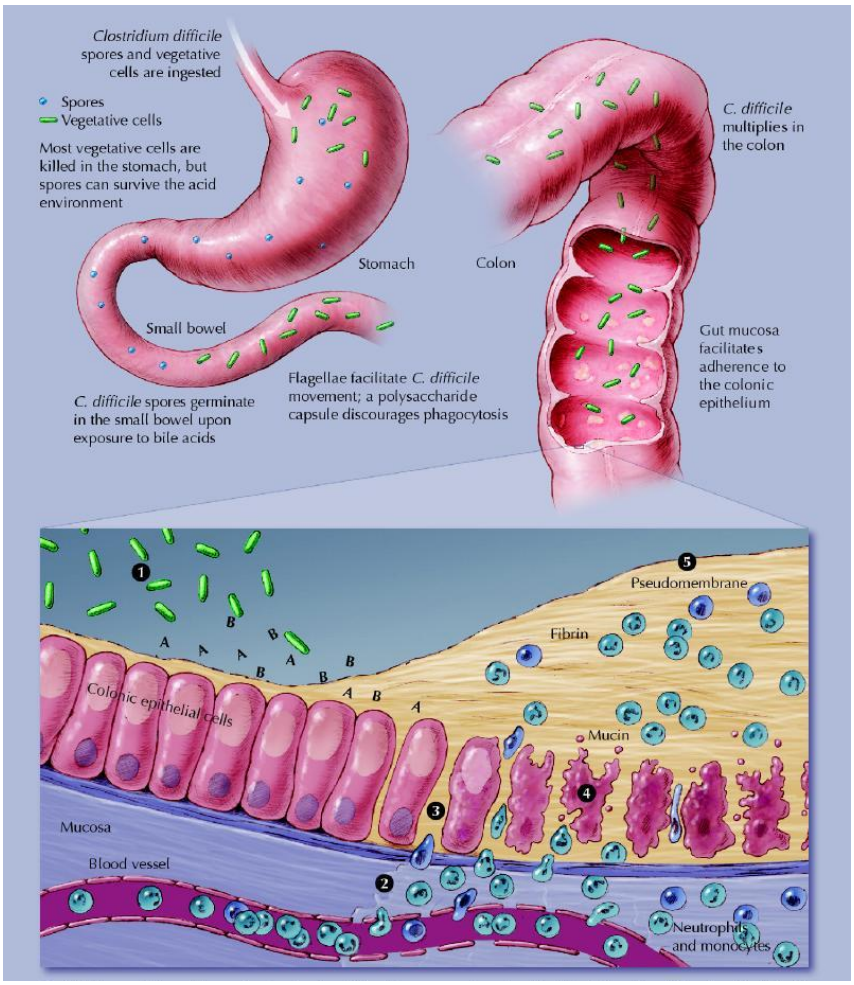


Fig. 2.2 | **Pathogenesis of C. difficile.** *C. difficile* vegetative cells produce toxins A and B and hydrolytic enzymes (**1**). Local production of toxins A and B leads to production of tumour necrosis factor-alpha and proinflammatory interleukins, increased vascular permeability, neutrophil and monocyte recruitment (**2**), opening of epithelial cell junctions (**3**) and epithelial cell apoptosis (**4**). Local production of hydrolytic enzymes leads to connective tissue degradation, leading to colitis, pseudomembrane formation (**5**) and watery diarrhea (Poutanen and Simor, 2004).

The primary bile salts produced by the liver consist mainly of cholate (CA) and chenodeoxycholate (CDCA) that can further conjugated with glycine or taurine (Sorg *et al.,* 2009). When the primary bile salts pass in the lower bowel, they are deconjugated by the normal flora. CA - and CA derivates - and glycine act as co-germinants and are sufficient to stimulate germination and outgrowth of *C. difficile* spores (Sorg *et al.,* 2008). Recent studies also demonstrated that *C. difficile* spore germination can be inhibited *in vitro* and *in vivo* by chenodeoxycholate and its derivates, that acts as competitive inhibitor of CA and its derivates with approximatately ten-times greater affinity than CA (Sorg *et al.,* 2009). Despite the identification of germinants and how they modulate germination of *C. diffcile* spores in the host's intestinal tracts, the identity of GRs remains unknown. There is kinetic evidence for a taurocholate and glycine GRs that suggests that these germinants bind to their receptors through a complex mechanism where the affinity of GRs for one co-germinant is affected by binding of the other (Ramirez *et al.,* 2010). However, extensive bioinformatics analyses have failed to identify GR homologs in the *C. difficile* genome, so further research is needed. Once germinants bind to these receptors, some signal must be traduced to downstream effectors. The nature of this event has not been identified. After germination, *C. difficile* evades immune responses, multiplies and produces toxins A and B, that are the major virulence factors responsible for the clinical symptoms and signs of CDI (Thelestam and Chaves-Olarte, 2000).

2.3 *C. difficile* virulence factors

2.3.1 Toxins

In the late 1970s, it was recognized that C. difficile toxins A (TcdA) and B (TcdB), are involved in AAD and PMC after colonization and proliferation of the pathogen in the human gut (Belyi and

Aktories, 2009). TcdA and TcdB are encoded together with TcdR (known as TcdD), which is an alterantive sigma factor that is involved in positive trascriptional regulation, TcdC, a negative regulator, and TcdE, a protein that has a similarity to phage holins, in a well-defined genetic element, the Pathogenicity locus (PaLoc) (**fig. 2.3**) (Rupnik *et al*., 2005). The PaLoc is present in the same chromosomal integration site in all the toxigenic *C. difficile* strains that have been analyzed to date. In non-toxigenic ($TcdA^-$ $TcdB^-$) strains, the locus is replaced by 115bp of non-coding sequence. The DNA sequence of the PaLoc is variable, and strains with changes in this region are defined as different toxinotypes (Rupnik, 2008). TcdA and TcdB are produced during the late log and stationary phases, and their production depends on the strain and environmental factors, such as nutrient levels, temperature and the presence of sub-inhibitory levels of antibiotics.

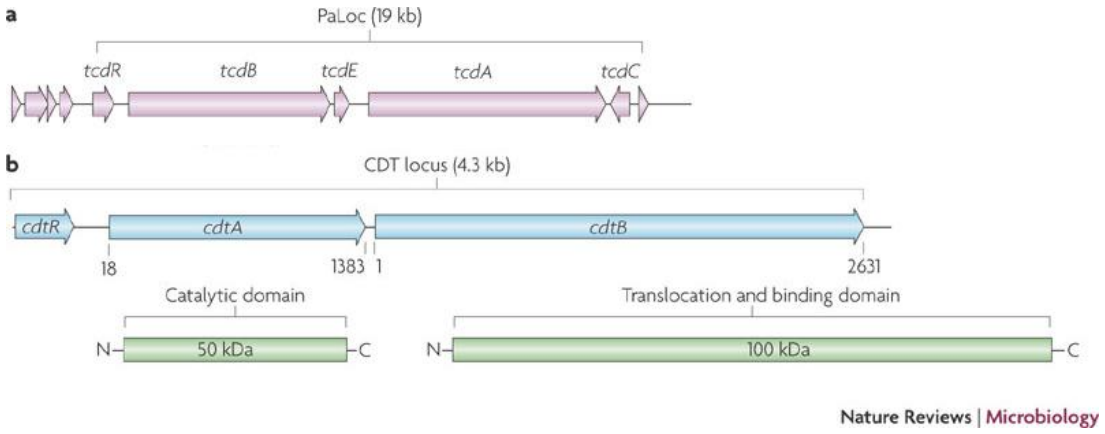


Fig. 2.3 | Toxins produced by Clostridium difficile. a) Two large toxins, toxin A and toxin B (TcdA and TcdB), are encoded on the pathogenicity locus (PaLoc), which comprises five genes. In non-toxigenic strains, this region is replaced by a short 115 bp sequence. **b)** A third toxin, the binary toxin or CDT, is encoded on a separate region of the chromosome (CdtLoc) and comprises three genes. The binary toxin is composed of two unlinked proteins, CdtB and CdtA. CdtB has a binding function and CdtA is the enzymatic component (Rupnik et al., 2009).

They are single-chain toxins of 308 and 270 kDa, respectively. They belong to the group of large clostridial toxins (LCT), that show a glucosyltrasferase (GT) activity and present a least four domains "ABCD", which are involved in biological activity (A-domain), receptor binding (B-domain), auto-proteolytic cleavage during toxin-processing (C-domain) and delivery of the A-domain into the cytosol (D-domain) (Belyi &Aktories, 2009) (**fig 2.4**). The B-domain contains polypeptide repeats called Combined Repetitive Oligopeptides (CROPs) and is located at the C-terminus of the toxins (Genth *et al*., 2008).

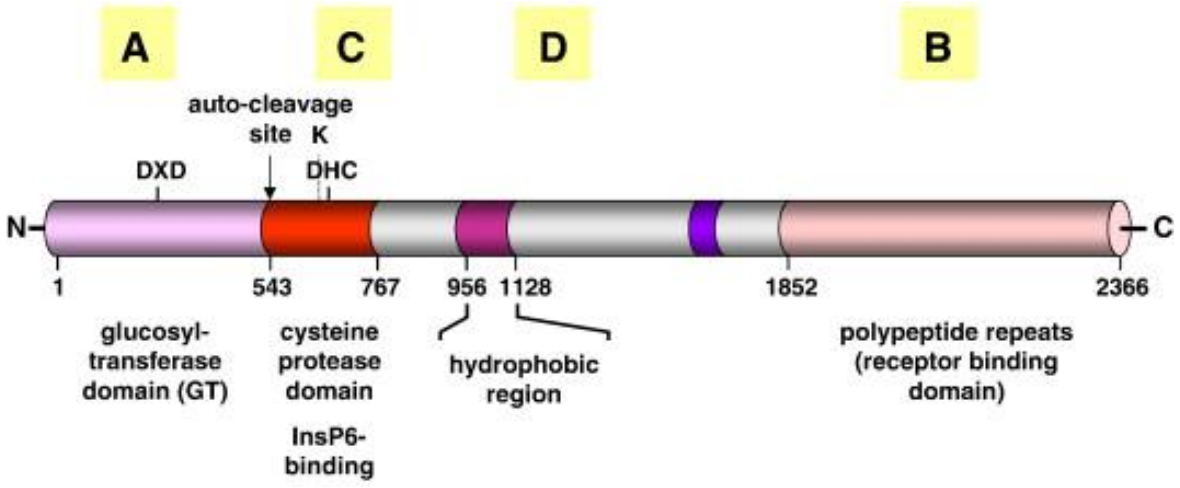


Fig. 2.4 | ABCD-model of clostridial glucosylating toxins. The clostridial glucosylating toxins are constructed of at least 4 domains. The A-domain covers the glucosyltransferase activity (note, *C. novyi* α-toxin possesses GlcNAcylation activity). The B-domain consisting of polypeptide repeats is involved in receptor-binding. The C-domain is responsible for the auto-catalytic cleavage of the toxins (arrow: cleavage site) and is a cysteine-protease with the catalytic residues DHC. Lysine-600 (K) was identified to be involved in InsP6-binding. InsP6 is necessary for activation of the cysteine protease. The D-domain is likely involved in the delivery of the A-domain into the cytosol. This domain contains a hydrophobic region (indicated) suggested to be important for insertion of the toxin into endosome membranes (Belvyi and Aktories, 2009).

The receptors of both toxins are unknown, but studies have shown that CROPs exhibit homology to carbohydrate-binding regions of glycosyltransferases. TcdA binds to carbohydrates, including α-Gal.(1,3)- β-Gal-(1,4)-β-GlcNAc on rabbit erythrocytes and hamster brush border membranes (Greco *et al*., 2006). While undefined, the receptor of toxin B seems to be ubiquitous (Voth and Ballard, 2005). After binding to their cellular receptors, toxins are taken up by endocytosis and end up in early acidic endosomes, from where only the active domains (A-domains) are traslocated into the cytosol (Belvyi and Aktories, 2009).

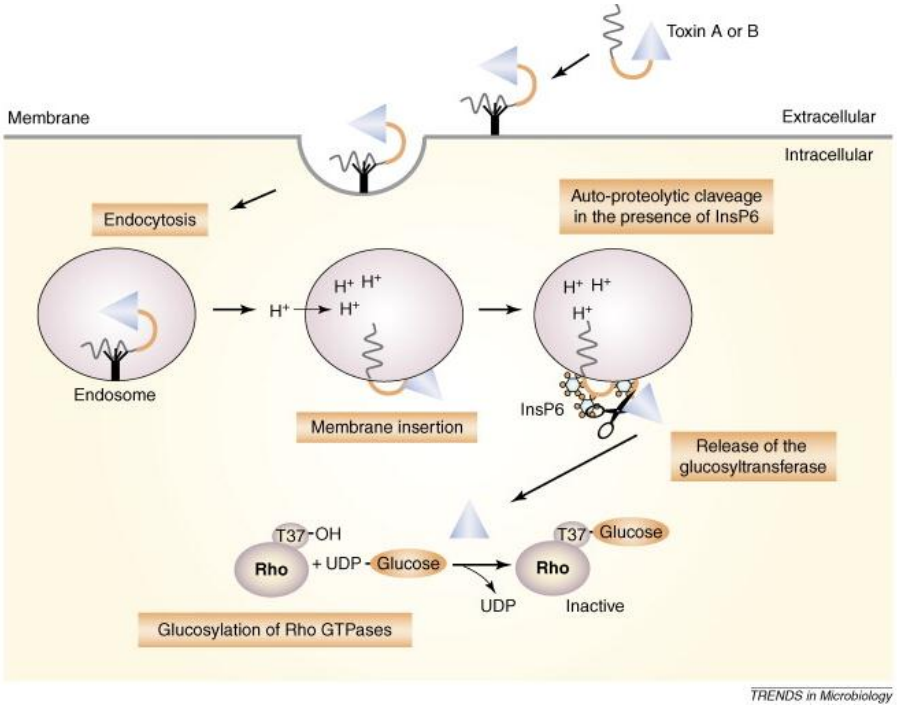


Fig. 2..5 | Processing of clostridial glucosylating toxins. Clostridial glucosylating toxins bind to the cell membrane through the C terminus. After binding, the toxins are endocytosed to reach an acidic endosomal compartment. Here, conformational changes occur enabling insertion of the toxin into the endosomal membrane and subsequent pore-formation. The toxins are processed by autocatalytic cleavage, which in the case of toxin B depends on the presence of inositol hexakisphosphate (InsP6). Only the glucosyltransferase domain of the N terminus of the toxins is released into the cytosol. In the cytosol, Rho GTPases are glucosylated at Thr37 (e.g. RhoA) or at Thr35 (e.g. Rac or Cdc42) (Jank and Aktories, 2008).

The autocatalytic cleavage of toxins occurs by a cysteine protease activity involving the C-domain, directly downstream of the glucosyltransferase domain (Egerer *et al*., 2007). The cysteine protease activity is enhanced by the inositol hexakisphosphate (InsP6) that binds the C-domain causing a conformational change inducing the auto-catalytic activity (Egerer *et al*., 2009). InsP6 is an eukaryotic host factor involved in the regulation of a large variety of the eukaryotic cells processes in the cytoplasm. The D-domain had been suggested to partecipate in the formation of transmembrane structure during pore formation, leading to the traslocation of toxins into the cytosol (Voth and Ballard, 2005). The traslocation mechanism would be dependent on the low pH of endosomes (**fig. 2.5**).

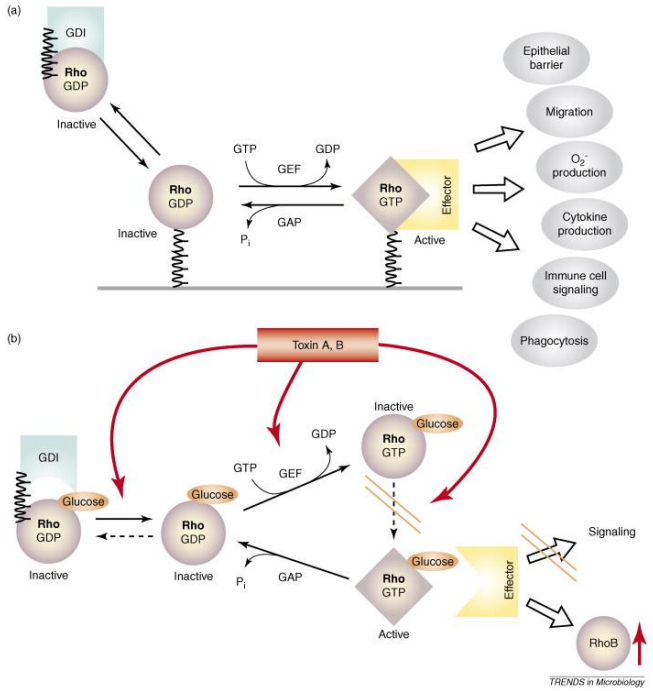


Fig. 2.6 | Rho sognalling pathway. a) Regulation and signaling of Rho proteins and **b)** functional consequences of the modification of Rho proteins by clostridial glucosylating toxins (Jank andAktories, 2008).

Once in the cytosol, TcdA and TcdB glucosylate small GTPases of the Rho and Ras superfamily (Just et al., 1995a-b). Small GTPases proteins in eukaryotic cells are involved in signal transduction associated with apoptosis and the maintenance and regulation of the cell actin cytoskeleton. They bind and hydrolyze GTP to GDP and it is the alternation between the GTP-bound (active, membrane associated) and GDP-bound (inactive, cytosol) states that regulates their activity (**fig. 2.6**). Glucosylation of Rho or Ras GTPases inhibits their interaction with their effectors, loss of this regulation at cellular level results in shrinking and rounding of cells via retraction of neurite-like fibers and finally, cell death (Voth and Ballard, 2005; Jank and Aktories, 2008). Cordially, this cytoskeletal perturbation and loss of cell polarity results in breakdown of tight junctions, resulting in increased paracellular permeability of mucosal surfaces

The gut damage and toxin induce inflammation results in CDI symptoms. In presence of the toxins, there is massive infiltration of neutrophils, the production and release of various inflammatory mediators from mast cells and macrophages, which includes prostaglandins, leukotrienes, IL8 nd TNFα. Moreover toxin A activates NF-κB, with subsequent release of various cytokines. Similarly, intestinal epithelia cells and macrophages are activated to release macrophage inflammatory protein 2 (MIP2). Toxins A and B also seem to activate intestinal nerves to release the neuropeptide substance P, which has a proinflammatory properties (Aktories and Babieri, 2005; Genth *et al*. 2008).

In addition to toxins A and B, some *C. difficile* strains, including 027 epidemic strains, produce a binary toxin CDT, that belongs to the clostridial binary toxins, a group of toxins that are unrelated to TcdA and TcdB, and is composed of two subunits, CdtA and CdtB. CdtB binds to host cells and traslocates CdtA, the catalytic component, into the cytosol where it ADP-rybosylates actin molecules. The genes that encodes for CdtA and CdtB are located in the binary toxin locus (CdtLoc), together with another gene, that codify for the regulatory factor CdtR (Carter *et al*., 2007) (**fig. 2.3**). The role of CDT

in disease is not well understood, it is demonstrated that wild-type strains that produce CDT but do not produce TcdA or TcdB colonize but do not kill hamsters (Geric et al., 2006). Recent studies have also shown that CDT induces the redistribution of microtubules and formation of long microtubule-based protrusions at the surface of intestinal epithelial cells, increasing the adherence of *C. difficile* (Schwan et al., 2009).

2.3.2 Colonization and adhesion factors

After germination, *C. difficile* must be implanted in the gut and attached to epithelial cells, which are protected by a layer of dense mucus, in order to induce its pathogenicity (Pechine *et al*., 2007). *C. difficile* can adhere to the mucus, the first barrier encountered during colonization, thanks to flagellar components such as cap protein (FliD), and flagellin (FliC), which possesses immunogenic properties (Tasteyre *et al.*, 2001).

Surface layer proteins (SLPs) are additional potential virulence factors. These proteins mediate adherence to the mucus and/or to the intestinal epithelial cells. They are within the S-layer, a paracristalline proteinaceous array, in the outer cell surface that coats the bacterium. The S-layer is formed of two proteins: the high-molecular-weight P47 (HMW) and the low-molecular-weight P36 (LMW). Both subunits are encoded by the *slpA* gene and are produced by posttranslational cleavage of the SlpA precursor (**fig. 2.7b**). After the cleavage, the resulting HMW and LMW SLPs interact via defined domains to form a stable heteromeric complex. Interestingly, while the HMW SLP is localized in the internal surface of the bacteria and is immunologically conserved, the LMW SLP localized to the external surface of the bacteria and is immunologically variable, suggesting a possible role of LMW in evasion of immune system (Fagan *et al.*, 2009) (**fig. 2.7a**).

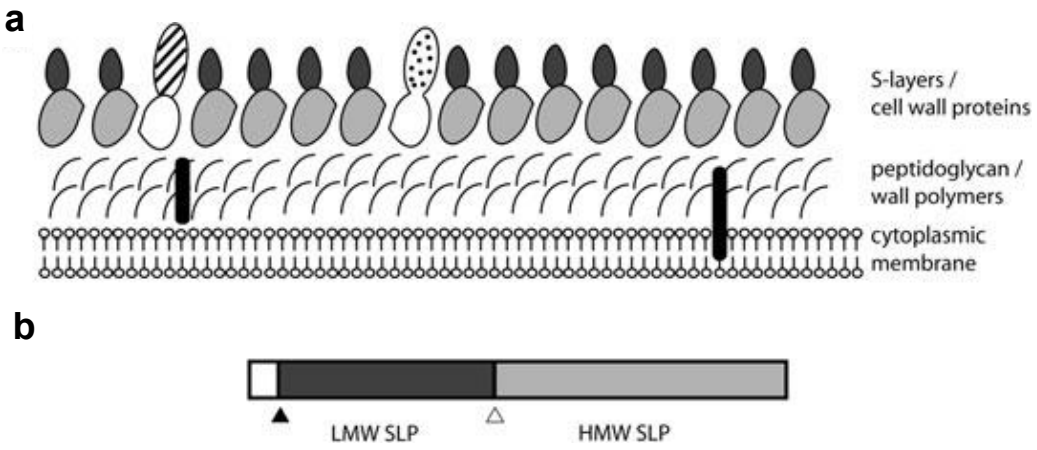


Fig. 2.7 | Model of the cell wall of *C. difficile*. a) The two SLPs are shown above the peptidoglycan layer: the HMW SLP (light grey), the LMW SLP (dark grey). Other minor cell wall proteins are shown as two-lobed structures (white); the filled areas, indicating domains predicted to be exposed to the environment, are variable between these proteins. Putative cell wall polymers (Ganeshapillai *et al*., 2008) including putative lipid-containing polymers (Poxton and Cartmill, 1982) are shown as vertical black bars. **b)** The precursor protein SlpA showing the cleavage sites generating the signal peptide (▲) and the mature HMW SLP and LMW SLP (△) (Fagan et al., 2009).

Indeed, the hypervirulent C. difficle 027 and 001 strains have a LMW SLP variant with unique immunogenic properties (Spigaglia *et al.,* 2011). Interestingly, HMW P47 shows strong and specific binding to gastrointestinal tissues of both human and mouse and some extracellular matrix proteins, like type I collagen, thrombospondin and vitronectin (Emerson *et al*., 2009; Janoir *et al*., 2007), yet the precise host receptor has not been identified.

Besides the SLP, *C. difficile* strain 630 genome contains 28 paralogs of HMW SLP, which are known as the family of cell wall protein (CWP). All of the factors that belong to this family contain up to three copies of the cell wall-binding motif and a variable motif that might be function-specific (Sebaihia *et*

al., 2006). Among these paralogs, there are the Cwp66 and CwpV, which have been fairly well studied. The C-terminal of Cwp66 seems mediate *in vitro* adherence to Vero cells; the CwpV is regulated by phase variation and had the ability to autoaggregate forming denser and more randomly packed cellular organizations (Reynolds *et al*., 2011). Moreover, *C. difficile* displays some surface proteolytic proteins that seem to contribute to the release of essential nutrients and therefore promote establishment of the pathogen in the gut (Seddon *et al.*, 1990). In particular the most characterized protease of *C. difficile*, is Cwp84, a cysteine protease, which degrades several extracellular matrix components thus contributing to the disruption of host tissue integrity (Janoir et al., 2007). Another putative cysteine protease, Cwp13, shares 62% amino acid similarity to Cwp84, but play an auxiliary role in the assembly of the S-layer (De La Riva et al., 2011). Unfortunately, the lack of information on the other CWPs paralogs make difficult to understand the role of these cell surface proteins in the pathogenesis of *C. difficile*.

Among the additional surface proteins, the heat shock protein GroEL has shown to have a role in adherence. Immunoelectron microscopy reveals that is localized in the spore surface, predominantly membrane bound, and released extracellularly after heat shock (Hennequin *et al*., 2001).

Finally, several pathogens are able to use extracellular matrix (ECM) components through bacterial adhesins localized on the cell surface. ECM, in addition to serving a structural function in tissues, is required for multiple cellular functions during development such us cell adhesion, proliferation and migration. *C. difficile* is able to interact with several proteins of ECM, such as fibronectin, fibrinogen, laminin and Collagen I, III, IV and V (Cerquetti *et al*., 2002), suggesting that vegetative cells use them as molecular bridges to increase their adherence to the colonic mucosa. One of the *C. difficile* ECM-binding factors that is well studied is the fibronectin-binding protein Fbp68. Studies demonstrated the ability of this protein to bind fibronectin in manganese-dependent manner (Lin *et al.*, 2011), fibrinogen and, to a lesser extent, vitronectin (Hennequin *et al*., 2003).

2.4 MSCRAMMs family

The class of cell surface adhesins that specifically interact with ECM components is designed as MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules). First, to be classified as a component of this family, the adhesin must be localized to the microbial cell surface. Second, the microbial component must recognize a component of ECM. Third, this interaction must be of high affinity and specificity. A single MSCRAMM can bin several ECM ligands: for example the collagen-binding adhesin YadA of enteropathogenic yersiniae, can also bind laminin and fibronectin (Emody *et al*., 1989). In addition, a microorganism can express several MSCRAMMs that recognize the same ECM components: for example, *Staphylococcus aureus* express several fibrinogen-binding molecules (Boden and Flock, 1989). Usually, the surface proteins in Gram-positive bacteria present at the C-terminal an LPXTG or LPXTG-like motif, located just outside a hydrophobic segment that is the membrane-spanning domain, which is necessary for the anchoring to the cell wall peptidoglycan by specific enzymes, the sortases. At N-terminal, following the signal peptide, there are the domains involved in the interaction with ECM (Patti *et al.*, 1994). Several studies demonstrated that the fibronectin-binding MSCRAMMs on Gram-positive bacteria have structures that are similar overall, although the amino acid sequences may vary considerably. The ligand-binding domains are localized to segment composed of motifs repeated several times and positioned just outside the cell-wall spanning domains (Patti *et al.*, 1994). The collagen-binding MSCRAMMs present repeated domain in the portion of the protein that protrudes outside the bacterial surface and the most characterized collagen-binding MSCRAMM is the *S. aureus* CNA protein. It contains from the N-terminus, a signal peptide, an A domain followed by 1–4 B repeats, the cell-wall anchoring region, a transmembrane segment, and a short cytoplasmic tail. The A domain is responsible for binding to several types of collagen, whereas the B repeats have no known effect on binding (**Fig. 2.8**) (Patti *et al*., 1993). The function of the B

repeats is unknown, but this region is thought to form a stalk that displays the A domain on the cell surface (Rich *et al*., 1998).

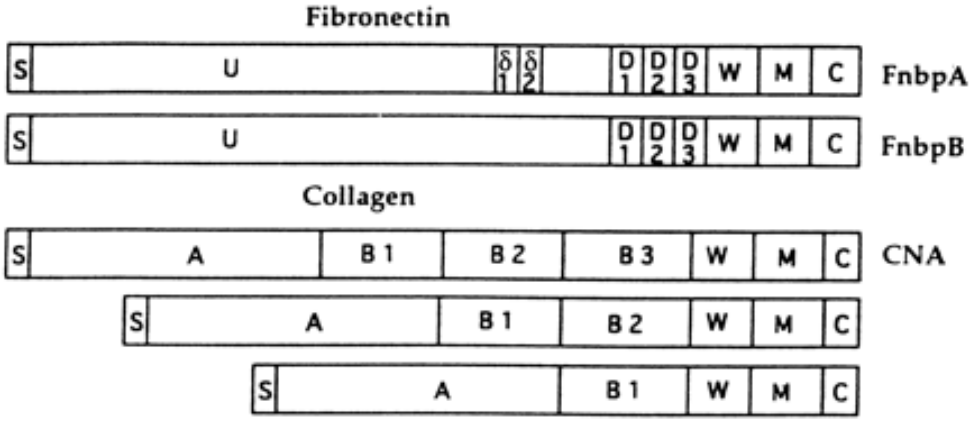


Fig. 2.8 | Schematic models of MSCRAMMs from *S. aureus*. The MSCRAMMs shown are the ones with affinity for fibronectin (FnbpA and FnbpB), collagen (CNA). S, signal sequence; U or A, unique nonrepetitive sequence; δ, upstream repeat sequences; D, B, or R: repeated domains; W, cell wall-spanning domain; M, hydrophobic membrane-spanning domain; C, positively charged carboxy terminus (Patti *et al.*, 1994).

Recently, CNA-like collagen-binding MSCRAMMs 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 *et al.*, 2003), and CNE (collagen-binding protein from *Streptococcus equi*) (Lannergard *et al*., 2003). These proteins all share a domain organization similar to that of CNA, in which the A domain is responsible for their collagen-binding activities; however, the specific binding mechanisms for each collagen adhesin may be somewhat different. Besides CNA, some other proteins involved in collagen binding have been characterized, nevertheless the domain responsible for specific adhesion to collagen still remains to be determined, e.g. Lsa63

(leptospiral surface adhesin of 63 kDa) (Vieira et al., 2010) or EhaB (enterohemorrhagic *Escherichia coli* autotrasporter B) (Wells et al., 2009). The importance of MSCRAMMs as adhesins and their role in the colonization process can be established in few cases, because in an intact healthy organism, most of ECM is not exposed to environment and so is not accessible for interactions. However, these ligands may be exposed after tissue damage due to mechanical or chemical injury, primary infectious injury or early cytophatic changes associated with infection. So MSCRAMMs have an important role in particular in infections caused by opportunistic pathogens or microorganism using multiple adhesion mechanisms.

The ECM has two basic forms in tissue: the basement membrane (also called basal lamina) and interstitial matrix. In some connective tissue such as blood or lymph tissue, the matrix is fluid and called plasma. The basement membrane is present at the basis of epithelial and connective tissues; it is composed by laminin, proteoglycans and collagens, especially the non-fibrillar-forming collagen IV, and collagen VII serves as an anchor to it (Amano *et al.*, 2001). The interstitial matrix composition depends on the tissue, but contains principally collagens, in connective tissue, or fibronectin and vitronectin, in blood tissue. In dense connective tissues, such as cartilage and tendon, the principal component is type II collagen. Instead, collagen I is the most abundant collagen protein of the non-cartilaginous tissues and Type V collagen is widely distributed in tissue as minor component. Collagen V is thought to be combined with collagen I in heterotypic fibers and to regulate the initiation of fibril assembly (Birk *et al.*, 1988; Wenstrup *et al.*, 2004).

Genome-based analysis of *C. difficile* demonstrated the presence of putative and known surface factors that could be involved in the interaction with ECM components (Sebaihia *et al.*, 2006, Monot *et al.*, 2011) (**table 2.1**). Among them, there is the known Fbp68 that already is classified as MSCRAMM (Lin *et al.*, 2011). Instead, the real localization and the role of the other factors, included in the table 1, remain unknown.

Annotation	Locus Tag	Strain	Classification
putative collagen-binding protein	CD3392	630	cnaB-containing protein
putative collagen-binding protein	CD2831	630	cnaB-containing protein
putative collagen-binding protein	CD0386	630	cnaB-containing protein
putative collagen-binding protein	orf00568	ATCC43255	cnaB-containing protein
putative serine-aspartate repeat-containing protein SdrF	CD3145	630	Collagen binding protein
Fbp68- FbpA	CD2592	630	Fibronectin binding protein
hypothetical protein	CD2797	630	Fibronectin binding protein

Table 2.1 | *C. difficile* surface factors potentially involved in ECM interaction.

3. Aim

In this study we functionally characterized a new protein of *C. difficile*, that we named CbpA, having features of typical MSCRAMMs to understand its role in the intestinal colonization process.

4. Results

4.1 Domain organization of CbpA/CD3145 protein

Collagen binding protein A (CbpA), encoded by the *CD3145* gene of *C. difficile* strain 630, is a predicted protein of 1190 amino acids annotated as serine-aspartate repeat-containing protein SdrF (Sebaihia *et al.*, 2006, Monot *et al.*, 2011). The protein has an N-terminal putative leader peptide and a C-terminus region comprising an NVQTG-motif followed by a hydrophobic membrane-spanning domain and a series of positively charged residues (**Fig.1**).

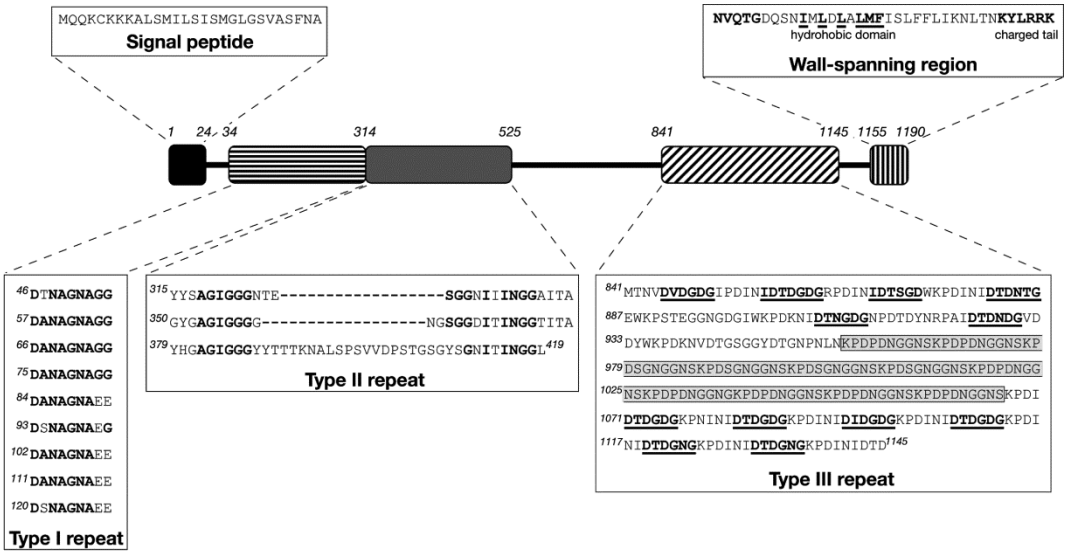


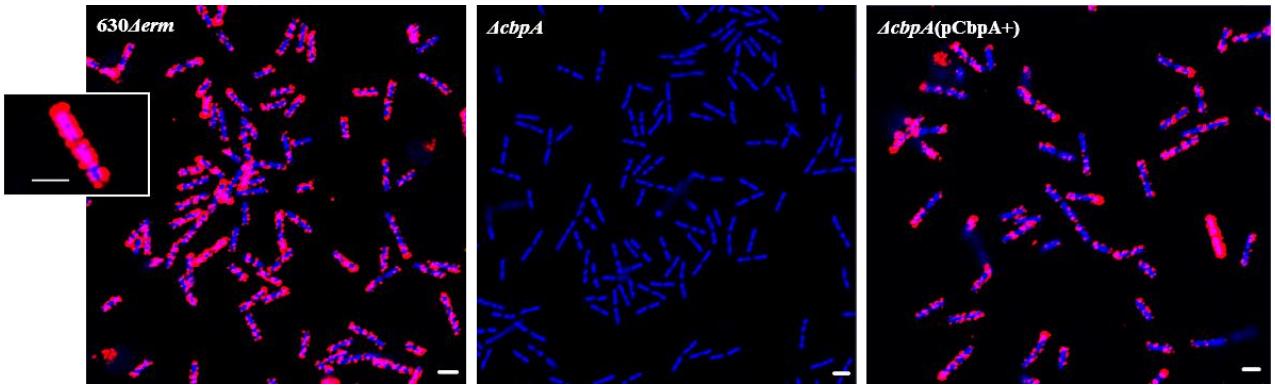
Fig. 4.1 | Schematic representation of CbpA (CD3145) protein organization.

These features suggest that CbpA is a putative substrate of *C. difficile* sortase B (Sebaihia *et al.*, 2006) and belongs to the family of Gram-positive proteins anchored to the peptidoglycan. The mature polypeptide has a predicted molecular weight of 118,400 Da. Sequence analysis revealed the presence of three distinct repeat types (**Fig.1**): type I (amino acids 34-314), type II (amino acids 315- 525) and type III (amino acids 841-1145); the latter comprise a central core of consecutive KPDPDNGGNS motifs flanked by DXDGDG signatures which are similar to the calcium-binding sequences found in the trombosphondin-like proteins (Kvansakul *et al.*, 2004, Rigden *et al.*, 2004).

4.2 Surface localization of CbpA

The predicted external localization of CbpA was investigated by confocal microscopy of 630 strain using antibodies raised against the recombinant $CbpA_{I-II}$ protein, containing type I and II repeats (amino acids 29-841, **Fig. 1**). As shown in **Fig. 2A**, the protein was homogeneously detected on the bacterial surface of strain 630 (**Fig. 2A**). To further analyse CbpA cellular distribution phenotype, we created an insertional mutant within *cbp,* designated Δ*cbpA*, using ClosTron technology (Heap *et al.*, 2010). The *cbpA* mutant did not express CbpA on its surface, showing the specificity of the labelling. Complementation of the mutant strain with a plasmid-encoded *cbpA* gene restored the surface expression of the protein (**Fig. 4.2A**). CbpA was present in the cell wall fraction as shown by Western blot analysis. Protein was detected in wild-type and complemented strains, but not in the *cbpA* mutant strain (**Fig. 4.2B**).

A



B

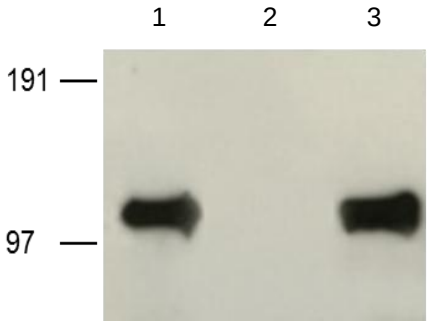


Fig. 4.2 | Surface exposure of CbpA protein in 630 strain
A: Confocal microscopy analysis of surface-expression using anti-$CbpA_{I-II}$ antibody and a secondary fluorescent antibody (red). DNA was stained with DAPI (blue). Scale bars: 2 µm.
B: Western blot analysis on cell-wall fractions obtained by mutanolysin extraction from 630Δ*erm* (lane 1), Δ*cbpA* (lane 2) and Δ*cbpA*(pCbpA+) (lane 3) strains. The Δ*cbpA* strain was used as negative control. Complementation of the mutant strain with a plasmid harboring the *cbpA* gene (Δ*cbpA*(pCbpA+) strain) restored the surface expression of the protein.

4.3 Recombinant $CbpA_{I-II}$ binds to extracellular matrix components *in vitro*

The presence of CbpA on the surface of *C. difficile* led us to hypothesize a contribution of the antigen in the interaction of the pathogen with host tissues. ECM components present at the basolateral surface of organs are important in maintaining tissue integrity and known to be target of several surface-exposed proteins contributing to bacterial colonization (Patti *et al.*, 1994). The propensity of CbpA to interact with ECM was evaluated by ELISA using a recombinant $CbpA_{I-II}$ polypeptide incubated in the presence of several ECM proteins including fibronectin, laminin and multiple collagen types. By this approach, we observed that $CbpA_{I-II}$ was able to bind in a concentration-dependent and saturable fashion only to collagen I and V (**Fig 4.3**). The K_d values, defined as the concentration able to saturate 50% of putative receptors, were $3x10^{-8}$M and $8x10^{-9}$M, respectively. No significant binding was reported for fibronectin, laminin and collagens II, IV and VI (**Fig. S1**).

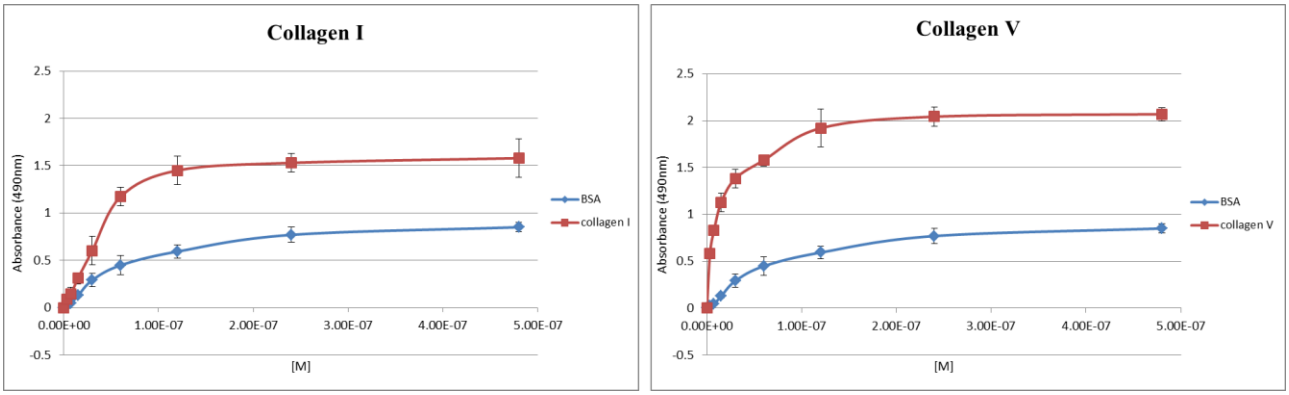


Fig.4.3 | Interaction of CbpA with immobilized ECM
ELISA plates were coated with 10 µg ml^{-1} of purified ECM components and incubated with serially diluted recombinant $CbpA_{I-II}$ protein ranging from 4 nM to 0.23 uM. Binding of the protein was detected by anti-$CbpA_{I-II}$ antibody followed by HRP-conjugated-secondary antibody. BSA protein was used as negative control.

To demonstrate that the binding of CbpA to collagens is relevant to the interaction of *C. difficile* to the ECM components produced by host cells, we used a human fibroblast cell line (IMR-90) presenting a complex fiber organization mainly composed by fibronectin and collagens (data not shown). As shown in **Fig. 4.4**, confocal microscopy analysis of IMR-90 cells incubated with 20 µg/ml of recombinant $CbpA_{I-II}$ conjugated to Alexa-Flour 488 dye, revealed a spatial co-localization of the protein with collagens I and V, supporting the hypothesis of a cognate interaction of the antigen with such host determinants. As control, pre-incubation of cells with an excess of unlabelled $CbpA_{I-II}$ prevented the binding to collagens, confirming the specificity of this interaction (**Fig. 4.4**).

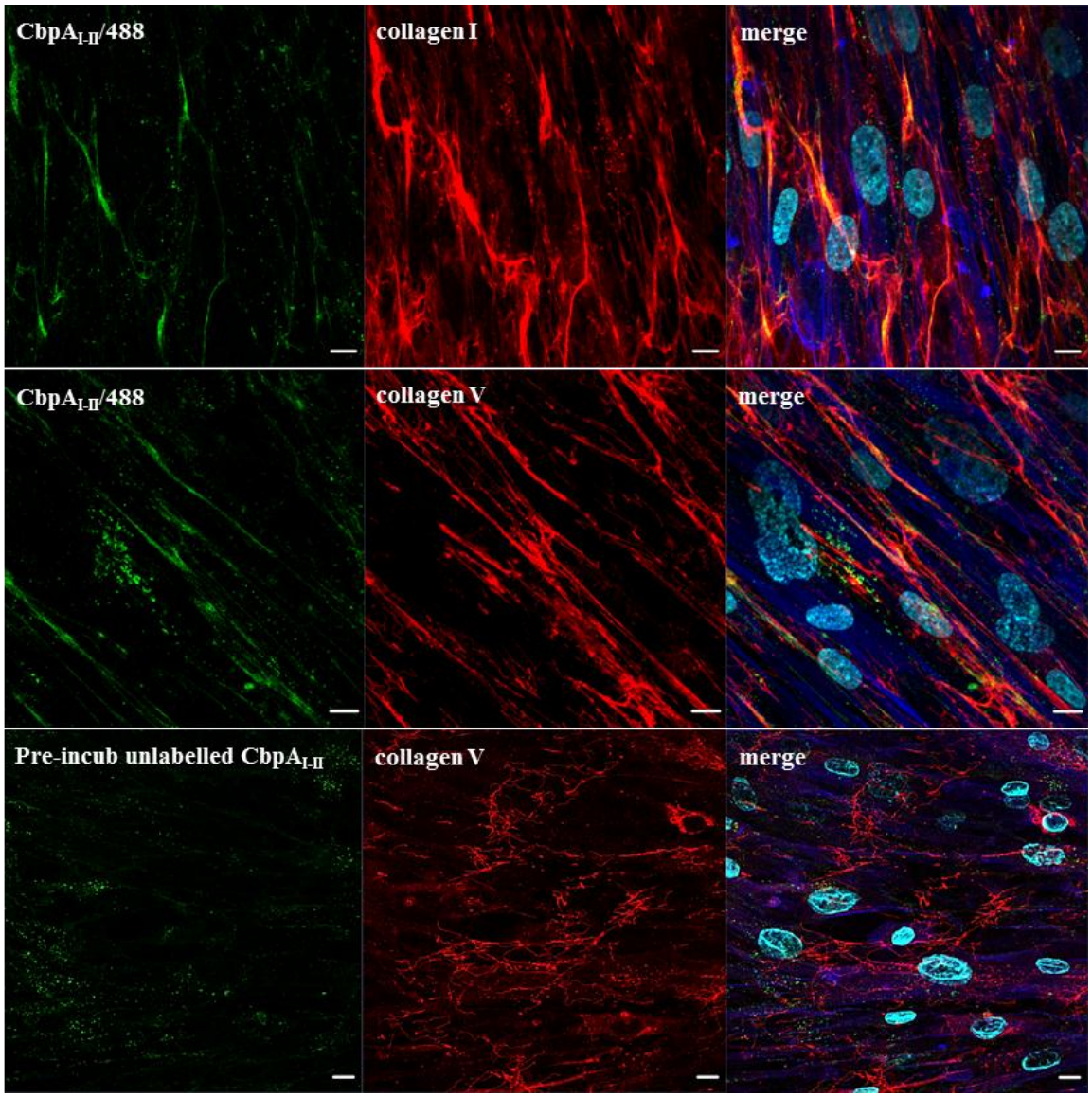
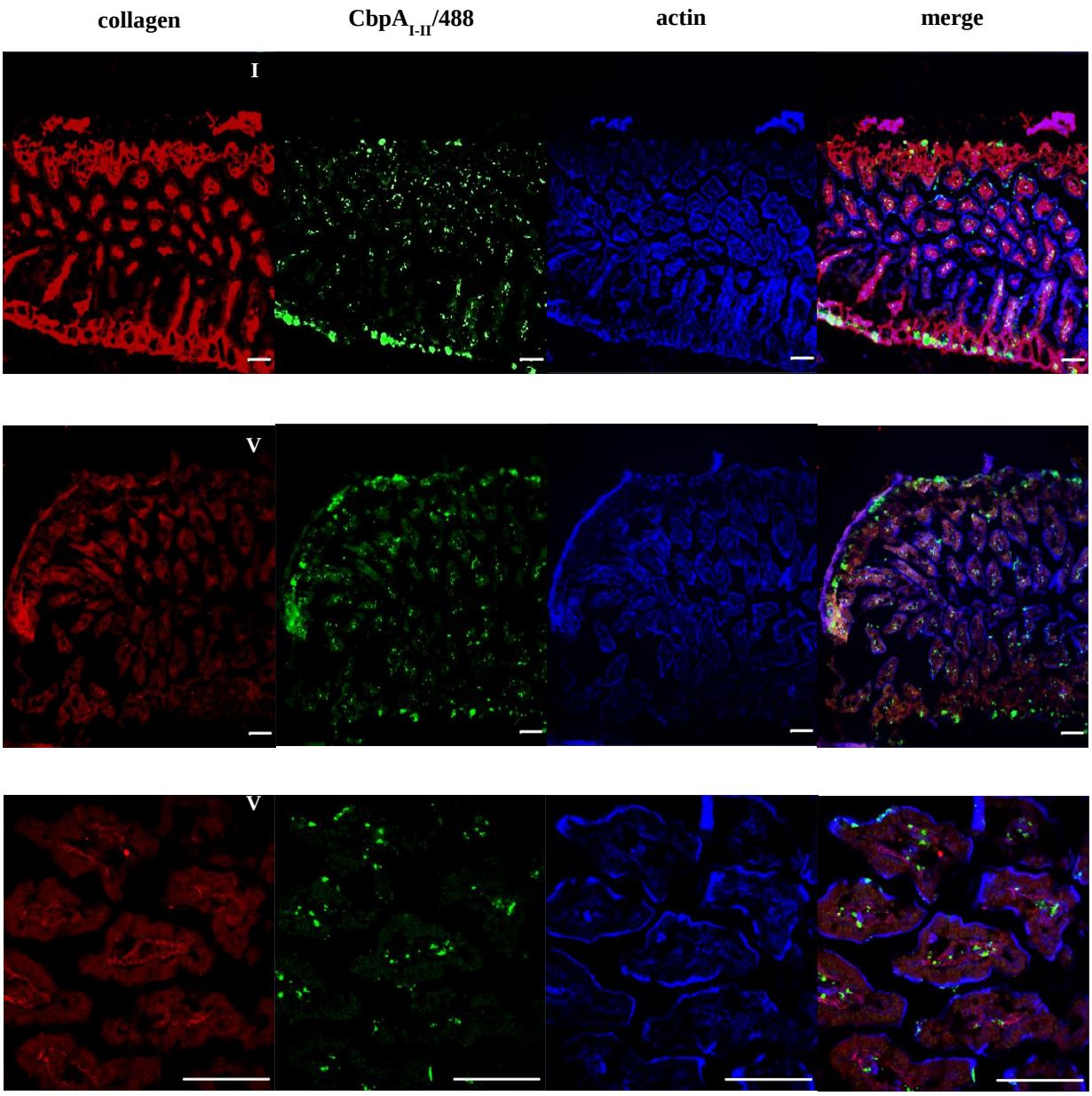


Fig. 4.4 | Interaction of CbpA with ECM-producing human fibroblasts.
Cells were incubated with 10 µg ml^{-1} of CbpA$_{I-II}$ protein conjugated with Alexa-488 dye (green) for 1 h at 37°C. Collagens I (upper panel) and V (middle panel) were labelled using specific antibody (red). DNA and actin were stained with DAPI (cyan) and Phalloidin-Alexa647 (blue), respectively.
In control experiments, cells were pre-incubated with an excess of unlabelled CbpA before binding to CbpA/488 (lower panel). Scale bars: 10 µm.

4.4 Recombinant $CbpA_{I-II}$ is able to bind to murine intestine

In order to further investigate the intrinsic adhesive properties of the CbpA protein to host targets, we isolated different tracts of CD1 mice intestine (ileum, jejunum, duodenum and colon) and prepared cryo-sections for examination in binding experiments. Briefly, sections were incubated with 20 µg/ml of recombinant $CbpA_{I-II}$ conjugated to Alexa-Flour 488 dye and collagens I/V stained. BSA was used as control sample, while F-actin staining was used to monitor tissue morphology. Confocal microscopy analysis revealed a large distribution of collagen I and V in all intestinal tracts, with $CbpA_{I-II}$ showing a diffuse localization in all of them (data not shown) with a strongest affinity to portions of small intestine (**Fig. 4.5**). In particular, magnification of longitudinal section of villi of jejunum showed a preferential localization of the protein in the submucosa underlying the surface epithelium (**Fig. 4.5,** 40x magnification).

Jejunun



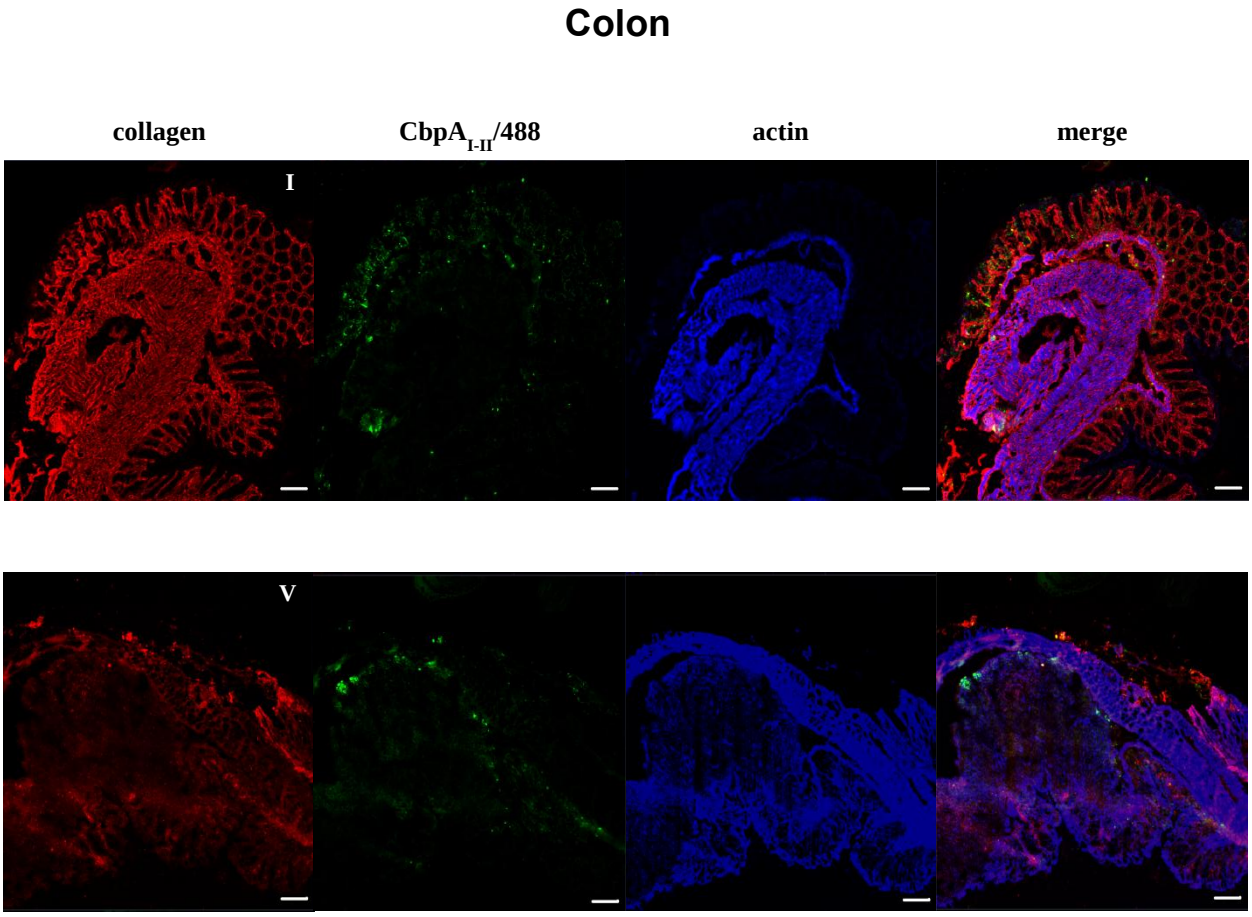


Fig.4.5 | Binding of recombinant $CbpA_{I-II}$ to mouse gut crio-sections.
Sections were incubated with the recombinant $CbpA_{I-II}$ conjugated to Alexa-Flour488 dye (green) for 2 h at room temperature. Collagens I and V were labelled using specific antibodies (red) and actin was stained with Phalloidin-647 dye (blue). Scale bars: 100 μm.

4.5 Engineering of *L. lactis* for surface-display of CbpA results in an increased adherence to collagen and ECM-producing human fibroblasts

To understand the impact of CbpA protein on binding of *C. difficile* to collagens, the ability of the *CbpA* knock-out strain to bind immobilized collagen V and to adhere to IMR-90 cells was compared to the wild-type strain. As shown in **Fig. 4.6**, in both collagen and cell binding assays, we observed no significant difference between the 630Δ*erm* and Δ*cbpA* strain, suggesting that redundancy of bacterial proteins potentially interacting with ECM components may mask the contribution of CbpA to collagen V binding.

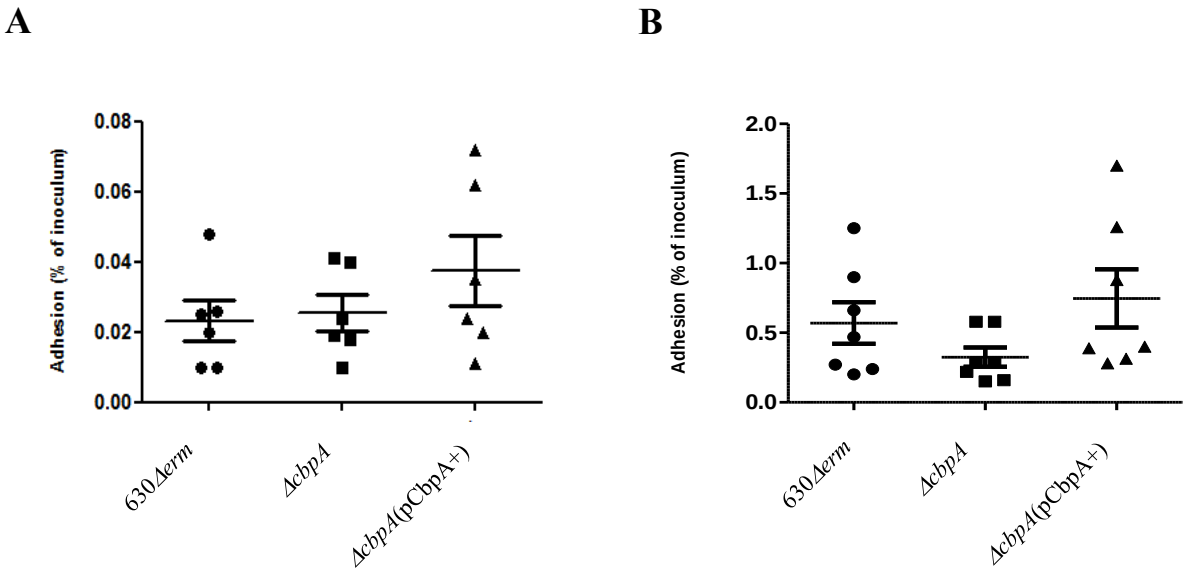


Fig.4.6 | Binding activity of *C. difficile* strains on immobilized collagen V and on IMR-90.
A: Plates were coated with collagen V and adherence of 630*Δerm* (●), Δ*cbpA* (■) and Δ*cbpA*(pCbpA+) (▲) strains was measured by detaching bacteria with trypsin and plating. Adhesion was expressed as percentage of total bacteria. P-values of Δ*cbpA* strain compared with 630*Δerm* and Δ*cbpA*(pCbpA+) strains are 0.31 and 0.78, respectively.
B: IMR-90 cells were incubated for 2 h with 630*Δerm* (●), Δ*cbpA* (■) and Δ*cbpA*(pCbpA+) (▲) strains at an MOI of 100:1. After washing and saponin treatment, lysates were plated and the number of total adherent bacteria was expressed as percentage of total bacteria in starting infection. P-values of Δ*cbpA* strain compared with 630*Δerm* and Δ*cbpA*(pCbpA+) strains are 0.15 and 0.07, respectively.

To prove that the lack of an adhesive phenotype by the Δ*cbpA* mutant strain was due to a real cell-wall protein redundancy, we employed *Lactococcus lactis* as a heterologous system for the constitutive expression of CbpA. Indeed, this bacterium is known to have been frequently used as a tool to express heterologous adhesins and show their functionality (Buccato *et al.*, 2006, Arrecubieta *et al.*, 2007, Sinha *et al.*, 2000). Since the cell wall anchoring motif of CbpA is not predicted to be recognized by sortase A of *L. lactis* (Dieye *et al.*, 2010), we substituted it with a previously described motif (Edwards *et al.*, 2008), to ensure CbpA exposure on *L. lactis* surface. As shown in **Fig. 4.7**, the successful export of CbpA onto the lactococcal cell surface was then assessed by confocal immunofluorescence (**Fig. 4.7A**) and Western blot analysis (**Fig. 4.7B**).

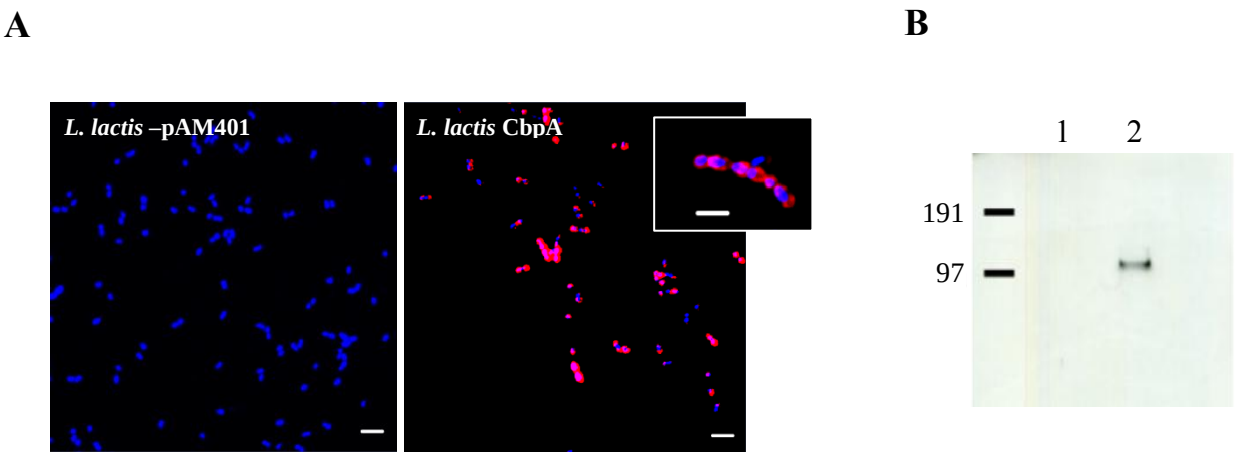


Fig.4.7 | Expression of CbpA in *L. lactis*.
A: Confocal microscopy analysis of surface-expression in *L. lactis*-CbpA strain using anti-$CbpA_{I-II}$ antibodies and a secondary fluorescent antibody (red). DNA was stained with DAPI (blue). An *L. lactis* strain carrying the empty vector (*L. lactis*-pAM401) was used as negative control. Scale bars: 2 µm.
B: Western blot analysis of cell-wall fractions from *L.lactis*-pAM401 (1) and *L. lactis*-CbpA strains (2).

Notably, surface expression of CbpA conferred to *L. lactis* a significant increased ability to adhere to immobilized solid-phase collagen V compared to *L. lactis* carrying the pAM401 empty vector (*L. lactis*-pAM401) (**Fig. 4.8A**). The binding was inhibited by the anti-$CbpA_{I-II}$ serum, demonstrating the specificity of the activity (**Fig. 4.8B**).

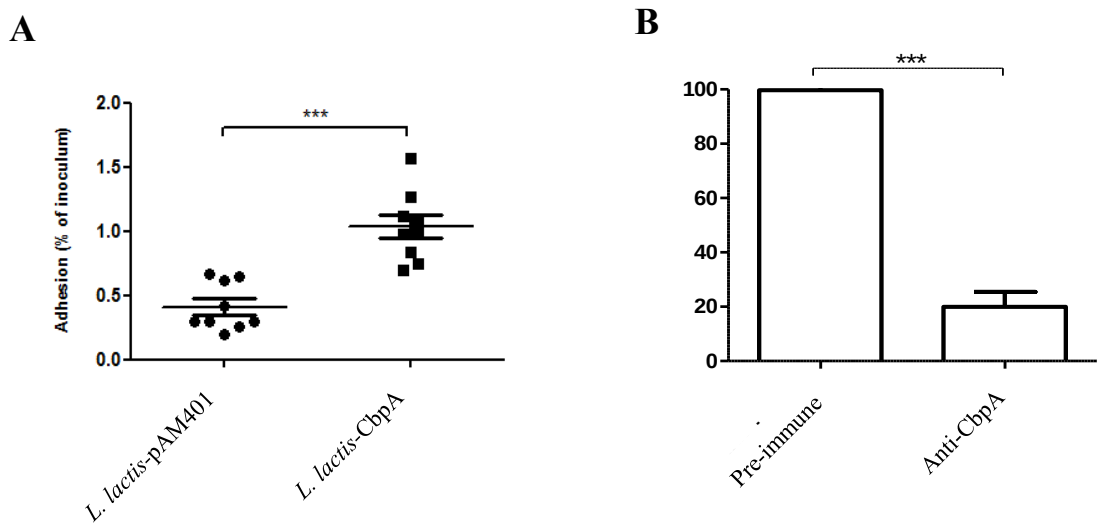


Fig.4.8 | Binding activity of *L. lactis*-CbpA strain to immobilized collagen V.
A: Plates were coated with collagen V and adherence of *L. lactis*-pAM401 (●) and *L. lactis*-CbpA (■) was measured by detaching bacteria with trypsin and plating. Adhesion was expressed as percentage of total bacteria in starting infection.
B: Adherence of *L. lactis*-CbpA strain to collagen V was inhibited by anti-$CbpA_{I-II}$ serum. Adhesion of *L. lactis*-CbpA strain treated with equally diluted pre-immune serum was assumed as 100% of adhesion.
Values represent the mean ± SE; ***$p<0.001$.

We also tested the ability of *L. lactis*-CbpA to adhere on IMR-90 cells by using both global quantification of viable adherent bacteria and confocal microscopy analysis. CFU counting of *L. lactis*-CbpA strain incubated with IMR-90 fibroblasts for 2h, clearly showed a 2.5 fold increase in cell adherence compared to control cells (**Fig. 4.9A**). As for the binding to collagen, the adhesion of *L.*

lactis-CbpA strain to fibroblasts was inhibited by pre-incubation of bacteria with anti-CbpA antibody (**Fig. 4.9B**). These data were confirmed by immunofluorescence confocal microscopy analysis (**Fig. 4.9C**).

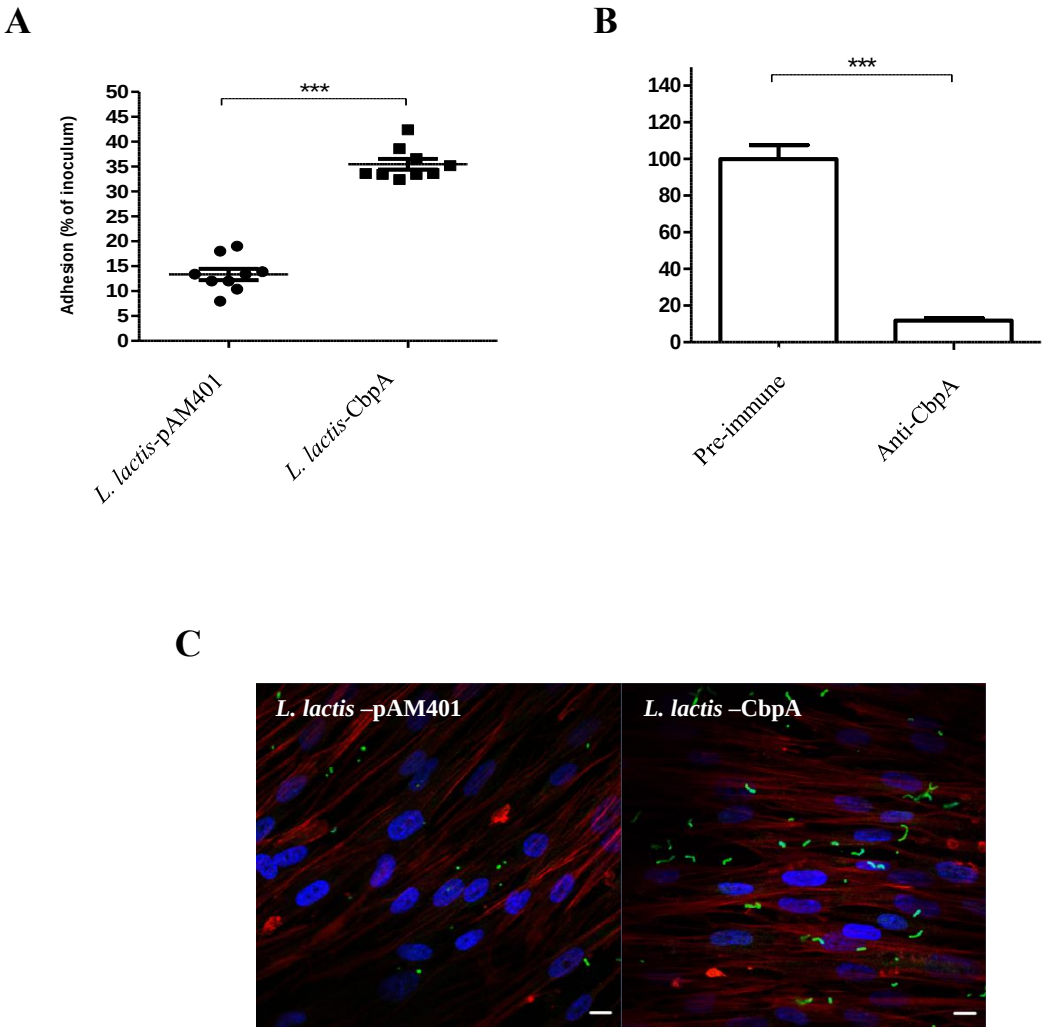


Fig. 4.9 | Adhesion of *L. lactis*-CbpA strain to IMR-90 human fibroblasts.
A: IMR-90 cells were incubated for 2 h with *L. lactis*-pAM401 (●) and *L. lactis*-CbpA (■) strains at an MOI of 100:1. After washing and saponin treatment, lysates were plated and the number of total adherent bacteria was expressed as percentage of total bacteria in starting infection.
B: Adherence of *L. lactis*-CbpA strain to IMR-90 cells was inhibited by anti-CbpA serum. Adhesion of *L. lactis*-CbpA strain treated with equally diluted pre-immune serum was assumed as 100% of adhesion.
Values represent the mean ± SE; ***p<0.001
C: IMR-90 cells were infected as in A and, after washing, were fixed and stained for confocal immunoflurescent microscopy. Bacteria were labeled by α-whole bacteria serum and a secondary fluorescent antibody (green). DNA and cellular actin were stained with DAPI (blue) and Phalloidin-Alexa568 (red), respectively. Scale bars: 10 µm.

5. Discussion

Antibiotic-induced disruption of the normal intestinal flora is a pre-requisite for *C. difficile* colonization of the gut and onset of disease (Rupnik *et al.*, 2009), with toxins A and B being the major virulence factors responsible for the clinical symptoms and signs of infection (Thelestam *et al.*, 2000). However, since adhesion to host tissues is considered a crucial step which allows pathogenic bacteria to persist in specific niches, it has been proposed that, other than toxins, surface-associated determinants are likely to be important in *C. difficile* pathogenesis (Rupnik *et al.*, 2009). Bioinformatic analysis of predicted cell wall-anchored proteins has led us to the selection of the *CD3145* gene (*cbpA*) coding for a polypeptide carrying at the C-terminus a NVQTG-motif. Based on functional evidences, CbpA can be ascribed to the MSCRAMM family which includes bacterial adhesins specifically interacting with ECM components (Patti *et al.*, 1994). Direct binding assays using recombinant CbpA on immobilized ECM components or human fibroblasts demonstrated the ability of the protein to bind with high affinity to collagen I and V. The binding of CbpA conjugated with Alexa-Fluor 488 dye to fibroblasts, was impaired by pre-incubation of cells with unlabeled protein, indicating the specificity of the interaction. Interestingly, the protein showed a tropism to gut tissues in accordance to the wide distribution of collagens in the submucosa. To further evaluate the role of CbpA as adhesin in the bacterial background, we employed *L. lactis* as heterologous system. This model was used for the expression of other MSCRAMMs from several pathogens, such as *Staphylococcus epidermidis* or *Staphylococcus aureus*, to evaluate their adhesive capacity (Arrecubieta *et al.*, 2007, Sinha *et al.*,

2000). *L. lactis* displaying CbpA on the membrane acquired a potentiated ability to interact to immobilized collagen V and to adhere to human fibroblasts, and specific serum against the recombinant protein inhibits this capacity.

Collagens are the major structural component of the ECM and participate in many cellular processes such as cell attachment, differentiation and migration (Hay, 1991). There are more than 20 types of collagen of which type I is the most abundant and in combination with type V is the main component of fibrils (Wenstrup *et al.*, 2004). Many bacteria have evolved to express adhesins to interact with collagens (Foster *et al.*, 1998, Arrecubieta *et al.*, 2007). In particular, type I and V collagens have been identified as the target of enteropathogenic *E. coli* (Ljungh *et al.*, 1990), enterobacteria (Tarkkanen *et al.*, 1990) and *Bifidobacterium adolescentis (*Mukai e*t al*., 1997*).* However, when we studied the role of CbpA in the clostridial background, we were not able to observe a significant contribution to the adhesion process. This result can be explained by the redundancy of the proteins targeting the ECM. Indeed, CbpA is one of a number of clostridial proteins sharing the common function of binding to the ECM. Examples are the high-molecular-weight subunit of SLP, which is able to mediate the attachment to collagen I, thrombospondin and vitronectin (Calabi *et al.*, 2002) and the manganese-binding protein Fbp68, having binding activity toward fibronectin (Hennequin et al 2003; Lin et al, 2011). Moreover, several genes containing ECM–binding domains were predicted in the genome of *C. difficile* strain 630 as potential virulence factors (Sebaihia *et al.*, 2006). Thus, the apparent common function of predicted ECM adhesins highlights that, in *C. difficile* as in many other human pathogens, a diverse adhesive repertoire could be an added value in the competition for specific niches, in which affinity for specific substrates and their availability might be determinant to a successful colonization. On the other hand, the presence of multiple factors sharing functional similarity makes difficult to determine the relative contribution of individual adhesin to the colonization processes.

The expression level of CbpA in our *in vitro* experimental conditions could affect the evaluation of the contribution of the protein in the adhesion process. Indeed, it has been recently reported that the protein is up-regulated in a pig ligated loop model, set up to analyze bacterial genes involved in pathogenesis (Scaria *et al.*, 2011). In particular, in this study, microarray analysis of strain *C. difficile* 630 transcriptome at different time points after infection, revealed the up-regulation of *cbpA*/*CD3145* transcription at 8 and 12 hours. Interestingly, *fbp68* and *slpA* are early up-regulated and other putative collagen binding proteins resulted up-regulated at 12 hours (e.g *CD0386*, *CD2831* and *CD3392*). We can then hypothesize that variable expression of the protein may modulate the binding characteristics of the bacterium in response to environmental changes encountered within the host during the colonization.

The interaction of bacterial pathogens with the ECM is an important virulence mechanism contributing to host colonization. While the ECM is not accessible to pathogens under normal conditions, it may be exposed to pathogen as consequence of tissue damage. Indeed, it has been reported that *C. difficile* has an intrinsic ability to bind fibronectin, fibrinogen, vitronectin and collage types I, III, IV and V (Cerquetti *et al.*, 2002). Based on the current knowledge of *C. difficile* molecular mechanisms of pathogenesis and the data reported in this study, we propose a model of gut colonization in which the disruption of tight junctions mediated by TcdA and B (Nusrat *et al.*, 2001), together with the degradative activity toward ECM components displayed by Cwp84 (Janoir *et al.*, 2007) and other hydrolytic enzymes (Seddon *et al.*, 1990, Seddon *et al.*, 1992, Poilane *et al.*, 1998), could cause destabilization of intestinal mucosa and allow *C. difficile* to gain access to ECM. This interaction may allow bacteria to use the submucosal matrix as a molecular bridge to increase their adherence to host tissues. In this context, surface determinants such as CbpA would play a fundamental role in the multi-factorial colonization process of the intestinal mucosa

6. Experimental procedures

6.1 Bacterial strains and growth conditions

L. lactis MG1363 strain was cultured statically at 30°C in M17 growth medium supplemented with 0.5% glucose and, when required, 20 ug ml^{-1} chloramphenicol.

C. difficile strains were grown in anaerobiosis at 37°C in Brain-Heart Infusion (BHI) broth. *Escherichia coli* HK100 cells and BL21(DE3)$T1^{R}$ strains, used to clone and express $CbpA_{I-II}$, were cultured at 37°C in Luria-Bertani (LB) broth supplemented with 100 ug ml^{-1} ampicillin.

6.2 Plasmid construction, DNA manipulation and mutant generations

For the expression of recombinant $CbpA_{I-II}$ (amino acids 29-841), the corresponding sequence was amplified by PCR using chromosomal DNA from *C. difficile* 630 strain as template. The PCR product was cloned in pET15b vector (Novagen) using the PIPE method. BL21(DE3)$T1^{R}$ chemically competent cells were used for expression of the N-terminal His-tag protein.

A *cbpA* mutant was generated in *C. difficile* 630 Δ*erm* strain by insertion of a bacterial group II intron containing a retrotransposition-activated marker (RAM) conferring erythromycin resistance using the ClosTron clostridial gene knockout system (Heap *et al.*, 2010). pMTL007C-E5 *CD3145*-18Ua

(pHAS018) was generated to insert the group II intron 18 bp upstream of the transcriptional start site as no insertion sites were predicted within the gene sequence. CA434 *E. coli* transformed with pHAS018 were grown in LB with 15 μg ml^{-1} chloramphenicol and conjugated into *C. difficile* strain Δ*erm* grown in TY media overnight. Conjugation was incubated overnight at 37°C under anaerobic conditions on non-selective Brazier's agar. pHAS018 positive *C. difficile* were selected with thiamphenicol (15 μg ml^{-1}), with Bioconnection's cefoxitin and cycloserine counter-selection against *E. coli*. Transconjugates were tested for intron insertion by selection on Brazier's agar with erythromycin selection (5 μg ml^{-1}) and for thiamphenicol sensitivity.

For complementation of the *cbpA* mutant the *CD3145* gene was amplified from the predicted signal peptide cleavage site from *C. difficile* strain 630 genomic DNA. pRPF144 was modified to contain the *slpA* Sec signal sequence followed by a Strep II tag and an XhoI restriction site, producing pHAS007. *CD3145* was ligated between XhoI and BamHI sites thus under control of the *Cwp2* promoter, with an N-terminal Strep II tag generating pHAS033.

For the construction of *L. lactis* strain surface-expressing CbpA, the *CD3145* gene was amplified by PCR using chromosomal DNA from *C. difficile* 630 strain as template (primer1: 5'-GAGGTTAAGGCTAACGGTTCAAATACTTTCGCAGATACTATAGAAGAAAG-3' and primer2: 5'-ACCTGTTGATGGTAATTGACCTACATTACTATCTAAACTTTCATCTG-3') and cloned in pAM401 expression vector (Buccato *et al.*, 2006). The sequence coding for the leader peptide (1-72 bp) and the NVQTG-motif at C-terminal (3463-3573 bp) were substituted with the same regions of the GAS M1 gene (1-129 bp and 1348-1455 respectively), to ensure the surface exposure in *L. lactis* as described by (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'). HK100 cells

were used to clone the two PCR products using the PIPE method. The new assembled vector was used to transform *L. lactis* subspecies *cremoris* MG1363 strain by electroporation.

6.3 Expression and purification of recombinant CbpA

A single colony of *E. coli* BL21(DE3)T1^{R} strain expressing $CbpA_{I-II}$ was inoculated in LB containing 100 µg ml^{-1} ampicillin and grown overnight at 37°C. Bacterial suspension was diluted in fresh HTMC medium supplemented with 200 µg ml^{-1} ampicillin and grown for 30 h at 25°C. Recombinant $CbpA_{I-II}$ His-tagged protein was purified by affinity chromatography on Ni^{2+}-NTA Superflow (Qiagen). The purity of the protein was checked by SDS-PAGE followed by Coomassie Blue staining and the protein concentration was determined by BCA reagent (Thermo Scientific).

6.4 Production of a polyclonal antibody against CbpA

5-week-old CD1 female mice were inoculated intraperitoneally three times (day 0, 21 and 35) with 10 µg of recombinant $CbpA_{I-II}$ in the presence of Aluminum hydroxyde. The immune serum was obtained 14 days after the last inoculation. The treatments were performed in accordance with internal animal ethical committee and institutional guidelines.

6.5 Cell fractionation and protein analysis

To obtain cell wall-associated proteins of *C. difficile* strains, bacteria were grown until exponential phase, harvested by centrifugation at 3000 × *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^{-1} of mutanolysin (Sigma) and digestion was allowed to proceed for 2 h at 37°C with gentle agitation. The majority of intact protoplasts were removed by centrifugation at 3000 × *g* for 30 min. The supernatant was then subjected to microcentrifugation at 17 000 × *g* for 30 min to remove cell debris and any remaining protoplasts. The supernatant containing the solubilized cell surface proteins was recovered.

To obtain cell wall-associated protein of *L. lactis* strains, bacteria were grown to exponential phase, centrifuged and resuspendend in protoplast buffer (0.1M KPO_4 pH 6.2, 40% sucrose, 10 mM $MgCl_2$ and protease inhibitor) with 100 U $µl^{-1}$ mutanolysin for 1 h at 37°C. The majority of intact protoplasts were removed by centrifugation at 3000 × *g* for 30 min. The supernatant containing the solubilized cell surface proteins was recovered.

Proteins were separated by SDS-PAGE electrophoresis using NuPage Gel System, according to the manufacturer's instructions. Samples were transferred to nitrocellulose membranes for Western blot analysis. Membranes transferred proteins were blocked with PBS containing 0.05% Tween 20 (PBST) and 10% skim milk powder (MERK). Proteins on nitrocellulose membranes were detected with α-$CbpA_{I-II}$ serum followed by the HRP-conjugated secondary antibodies (DAKO) diluted in PBST and 3% skim milk powder. Bands were visualized with Super Signal® West Pico Chemiluminescent Substrate (Thermo Scientific).

6.6 Cell culture

IMR-90 cells (Human fibroblasts, ATCC) were maintained in Eagle's Minimum Essential Medium (EMEM, ATCC) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) and antibiotics. Cells were grown at 37°C with 5% CO_2.

6.7 Immunofluorescence analysis

To verify the presence of CbpA on bacterial surface of *C. difficile* and *L. lactis* strains, bacteria were grown until exponential phase, washed with PBS and fixed in 3.7% paraformaldehyde (Sigma). After multiple washings, bacteria were spread on polylisine-coated slides and blocked with PBS +3% Bovine Serum Albumin (BSA) (Sigma) for 1 h at room temperature. Samples were washed and incubated with anti-$CbpA_{I-II}$ serum (1:500) for 1 h at room temperature. Bacteria were washed several time with PBS and incubated with Alexa Fluor 488 goat anti-mouse IgG (1:500) (Molecular Probes). Labeled samples were mounted with ProLong® Gold antifade reagent with DAPI (Molecular Probes) and analyzed with Zeiss LSM710 confocal microscope.

6.8 Labeling of recombinant protein

Purified recombinant $CbpA_{I-II}$ was labeled with Alexa Fluor® 488 Microscale Protein Labeling Kit (Molecular Probes), according to the manufacturer's instructions.

6.9 Binding assay with recombinant protein on eukaryotic cells

IMR-90 cells were seeded on 8-well chamber slides coated with collagen I (2.5×10^4/ well) (BD BioCoat) and cultured for 3 days. Cells were then incubated with 20 µg ml^{-1} of recombinant $CbpA_{I-II}$ conjugated with Alexa Fluor 488 dye for 2 h at 37°C. After washings to remove unbound protein, cells were fixed with 3.7% paraformaldehyde. Samples were washed extensively and incubated with rabbit monoclonal anti-ECM components antibodies (1:50) for 1 h at room temperature. After multiple washings, samples were incubated with Alexa Fluor 568 goat anti-rabbit IgG (1:500) and Alexa Fluor 647-conjugated phalloidin (1:200) (Molecular Probes). Glass coverslips were mounted with ProLong® Gold antifade reagent with DAPI and analyzed with Zeiss LSM710 confocal microscope. To perform the competition assay, cells were incubated with 50 µg ml^{-1} of unlabeled $CbpA_{I-II}$ for 2 h at 37°C. After pre-incubation, wells were washed with PBS and were used for the binding assay described above.

6.10 Binding assay with recombinant protein on mouse gut cryo-sections

Samples from different intestinal tracts (duodenum, jejunum, ileum and colon) were collected from CD1 mice and stored at -80°C. 8 µm cryo-sections were fixed with 3.7% paraformaldehyde and, after multiple washings, were incubated with 20 µg ml^{-1} of recombinant $CbpA_{I-II}$ - Alexa Fluor 488 for 2 h at 37°C. Samples were washed extensively and ECM and actin stained as described above. Glass coverslips were mounted with ProLong® Gold antifade reagent with DAPI and analyzed with Zeiss LSM710 confocal microscope.

6.11 Enzyme-linked Immunosorbent Assays

Flat bottom 96-well microtiter plates (Greiner Bio-One) were coated with 10 µg ml^{-1} of each ECM component (collagen I, II, III, IV, V, VI, laminin, fibrinogen and fibronectin) or BSA overnight at 4°C. Wells were washed with PBS + 0.05% Tween and blocked them for 2 h at 37°C with Polyvinylpyrrolidone (PVP, SERVA); after several washings plates were incubated overnight at 4°C. $CbpA_{I-II}$ was serially diluted (from 20 to 0.156 ug ml^{-1} and incubated for 2 h at 37°C. To detect the binding, plates were washed and incubated with anti-$CbpA_{I-II}$ serum for 1 h at 37°C followed by incubation with HRP-conjugated anti mouse antibodies. Wells were developed with o-Phenylenediamine dihydrochloride (SigmaFast OPD; Sigma). The reaction was stopped by adding 12.5% H_2SO_4 and finally the absorbance at 490 nm was measured. BSA was used as negative control.

6.12 Bacterial adherence to immobilized collagen V

Flat bottom 96-well microtiter plates were coated with 50 µg ml^{-1} of collagen V overnight at 4°C. After several washings with PBS, wells were blocked with PBS containing 2% skim milk powder (MERK) for 1 h. *L. lactis* or *C. difficile* strains were grown until exponential phase, resuspended in DMEM medium adjusted to an OD_{600} of 1 and added to the microtiter wells. After 2 h of incubation at 37°C, plates were washed with PBS and adherent bacteria were collected by treatment with Trypsin/EDTA for 5 min at 37°C. Bacterial suspensions were serially diluted and plated for CFU counting. BSA coating was used as negative control. To perform the inhibition of adherence to Collagen V, *L. lactis* cells were pre-incubated with anti-$CbpA_{I-II}$ serum (1:500) for 1 h at 37°C. As negative control, bacteria were incubated with the same concentrations of a pre-immune serum. After pre-incubation, bacteria

were centrifuged and re-suspended in DMEM without glucose and were used for the adherence assay protocol described above.

6.13 Quantification of bacterial adhesion on eukaryotic cells by viable counting and immunofluorescence analysis

IMR-90 cells were seeded on 96-well tissue culture plates coated with collagen I (10^4 cells / well) and cultured for 3 days. The day before infection, cells were incubated for 24 h in an antibiotic-free medium. *L. lactis* or *C. difficile* strains were grown to exponential phase and added at multiplicity of infection (MOI) of 100:1 in DMEM without glucose for 2 h at 37°C in 5% CO_2. After several washings to remove non-adherent bacteria, cells were lysed with 1% saponin (Sigma), bacterial suspensions were serially diluted and plated onto appropriated culture solid medium for CFU counting. To perform the inhibition of adhesion, *L. lactis* cells were pre-incubated with α-$CbpA_{I-II}$ serum (1:500) for 1 h at 37°C. As negative control, bacteria were incubated with the same concentrations of a pre-immune serum. After pre-incubation, bacteria were centrifuged and re-suspended in DMEM without glucose and were used for the adhesion assay as described above. For immunofluorescence analysis, cells seeded on chamber slides coated with collagen I were incubated with bacteria as described above. After incubation, wells were washed with PBS and fixed with 3.7% paraformaldehyde. Subsequently, samples were blocked with PBS containing 3% BSA for 1 h at room temperature and incubated with mouse polyclonal serum against PFA-fixed whole bacteria (1:1000) for 1 h at room temperature. Wells were washed several time with PBS and incubated with Alexa Fluor 488 goat anti-mouse IgG (1:500) and Alexa Fluor 568-conjugated phalloidin (1:200). Labeled samples were mounted with ProLong® Gold antifade reagent with DAPI and analyzed with Zeiss LSM710 confocal microscope.

6.14 Statistical analysis

Results were assessed by the Student *t* test for unpaired data. $P \leq .05$ was considered statistically significant (*,$P \leq .05$; **, $P \leq .01$; ***, $P \leq .001$).

7. Supplementary figures

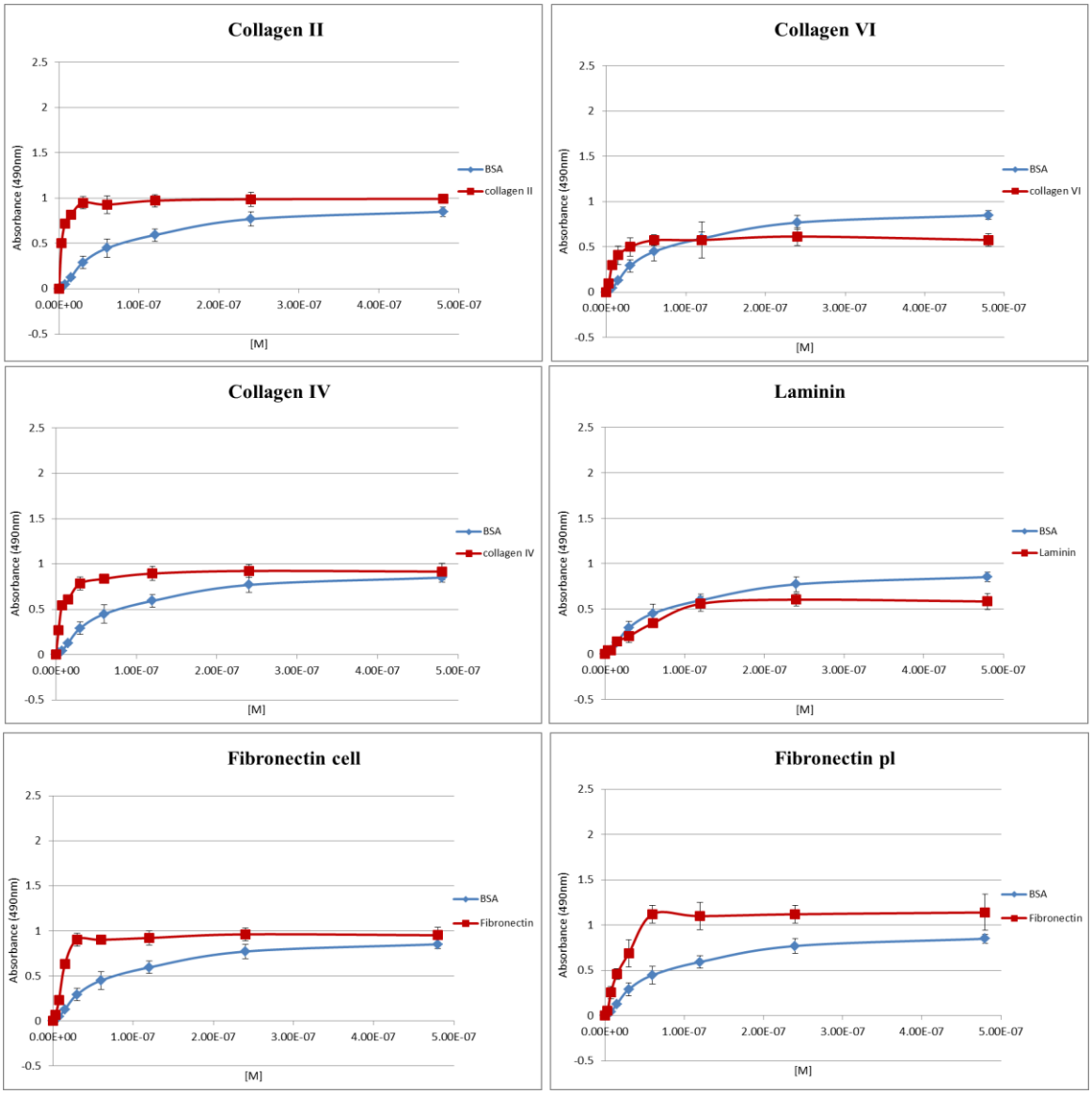


Fig. S1 | Interaction of CbpA with immobilized ECM
ELISA plates were coated with 10 µg ml^{-1} of purified ECM components and incubated with serially diluted recombinant $CbpA_{I-II}$ protein ranging from 4 nM to 0.23 uM. Binding of the protein was detected by anti-$CbpA_{I-II}$ antibody followed by HRP-conjugated-secondary antibody. BSA protein was used as negative control.

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