Localization of the *Clostridium difficile* cysteine protease Cwp84 and insights into its maturation process

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Running title: Localization and maturation process of Cwp84 of *Clostridium difficile*

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Abstract

*Clostridium difficile* is a nosocomial pathogen involved in antibiotic-associated diarrhea. *C. difficile* expresses a cysteine protease, Cwp84, which has been shown to degrade some proteins of the extracellular matrix and play a role in the maturation of the precursor of the S-layer proteins. We sought to analyze the localization and the maturation process of this protease. Two identifiable forms of the protease were found to be associated in the bacteria: a form of about 80 kDa and a cleaved one of 47 kDa, identified as the mature protease. They were found mainly in the bacterial cell surface fractions, and weakly in the extracellular fraction. The 80 kDa protein was non covalently associated to the S-layer proteins, while the 47 kDa form was found to be tightly associated with the underlying cell wall. Our data supported that the anchoring of the Cwp84 47 kDa form is presumably due to a re-association of the secreted protein. Moreover, we showed that the complete maturation of the recombinant protein Cwp84<sub>30-803</sub> is a sequential process beginning at the C-terminal end, followed by one or more cleavages at the N-terminal end. The processing sites of recombinant Cwp84 are likely to be residues Ser-92 and Lys-518. No proteolytic activity was detected with the mature recombinant protease Cwp84<sub>92-518</sub> (47 kDa). In contrast, a fragment including the pro-peptide (Cwp84<sub>30-518</sub>) displayed proteolytic activity on azocasein and fibronectin. These results showed that Cwp84 is processed essentially at the bacterial cell surface, and that its different forms may display different proteolytic activity.
INTRODUCTION

Clostridium difficile, a Gram-positive spore forming anaerobic bacteria, is the leading bacterial cause of nosocomial intestinal infection worldwide, and is responsible for illness ranging from mild-diarrhea to life-threatening pseudomembranous colitis (8, 16). The clinical relevance of C. difficile has increased significantly during the past few years, particularly since 2003, when hypervirulent PCR-ribotype 027 strains have been involved in outbreaks and have been associated with severe disease in North America and Europe (39).

As in other pathogenic bacteria, C. difficile expresses several virulence factors. The two large clostridial toxins A (TcdA) and B (TcdB) are the most important and the best characterized virulence factors of C. difficile (24, 26, 30, 37), leading to clinical manifestations by disorganizing the cell actin cytoskeleton. At present, the interactions between C. difficile and the host cell surface are not fully understood, even if several cell surface proteins including adhesins and flagella have been shown to mediate bacterial attachment (5, 17, 18, 35, 38). The S-layer is a paracrystalline array on the outer cell surface which completely coats the bacterium; it is composed of two proteins, the high molecular weight S-layer protein (HMW-SLP) and the low molecular weight S-layer protein (LMW-SLP), derived from a common precursor, SlpA (6). These two proteins are the major surface proteins in C. difficile, and have been shown to play a role in the intestinal colonization and in the inflammatory process (1, 5). However, the colonization step needs to be further characterized in order to better understand the whole pathogenesis process of C. difficile.

Proteolytic and hydrolytic enzymes also play a role in the pathogenesis of several diseases (27, 32). Cwp84 is a surface-associated cysteine protease, which displays a proteolytic activity towards several proteins of the extracellular matrix (ECM) such as fibronectin, laminin, and
vitronectin (20). As a member of papain-like proteins, Cwp84 possesses the catalytic triad
cysteine, histidine and asparagine (34). It has been recently shown to play a key role in the
maturation of the precursor SlpA into two components of the S-layer, the HMW and the LMW-
SLP (10, 23). The translational product of cwp84 gene is a preproenzyme (Cwp841-803, 803 amino
acid residues, 84 kDa) containing a hydrophobic signal peptide of 32 amino-acid residues, a N-
typical domain of 338 amino-acid residues (33-370) containing the catalytic triad, and a C-
typical domain with three Pfam 04122 motifs, presumed to serve as an anchoring domain to the
underlying cell wall. Previously, we have shown that Cwp84 is matured presumably by an
autoprotoelectolytic cleavage (20). We also showed that cwp84 is highly conserved in C. difficile
strains of different toxinotypes or serotypes (34). Furthermore, this protease induces an immune
response during the course of the infection, as shown by the presence of specific antibodies in
patients with CDI (29, 41). These observations suggested that Cwp84 could play a role in C.
difficile pathophysiology.

The aim of our study was to investigate the localization of Cwp84 in the bacterium and its
maturation process, in relation to its putative role in C. difficile virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Clostridium difficile strain 630 was cultured at 37°C
in an anaerobic chamber (Jacomex, France) in Tryptone Yeast extract infusion broth, with (TYG)
or without glucose (TY) (Difco Laboratories), pH 7.4. Bacillus subtilis 168 strain was grown
aerobically in brain-heart infusion broth or agar (Difco Laboratories). The Escherichia coli
recombinant strains BL21/pET-28a(+)Ω\textit{cwp}84_{30-803} (20), BL21/pET28a(+)Ω\textit{cwp}84_{92-518},
BL21/pET28a(+)Ω\textit{cwp}84_{30-518} and BL21/pET28a(+)Ω\textit{cwp}84_{C116A} were grown in LB agar or in
broth (Difco Laboratories) supplemented with 50 µg/ml kanamycin to maintain the pET plasmid,
at 37°C. The \textit{cwp84} mutant, 630\textDelta\textit{erm} \textit{cwp84}_{347a::erm} strain, a generous gift from Neil
Fairweather, Imperial College of London, England, and the 630\textDelta\textit{erm} \textit{fbpA}_{640a::erm} were cultured
in Brain Heart Infusion (BHI) supplemented with 5µg/ml erythromycin.

\textbf{Animal model.} Eight germ free mice (purchased from CNRS, Orléans) were inoculated orally
with 5x 10^5 vegetative cells of \textit{C. difficile} strain 630, and were sacrificed 40 hours post-challenge.
The caecal contents were collected and protease inhibitor cocktail (Sigma) was immediately
added. Bacteria were pelleted and bacterial proteins were extracted from different fractions.

\textbf{\textit{C. difficile} protein extraction.} \textit{C. difficile} proteins were extracted from bacteria grown in
different media or from bacteria collected from mouse caeca 40 hours post-challenge.

\textbf{S-layer proteins.} The S-layer proteins (SLPs) were prepared by the low-pH glycine
extraction method, as previously described (6).

\textbf{Cell surface-associated proteins.} Surface-associated proteins were extracted using the
method of Wexler \textit{et al.} (40) with some modifications. Briefly, harvested and washed cells were
resuspended in 1mM Tris pH 6.8 with 60 µg/ml mutanolysin and incubated at 37°C for 30 min.
After mixing with a vortex (30 sec) at room temperature, the cells were harvested by
centrifugation at 20000g for 20 min and the supernatant containing the cell wall associated
proteins was retained. In some experiments, trans-Epoxysuccinyl-L-leucylamido (4-guanidino)
butane (E-64) (Sigma), a specific cysteine protease inhibitor was added to the Tris buffer at the final concentration of 10µM.

**Secreted proteins.** To collect the extracellular proteins from broth culture, the bacteria were removed by centrifugation (4500g, 20 min, 4°C) and the supernatant was filtered by passing through a 0.45 µm sterile filter. The proteins in the supernatant were precipitated by adding trichloroacetic acid (TCA, Invitrogen) 10% with gentle shaking at 4°C for 15 hours. The proteins were then pelleted by centrifugation (5000g, 60 min, 4°C) and suspended in 8M urea.

Protein concentration was determined by the Bradford method, using bovine albumin (1 mg/ml) as a standard. Protein extraction for each growth condition was performed in triplicate.

**SDS-PAGE and Immunoblotting.** The proteins from different extracts were separated by SDS-PAGE on a 12% polyacrylamide separating gel, and transferred onto a PVDF membrane (Amersham Biosciences) for immunoblotting with specific anti-Cwp84 antibodies (used as 1:5,000 dilution in blocking buffer). Primary antibodies were detected using alkaline phosphatase conjugated goat anti-rabbit immunoglobulin G (1:20,000 dilution; Sigma), and BCIP/NBT substrate (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; Sigma). In experiments detecting the His tag of the recombinant protein Cwp84<sub>30-803</sub>, anti-His tag antibodies were used as a 1:10,000 dilution in blocking buffer, and detection was performed as indicated above.

**Recombinant Cwp84 proteins production and purification.** Four recombinant proteins were produced. They correspond respectively to the proprotein form Cwp84<sub>30-803</sub>, the cleaved mature form Cwp84<sub>92-518</sub>, the mature form including the propeptide Cwp84<sub>30-518</sub> and the Cwp84<sub>30-803</sub> containing the substitution C116A (named Cwp84<sub>C116A</sub>). The fragment corresponding to the
processed cleaved form Cwp84_{92-518}, and the partially cleaved form Cwp84_{30-518} (incorporating
the N-terminal propeptide of the protease) were cloned into pET28a(+) (Novagen) and expressed
in E. coli BL21 Star™(DE3) (Invitrogen) under the control of IPTG. Primers used to amplify by
PCR the fragment from nucleotide 274 to 1554 were Cwp84_{92}(F) (5’-
CTAGCTAGCTCAAGTGTAGCATACAACCC-3’) and Cwp84_{518}(R) (5’-
CCGGAATTC
TGAGCTAGCGCAGAAAAACCATAAAACTCTAGATG-3’), incorporating also the
NheI
restriction site, and Cwp84_{518}(R). The resulting NheI/EcoRI digested PCR products were each
inserted into the linearized pET28a(+) to create the N-terminal His-tag proteins Cwp84_{92-518}, and
Cwp84_{30-518}. The resulting constructions were transformed in E. coli BL21 according to the
manufacturer’s instructions. The nucleotide sequences of the junctions between the vector and
insert were confirmed.

To generate site-directed mutagenesis of Cwp84, we used the following oligonucleotides 5’-
CAAGGAAGTCTTAATACAGCATGGTCTTTTTCAGGTATG-3’ and 5’-
CAACCTGAAA
AAGACCATGCTGTATTAAGACTTCCTTG-3’. Briefly, a PCR of 18 cycles was performed
using pET28a(+)Ω_cwp84_{30..803} as template and the phusion polymerase (Finnzymes). The parental
plasmid was digested by DpnI and the PCR product transformed into E. coli BL21. The active
residue Cys-116 was then substituted with an Ala residue.

Recombinant Cwp84_{30..803} (previously rCwp84) was purified as already described (20); in one
experiment, Cwp84_{30..803} purification was modified by adding E-64 in the purification buffers, at
a final concentration of 50 µM to inhibit, at least partially, the putative automaturation of the
protease. The recombinant Cwp84\textsubscript{30-518}, Cwp84\textsubscript{92-518} and Cwp84\textsubscript{C116A} were purified using the same protocol with some modifications. Briefly, clones were grown in 1 liter of LB supplemented with 50 µg/ml kanamycin at 37°C to an optical density at 600nm of 0.6-0.8. Protein expression was then induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM, at 37°C, and cells were harvested 4h post-induction for Cwp84\textsubscript{30-518} and Cwp84\textsubscript{C116A} and 6h post-induction for Cwp84\textsubscript{92-518}. The recombinant forms of the protease were then purified by affinity chromatography employing BD TALON cobalt affinity resin (Clontech Laboratoires, Inc) in accordance with the standard protocol provided by the manufacturer. Elution was performed with a gradient of imidazole (10 mM to 1 M), and elution fractions were dialyzed overnight against phosphate buffer saline (PBS) pH 7.4. Elution fractions were then aliquoted and frozen at -80°C for storage until use. These fractions were analyzed on 12% SDS-PAGE.

Proteolytic activity of recombinant Cwp84s. The proteolytic activity of Cwp84\textsubscript{30-803}, Cwp84\textsubscript{30-518}, Cwp84\textsubscript{92-518} and Cwp84\textsubscript{C116A} was determined using azocasein (Sigma) as a substrate, as previously described (20). Proteolytic activity of active forms of the protease was further assayed on fibronectin (Sigma), at an enzyme to substrate ratio 1:1 or 1:10 at 37°C for 16 h in 25 mM Tris buffer, pH 7.5. Experiments were performed with 2 mM DTT, a reducing agent known as cysteine protease activator and the samples were analyzed on 8% SDS-PAGE. Moreover, proteolytic activity of Cwp84\textsubscript{30-803} on Cwp84\textsubscript{C116A} was analyzed at an enzyme to substrate ratio 1:100 at 37°C for 0, 2, 4, 6 and 24 h in 200 µl of PBS with 2 mM DTT; Cwp84\textsubscript{C116A} was incubated alone in the same conditions.
Reassociation of recombinant Cwp84 with the *C. difficile* and *B. subtilis* cell surface. The *cwp84* mutant and *B. subtilis* were grown to stationary growth phase, in TYG and BHI respectively. In a first set of experiment, the *cwp84* mutant was washed once in PBS, and then incubated 1 h at 37°C in anaerobiosis, with 20 µg of Cwp84_{92-518}. Bacteria were washed five times with PBS Tween 0.05% in order to eliminate the recombinant protease that has not been associated tightly with the bacterial surface. As a negative control *C. difficile* 630fbpA_{640a::erm} and purified recombinant FbpA protein were incubated, in the same conditions. To analyze if the re-association was specific, we performed a second set of experiments, where we varied the amount of bacteria while maintaining the concentration of protein constant, as previously described (22). Thus, different dilutions of bacteria were incubated in 100 µl of PBS with 2µg of Cwp84_{92-518} for 1 h at 37°C. The samples were centrifuged at 10000g for 5 min, and the pellet was resuspended in 100 µl of PBS. The potential reassociation of Cwp84_{92-518} with *C. difficile* and *B. subtilis* cell surface was tested in the same condition, except that *B. subtilis* was grown in aerobic condition. Analysis was performed by dot-blot using specific anti-Cwp84 antibodies (1:5,000 dilution) to detect Cwp84 that have reassociated with bacterial cells.

**Immunofluorescence microscopy.** The localization of Cwp84 was examined by immunofluorescence microscopy. Strains 630 and 630Δerm *cwp84*_{347a::erm} were cultured in TY for 15 h at 37°C. Cells were washed with PBS, and 10^8 colony-forming units per ml (CFU ml⁻¹) were fixed on a slide by ethanol and 5% BSA in PBS was used as blocking agent. Incubation with first antibody (specific anti-Cwp84, 1:200) was performed for 1 h at room temperature in blocking buffer. Then, samples were washed with PBS and incubated with 1:500 dilution of fluorescein-conjugated Alexa Fluor 488 goat anti-rabbit IgG as the secondary antibody.
(Molecular Probes, Invitrogen) for 1 h at room temperature. Cells were washed again and stained with the nuclear stain 4',6'-diamidino-2-phenylindole (DAPI; Molecular Probes). Controls were carried out similarly; rabbit anti-FliC (1:200 dilutions) was used as a control of another surface protein, the bacteria were incubated without primary or secondary antibody as negative controls.

Fixed samples were analyzed by epifluorescence using a Nikon Eclipse 80i microscope, and the images were analysed using the ImageJ software (31).

MALDI/TOF-MS, LC-MS/MS and N-terminal protein sequencing. Purified Cwp8430-803 was incubated with 2 mM DTT for 4 h, in order to collect the mature cleaved form of the protease (mCwp84). This form was subjected to MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) mass spectrometry for the determination of molecular weight using a Perceptive Biosystems/Applied Biosystems/ABI Voyager DE-STR MALDI-TOF mass spectrometer. Automated Edman sequence analysis of mCwp84 was performed on the proteins transferred onto polyvinylidene difluoride (PVDF) membrane (Amersham, GE Healthcare). The blots were stained in 0.1% Coomassie Blue, 1% acetic acid, and destained with 50% methanol. Dry membranes were stored at -20°C. N-terminal sequencing was carried out at the Institute of Biochemistry and Molecular and Cellular Biophysics (IBBMC-UMR CNRS 8619), Team Chemistry of proteins, University Paris-Sud 11, on an Applied Biosystems Procise sequenator using established protocols. The LC-MS/MS analyses were performed using a 1200 Series nano-LC system (Agilent Technologies), coupled with an Agilent 6330 XCT Ion Trap equipped with a nanospray Chip Cube ion source, at the proteomic platform of IFR 141, University Paris-Sud 11.
RESULTS

Localization of Cwp84. In vitro experiments. In a previous work, we showed by immunoblotting that Cwp84 was surface associated, and was recovered mainly in the glycine extract associated with the S-layer (20). To confirm that the protease was located on the cell-surface, immunofluorescence analysis was performed in this study on C. difficile strain 630, after growth in TY medium. As shown in Fig. 1A, Cwp84 was localized on the cell surface. In the same conditions, flagella were observed at the bacterial surface of C. difficile (36) (data not shown). As expected, the 630Δerm cwp84Δ347a::erm strain (Fig. 1A) and the controls omitting the first or second antibodies were not labeled (data not shown), indicating that the fluorescent signal was Cwp84 specific.

To further analyze the exact localization of Cwp84, different fractions of C. difficile proteins, extracted after growth in TY or TYG media were studied by immunoblot analysis. In the glycine extract, regardless of growth medium, Cwp84 was recovered mainly as a protein of approximately 80 kDa as previously observed (Fig. 1B, lane 1) (20). The glycine extraction method exploits the ability of low pH to remove the S-layer and associated proteins from the bacterial surface without producing changes in the SLP; proteins that are extracted by this method should be non-covalently associated with the underlying cell-wall.

In the cell surface-associated proteins extract (Fig. 1B, lane 2), anti-Cwp84 antibodies mainly revealed 3 different forms of the protease: the 80 kDa form, an intermediate form of about 60 kDa and a mature form of about 50 kDa as determined by electrophoretic migration. However, some differences were observed in the relative amount of these three protease forms according to the growth medium. For example, the mature 50 kDa-Cwp84 was recovered especially in the
TYG medium. The method used to collect these extracts combines the use of mutanolysin to gently disorganize the cell wall with an abrasive method and consequently promotes the release of tightly cell wall-associated proteins. To ensure that these cleaved (intermediate and mature) forms of Cwp84 were not the result of a proteolytic process of the 80 kDa protein during the extraction steps, experiments were performed with E64 in the extraction solutions. No difference was observed (data not shown), suggesting that these different forms are truly associated with the bacteria. Moreover, no reaction was observed with the surface-associated proteins from the cwp84 mutant, indicating that detection of lower molecular weight proteins was not due to a cross reaction. These results suggest that Cwp84 could exist at the C. difficile surface under different forms anchored to the bacterial cell surface in different ways.

In the extracellular fraction, we observed both the 80 kDa form and the mature 50 kDa-form (Fig. 1B, lanes 3).

In vivo experiments. After C. difficile challenge in mice, bacteria expressed Cwp84 at the cell surface, mainly associated with the S-layer proteins in the glycine extract (Fig. 1C, lanes 1 and 2). The cysteine protease was detected as the 80 kDa form, processed intermediates forms and the mature cleaved 50 kDa form. Cwp84 was not detected in the extracellular fraction.

Proteolytic processing of recombinant Cwp8430-803. In our first Cwp84 study, we observed that Cwp8430-803 was processed into a cleaved form, called mCwp84 (20). The mCwp84 migrates on SDS-PAGE at approximately 50 kDa similarly to the cleaved mature form found mainly in the cell surface extract. So we hypothesized that the cleavage was due to an auto- proteolytic process.
and we observed that this process began during the metal affinity purification and the dialysis steps.

Therefore, to be able to observe the intermediates forms, we slowed down the maturation process and consequently we performed purification of Cwp84\textsubscript{30-803} under conditions of partial proteolytic inhibition. SDS-PAGE analysis showed that at 0, 2 and 4 h post-incubation with 2 mM DTT, intermediate forms of Cwp84\textsubscript{30-803} were detected, and the majority of Cwp84\textsubscript{30-803} had been converted in the mature form (mCwp84) at 6 h (Fig. 2A, lane 5). Whereas, for Cwp84\textsubscript{30-803} purified without the cysteine protease inhibitor, the protease was quite fully matured after the dialysis (0h post-incubation) and intermediate forms were detected only at very low levels (Fig. 2A, lane 6).

Immunoblot analysis of protease fractions purified with E64 revealed that anti-His tag antibodies reacted with distinct protein bands that were considered to correspond to the whole recombinant Cwp84\textsubscript{30-803} and some of its processed intermediate forms (Fig. 2B). The histidine tag at the N-terminal end was still present in Cwp84 cleaved forms, after 2 h of incubation with 2 mM DTT (Fig. 2B, lane 3) suggesting that the maturation process of recombinant Cwp84\textsubscript{30-803} begins at the C-terminal end. Since no His tag was detected after 4 h (Fig. 2B, lane 4), the maturation of Cwp84\textsubscript{30-803} seems to proceed via cleavage at the N-terminal end. These results suggested that the maturation process of Cwp84\textsubscript{30-803} occurred via sequential cleavage beginning at the C-terminal end, followed by one or more cleavages at the N-terminal end.

To determine the cleavage sites, the band corresponding to the mature form mCwp84 (of about 50 kDa as determined by SDS-PAGE) was analyzed using MALDI-TOF mass spectrometry, MS/MS and N-terminal Edman sequencing. The molecular mass obtained by MALDI-TOF was 47,136 Da. N-terminal sequencing revealed that a major cleavage site at the N-terminal part of
the Cwp84\textsubscript{30-803} occurred between the Lys-91 and the Ser-92 with an N-terminal amino acid sequence being SSVAYN (Fig. 3). This cleavage seemed to be heterogeneous with a minor site occurring between the Ser-93 and the Val-94 residues. By MS/MS, we identified a cleavage at the C-terminal end at Lys-491, and we confirmed the cleavage at the N-terminal end at Ser-92. Therefore the predicted molecular mass of this cleaved form (Ser 92 – Lys 491) was 44,500 Da calculated using ExPaSy software, which differed from the molecular mass calculated, 47,136 Da, by over 2,600 Da. This discrepancy indicated that the cleavage at the C-terminal end might take place downstream of this site, most likely between Lys-518 and Val-519 (Fig. 3), and that it has been hidden by trypsin digestion performed for MALDI analysis.

To assess if maturation of Cwp84 was an autoproteolytic process, we constructed an active site substitution, Cwp84\textsubscript{C116A}. As expected, Cwp84\textsubscript{C116A} displayed no proteolytic activity against azocasein. Surprisingly, after purification we did not observe as expected only the total protease form, but four bands corresponding to the total and intermediate forms, suggesting that these forms could finally be due to degradation during purification steps. The incubation of Cwp84\textsubscript{C116A} with 2mM DTT over 24 h did not lead to additional cleavage (Fig. 4 A), suggesting that the mutated protease was not able to complete its own cleavage process. In contrast, incubation with Cwp84\textsubscript{30-803} led to the conversion to intermediate and mature forms at 2, 4, 6 and 24 h (Fig 4B), similarly to the autocatalytic process of Cwp84\textsubscript{30-803}. This, together with the previous results, suggested that the complete maturation process of Cwp84 proceeds, at least partially, by an autocatalytic mechanism involving a trans-maturation.

**Purification and characterization of the proteolytic activity of Cwp84\textsubscript{30-803}, Cwp84\textsubscript{92-518} and Cwp84\textsubscript{30-518}**. In our previous study, we could not be certain which protein form was responsible
for the proteolytic activity (20). Therefore, we cloned the cwp84 gene region corresponding to the mature form (Cwp84\(^{92-518}\)), deduced from previous analysis, into a pET expression vector. The purified recombinant Cwp84\(^{92-518}\) was tested for proteolytic activity on azocasein and fibronectin. However, Cwp84\(^{92-518}\) did not show any proteolytic activity on these substrates, suggesting that the mature form was expressed as a non-active enzyme. Thus, a larger DNA fragment corresponding to the mature form including the pro-peptide of Cwp84 was cloned into pET expression vector. This form, named Cwp84\(^{30-518}\), was expressed and purified by affinity column. However, after SDS-PAGE analysis, two forms of the protease, the Cwp84\(^{30-518}\) and another form with a lower molecular weight, corresponding presumably to the mature Cwp84\(^{92-518}\) were detected (data not shown). This purified fraction displayed proteolytic activity on azocasein. As shown in the Fig. 5, the recombinant proteases Cwp84\(^{30-518}\) (lanes 4 and 5), and Cwp84\(^{30-803}\) (lanes 2 and 3) were able to degrade fibronectin. 

Reassociation of Cwp84 at the C. difficile surface. The existence of the mature form of the protease both as a surface associated protein and as an extracellular protein raises the hypothesis of a putative re-association of this form to the bacterial surface. As shown in Fig. 6A, incubation of 20 µg of recombinant Cwp84\(^{92-518}\) with the cwp84 mutant bacteria resulted in detection of labeled bacterial cells after immunodetection with specific anti-Cwp84 antibodies. No labeling was observed after incubation in the same conditions of another surface protein FbpA with the corresponding knock-out strain 630\(\Delta\)erm fbpa\(_{640::erm}\). This suggests that the protease is able to re-associate with the C. difficile surface.

To address whether Cwp84 is able to re-associate specifically to the bacterial surface of C. difficile, or whether it may associates any bacterial surface, different concentration of bacteria

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were incubated with 2 µg of Cwp84<sub>92-518</sub>. As shown in Fig. 6B, an efficient association of recombinant protein with the surface of *C. difficile* was detected in a concentration dependent manner, the level of re-association of recombinant protein decreased in parallel with the decrease of the bacterial amount. When *B. subtilis* was incubated with Cwp84<sub>92-518</sub> under identical conditions, a labeling in a non uniform manner was observed (Fig. 6B), suggesting that the association at the surface of *B. subtilis* is non specific. These results argued for the presence of a binding site for Cwp84<sub>92-518</sub> at the bacterial surface of *C. difficile*.

**DISCUSSION**

Cysteine proteases represent a large group of proteolytic enzymes that have been shown to contribute to a large variety of complex functions. Bacterial proteases play a critical role in the pathogenesis of several human and animal diseases by direct damage to host tissues, by activating zymogens, and often also participate in the turnover of bacterial proteins (25, 33). Cwp84 is the first characterized protease of *C. difficile*; this cysteine protease plays a key role in the maturation of S-layer proteins (10, 23). Cwp84 has also been shown to possess *in vitro* degrading activity against human ECM proteins (20), but the physiological relevance of this property is not yet known. In addition, it was hypothesized that Cwp84 could undergo autocatalytic maturation, as previously observed for numerous papain family cysteine proteases (32). The aim of the present study was to examine the localization of Cwp84 in the bacterial cell and its maturation process.

We have shown that the maturation process of recombinant Cwp84<sub>30-803</sub> proceeds in sequential cleavage reactions, beginning at the C-terminal domain. The cleavage at the N-terminal domain is
heterogeneous, with the major cleavage site identified at Lys-91, in accordance with the recent results from de la Riva et al. (11). It is noteworthy that the consensus cleavage site of SlpA into the mature S-layer proteins is also located after a serine residue (13, 14), but no other consensus motif has been recovered in Cwp84. The C-terminal cleavage site has been presumably identified at Lys-518. The molecular mass of the mature recombinant Cwp84 has been determined to be 47 kDa. Some members of the M4 family of metalloproteases require processing at the N-terminal end, and at the C-terminal end to become fully active (12). Since we observed a maturation process on purified recombinant protease, it is likely that these cleavages occurred via an autocatalytic mechanism. However, purification of the inactive mutated Cwp84\textsubscript{C116A} showed that some of the Cwp84 cleavages, leading to intermediary forms of the protease, could be due to a degradation process. Nevertheless, conclusive proof that ultimate conversion to the 47 kDa form proceeds via an autocatalytic mechanism was obtained from our processing experiments, where the different forms of the mutated Cwp84\textsubscript{C116A} protease were cleaved by active Cwp84\textsubscript{30-803}, leading to accumulation of the mature form (Fig. 4). Autocatalytic maturation of cysteine proteases could be due to \textit{cis} or \textit{trans} processing (7). It is therefore possible that first cleavages could be due to the activity of an exogenous protease, as recently suggested by de la Riva \textit{et al.} for the removal of the propeptide leading to the 77 kDa form associated to the S-layer; then, the following cleavages could result from an autoproteolytic mechanism. Maturation of protease involving both exogenous and autocatalytic processing has already been described (28).

In the mouse model, we found that Cwp84 is associated with the bacterial surface in different forms, suggesting that the proteolytic maturation of Cwp84 occurs in the gut. The absence of Cwp84 in the extracellular fraction could be due to the dilution of the caecal contents.
In this study, we showed that Cwp84 was expressed as an extracellular protein and mostly localized at the bacterial surface. Surface localization is not usual for bacterial cysteine protease, but has been already reported for the SpeB protease of *Streptococcus pyogenes* (19). Here, we confirmed that Cwp84 exists at the bacterial cell surface associated with the S-layer proteins mainly as a ~80 kDa protein, and this is consistent with the recent results from de la Riva *et al.* (11). As we had not precisely determined the molecular weight of this form in the bacterial cell, it could correspond either to the proprotein (amino acids 33 to 803), or to a form without the propeptide (amino acids 92 to 803). The association of the protease with the S-layer proteins is not unexpected, since these latter are substrate for Cwp84 (23). Moreover, the C-terminal part of Cwp84 contains three PF04122 Pfam « cell wall binding » motifs, which could mediate anchoring to the cell wall (6, 21). These motifs are also found in three copies in the HMW-SLP (15), suggesting that these two proteins may share a common binding mechanism to the *C. difficile* surface. This mechanism is not yet characterized, but is likely to involve a non-covalent attachment mode. Furthermore, we also recovered the protease as a lower molecular weight form, both in the extracellular and in the cell wall-associated protein fractions, especially when *C. difficile* was grown in the TYG medium. This form shared the same molecular weight as the cleaved one, which resulted from maturation of the recombinant Cwp84_{30-803}, and we assume that it is the same mature form of the protease. The mature protease could be secreted into the extracellular fraction after cleavage at the C-terminal part. However, it is surprising to recover this mature form tightly associated with the underlying cell wall. It may be noted that this last result is consistent with a previous proteomic study, where Cwp84 was recovered in the extracts collected after enzymatic degradation of the peptidoglycan (42). One hypothesis is that the secreted cleaved protease could
be re-associated with the bacterial surface, as it has been shown for some surface proteins in other bacteria (2, 9), and previously proposed as an anchoring mechanism of GroEL at the \textit{C. difficile} surface (18). To further investigate this hypothesis, the ability of recombinant Cwp84_{92-518} to re-associate at the surface of \textit{C. difficile} was explored. This recombinant protein was able to specifically re-associate at the surface of the $630\Delta_{erm}\ cwp84_{347a::erm}$ mutant; this specific reassociation was not observed with \textit{B. subtilis}. The recombinant protein Cwp84_{92-518} does not possess the three complete PF04122 Pfam motifs, but only eleven amino acids of the first motif. Therefore, the Pfam motifs may not be necessary to the re-association of the mature protease. Neither GW nor LysM conserved anchoring motifs have been recovered (4). Thus, the Cwp84 anchoring domain to the underlying cell surface is still unknown but could be mediated by interactions either with the peptidoglycan or lipoteichoic acids.

Cwp84 is located at the bacterial surface and also released extracellularly, and may contribute to the pathogenicity of \textit{C. difficile} by several mechanisms. Cwp84 participates in the turnover of S-layer proteins (23). It is therefore possible that the different forms of Cwp84 may promote either cleavage of SlpA or additional functions in the development of the infection. We did not explore in this study, which form of the protease could be responsible for the maturation of the S-layer protein, but it is likely to be the form Cwp84_{30-803} associated to the S-layer. In contrast, degrading activity against human tissues could be expressed by the mature extracellular Cwp84. Indeed, in a previous study, we showed that Cwp84 has a degrading activity on some ECM proteins (20), but we could not assign this role to one defined form of the protease. Here, purification of the cleaved forms helped us to answer this question. We observed that the purified recombinant mature Cwp84_{92-518} had no proteolytic activity on tested substrates, whereas the purified Cwp84_{30-518} expressed catalytic activity against azocasein and fibronectin. The inactivity of mature Cwp84_{92-518}
may be due to a defective folding of the active site of this recombinant protein. Other recombinant cysteine proteases have been purified directly under mature form and have also been shown to be inactive, suggesting that the pro-peptide may play a role in the maturation and correct folding of the catalytically mature protein (3). In fact, the recombinant Cwp84$_{30-518}$ was also cleaved during the purification in the mature protease; therefore we could not exclude that the proteolytically active form is the Cwp84$_{92-518}$ and that presence of pro-peptide is essential for correct folding before maturation of the active mature protease.

In which subcellular compartment Cwp84 maturation occurs remains to be found. However, according to our results, we could hypothesize that, after the peptide signal removal from the preproprotein and translocation across the cytoplasmic membrane, the 84 kDa proprotease or the 77 kDa form (without pro-peptide) is associated to the S-layer. Cleavage at the C-terminal domain leads finally to secretion of mature Cwp84, where it can cleave host proteins and also be re-associated to the bacterial surface (Fig. 7). These different phases may require stringent regulation by environmental conditions.

**ACKNOWLEDGEMENTS.**

Diana Chapeton Montes is supported by a doctoral fellowship from the French Ministry of Higher Education and Research. We thank Celine Boursier for her kind assistance in proteomic analyses (proteomic platform, IFR 141, Université Paris-Sud), and Séverine Péchiné for her valuable advice. Special thanks also to Neil Fairweather for the gift of the cwp84 mutant strain and his appreciation of this manuscript.
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**Figure legends**

**Fig. 1. Localization of Cwp84 in *C. difficile*.** (A) Immunofluorescence microscopy of Cwp84 (green) by Alexa Fluor 488 in whole bacteria, comparing the 630 and the 630Δerm cwp84Δerm strains; the nucleus was stained by DAPI (blue). (B) Immunoblot analysis using Cwp84 antibodies was carried out on protein extracts collected from bacteria grown in TY and
Fig 2. Proteolytic process of Cwp84\textsubscript{30-803}. The recombinant protease Cwp84\textsubscript{30-803} was purified in the presence or absence of 50 µM E-64, and then incubated under reducing conditions, with 2 mM DTT. (A) SDS-PAGE analysis. Purification of Cwp84\textsubscript{30-803} in presence of the cysteine protease inhibitor (lanes 2 to 5) resulted in the detection of intermediates forms at 0, 2 and 4 h, and the mature form at 6 h, whereas the purification in absence of E64 (lanes 6 to 9) resulted in the detection of the mature form from 0 h. Lane 1 corresponds to the protease before dialysis. (B) Immunoblot analysis using anti-His tag antibody. In the presence of E64, the histidine tag fused to the N-terminal end was detected in the total and some of the processed intermediate forms of Cwp84; however the mature form was not detected, suggesting that proteolytic process of Cwp84 begins at the C-terminal end, and follows at the N-terminal end. The molecular weight standard Bio-Rad was used.

Fig 3. Schematic representation of Cwp84 proteolytic processing. cwp84 encodes a protein of 803 amino acid residues. The peptide signal is shown dark, the catalytic domain is clear, and the domain corresponding to the anchoring domain is shaded. The scheme shows the three Pfam 04122 motifs and the catalytic triad (Cys-116, His-262 and Asn-287). The amino acids involved in the proteolytic cleavage are indicated.
Fig 4. Purification of Cwp84C116A and trans-maturation by Cwp8430-803. The mutated protease Cwp84C116A was incubated at 37°C alone or with Cwp8430-803 under reducing conditions, for 0, 2, 4, 6 and 24 h. (A) Cwp84C116A alone with 2mM DTT do not show the maturation process, while (B) incubation of Cwp84C116A with Cwp8430-803, at an enzyme to substrate ratio 1:100 resulted in the increase of intermediate forms at 4 and 6 h, and detection of mature form at 24 h. The molecular weight standard used was purchased from Fermentas.

Fig 5. Proteolytic activity of Cwp8430-803 and Cwp8430-518 on fibronectin. The recombinant proteases were incubated 16 h with the fibronectin, with an enzyme: substrate ratio 1:1 and 1:10. All experiments were performed under reducing conditions (2 mM DTT). Lane 1, fibronectin control (5 µg); lane 2, Cwp8430-803: fibronectin (1:1); lane 3, Cwp8430-803: fibronectin (1:10); lane 4, Cwp8430-518: fibronectin (1:1); and lane 5, Cwp8430-518: fibronectin (1:10). The molecular weight standard used was Fermentas.

Fig. 6. Reassociation of cwp84 protein. Bacteria were loaded on a nitrocellulose membrane and treated with specific anti-Cwp84 or anti-Fbp68 antibodies. (A) C. difficile 630Δerm cwp843475::erm and 630Δerm fbpA640a::erm were incubated with 20 µg of Cwp8492-518 (lane 3); lane 1 corresponds to the positive control Cwp8492-518; lane 2 corresponds to the negative control 630Δerm cwp843475::erm and 630Δerm fbpA640a::erm non incubated with the recombinant protease. (B) Different amounts of C. difficile 630Δerm cwp843475::erm and B. subtilis: 5x10^8 (lane1), 10^8 (lane 2), 10^7 (lane 3), 10^6 (lane4), 10^5 (lane 5) and 10^4 (lane 6) were incubated with 2 µg of Cwp8492-518. The samples were centrifuged and the re-association of recombinant Cwp84 was analyzed by dot blot.
**Fig. 7. Cwp84 localization and maturation process model.** The Cwp84 native form is composed of a signal peptide, an N-terminal pro-peptide (dash line), a N-terminal part (empty circle) and a C-terminal part (part-filled circle). The native protein is exported to the S-layer probably via the SecA secretion system where the signal peptide is removed. Cwp84_{30-803} is then associated with the S-layer where the N-terminal peptide (dash line) is potentially removed. At this sub-cellular position, it is likely that Cwp84_{30-803} cleaves the SlpA protein and participates in the S-layer turnover. When Cwp84_{30-803} is released from the bacterial surface, first it is cleaved at the C-terminal part leading to Cwp84_{30-518} and second after the N-terminal propeptide leading to Cwp84_{92-518}. According to our observations in *E. coli*, at least the last cleavage is probably due to an automaturation mechanism. The mature Cwp84_{92-518} is thereafter reassociated with the underlying cell wall. When the mature form is not surface bound, it potentially degrades the ECM host proteins.