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Roles of Cysteine Proteases Cwp84 and Cwp13 in Biogenesis of the Cell Wall of *Clostridium difficile*▽†

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Clostridium difficile **expresses a number of cell wall proteins, including the abundant high-molecular-weight and low-molecular-weight S-layer proteins (SLPs). These proteins are generated by posttranslational cleavage of the precursor SlpA by the cysteine protease Cwp84. We compared the phenotypes of *C. difficile* strains containing insertional mutations in either *cwp84* or its paralog *cwp13* and complemented with plasmids expressing wild-type or mutant forms of their genes. We show that the presence of uncleaved SlpA in the cell wall of the *cwp84* mutant results in aberrant retention of other cell wall proteins at the cell surface, as demonstrated by secretion of the proteins Cwp66 and Cwp2 into the growth medium. These phenotypes are restored by complementation with a plasmid expressing wild-type Cwp84 enzyme but not with one encoding a Cys116Ala substitution in the active site. The *cwp13* mutant cleaved the SlpA precursor normally and had a wild-type-like colony phenotype. Both Cwp84 and Cwp13 are produced as proenzymes which are processed by cleavage to produce mature enzymes. In the case of Cwp84, this cleavage does not appear to be autocatalytic, whereas in Cwp13 autocatalysis was demonstrated as a Cys109Ala mutant did not undergo processing. Cwp13 appears to have a role in processing of Cwp84 but is not essential for Cwp84 activity. Cwp13 cleaves SlpA in the HMW SLP domain, which we suggest may reflect a role in cleavage and degradation of misfolded proteins at the cell surface.**

Clostridium difficile is a Gram-positive, spore-forming anaerobe that can cause serious gastrointestinal infections in humans and animals (8, 34). *C. difficile* infection (CDI) is most commonly seen in hospital environments and is associated with antimicrobial therapy that disrupts the normal microbiota (1). Clinical symptoms of disease range from mild or severe diarrhea to serious inflammatory conditions including pseudomembranous colitis (34). Although the elderly population still remains the largest at-risk group, CDI is increasingly being seen in younger patients and in patients in the community (16). The spores are the infectious form of the bacterium, as recently demonstrated in a mouse model of transmission (25).

Symptoms of disease are primarily caused by two secreted virulence factors, the toxins TcdA and TcdB. The mechanisms of action of these toxins have been well described, with both toxins exhibiting glucosyltransferase activity which inactivates Rho GTPases within host cells (21). This causes pleiotropic effects, including disruption of the actin cytoskeleton and tight junctions, induction of apoptosis, fluid accumulation, and destruction of the epithelium. Recent studies using *C. difficile* toxin knockout strains in the hamster model of infection have examined the essential nature of these toxins in disease (24, 27). Although the results of these studies were not in complete agreement, it appears that toxin A-negative strains were more virulent than toxin B-negative strains.

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C. difficile has a cell wall typical of Gram-positive bacteria, comprising a cytoplasmic membrane and a thick peptidoglycan layer that may contain teichoic acids and other secondary cell wall polymers (31). In common with many bacteria, *C. difficile* expresses an S-layer, a two-dimensional proteinaceous array that coats the outer surface of the bacterium. The S-layer is composed primarily of two proteins, the high-molecular-weight S-layer protein (HMW SLP) and the low-molecular-weight (LMW) SLP (5). The SLPs can be removed from the cell by treatment with low pH glycine (5), which also removes other cell wall proteins (CWPs) present in relatively low amounts within the cell wall. The HMW SLP and the CWPs each contain three cell wall-binding motifs (Pfam 04122 [http://pfam.sanger.ac.uk/]) that appear to mediate noncovalent binding to the underlying cell wall by an uncharacterized mechanism. The majority of the CWPs have a second unique domain that in some cases specifies, or is predicted to specify, a function (11). Examples include Cwp66, a putative adhesin (41), CwpV, a phase-variable protein (12), and Cwp84, a cysteine protease (20).

The SLPs are present as a heterodimeric complex within the S-layer, and structural analysis has revealed the HMW SLP and LMW SLP interact through highly conserved domains present at the C terminus of the LMW SLP and the N-terminal domain of the HMW SLP (14). The remaining portions of the SLPs exhibit sequence divergence, in particular the LMW SLP, which shows immunological diversity between many *C. difficile* strains (4). The SLPs are derived from a precursor protein, SlpA, by proteolytic cleavage which removes the signal peptide, followed by a second cleavage resulting in the mature SLPs (5, 22). Recently, using both chemical and genetic techniques, the cysteine protease Cwp84 was shown to mediate

TABLE 1. Bacterial strains and plasmids used in this study

Strain of plasmid	Relevant characteristics	Source or reference
C. difficile strains		
630Δ*erm*	Also named WT in this study	19
NF2184	630Δ*erm* $cwp84_{347a}$::*erm*; also named *cwp84* mutant in this study	This study
NF2233	630Δ*erm* $cwp13_{833a}$::*erm*; also named *cwp13* mutant in this study	This study
Plasmids		
pMTL960	*E. coli-C. difficile* shuttle vector	32
pCBR023	Contains modified *gusA* gene under the control of the *cwp2* promoter; pUC19 backbone	12
pMTL007C-E2-*cwp84*-347/348a	pMTL007C-E2 with the Ll.ltrB intron retargeted to insert after base 347 in the 630Δ*erm* *cwp84* ORF	18
pLRP024	Contains the *cwp84* gene under the control of the *cwp2* promoter; pUC19 backbone	This study
pLRP025	Contains a derivative of the *cwp84* gene, with a mutation for generating the substitution Cys116Ala, under the control of the *cwp2* promoter; pUC19 backbone	This study
$pCwp84_{WT}$	Insert from pLRP024 cloned into pMTL960	This study
$pCwp84_{C116A}$	Insert from pLRP025 cloned into pMTL960	This study
pMTL007C-E5-*cwp13*-833/834a	pMTL007C-E5 with the Ll.ltrB intron retargeted to insert after base 833 in the 630Δ*erm* *cwp13* ORF	18
pLRP033	Contains the *cwp13* gene under the control of the *cwp2* promoter; pUC19 backbone	This study
pLRP035	Insert from pLRP033 cloned into pMTL960	This study
$pCwp13_{WT}$	pMTL960 containing the *cwp13* gene, with the RBS-ORF spacer region of *cwp84* and a mutation for generating the substitution Val1Met, under the control of the *cwp2* promoter	This study
$pCwp13_{C109A}$	pMTL960 containing the *cwp13* gene, with the RBS-ORF spacer region of *cwp84* and two mutations for generating the substitutions Val1Met and Cys288Ala, under the control of the *cwp2* promoter	This study
pCBR044	pMTL960 containing the full-length *cwpV* gene from 630 under the control of *cwp2* promoter	12

cleavage of the mature SlpA precursor (9, 23). Cwp84 has also been implicated in degradation of extracellular matrix proteins such as fibronectin, laminin, and vitronectin (20). Neither chemical inhibition of Cwp84 (9) nor inactivation of the *cwp84* gene (23) resulted in lethality, although severe growth defects were seen in both cases. These results indicate that correct processing of SlpA is important to retain healthy bacterial cells and suggest that perturbation of processing may affect the ability of bacteria to compete with other bacterial species in certain environments, for example, in the complex microbiota of the intestine. Interestingly, in the hamster model of acute infection, a *C. difficile cwp84* mutant was not attenuated for virulence, which may be due to endogenous proteases within the hamster that could substitute for Cwp84 in cleavage of SlpA (23).

In addition to Cwp84, *C. difficile* 630 encodes a second, homologous protein termed Cwp13 (CD1751). *cwp13* exhibits 70.4% nucleotide identity to *cwp84*, and the Cwp13 protein exhibits 63.2% amino acid identity to Cwp84. *cwp13* is located outside the gene cluster that contains *slpA*, *cwp84*, and 10 other CWP genes. Cwp84 and Cwp13 are predicted to be members of the family of CA1 cysteine proteases (33). Proteases in this family (also termed papain proteases) possess a catalytic triad which in Cwp84 is proposed to comprise Cys116, His262, and Asn287 (37). We recently showed that Cwp84 containing the substitution Cys116Ala does not cleave SlpA in an *Escherichia coli*-based coexpression assay, confirming Cys116 as a catalytic residue (9). Papain peptidases are typically composed of an N-terminal signal peptide, a propeptide, and the catalytic domain (reviewed in reference 42). After removal of the signal peptide by a signal peptidase, the proenzyme undergoes self-cleavage, removing the proregion and generating the mature, active enzyme. The main function of the propeptide is to inhibit the activity of the proenzyme (15), which is potentially active in a manner independent of substrate binding due to the ionized state of the nucleophilic Cys. Furthermore, it has been proposed that the propeptide ensures the correct folding of the protein (6). There are, however, some papain peptidases that do not undergo autoprocessing, including human cathepsins X and C (29). Previous studies have indicated that Cwp84 undergoes autoprocessing based on two findings. First, an attempt to purify full-length Cwp84 from *E. coli* resulted in degradation of the recombinant protein. However, degradation was not prevented by treatment with cysteine protease inhibitors (37). Second, proteins of various sizes were purified from *E. coli* containing a plasmid designed to produce Cwp84 without its signal peptide (84 kDa) and treatment with dithiothreitol or trypsin resulted in purification of a single, active species of 61 kDa (20).

We examine here in detail the phenotype of a *C. difficile cwp84* mutant and show that some other CWPs are dependent on Cwp84 activity for correct anchoring to the cell wall. Using a plasmid-localized copy of the wild-type gene in a *cwp84* background, we characterized the processing of *cwp84*. We have created a mutant in *cwp13* and show that this has subtle effects of the processing of Cwp84. Finally, we have analyzed the role of Cwp84 and Cwp13 in the cleavage of SlpA and of Cwp13 in the processing of the phase-variable protein CwpV (12).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *E. coli* strains Top10 (Invitrogen) and NovaBlue (Novagen) were used routinely for cloning and propagation of plasmids. *E. coli* strain CA434 was used for conjugating plasmids into *C. difficile*. *E. coli* strains were routinely grown in LB broth (2) or on LB agar supplemented with appropriate antibiotics: carbenicillin at 50 μg/ml for the

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)[a]	Relevant characteristic	Source or reference
NF722	ACGCGTTATATTGATAAAAATAATAATAGTGGG	ErmRam-F	17
NF723	ACGCGTGCGACTCATAGAATTATTTCCTCCCG	ErmRam-R	
NF1063	CGAAATTAGAAACTTGCGTTCAGTAAAC	EBS Universal	17
NF1163	CCATAAAACTCTAGATGGAG	Fw primer used for screening of insertion of the Ll.ltrB intron after base 347 in the 630Δ*erm cwp84* ORF	This study
NF1165	GTTACATTTGAACTCCCTG	Rv primer for NF1163	This study
NF1225	AGTCTTAATACA**GCA**TGGTCTTTTTCAG	Fw primer used for introducing the Cys116Ala mutation in Cwp84 by inverse PCR from plasmid pLRP024	9
NF1226	TCCTTGATTTTTTGCTGGTGTAGTAAGAC	Rv primer for NF1225	9
NF1250	CCG*GAATTC*ATACATATAAGGGGGTAAACATGAG	Fw primer for amplification of *cwp84*	This study
NF1251	CGC*GGATCC*CTAGTGGTGGTGGTGGTGGTGCTCGAG TTTTCCTAAAAGAGTATTTAG	Rv primer for NF1250	This study
NF1462	CCG*GAATTC*CTCAAAATAAGGGGGAGAAAGCGTG	Fw primer for amplification of *cwp13*	This study
NF1463	CGC*GGATCC*CTAGTGGTGGTGGTGGTGGTGCTCGAG TTTAGCACTTTTTAATTTTACTAACTCT	Rv primer for NF1462	This study
NF1479	**TAAACATG**AAAAAATTTACTTCAAAAAAAGTAACA	Fw primer used for replacing the RBS-ORF spacer region of *cwp13* by that of *cwp84* and introducing a Val1Met substitution	This study
NF1480	CCCCCTTATTTTGAGGAATTCTTAT	Rv primer for NF1479	This study
NF1487	CTGTGTGTGTAAGTAATGACATAAATTCTAC	Fw primer for screening of insertion of the Ll.ltrB intron after base 833 in the 630Δ*erm cwp13* ORF	This study
NF1488	GTTAAGAACATTACAGATTTTAGAGTTTCATCGCCTC TATTG	Rv primer for NF1487	This study
NF1489	GATTTAGGAATC**GCA**TGGGATTTTG	Fw primer used for substituting Cys109 by Ala in Cwp13	This study
NF1490	CTCTTGATTTTTTATTGATGTCATAAGAC	Rv primer for NF1489	This study

[a] Restriction endonuclease sites (EcoRI or BamHI) are italicized. Sequences encoding the Leu-Glu-His_6 tag are underlined. Mutations introduced by primers are indicated in boldface and are described in detail in column 3.

selection of derivatives of pUC19 and chloramphenicol at 12.5 μg/ml for the selection of derivatives of pMTL007 or pMTL960. The *C. difficile* strains and plasmids used in the present study are described in Table 1. *C. difficile* was routinely grown on blood agar base (Oxoid) supplemented with 7% defibrinated horse blood (TCS Biosciences), on brain heart infusion (BHI) agar (Oxoid), or in BHI broth (Oxoid). Thiamphenicol at 15 μg/ml was used for selection of derivatives of pMTL007 or pMTL960 in *C. difficile*. Growth was carried out under anaerobic conditions (10% H_2, 10% CO_2, 80% N_2) at 37°C. Plasmids were introduced by conjugation into *C. difficile* as described previously (32) using LB medium for washing the pellets of the overnight cultures of the donor strains and BHI instead of phosphate-buffered saline (PBS) for scraping the transconjugants off BHI plates.

Plasmid construction. DNA manipulations were performed according to standard techniques (35). Primers used in the present study are detailed in Table 2. Inserts for cloning were amplified from genomic DNA, which was isolated as described previously (5), using KOD Hot Start DNA polymerase (Novagen), which was also used for inverse PCR. Standard PCR was performed using *Taq* DNA polymerase (Sigma). Restriction enzymes were provided by New England BioLabs. Ligations were carried out using Quick-Stick ligase (Bioline) according to the protocol given by the manufacturer. All recombinant DNA molecules that were generated in the present study were confirmed to be correct by sequencing (GATC Biotech).

$pCwp84_{WT}$ and derivatives were constructed as follows. *cwp84* was amplified by using the primers NF1250 and NF1251 to introduce a C-terminal His_6 tag. The fragment was cloned into the pCBR023 backbone, replacing the *gusA* gene (12) using EcoRI and BamHI sites, to generate plasmid pLRP024. Inverse PCR was carried out on pLRP024 by using the primers NF1225 and NF1226 as described previously (14) to obtain plasmid pLRP025, which contains a mutation substituting Cys116 in Cwp84 by Ala. The P_{cwp2}-$cwp84_{WT}$ and P_{cwp2}-$cwp84_{C116A}$ fragments were subcloned into pMTL960 using the Acc65I and BamHI sites to obtain the plasmids $pCwp84_{WT}$ and $pCwp84_{C116A}$, respectively.

$pCwp13_{WT}$ and derivatives were constructed as follows. c*wp13* was amplified by using the primers NF1462 and NF1463 to introduce a C-terminal His_6 tag. Plasmid pLRP033 was obtained in the same way as pLRP024 and pLRP025. The P_{cwp2}-*cwp13* fragment was subcloned into pMTL960 as detailed above to obtain plasmid pLRP035. Inverse PCR was carried out using pLRP035 and primers NF1479 and NF1480 to replace the ribosomal binding site (RBS), RBS-open reading frame (ORF) spacer and Val1 encoding GTG of *cwp13* with the RBS, RBS-ORF, and Met1 encoding ATG of *cwp84*, obtaining plasmid $pCwp13_{WT}$. Plasmid $pCwp13_{C109A}$ was generated by inverse PCR from $pCwp13_{WT}$ using the primers NF1489 and NF1490.

Construction of mutants in *C. difficile* 630Δ*erm*. The ClosTron gene knockout system (17) was used to obtain insertional mutants in *cwp84* (CD2787) and *cwp13* (CD1751) genes. *cwp84* and *cwp13* were analyzed by the Intron Design Tool (USG, University of Nottingham, Nottingham, United Kingdom) to identify insertion sites for the Ll.ltrB intron. The Ll.ltrB intron was inserted after base 347 in *cwp84* and after base 833 in *cwp13*, both in the antisense orientation, using the retargeted plasmids pMTL007C-E2-*cwp84*-347/348a and pMTL007C-E5-*cwp13*-833/834a. The plasmids were synthesized (DNA2.0, Menlo Park, CA) and transformed into *E. coli* CA434 prior to conjugation into *C. difficile* 630Δ*erm*. The following protocol for generating and selecting mutants was kindly provided by Lisa Dawson (London School of Hygiene and Tropical Hygiene, London, United Kingdom). First, 1 ml of an overnight culture of the donor strain was harvested by centrifugation at 1,700 × *g* for 5 min. The pellet was washed once in LB broth and then resuspended in 200 μl of a culture of the recipient strain in exponential phase of growth. Then, 20-μl drops of the bacterial suspension were spotted onto Brazier's agar (Bioconnections) supplemented with 4% egg yolk (Bioconnections) and 1% defibrinated horse blood (TCS Biosciences), followed by incubation overnight. Bacterial growth was scraped off the plates with 600 μl of PBS and were spread onto Brazier's agar supplemented with egg yolk, blood, 15 μg of thiamphenicol/ml, and cefoxitin-cycloserine supplements (Bioconnections). Transconjugants were allowed to grow for 2 to 3 days before single colonies were purified twice on the same medium. Pure transconjugants were resuspended in 200 μl of PBS, and 100-μl samples of undiluted bacterial suspension and of 1:100, 1:1,000, and 1:10,000 dilutions were spread onto Brazier's agar supplemented with egg yolk, blood, and 5 μg of erythromycin/ml. Erythromycin-resistant colonies were streaked twice onto the same medium. For PCR screening of the insertion of the intron within the target gene, genomic DNA was extracted

and used as a template for the following PCRs (see Fig. S1 in the supplemental material). (i) Splicing of the group I intron from the group II intron was confirmed by using the primers NF722 and NF723. (ii) Integration of the Ll.ltrB intron was confirmed by using primers flanking the insertion site. The primers NF1163 and NF1165 were used in the case of the *cwp84* mutant, and NF1487 and NF1488 were used in the case of the *cwp13* mutant. The *cwp13* gene in the *cwp84* mutant and the *cwp84* gene in the *cwp13* mutant were also analyzed to confirm insertion of the Ll.ltrB intron in the specific target gene in each case. (iii) Integration in the antisense orientation was confirmed by using the intron-specific primer EBS Universal (17) and one of the two flanking primers.

Protein analysis. Cell wall proteins were extracted from *C. difficile* cells using the low-pH glycine extraction method described previously (4). Culture supernatants were concentrated using Amicon 10-kDa cutoff centrifugal filter units (Millipore) according to the instructions given by the manufacturer. SDS-PAGE was carried out as described previously (13). Acrylamide in the resolving gel was used at 10% unless otherwise specified. For immunoblot analysis, proteins were transferred to Immobilon-PVDF membranes (Millipore) using a three-buffer semidry method according to the instructions provided by the manufacturer. The rabbit antibodies to HMW SLP and LMW SLPs were described previously (14). Antisera against Cwp84, Cwp66-Cter, and Cwp2 were raised in mice. Anti-Cwp84, anti-Cwp66, anti-Cwp2, anti-HMW SLP, and anti-LMW SLP were used at the dilutions 1:4,000, 1:20,000, 1:50,000, 1:100,000, and 1:200,000, respectively. Primary antibodies were detected by using horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody at 1:2,000 or rabbit anti-mouse antibody at 1:1,000 (Dako) and the SuperSignal West Pico chemiluminescent substrate (Thermo Scientific Pierce). His_6 tag was detected using HRP-conjugated anti-histidine tag (Sigma).

N-terminal sequences were determined by Edman degradation at the Protein and Nucleic Acid Chemistry Facility of the University of Cambridge, Cambridge, United Kingdom. Samples were prepared according to the instructions given on http://www.bioc.cam.ac.uk/pnac/proteinsequencing.html. For a better resolution of the 77-kDa Cwp84 band and the 78-kDa and 84-kDa Cwp13 bands, proteins were separated using bis-Tris gels (http://openwetware.org/wiki/Sauer:bis-Tris_SDS-PAGE,_the_very_best) with 8% acrylamide.

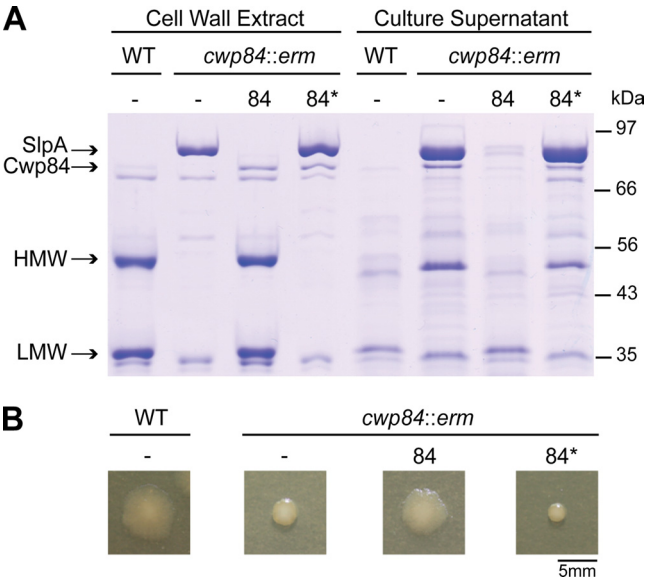

FIG. 1. Phenotype and complementation of a *cwp84* mutant *of C. difficile* 630Δ*erm*. (A) SDS-PAGE analysis of cell wall proteins and culture supernatants. Bacteria containing plasmids (see below) were grown in BHI broth. Cell wall proteins were extracted, and culture supernatants were concentrated from bacterial cultures at late exponential phase (OD_{600} = 0.5). Plasmids: –, pMTL960; 84, $pCwp84_{WT}$; 84*, $pCwp84_{C116A}$. (B) Colony morphologies. Serial dilutions (100 μl) of overnight cultures were plated on BHI agar supplemented with 15 μg of thiamphenicol/ml and grown for 2 days, and representative colonies were photographed.

RESULTS

Characterization of a *C. difficile cwp84* mutant. To examine the role of Cwp84 in cell wall biogenesis, we generated an insertionally inactivated mutant of *cwp84* in *C. difficile* 630Δ*erm* (WT) using targeted mutagenesis (17). Several independent mutants were analyzed and all exhibited the same genotype and phenotype. In the *cwp84* mutant (Fig. 1A), the full-length SlpA precursor was the predominant band in the cell wall extract, and the mature HMW and LMW SLPs were not present, confirming the phenotype observed previously (23). In order to complement the mutation and confirm in *C. difficile* that Cys116 in Cwp84 is a catalytically active residue as described previously in *E. coli* (9), we used the shuttle plasmid pMTL960 to introduce into the *cwp84* mutant strain either the wild-type *cwp84* gene ($pCwp84_{WT}$) or a derivative containing a Cys116Ala mutation ($pCwp84_{C116A}$). In these plasmids the *cwp84* gene was placed under the control of P_{cwp2}, a promoter we used previously to obtain constitutive expression of cell wall proteins in *C. difficile* (12). Both Cwp84 proteins incorporated a C-terminal His_6 tag to aid identification.

The plasmid-encoded wild-type *cwp84* gene completely restored cleavage of SlpA in the *cwp84* mutant strain, as judged by the appearance of the mature SLPs in the cell wall extracts (Fig. 1A), whereas the strain harboring the *cwp84*(C116A) allele was indistinguishable from the *cwp84* mutant. Analysis of the culture supernatants showed that the *cwp84* mutant harboring either the vector pMTL960 or $pCwp84_{C116A}$ secreted large amounts of unprocessed SlpA into the culture medium, in contrast to the *cwp84* mutant *c*omplemented with $pCwp84_{WT}$ or the WT strain, where the secretion of SlpA was barely detectable.

Previously, it was noted that deletion of *cwp84* resulted in a small-colony phenotype (23). As shown in Fig. 1B, *cwp84* mutant colonies lack the rough, irregular edges typical of *C. difficile* and instead are shiny and have a defined edge and a raised center. Complementation of the *cwp84* mutant with $pCwp84_{WT}$ restored the colony morphology seen with *C. difficile* 630Δ*erm*, whereas the small-colony morphology was retained in the *cwp84* mutant carrying $pCwp84_{C116A}$.

Deletion of *cwp84* results in instability of the cell wall. Since the colony morphology of the *cwp84* mutant was so different from the WT strain, we investigated whether there were any other changes to the cell wall composition of this strain. Cell wall extracts and supernatants were prepared from WT and *cwp84* mutant cultures containing plasmids $pCwp84_{WT}$ or $pCwp84_{C116A}$ and analyzed by Western blotting. Using antibody prepared against the C-terminal domain of Cwp66, a cell wall protein described as an adhesin (41), a band of ~70 kDa can be seen in the cell wall extracts of 630Δ*erm* (WT) (Fig. 2, anti-Cwp66). In contrast, this band was absent in the *cwp84* mutant but present when it harbored $pCwp84_{WT}$ but not $pCwp84_{C116A}$. Analysis of the culture supernatants of the *cwp84* mutant containing the vector pMTL960 or the $pCwp84_{C116A}$ plasmid revealed two bands, one corresponding to the full-length Cwp66 and a second of ~45 kDa which is presumably a degradation product (Fig. 2, anti-Cwp66). These results indicate that the absence of active Cwp84 in the cell wall, as well as the consequential lack of mature HMW and

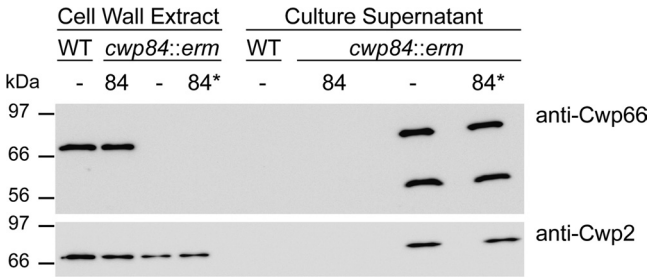

FIG. 2. Cell wall protein localization is defective in a *cwp84* mutant. *C. difficile* 630Δ*erm* (WT) and the *cwp84* mutant harboring plasmids (see below) were grown in BHI broth to late exponential phase (OD_{600} = 0.5). Cell wall extracts and culture supernatants were prepared and analyzed by SDS-PAGE, followed by Western blotting with antibodies against Cwp66 and Cwp2. Plasmids: –, pMTL960; 84, $pCwp84_{WT}$; 84*, $pCwp84_{C116A}$.

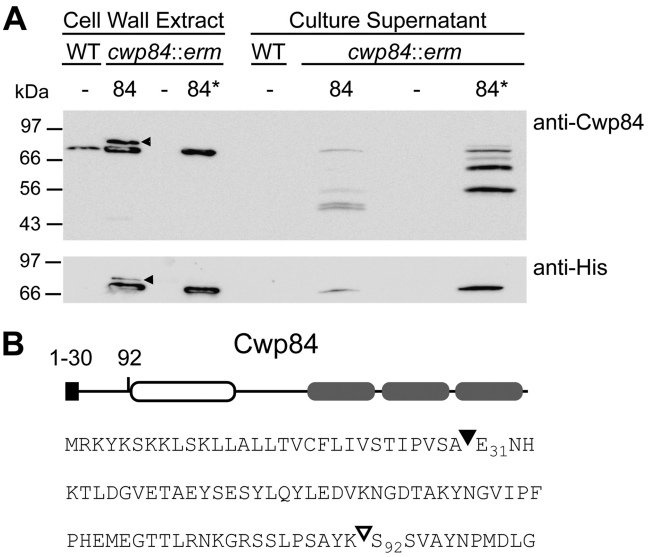

FIG. 3. Processing of Cwp84. (A) Detection of Cwp84 in the *cwp84* mutant overexpressing either WT or the Cys116Ala protein. *C. difficile* 630Δ*erm* (WT) and the *cwp84* mutant harboring plasmids as indicated were grown in BHI broth to late exponential phase (OD_{600} = 0.5). Cell wall extracts and culture supernatants were prepared and analyzed by SDS-PAGE, followed by Western blotting with antibodies against Cwp84 and the C-terminal His_6 tag. Plasmids: –, pMTL960; 84, $pCwp84_{WT}$; 84*, $pCwp84_{C116A}$. The upper band (84 kDa) recognized by these antibodies in the *cwp84* mutant carrying $pCwp84_{WT}$ is indicated (◀). The N-terminal sequence of the 77-kDa protein corresponding to the lower band was determined as SSVAY. (B) Line diagram of domain structure of Cwp84 and location of the signal peptide and propeptide domains. At the top is the domain structure of Cwp84 showing the location of the signal peptide (black rectangle), as predicted by SignalP, and the cysteine protease domain (white box), as predicted by Pfam (PF00112). The three cell wall binding motifs (PF04122) are shaded in gray. Below is the N-terminal amino acid sequence of Cwp84 showing the sites of cleavage to release the signal peptide (▼) and the mature protein (▽).

LMW SLPs, results in a defect in retention of Cwp66. We repeated these experiments using an antibody against Cwp2, a cell wall protein that is conserved in a majority of *C. difficile* strains (4). A similar result was seen with Cwp2, although the effect was not as dramatic (Fig. 2, anti-Cwp2). Cwp2 was present in the cell wall extracts of 630Δ*erm* and the *cwp84* mutant and also in the supernatant of the *cwp84* mutant. The presence of plasmid $pCwp84_{WT}$, but not $pCwp84_{C116A}$, complemented the phenotype of shedding of Cwp2 to the supernatant in the *cwp84* mutant.

Processing of Cwp84. Previous studies have indicated that, when expressed in *E. coli*, Cwp84 may undergo posttranslational processing (20). Our recent work in *C. difficile* using biotinylated inhibitors of cysteine proteases in combination with streptavidin pulldown experiments identified two species of Cwp84, migrating with apparent masses of 84 and 77 kDa (9). We therefore investigated processing of Cwp84 in *C. difficile* using an antibody raised against recombinant (*E. coli*-produced) Cwp84. In the WT strain, Cwp84 is visible in the cell wall as a single species of 77 kDa (Fig. 3A, anti-Cwp84). We infer this to be the active form of the enzyme. When WT *cwp84* is expressed from plasmid $pCwp84_{WT}$ in the *cwp84* mutant, an additional band at 84 kDa is visible (Fig. 3A, anti-Cwp84). Interestingly, this 84-kDa band is not visible when $Cwp84_{C116A}$ is expressed from the plasmid. Most probably, this protein is the 84-kDa species we previously identified as Cwp84 (9). The 77- and 84-kDa bands are also visible when probed with an anti-histidine tag antibody, which recognizes the C-terminal His_6 tag present on these proteins (Fig. 3A, anti-His), indicating that the two forms of Cwp84 must differ at their N termini. In order to determine whether the 77-kDa band, which is also detected by Coomassie blue staining (Fig. 1A), is a processed form of Cwp84, we determined its N-terminal sequence. The N-terminal sequence of the 77-kDa band from the *cwp84* mutant carrying $pCwp84_{WT}$ was determined to be SSVAY. Unfortunately, the 84-kDa protein could not be sequenced due to insufficient protein on the gel. The sequence SSVAY is located 92 residues downstream of the N-terminal Met residue and 61 residues downstream of the predicted site of cleavage by a signal peptidase (Fig. 3B). These results strongly suggest that the mature form of Cwp84 present on the cell wall results from two proteolytic processing events; a signal peptidase removes the signal peptide generating the 84-kDa proenzyme, followed by a second cleavage to generate the 77-kDa mature active enzyme. The 77-kDa band is also observed in the *cwp84* mutant expressing the $Cwp84_{C116A}$ protein, indicating that the cleavage event generating the mature protease is unlikely to be due to self-cleavage. In the supernatant of the *cwp84* mutant overexpressing the WT or mutant Cwp84, several fragments of Cwp84 were detected which are presumed to be degradation products.

Characterization of Cwp13, a Cwp84 paralog. *C. difficile* 630 contains a second gene, *cwp13*, that encodes a very similar protein to Cwp84 (see the introduction). It was of interest to investigate whether Cwp13 contained cysteine protease activity and, if so, what the substrate(s) of this protease might be. Using insertional mutagenesis, we constructed a mutant of *cwp13* in strain 630Δ*erm* and analyzed the phenotype of this strain. We first investigated whether the *cwp13* mutant had any defect in processing of SlpA. In contrast to the *cwp84* mutant, the *cwp13* mutant appeared to process SlpA normally, as seen by the appearance of the HMW and LMW SLPs in the cell wall (Fig. 4A, Coomassie blue stain), and it retained the large-colony morphology of the parental strain (data not shown). The WT *cwp13* gene and a derivative encoding a mutation at

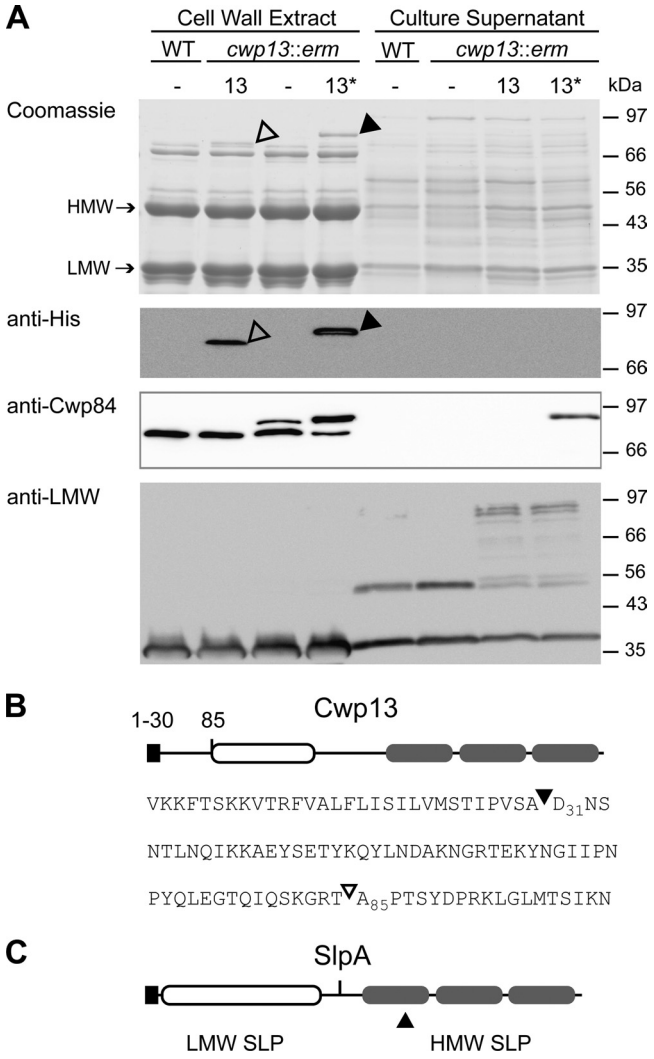

FIG. 4. Characterization of a *cwp13* mutant and processing of Cwp13. (A) Analysis of the effects of an insertional mutation in *cwp13* by SDS-PAGE and Western blotting. *C. difficile* 630Δ*erm* (WT) and the *cwp13* mutant harboring plasmids as indicated were grown in BHI broth supplemented with 15 μg of thiamphenicol/ml to late exponential phase (OD_{600} = 0.5). Cell wall extracts and culture supernatants were prepared and analyzed by Coomassie blue staining (top) and Western blotting with antibodies against the C-terminal His_6 tag (6% acrylamide), Cwp84 (6% acrylamide) and LMW SLP. Plasmids: –, pMTL960; 13, $pCwp13_{WT}$; 13*, $pCwp13_{C109A}$. The 77-kDa $Cwp13_{WT}$ and 84-kDa $Cwp13_{C109A}$ proteins detected by Coomassie blue staining and the anti-His_6 tag are indicated (◁ and ◀), and their N-terminal sequences were determined as DNSNT and APTSY, respectively. (B) Domain structure of Cwp13 and location of the signal peptide and propeptide domains. At the top is domain structure of Cwp13 showing the location of the signal peptide (black rectangle) and the cysteine protease domain (white box), as predicted by Pfam (PF00112). The cell wall anchoring domains (PF04122) are shaded in gray. Below is the N-terminal amino acid sequence of Cwp13 showing the sites of cleavage to release the signal peptide (▼) and the mature protein (▽). (C) Domain structure of SlpA. The vertical bar indicates the cleavage site of Cwp84 to produce the mature HMW SLP and LMW SLP. The black triangle (▲) shows the approximate site of cleavage, within a cell wall binding domain, by Cwp13 to generate a 47-kDa N-terminal product.

the predicted catalytic cysteine (Cys109Ala substitution) were introduced into the *C. difficile cwp13* mutant using the plasmids $pCwp13_{WT}$ and $pCwp13_{C109A}$, respectively. Cell wall extracts and culture supernatants from these strains showed very similar profiles with the HMW SLP and LMW SLP clearly visible, indicating cleavage had occurred mediated by Cwp84 (Fig. 4A, Coomassie). Interestingly, wild-type Cwp13 migrated at 78 kDa, whereas the Cys109Ala mutant protein migrated at 84 kDa. Since the Cwp13 proteins introduced on these plasmids contain a C-terminal His_6 tag, the processing of this protein could be assessed by using Western blotting. (Fig. 4A, anti-His). Both proteins were recognized by the anti-His tag antibody showing that the proteins differed at their N termini. The N-terminal sequences of these two proteins were identified as APTSY for the 78-kDa fragment of Cwp13 and DNSNT for the 84-kDa fragment of $Cwp13_{C109A}$. These results show that the signal peptide of Cwp13 consists of the first 30 amino acids, as predicted by SignalP. They also reveal that Cwp13 undergoes autocleavage between Thr84 and Ala85, but this processing does not occur in the $Cwp13_{C109A}$ protein (see Fig. 4B).

Another interesting observation was the detection of unprocessed Cwp84. In the *cwp13* mutant harboring either pMTL960 or $pCwp13_{C109A}$, both the proenzyme migrating at 84 kDa and the mature 77-kDa species were observed. In contrast, in the cell wall fraction of the 630Δ*erm* parental strain harboring the vector pMTL960 and in the *cwp13* mutant harboring $pCwp13_{WT}$ only the processed form of Cwp84, migrating at 77 kDa, was seen. The Cwp84 preprotein was also observed in the supernatant of the *cwp13* mutant harboring the $pCwp13_{C109A}$ plasmid (Fig. 4A, anti-His), but not in supernatants from the other strains. These results indicate that Cwp13 may have a role in cleaving and/or processing Cwp84.

Finally, we analyzed in greater detail the effects of the c*wp13* mutation on processing of SlpA using an antibody recognizing the LMW SLP. Mature LMW SLP (34 kDa) was seen in the cell wall in all strains, with no full-length SlpA detected, in agreement with the stained gel (Fig. 4A, anti-LMW). In the culture supernatants of 630Δ*erm* carrying pMTL960 or in the *cwp13* mutant carrying $pCwp13_{WT}$, a band of ~47 kDa is seen. However, in the c*wp13* mutant carrying either pMTL960 or $pCwp13_{C109A}$ this band is decreased in intensity and full-length SlpA is visible. This 47-kDa protein was investigated further (see below).

Cwp13 cleaves SlpA and does not complement the *cwp84* mutant. In order to better understand whether Cwp13, which shares 63.2% sequence identity with Cwp84, plays any role in the processing of SlpA we introduced plasmids $pCwp13_{WT}$ and $pCwp13_{C109A}$ in the *cwp84* mutant by conjugation. Cell wall extracts and culture supernatants of these strains were analyzed by SDS-PAGE, followed by either Coomassie staining or Western blotting (Fig. 5). Corresponding samples of *cwp84* mutant overexpressing $Cwp84_{WT}$ and $Cwp84_{C116A}$ were prepared alongside for comparison. Wild-type Cwp13 was not detected on the stained gel, but, interestingly, a small amount of the $Cwp13_{C109A}$ derivative was detected (Fig. 5, Coomassie), which was confirmed by Western blotting (data not shown). However, the size of $Cwp13_{C109A}$ when overexpressed in the *cwp84* mutant is 78 kDa instead of the expected 84 kDa that was observed in the *cwp13* mutant background (Fig. 4, Coomassie). This indicates that recombinant $Cwp13_{C109A}$ is

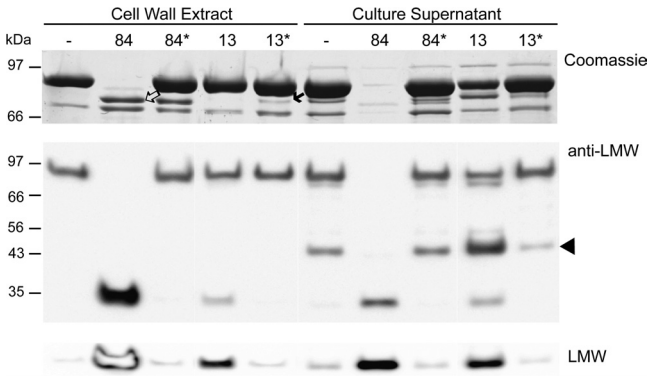

FIG. 5. Cwp13 cleaves SlpA but does not complement the *cwp84* mutant. *C. difficile cwp84* mutant harboring different plasmids was grown in BHI broth supplemented with thiamphenicol (15 μg/ml) till late exponential phase (OD_{600} = 0.5). Cell wall extracts and culture supernatants were prepared and analyzed by Coomassie blue staining (top) and Western blotting with antibody against the LMW SLP (middle and bottom). The bottom panel shows the 34-kDa LMW SLP species on an overexposed Western blot. Plasmids: –, pMTL960; 84, $pCwp84_{WT}$; 84*, $pCwp84_{C116A}$; 13, $pCwp13_{WT}$; 13*, $pCwp13_{C109A}$. Open arrow, 77-kDa Cwp84; solid arrow, 78-kDa $Cwp13_{C109A}$ processed by endogenous Cwp13; ◀, 47-kDa fragment of SlpA.

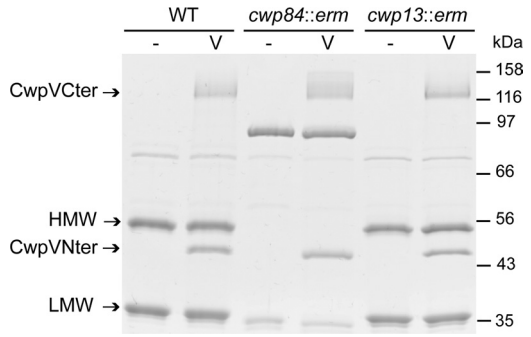

FIG. 6. Investigation of cleavage of CwpV by Cwp84 and Cwp13. *C. difficile* strains containing either pMTL960 vector or pCBR044 carrying *cwpV*+ were grown overnight in BHI broth supplemented with thiamphenicol (15 μg/ml). Cell wall proteins were extracted and analyzed by SDS-PAGE. Plasmids: –, pMTL960; V, pCBR044 CwpV+.

processed from 84 to 77 kDa in this strain, presumably by the chromosomally encoded *cwp13*.

We then investigated cleavage of SlpA by visualizing the LMW SLP using antibody. Interestingly, a small quantity of the LMW SLP was detected both on the cell wall (Fig. 5, anti-LMW) and in the culture supernatant (Fig. 5, anti-LMW SLP) of the *cwp84* mutant overexpressing $Cwp13_{WT}$ but not $Cwp13_{C109A}$. This result shows that Cwp13 is weakly active on SlpA and that Cys109 is a catalytic residue for this cleavage. However, this cleavage does not result in restoration of the WT colony morphology (data not shown) or in complete SlpA processing. When the Western blot with the anti-LMW SLP is overexposed (Fig. 5, bottom), very faint bands corresponding the LMW SLP are detected on the cell wall and culture supernatants of *cwp84* mutant containing pMTL960 or overexpressing $Cwp84_{C116A}$ or $Cwp13_{C109A}$. This could be due to the activity of Cwp13 expressed from the genomic copy of *cwp13*.

Surprisingly, a protein of ~47 kDa is detected by anti-LMW SLP predominantly in the culture supernatant of the *cwp84* mutant overexpressing $Cwp13_{WT}$ (Fig. 5, anti-LMW). The same protein is present but to a less extent in the culture supernatants of the *cwp84* mutant containing pMTL960 or $pCwp84_{C116A}$ and to an even lesser extent in the case of $pCwp13_{C109A}$. Most probably, that protein is the same one that was detected previously (Fig. 4A, anti-LMW SLP). The N-terminal sequence of the 47-kDa protein was determined to be ATTGT, which is located at the N terminus of the LMW SLP. Since the LMW SLP is only 34 kDa, cleavage must occur within the cell wall binding domains of the SlpA precursor (see Fig. 4C). Thus, either Cwp13 recognizes a second cleavage site in SlpA or activates another protease that catalyzes this cleavage. This cleavage occurs most efficiently in the absence of active Cwp84, i.e., in the presence of full-length SlpA. We propose a model that summarizes our findings on the maturation and activities of Cwp13 and Cwp84 (see Fig. 7).

Cwp84 and Cwp13 are conserved across multiple strains of *C. difficile*. The available complete or incomplete genome sequences of *C. difficile* strains 630, R20291, 196, BI1, CF5, M210, M68, 855, QCD32g58, and VPI10463 were searched for the *cwp13 and cwp84* genes. A gene coding for a protein with very high sequence identity (≥95%) to Cwp13 from *C. difficile* 630 was found in all genomes searched, in contrast to a previous report suggesting *cwp13* to be absent from the genome of strain R20291 (23). The predicted catalytic dyads within Cwp84 and Cwp13, which are Cys109 His256 and Cys116 His263, respectively, are fully conserved in all of these strains (data not shown). Furthermore, the site in Cwp84 at which the proenzyme is cleaved to produce the active enzyme (Lys91-Ser92 in strain 630) is conserved across Cwp84 from all of the strains. Notably, the 7-amino-acid sequence in Cwp84 containing the cleavage site and residues immediately upstream (Leu86 to Ser92) is absent in all Cwp13 sequences.

Cwp13 does not cleave CwpV. CwpV is the largest member of the family of cell wall proteins and is expressed in a phase variable manner (12). CwpV is detected in ca. 5% of cells in a *C. difficile* population under laboratory conditions. CwpV is processed: two fragments of 40 and 120 kDa, corresponding to the N-terminal cell wall anchoring domain and the C-terminal repeats domain, respectively, are detected on the cell wall of *cwpV*-expressing cells.

It was shown that Cwp84 is not responsible for cleavage of CwpV (23). We therefore investigated whether Cwp13 catalyzes the cleavage of CwpV by introducing plasmid pCBR044, which directs the constitutive expression of CwpV, into the *cwp84* and *cwp13* mutants by conjugation. As Fig. 6 shows, both domains of CwpV were detected in the wild-type and both mutant backgrounds, confirming that neither Cwp84 nor Cwp13 catalyzes the cleavage of CwpV.

DISCUSSION

A role for Cwp84 in cleavage of the S-layer protein precursor SlpA was demonstrated by using both chemical biology (9) and traditional genetic approaches (23). We have extended these studies here by using *C. difficile* strains containing gene knockouts in *cwp84* or *cwp13* coupled with plasmid-based ex-

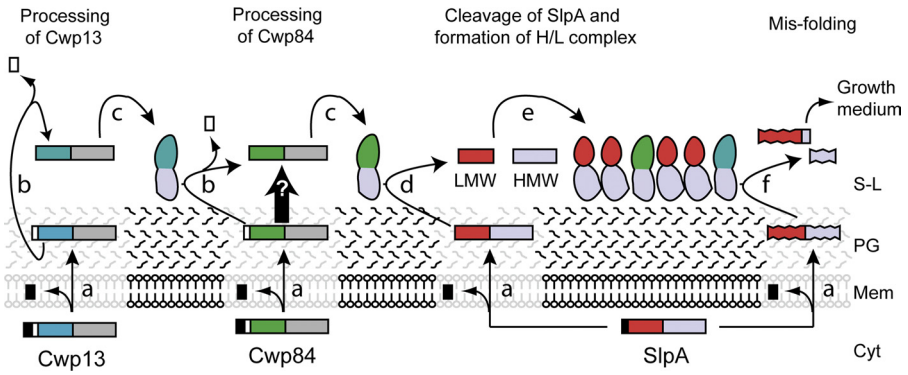

FIG. 7. Model for processing and activities of Cwp84 and Cwp13. SlpA, Cwp84, and Cwp13 are produced as preproteins containing signal peptides that are removed during processing by the *sec* system (step a). The propeptides of Cwp84 and Cwp13 are removed (step b), either by autocatalysis in the case of Cwp13 or by an unknown activity together with Cwp13 activity in the case of Cwp84, to form the active enzyme species that are incorporated into the S-layer (step c). Mature Cwp84 cleaves the SlpA precursor (step d), which results in the formation of the H/L complex (step e). Misfolded proteins are recognized by Cwp13 and are cleaved in their cell wall binding domains to prevent incorporation into the S-layer, resulting in detachment from the cell and deposition into the growth medium (step f). S-L, S-layer; PG, peptidoglycan; Mem, membrane; Cyt, cytoplasm.

pression of WT or mutant versions of the enzymes. These studies allowed us to confirm the phenotype of a *cwp84* knockout and to demonstrate that the conserved Cys116 residue is a required catalytic residue. Importantly, complementation of the *cwp84* mutant restored both SlpA cleavage and the WT colony phenotype, demonstrating that neither defect is due to second site mutations within the genome.

An unexpected consequence of the *cwp84* mutation is defective localization of other CWPs. Analysis of the culture supernatants by Western blotting revealed detachment of Cwp66 and Cwp2 from the cell wall of the *cwp84* mutant. It is possible that other members of the CWP family are affected in a similar way. Lack of cleavage of the S-layer also has a dramatic effect on colony morphology. The reasons why certain cell wall proteins do not fully associate with the cell wall and why the *cwp84* mutant displays such a characteristic colony morphology are likely to lie in the altered nature of the S-layer rather than being a direct effect of the lack of Cwp84 cysteine protease activity. Clearly, full-length, uncleaved SlpA is tolerated on the cell wall, but it would appear that the S-layer is incorrectly formed. Our previous structural studies showed SlpA to be an elongated molecule with the HMW SLP and LMW SLP interacting through domains located on either side of the cleavage site (14). The lack of cleavage almost certainly prevents correct interaction between the two SLPs leading to an abnormal S-layer. The mechanism of anchoring of the S-layer proteins and the CWPs to the underlying cell wall is currently unknown. However, it is probable that a common mechanism exists for the S-layer proteins and the extended family of CWPs since they all contain three Pfam 04122 cell wall binding domains. Our finding that some CWPs are shed from the cell wall in the absence of an incorrectly formed S-layer suggests a complex mechanism of attachment, perhaps involving interactions between the CWPs and the SLP subunits in addition to putative interactions with underlying cell wall polymers.

We show that Cwp84 undergoes posttranslational processing to form the active enzyme. After removal of the signal peptide, the 84-kDa proenzyme is processed to an active 77-kDa species. The N terminus of this active protein was localized at Ser92; hence, the protein contains a 61-residue propeptide after removal of the signal peptide. Consistent with this are results from our previous study using biotinylated compounds that inhibited SlpA cleavage; two bands of 84 and 77 kDa were labeled from a cell wall extract and were identified by proteomic techniques as derived from Cwp84 (9). The sequence of Cwp84 is highly conserved across *C. difficile* strains (26, 37), and it is likely that the Cwp84 species detected in 17 *C. difficile* isolates from CDI patients (30) is the active form of the enzyme. Surprisingly, mutation of the Cys116 catalytic residue to Ala did not prevent processing of the 84-kDa proenzyme to the 77-kDa species, indicating that processing may not be autocatalytic or that an alternative mechanism for processing may exist (Fig. 7). In fact the $Cwp84_{C116A}$ protein was found exclusively in the 77-kDa form with no evidence of a 84-kDa species.

Cwp13 is highly related to Cwp84 in primary sequence, but our experiments show it has distinct activities. Cwp13 shows very weak activity in cleavage of the SlpA precursor to produce the HMW and LMW SLPs. However, Cwp13 does contain proteolytic activity, as shown by autoprocessing and by cleavage of SlpA at a sequence distinct from the cleavage site recognized by Cwp84. Cwp13 is localized to the cell wall so lack of activity against SlpA must be due either to poor recognition of the substrate or absence from the immediate environment of SlpA as it translocates through the membrane and is localized in the cell wall.

Our data also show that Cwp13 is synthesized as a proenzyme that is processed to produce the active enzyme. However, in contrast to Cwp84, this processing does appear to be autocatalytic as it is not observed in a *cwp13* mutant expressing $Cwp13_{C109A}$. As stated above, Cwp84 does not appear to undergo auto-processing. However, our data using a *cwp13* mutant do point to a role for Cwp13 in processing Cwp84. Western blotting showed that the 84-kDa proenzyme form of Cwp84 was processed efficiently to the active 77-kDa species only in wild-type cells and in the *cwp13* mutant when complemented with WT Cwp13 but not with $Cwp13_{C109A}$. Where *cwp13* activity was absent, both the proenzyme and the mature

enzyme forms of Cwp84 were seen, indicating impaired processing of Cwp84. This suggests that Cwp13 has a role in cleavage of Cwp84 but that in the absence of Cwp13 cleavage of Cwp84 can still occur, albeit less efficiently.

The S-layer proteins are the major proteinaceous cell wall components and appear to be essential, as demonstrated by an apparent inability to create an *slpA* gene knockout (our unpublished data; Nigel Minton and Julian Rood, unpublished data). It might therefore be advantageous for the cell to have more than one mechanism to process Cwp84 to ensure correct processing of SlpA. It is possible that cleavage of Cwp84 may proceed via an unconventional self-processing route that is unrelated to its papain-like activity (3, 10). Alternatively, there could be a second protease that mediates cleavage of Cwp84.

Our results suggest that Cwp13 may have roles other than processing of Cwp84. In culture supernatants of a *cwp13* mutant, accumulation of the mature form of SlpA was seen, whereas in the mutant complemented with active Cwp13, a 47-kDa cleavage product of SlpA was seen. This protein was shown to be derived from the LMW SLP and a portion of the HMW SLP, indicating cleavage was within the HMW SLP. SlpA is naturally expressed at very high levels, and excess protein would need to be efficiently removed from the cell wall to allow correct localization and assembly of the HMW and LMW SLPs to form the S-layer.

It is possible that Cwp13 might have a more general role in the cleavage of misfolded proteins secreted to the cell wall (see Fig. 7). Gram-negative bacteria produce a number of periplasmic chaperones that prevent misfolding of periplasmic and outer membrane proteins, ensuring safe transit of proteins and avoiding attack by proteases that recognize unfolded proteins with the consequential activation of the stress response (38). SurA and Skp are two such chaperones, and DegP is a chaperone-protease complex and member of the large HtrA family (7, 39, 40). In *B. subtilis* several chaperones and proteases have been identified that are located external to the plasma membrane, possibly within the space between the membrane and the thick peptidoglycan layer, termed the "Gram-positive periplasmic space" (28). These proteins include homologs of DegP and the lipoprotein PrsA, a putative *cis-trans* peptidyl proyl *trans*-isomerase (reviewed in reference 36). It is likely that all Gram-positive bacteria, including the clostridia, have chaperones and proteases located external to the plasma membrane since folding of proteins secreted through the *sec* pathway and correct assembly of protein complexes must occur immediately after traversal of the plasma membrane. Whatever the natural substrate(s) of Cwp13, its activity must be regulated either spatially or kinetically to avoid unwanted cleavage of the HMW SLP which is essential for correct formation of the S-layer. Notably, Cwp13 cleaves full-length SlpA within the region of the HMW SLP containing the three cell wall binding domains that mediate attachment to the underlying cell wall. Cleavage in this region would disrupt attachment to the cell wall, and the protein would be released from the cell. This then would provide a mechanism for the release of incorrectly folded SlpA precursor from the cell wall.

Another possibility is that Cwp13 may function to digest extracellular substrates, such as extracellular proteins. Cwp84 has been shown to possess degradative activity toward extracellular matrix proteins fibronectin, laminin, and vitronectin (20). Perhaps Cwp13 also provides similar activities to aid in destruction of host tissues, colonization of the host, and dissemination of infection, as suggested previously (20).

In conclusion, we show that *C. difficile* contains two active cysteine proteases in the cell wall with distinct functions. Both are produced as inactive proenzymes that are processed to active enzymes, however, only Cwp13 appears to undergo autocatalytic cleavage. These proteins both function in assembly of the S-layer, but the activity of Cwp13 seems to be dispensable, whereas that of Cwp84 is essential. Our finding that incorrect assembly of the S-layer has profound consequences for the localization of other CWPs is significant since it points to important interactions between the S-layer proteins and the diverse CWPs that together are major components of the cell wall. The possible activity of Cwp13 as a protease involved the regulation of protein misfolding is currently under investigation.

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