Clostridium difficile Cell Wall Protein CwpV Undergoes Enzyme-Independent Intramolecular Autoproteolysis

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/// Introduction

C. difficile produces a proteinaceous array on its cell surface known as the S-layer, consisting primarily of the major S-layer protein SlpA and a family of SlpA homologs. CwpV is the largest member of this family and is post-translationally processed at a defined site to form a noncovalent, heterodimeric complex (Fig. 1).

No specific protease cleaving CwpV has been identified. Furthermore, cleavage is unaffected by protease inhibitors, suggesting that CwpV cleavage is enzyme-independent. Given that a threonine residue (**Thr-413**) is located directly downstream of the cleavage site, a plausible cleavage mechanism is an N-O acyl migration resulting in an ester intermediate which is then hydrolysed into two peptides. Here we use site site-directed mutagenesis to investigate this hypothesis.

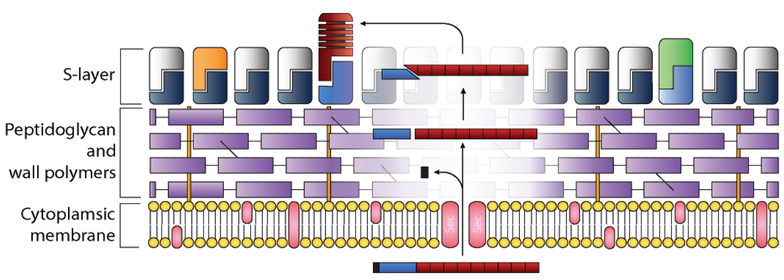

FIG 1. Schematic representation of *C. difficile* cell wall structure and CWP processing. SlpA (white/blue), CwpV (red/blue) as well as other, minor CWPs are idnicated.

/// Site-directed mutagenesis of Thr-413

Thr-413 was substituted with serine, cysteine, alanine or valine using inverse PCR on a plasmid pCwpV carrying a WT copy of CwpV. The resulting plasmids were introduced into a CwpV knock-out strain *via* conjugation.

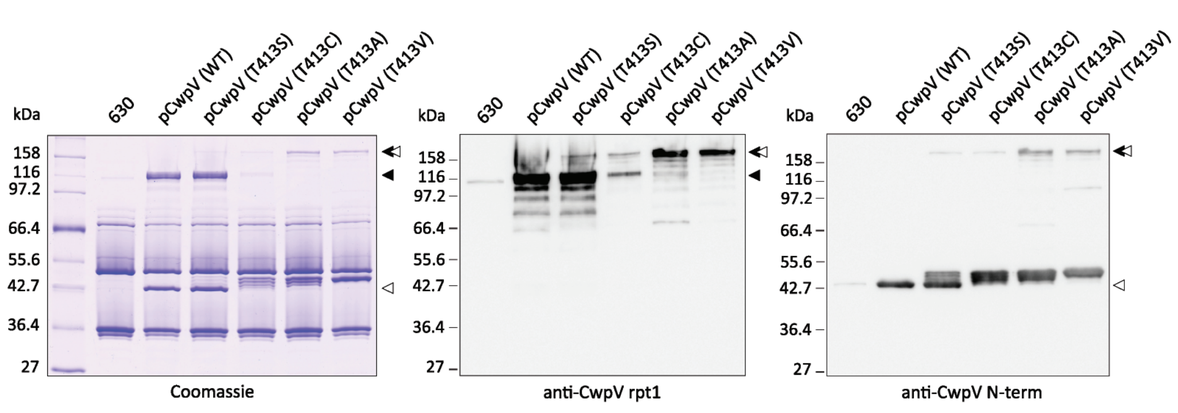

FIG 2. SDS-PAGE analysis of CwpV processing in Thr-413 mutants. ◁ anchoring domain; ◀ repeat domain

In all Thr-413 mutants, full-length CwpV was observed. Its amount was higher in strains carrying non-conservative mutations (**T413A** and **T413V**) than those carrying conservative mutations (**T413S** and **T413C**), indicating that the nucleophile-carrying residues of serine and cysteine can partially mimic the activity of Thr-413 in CwpV cleavage (Fig. 2).

/// Site-directed mutagenesis of Lys-410, Asp-411

A strained backbone conformation at the scissile peptide bond is critical for driving autoproteolysis through an N-O acyl shift. A small, tight turn consisting of three amino acids (**Lys-410, Asp-411, Gly-412**) was predicted to be located directly upstream of the CwpV cleavage site (Fig 5D). In an attempt to reduce the strain within the cleavage region, **Lys-410** and **Asp-411** were replaced with glycine (**GGG**) as described above.

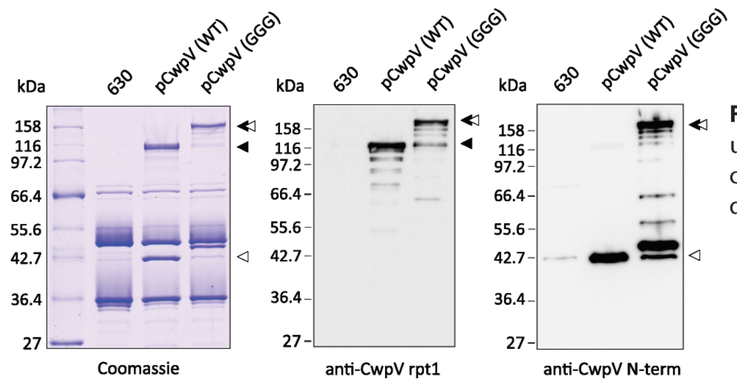

FIG 3. SDS-PAGE analysis of CwpV processing upon reduction of the strain within the cleavage site. ◁ anchoring domain; ◀ repeat domain

The **K410G/D411G** mutation significantly reduced the efficiency of CwpV cleavage and resulted in full-length protein. This is likely due to a loss of the critical base required to activate the nucleophile Thr-413 and/or relaxation of the scissile peptide bond and thus loss of the precise backbone conformation necessary to initiate a nucleophilic attack and a N-O acyl rearrangement (Fig. 3).

/// CwpV cleavage is enzyme-independent

In order to 'uncouple' CwpV from the host processing pathways, a *Strep*-tagged fragment of CwpV including the entire N-terminal anchoring domain and part of the C-terminal domain was expressed using the PURExpress *in vitro* protein synthesis kit (NEB).

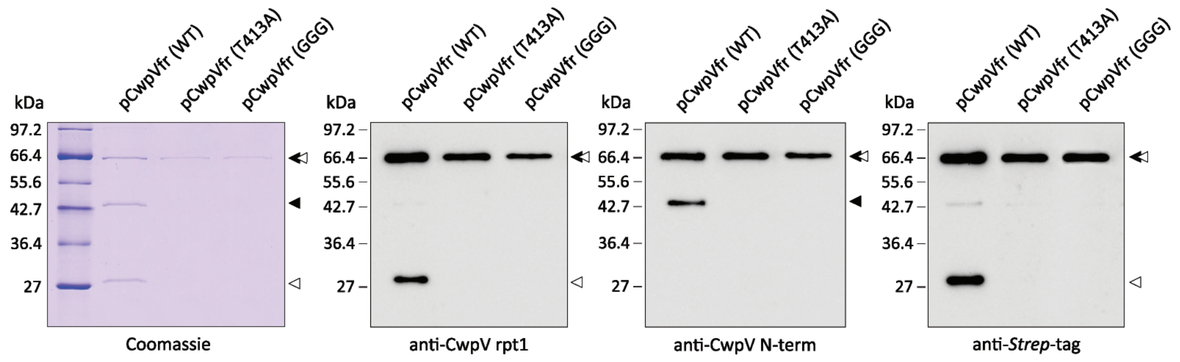

FIG 4. SDS-PAGE analysis of CwpV processing in an enzyme-free environment. ◁ anchoring domain; ◀ repeat domain

Cleavage occured in WT but not in T413 and K410G/D411G mutants indicating that CwpV cleavage is enzyme-independent (Fig 4).

/// Proposed mechanism of cleavage

Following Sec-mediated translocation of the precursor polypeptide across the cell membrane, the signal peptide is removed, and the protein is allowed to fold. The reactive hydroxyl of Thr-413 is deprotonated by the carboxylate group of Asp-411 and launches a nucleophilic attack on the α-carbonyl carbon of Gly-412 to form a hydroxazolidine intermediate. After a proton is transferred to the leaving amino group of Thr-413, the α-carbonyl of Gly-412 is shifted to the hydroxyl of Thr-413, leading to the ester intermediate *via* an N-O shift. The ester is then hydrolyzed to form two peptides, the anchoring domain and the repeat domain, which assemble into a noncovalent, heterodimeric complex (Fig. 5C).

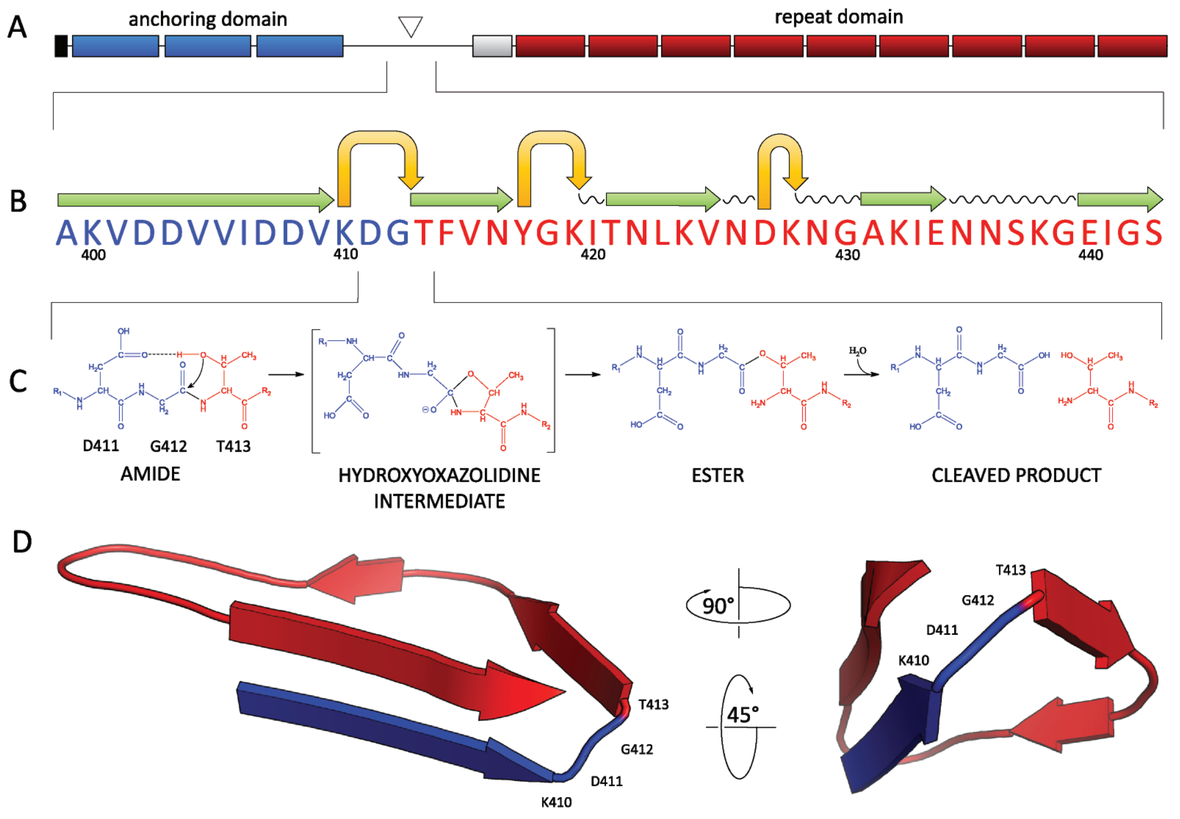

FIG 5. (**A**) domain structure of CwpV. The N-terminus of CwpV contains a signal sequence (black), followed by three PF04122 cell wall anchoring motifs (blue), and a region containing the cleavage site (triangle). A serine-glycine rich region (white) precedes a number of repeats (red). (**B**) predicted secondary structure of the region spanning the CwpV cleavage site generated using Phyre. (**C**) proposed mechanism of CwpV intramolecular autoprocessing. (**D**) three-dimensional model of CwpV cleavage site (106–135 amino acids). The tertiary structure of CwpV cleavage region was predicted using Phyre with an estimated precision of 80%. A tight turn can be observed directly upstream of Thr-413 and consisting of Gly-412, Asp-411, and Lys-410.

/// Conclusions

CwpV undergoes intramolecular autoprocessing which...

- is facilitated by a Thr-413 nucleophile-driven N-O acyl migration
- is dependent on a strained backbone conformation within the cleavage site
- requires neither cofactors nor auxiliary enzymes