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## Protein Composition of the Outermost Exosporium-like Layer of *Clostridium difficile* 630 Spores.

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### Abstract

*Clostridium difficile* spores are considered the morphotype of infection, transmission and persistence of *C. difficile* infections. There is a lack of information on the composition of the outermost exosporium layer of *C. difficile* spores. Using recently developed exosporium removal methods combined with MS/MS, we have established a gel-free approach to analyze the proteome of the exosporium of *C. difficile* spores of strain 630. A total of 184 proteins were found in the exosporium layer of *C. difficile* spores. We identified 7 characterized spore coat and/or exosporium proteins; 6 proteins likely to be involved in spore resistance; 6 proteins possibly involved in pathogenicity; 13 uncharacterized proteins; and 146 cytosolic proteins that might have been encased into the exosporium during assembly, similarly as reported for *Bacillus anthracis* and *Bacillus cereus* spores. We demonstrate through Flag-fusions that CotA and CotB are mainly located in the spore coat, while the exosporium collagen-like glycoproteins (i.e. BclA1, BclA2 and BclA3), the exosporium morphogenetic proteins CdeC and CdeM, and the uncharacterized exosporium proteins CdeA and CdeB are mainly located in the exosporium layer of *C. difficile* 630 spores. This study offers novel candidates of *C. difficile* exosporium proteins as suitable targets for detection, removal and spore-based therapies.

### Keywords

*Clostridium difficile*; Spores; Exosporium; spore surface proteins

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## 1. Introduction

*Clostridium difficile* is a Gram positive, anaerobic, spore-forming nosocomial pathogen that has established itself as a major nosocomial pathogen<sup>1</sup>. The severity of *Clostridium difficile* infections (CDI) can vary from mild diarrhea to life threatening toxic megacolon, with a mortality rate of ~ 5%<sup>2</sup>. Clinical symptoms are caused by main virulence factors, the enterotoxin TcdA and the cytotoxin TcdB<sup>3</sup>. A striking feature of CDI is the high rates with which those patients that have cleared from the first CDI episode suffer recurrence of CDI and can reach up to 20 to 30% of CDI patients<sup>4</sup>, which is attributed primarily to the persistence of the dormant spore morphotype<sup>5</sup>. Indeed, *C. difficile* strains that are unable to form spores, are unable to persist and disseminate in a mouse model, indicating that spores are essential for persistence and dissemination of *C. difficile*<sup>5</sup>.

Bacterial spores have unique structural features that enable them to survive for extended periods of time in adverse environments<sup>6</sup>. From a spore-host interaction perspective, the outermost layer gains particular importance as it has the sites that will interact with the host<sup>7, 8</sup>. Identification of the protein composition of this outermost layer will uncover novel targets for the development of detection and/or spore removal strategies. The outermost layer of several bacterial species (i.e. members of the *Bacillus cereus* group and *C. difficile*) is the exosporium layer<sup>7, 9</sup>, which in *B. anthracis* and *B. cereus* is a loosely-fitted layer composed by ~ 15 proteins and/or enzymes<sup>7</sup>, and plays a relevant role during infection<sup>8, 10</sup>. In contrast, several *C. difficile* strains produce spores with an exosporium layer that has organized hair-like extensions and is tightly attached to the spore coat, while other strains such as 630 have a hairy-less electron-dense exosporium-like layer that is also attached to the spore coat<sup>9, 11</sup>, supporting the notion that significant ultrastructural differences exist with the *Bacillus* exosporium layer<sup>11</sup>. Regardless of the differences in exosporium ultrastructure, both types of layers (i.e. *C. difficile* and *Bacillus* exosporium layers) share common properties: i) both layers contribute to the spore surface hydrophobicity; and ii) both have conserved markers (i.e. collagen-like exosporium glycoproteins) responsible for the hair-like nap of the exosporium layer of members of the *B. cereus* group, which in *C. difficile*, are encoded by *bclA1*, *bclA2* and *bclA3* genes that become expressed in the mother cell compartment uniquely during sporulation<sup>11-13</sup>. The correct assembly of the *C. difficile* exosporium layer depends on the exosporium morphogenetic protein, CdeC<sup>9</sup> indicating that the *C. difficile* exosporium layer has a unique assembly mechanism. Recent progress has been made in the identification of putative phenotypes for the exosporium layer of *C. difficile* spores, such as for adherence of *C. difficile* spores to epithelium cells *in vitro*<sup>14</sup> and colonization in a mouse model<sup>15</sup>, possible disruption of the phagosome's membrane during phagocytosis<sup>16</sup>, and for adherence to stainless steel<sup>17</sup>. The exosporium layer of *C. difficile* spores also seems to have some role in spore dormancy<sup>18</sup>. However, the proteins that are involved in these phenotypes remain elusive.

To date, only two studies have addressed the composition of *C. difficile* 630 spores<sup>19, 20</sup>; Lawley *et al.* (2009) used nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/-MS) to determine spore proteins previously fractionated on a NuPAGE gel, identifying ~ 336 proteins associated to *C. difficile* 630 spores<sup>19</sup>. However, their precise location in the different spore layers was unclear<sup>19</sup>. A recent study by Abhyankar *et al.*

(2013) also used LC-MS-MS to identify proteins present in the spore coat fraction of *C. difficile* 630 spores. Among the 54 protein species identified in *C. difficile* spore coat fractions, none of the three *C. difficile* exosporium collagen-like glycoproteins (i.e. BclA1, BclA2 and BclA3), and the exosporium morphogenetic protein, CdeC, were found<sup>20</sup>. Both of the aforementioned studies used proteinase K during the purification of *C. difficile* spores<sup>19, 20</sup>, which we recently showed to efficiently remove the exosporium layer of *C. difficile* spores, as well as the exosporium BclA1 and CdeC proteins<sup>9, 12, 18</sup>; explaining, at least in part, the absence of these and other known exosporium proteins in the proteomic analysis of proteinase K-treated *C. difficile* 630 spore coat<sup>20</sup>. Therefore, in an effort to contribute with tools to study the exosporium layer of *C. difficile* spores, we recently published<sup>18</sup> two different strategies based on sonication treatment and trypsin digestion. As demonstrated by transmission electron microscopy and spore surface hydrophobicity assay, both strategies remove most of the exosporium-like layer of *C. difficile* 630 spores, and apparently very little, if any, external spore coat material<sup>18</sup>. In the present work, in an effort to contribute towards the identification of proteins of the exosporium-like layer of spores of *C. difficile* strain 630, we extracted the exosporium layer by sonication and trypsin digestion and analyzed them by a highly sensitive “gel-free” ultra-high performance liquid chromatography (UPLC)-tandem MS/MS approach<sup>21</sup>. We have identified two exosporium proteins (i.e. BclA1 and CdeC), four previously described spore coat proteins (i.e. CotA, CotB, CotD, CotE) as well as several uncharacterized exosporium proteins including some with putative protective roles. Importantly, by creating translational fusions of a subset of these proteins with the FLAG epitope, we have demonstrated by western blot analysis of untreated and proteinase K-treated spores expressing Flag-fusions that, they localize to the exosporium-like layer of *C. difficile* 630 spores. These results provide the first proteomic study that focuses on the outermost exosporium-like layer of *C. difficile* 630 spores and when coupled with previous proteomic work on spores of strain 630, it provides comprehensive information of the proteins that are present in the outer layers of *C. difficile* 630 spores.

## 2. Methods

### Spore preparation and purification.

For *C. difficile* strain 630 (*tcdA*<sup>+</sup>, *tcdB*<sup>+</sup>, *tcdC*<sup>+</sup>, *ctdA*<sup>-</sup>, and *ctdB*<sup>-</sup>)<sup>22</sup> two independent biological spore crops were prepared as previously described on BHI agar plates<sup>14</sup>. Sporulating cultures were routinely washed with ice cold sterile distilled water, and free spores were separated using 50% HistoDenz and washed five times to eliminate traces of HistoDenz (Sigma). Spore suspensions were further washed 10 times with 150 mM NaCl in milliQ-grade water to remove cytosolic proteins from the exosporium layer. Suspensions were > 99% free of vegetative cells, sporulating cells and cell debris. To remove loosely bound proteins and intracellular contaminants, spores were washed 5 times with 1 M NaCl. Purified spores were quantified with a Neubauer chamber prior to storage at -80°C until use. This storage condition does not affect spore viability and ultrastructural properties (unpublished data).

### Trypsin digestion of the exosporium-like layer of *C. difficile* 630 spores.

To uniquely remove exosporium proteins, two biological replicates of *C. difficile* 630 spores were treated with trypsin that has been previously demonstrated to remove exosporium material<sup>18</sup>. Briefly, *C. difficile* 630 spores ( $2 \times 10^9$  spores) were resuspended in 50 mM  $\text{NH}_4\text{HCO}_3$  (pH 7.8)-0.4% Protease Max (Promega) with 10 mM DTT and incubated 30 min at 56°C. Protease Max is a surfactant that enhances trypsin digestion in solution. The reducing reaction was followed by an alkylation treatment with 55 mM iodoacetamide (IAA) in 100 mM  $\text{NH}_4\text{HCO}_3$  for 45 min at room temperature in the dark. Samples were immediately digested for 18 h at 37°C with 1 µg of trypsin (Trypsin Sequencing Grade Promega, Madison, WI, USA) using a 1:2 w/w protease to protein ratio (Fig. S1 and Fig. 1). Spores were collected by centrifugation and the tryptic digests were desalted using PepClean C18 spin columns (30 µg capacity, Pierce Biotechnology, IL, USA). Purified peptides were quantified at 205 nm with a Nanodrop ND1000 spectrophotometer and stored at -80°C until analyzed.

### Extraction of exosporium fragments by sonication.

Sonication was previously shown to remove the exosporium layer leaving the spore coats intact, thus exosporium fragments of two biological replicates were extracted by sonication<sup>14, 18</sup>. Briefly, *C. difficile* 630 spores were resuspended in 50 mM Dulbecco's Phosphate-Buffered Saline (DPBS)-0.5 mM EDTA buffer (pH 7.5), and sonicated with a Vibra-Cell VCX130 (Sonics & Materials, NewTown, CT, USA) in ice-cold conditions for 30 1-min bursts, separated by 3 min of cooling on ice. Sonicated spores were removed from the exosporium material by centrifugation, and the supernatant containing the exosporium fragments was filtered with syringe filters with 0.2 µm pores (Milipore, U.S.A.) to remove any remaining spores (Fig S1 and Fig. 1). Samples were concentrated by vacuum-centrifugation and resuspended in 50 mM  $\text{NH}_4\text{HCO}_3$  with 0.2% Protease Max (Promega) and trypsin-digested as described above. The tryptic digests were desalted in C<sub>18</sub> spin columns as described above and stored at -80°C until analyzed.

As an alternative strategy, the exosporium layer was biotinylated prior to sonication (Fig. 1). Briefly, two biological replicates of *C. difficile* spores ( $\sim 2 \times 10^9$  spores) were biotinylated with 1 mg of biotinamidohexanoic acid N-hydroxysuccinimide ester (Sigma-Aldrich, U.S.A.) in 0.1 M sodium bicarbonate (pH 8.2) for 45 min at room temperature. Biotin-labeled spores were washed 10 times with sterile phosphate buffered saline (pH 7.4) with glycine (10 mg/ml) to inactivate and remove any excess of unbound biotin succinimide ester and three times with PBS to remove excess glycine. Biotin-labeled exosporium fragments were removed by sonication as described above and affinity captured with magnetic streptavidin-coated C1 Dynabeads (Invitrogen) as described by the manufacturer. Dynabeads containing exosporium fragments were resuspended in 50 mM  $\text{NH}_4\text{HCO}_3$  with 0.2% Protease Max (Promega) and digested with trypsin as described above. Tryptic digests were desalted in a C<sub>18</sub> spin column (Pierce), and stored at -80°C until use.

### UPLC MS/MS analysis.

UPLC-MS/MS data were acquired with a LTQ-FT Ultra mass spectrometer with an IonMax ion source (Thermo, West Palm Beach, FL, USA) coupled to a nanoAcquity Ultra

performance LC system (Waters, Milford, MA, USA) equipped with a Michrom Peptide CapTrap column (Michrom) and a C<sub>18</sub> column (Zorbax 300SB-C18, 250 by 0.3 mm, 5- $\mu$ m; Agilent). Prior to analysis, the digests were lyophilized using speedvac centrifuge then and resuspended to a final concentration of approximately 500 ng/ $\mu$ l in mass spectrometry (MS) loading solution (95% H<sub>2</sub>O, 5% [vol/vol] acetonitrile [ACN], 0.1% [vol/vol] formic acid). A binary gradient system was used consisting of solvent A (0.1 % aqueous formic acid) and solvent B (ACN containing 0.1% [vol/vol] formic acid). Two  $\mu$ l of C<sub>18</sub> spin column-purified peptides was trapped and washed with 3% solvent B at a flow rate of 5 $\mu$ l/min for 3min. Trapped peptides were then eluted into an analytical column using a linear gradient from 3% B to 30% B at a flow rate of 4  $\mu$ L/min over 35 minutes. The column was maintained at 37°C during the run. The mass spectrometer was operated in a data-dependent mode. A full FT-MS scan (m/z 350 to 2,000) was alternated with collision-induced dissociation (CID) MS/MS scans of the 5 most abundant doubly or triply charged precursor ions. As the survey scan was acquired in the ion cyclotron resonance (ICR) cell, the CID experiments were performed in the linear ion trap where precursor ions were isolated and subjected to CID in parallel with the completion of the full FT-MS scan. CID was performed with helium gas at a normalized collision energy of 35 % and activation time of 30 ms. Automated gain control (AGC) was used to accumulate sufficient precursor ions (target value,  $5 \times 10^4$ /micro scan; maximum fill time 0.2 s). Dynamic exclusion was used with a repeat count of 1 and exclusion duration of 60 s. Data acquisition was controlled by Xcalibur (version 2.0.5) software (Thermo Scientific).

### Database search.

Thermo Scientific raw data files were processed with Proteome Discoverer version 1.2 using default parameters. A Mascot (version 2.2.04) search against a *Clostridium difficile* 630 database (obtained from UniProt; 3,982 sequences; 1,222,570 residues) was launched from Proteome Discoverer with the following parameters. The digestion enzyme was set to Trypsin/P and three missed cleavage sites were allowed as previously described<sup>19</sup>. The precursor ion mass tolerance was set to 5 ppm, while fragment ion tolerance of 0.8 Da was used. Dynamic modifications included: Deamidation (+0.984 Da) for asparagine and glutamine, oxidation (+15.9994 Da) for methionine, and carbamidomethyl (+57.0214 Da) for cysteine. For biotinylated exosporium extracts, an LC-biotin (+454.5) was also included to lysine and the N-terminus. Data from two experimental replicates were combined using Scaffold software (Proteome Software, Portland, OR) and applying the MudPIT (multidimensional protein identification technology) option, and the identified proteins from each sample were summarized with Scaffold 3 software. The inclusion of a protein in the final data set required that one or more unique peptides, with a score higher than 20, for that protein could be identified in at least one experimental condition. Only peptides that were identified with a confidence greater than 90% were utilized to calculate the overall probability of protein identification as specified by the Peptide Prophet algorithm<sup>23, 24</sup>.

### Functional classification of identified proteins.

Where a direct homologue between *C. difficile* and other *Clostridium* species existed, functional annotations were drawn from the NCBI website ([www.pubmed.com](http://www.pubmed.com)) or the BioCyc Database Collection (<http://biocyc.org>). Direct homologues were defined as proteins

with an identity higher than 60%. The presence and direct annotation of direct homologues for *C. difficile* proteins identified in this study are detailed in Table S1 in the supplemental material.

### Construction of plasmids.

To label exosporium proteins with the FLAG epitope at the C-terminal of selected genes, we used previously described plasmid pDP345 containing *cdeC*<sub>630</sub>-FLAG fusion as a template<sup>9</sup>. Briefly, to create a *cdeC*<sub>R20291</sub>-FLAG fusion, an NdeI-SalI fragment containing the promoter region and the entire *cdeC*<sub>630</sub> with the exclusion of its stop codon, was PCR amplified from genomic DNA of *C. difficile* strain R20291 with Phusion High Fidelity DNA Polymerase (Thermo Scientific) using primer pairs P113-P166 (Table S1). The PCR fragment was inserted into NdeI and SalI sites of pDP345, giving plasmid pDP375 (Table S2). The resulting plasmid was sequenced to confirm that no mutations were generated during PCR or cloning. Plasmid pDP375 was transformed into *E. coli* HB101(pRK24) and subsequently conjugated into strain *C. difficile* 630 *ermB* as previously described<sup>25</sup>. Sporulating cultures were obtained as described above and analyzed by Western blotting and immunofluorescence.

To construct FLAG fusions of the *cotA* (CD1613), *cotB* (CD1511), *bclA1* (CD0332), *bclA2* (CD3230), *bclA3* (CD3349), *cdeM* (CD1581), *cdeA* (CD2375) and *cdeB* (CD2752) genes, the promoter region and the entire ORF with the exclusion of the respective stop codons was PCR amplified from genomic DNA of *C. difficile* strain 630 with Phusion High Fidelity DNA Polymerase (Thermo Scientific) using primer pairs described in Table S1. To facilitate cloning we used a KpnI site upstream of NdeI. KpnI-SalI fragments were introduced into KpnI and SalI sites of pDP345 giving plasmids described in Table S2. All plasmids were sequenced to confirm correct insertion, and were subsequently introduced into *E. coli* HB101(pRK24) and subsequently conjugated into strain *C. difficile* 630 *ermB*. Sporulating cultures were obtained as described above and analyzed by Western blotting and immunofluorescence.

### Sarkosyl-proteinase K treatment of *C. difficile* 630 spores.

To remove the exosporium layer as previously described<sup>18</sup>, spores ( $5 \times 10^8$  spores) were incubated at 37°C at 200 rpm with 30  $\mu$ l of 1% sarkosyl, 0.3 mg/ml proteinase K, 25 mM phosphate buffer (pH 7.4) (PK) for 2 h. Treated spores were washed 10 times with sterile distilled water prior to storage at -80°C until use. To confirm that the exosporium layer was removed, hydrophobicity of PK-treated spores was measured as previously described and decreased from 60–70% to 2%, meaning that only 2% of the spores were in the organic phase<sup>18</sup>.

### Western blotting.

Samples (10 $\mu$ l) of SDS-PAGE loading buffer containing the spore coat and exosporium extracts from  $4 \times 10^7$  untreated and only the spore coat extracts from  $4 \times 10^7$  SPK treated spores of *C. difficile* 630 carrying plasmids pDP365 and pDP366 were electrophoresed on SDS-PAGE gels (12% acrylamide). Proteins were transferred to a nitrocellulose membrane (Bio-Rad) and blocked for 1 h at room temperature with 2% bovine serum albumin (BSA)–

Tris-buffered saline (TBS) (pH 7.4). Western blots were probed with a 1:1,000 dilution of anti-FLAG overnight at 4°C and then with a 1:5,000 dilution of anti-mouse horseradish peroxidase (HRP) conjugate (Promega) for 1 h at room temperature in PBS–1% BSA–0.1% Tween 20. HRP activity was detected with a chemoluminescence detection system (Fotodyne Imaging system) by using PicoMax sensitive chemiluminescent detection system HRP substrate (RockLand Immunochemicals). Each Western blot also included 2µl of a PageRuler Plus prestained protein ladder (Fermentas). Each Western blot was repeated at least 3 times with independent spore extracts, and analyzed by densitometry to quantify the relative amounts of protein by ImageJ as previously described<sup>9</sup>.

### 3. Results and Discussion

#### **Sonication and trypsin-digestion uniquely remove exosporium material of *C. difficile* 630 spores.**

The precise composition of the outermost layer of *C. difficile* spores remains unknown. Previously, Lawley *et al.* (2009) provided the first proteomic study of *C. difficile* 630 spores<sup>19</sup>, while Abhyankar *et al.* (2013) defined the spore coat proteome of *C. difficile* 630 spores<sup>20</sup>. However, spores used in those studies were purified in the presence of proteinase K, which has been recently shown to remove the exosporium-like layer of *C. difficile* 630 spores<sup>18</sup>. Therefore, to pursue the identification of proteins of the outermost exosporium-like layer of *C. difficile* 630 spores, we used spores purified in the absence of proteases. To remove the electron-dense exosporium-like layer of *C. difficile* 630 spores (Fig. S1), we used two previously reported experimental approaches<sup>18</sup>. As expected, sonication and 18 h trypsin-digestion treatments removed most of the electron-dense exosporium-like layer of *C. difficile* 630 spores without apparent effect on the underlying layers (Fig. S1) as we have previously described<sup>18</sup>. However, we cannot rule out that some coat material could have been degraded without changing the ultrastructural appearance of the spore coat.

In addition to transmission electron micrographs, spore hydrophobicity was used as an additional marker for exosporium removal as we have previously described<sup>18</sup>. Untreated *C. difficile* 630 spores have a notional surface hydrophobicity value of 62%, meaning that nearly 62% of the spores are in the organic phase. As expected, 30 pulses of 1 min of sonication reduced the hydrophobicity of *C. difficile* 630 spores to 11%. Similarly, a trypsin-digestion treatment of 18 h at 37°C reduced the hydrophobicity to 9%. These results are consistent with most of the exosporium layer being removed by sonication and trypsin treatments as previously described<sup>18</sup>. The integrity of the spore coat of sonicated and trypsin-digested spores was further confirmed by lysozyme digestion. Phase contrast microscopy analysis of ~ 1,000 spores demonstrated that > 99% of either sonicated or trypsin-digested spores remained phase bright after lysozyme treatment. These results indicate that the spore coat of sonicated and trypsin-treated spores retained its permeability properties, but does not rule out that some spore coat material might have been removed. Collectively, these results suggest that these treatments remove sufficient exosporium material without affecting the spore coat properties.

## Gel-free experimental approach to determine the proteome of the exosporium-like layer of *C. difficile* 630 spores.

The aforementioned treatments were used to remove the exosporium-like layer for MS/MS analysis in an attempt to define the exosporium proteome of *C. difficile* 630 spores (Fig. 1). First, we used a trypsin digestion treatment to remove the exosporium-like layer of *C. difficile* 630 spores and detected a total of 101 protein species (Fig. 1 and Fig. 2). By reasoning that trypsin might encounter steric hindrance when digesting the intact exosporium-like layer, we also used an alternative approach where the exosporium-like layer was extracted by sonication and subsequently subjected to trypsin digestion (Fig. 1 and Fig. 2). This approach allowed the assignment of 67 polypeptides with high confidence, of which 30 polypeptides were not identified by trypsin-digestion of *C. difficile* 630 spores (Fig. 1 and Fig. 2). Biotinylation of bacterial surfaces prior to protein extraction has been used to identify the surface proteome in several species<sup>26</sup>. Consequently, we also included this alternative approach in our effort to identify the exosporium proteome. *C. difficile* 630 spores were biotinylated prior to exosporium extraction by sonication and sonicated-biotinylated-exosporium extracts were affinity captured and trypsin digested prior to MS/MS analysis (Fig. 1). This approach identified a total of 116 polypeptide species (Fig. 2) of which 53 were unique to this approach (Fig. 2). Combining these results, the exosporium of *C. difficile* 630 spores seems to harbor at least 184 polypeptide species (Table S1) of which 61 had not been found previously in proteomic studies with *C. difficile* 630 spores<sup>19, 20</sup>. Most of these proteins seem to be vegetative cell contaminants that became entrapped during exosporium assembly. The difference in the spectra of proteins detected by each treatment might rely in that some proteins were lost while others became accessible to trypsin during sonication providing a complementary protein spectrum to that of trypsin digestion of the entire spore.

## Functional classification of exosporium proteins.

To analyze the exosporium-associated peptides, these were classified according to the functional scheme used for the *C. difficile* 630 genome<sup>27</sup> (Fig. 3). Surprisingly, the most common functional classes found in the exosporium-like layer of *C. difficile* 630 spores were proteins involved in energy metabolism, with a total of 43 proteins (23.5 %) (Fig. 3); most likely these proteins were trapped during the assembly of the exosporium-like layer as has been similarly observed for the exosporium layer of spores of the *B. cereus* group<sup>28, 29</sup>, and easily detected by the highly sensitive UPLC MS/MS approach. The identified proteins found in the exosporium were divided into various categories based on their demonstrated and/or predicted functions: i) previously characterized; ii) putative roles in protective response; iii) possibly involved in pathogenesis; iv) uncharacterized proteins; v) other spore proteins.

**1. Characterized exosporium proteins.**—Recent work has characterized several *C. difficile* exosporium proteins<sup>9, 12, 30, 31</sup>. Polypeptides of the exosporium morphogenetic cysteine rich protein, CdeC<sup>9</sup> (Table 1), were 10-fold more abundant than that of other species in the extracts from the trypsin digestion treatment. The high abundance of cysteine residues (9%) suggests that this protein might contribute to the crosslinking of the exosporium layer of *C. difficile* spores. CdeC is unique to *C. difficile* and thus may be used



as a biomarker for *C. difficile* spores. Another spore morphogenetic factor, required for correct spore assembly, CotA, was also present in the exosporium extracts (Table 1 and S1). Polypeptides of the exosporium protein, CotB, which plays a slight role in spore resistance and the manganese catalase (CotD)<sup>31</sup> were also found in the exosporium proteome of *C. difficile* 630 spores (Table 1 and Table S1).

Three orthologues of exosporium collagen-like BclA glycoprotein of the *B. cereus* group, are encoded in the 630 genome (i.e. BclA1, BclA2 and BclA3). Despite the fact that all three orthologs are expressed during sporulation<sup>12, 13, 32</sup> and localized to the surface of *C. difficile* 630 spores<sup>12</sup>, we could only detect peptides derived from BclA1, but not of BclA2 and BclA3 (Table 1 and Table S1). Analysis of the amino acid sequence of BclA2 and BclA3 shows the presence of 6 and 9 trypsin cleavage sites, respectively, suggesting that the BclA2 and BclA3 cleavage sites might not be accessible to trypsin. In previous work using in-gel trypsin digestion of electrophoresed spore coat and exosporium fractions, followed by UPLC MS/MS, we were not able to detect BclA2 and BclA3 derived peptides, yet detected significant peptides derived from the trypsin-digestion of BclA1<sup>12</sup>. It was also surprising that the exosporium proteins CotF and CotG as well as the coat and exosporium protein CotCB<sup>31</sup> were not detected in either of the three extraction methods (Table 1 and Table S1). These three proteins also have predicted trypsin-cleavage sites, suggesting that they might not be accessible to trypsin digestion. Alternatively, is also likely that these proteins might not be abundant enough to be detected using any of the approaches employed in this study.

Polypeptides of the uncharacterized protein CD1581, recently demonstrated to be highly expressed during *in vivo* sporulation, and which plays a role in persistence of *C. difficile* in a mouse model<sup>33</sup>, were present in the exosporium proteome (Table 1); however, whether it is uniquely localized in the exosporium layer and plays a role in the exosporium assembly or acts as a spore adhesin remains unclear. Polypeptides of *C. difficile* CotE, which has *in vitro* peroxiredoxin reductase and chitinase activity, should not be confused with the *B. subtilis* spore coat morphogenetic factor CotE, and were also detected in the exosporium proteome (Table 1)<sup>30, 31</sup>. Previous work localized *C. difficile* CotE to both the spore coat and the exosporium-like layer of *C. difficile* 630 spores<sup>31</sup>, suggesting that it might play a role during the assembly of both layers, albeit this role seems not noticeable since no ultrastructural defects were observed in spores of a *cotE* mutant<sup>31</sup>.

**2. Exosporium proteins with roles in protective response.**—In anaerobic organisms, such as *C. difficile*, defense against oxidative stress is of prime importance. Given the fact that *C. difficile* spores are metabolically dormant and possess similar resistance properties as those of *B. subtilis* that prevent small molecules from entering the spore core<sup>9, 31</sup>, they are naturally resistant to anaerobic conditions and germinate well in the presence of oxygen<sup>34</sup>. However, during the release of the nascent cell during spore outgrowth, proteins able to remove reactive oxygen species present in the exosporium of *C. difficile* spores might be of importance to the survival of the nascent cells. This may be the case of the *C. difficile* 630 exosporium-like layer, as three non-haem iron ruberythrin proteins (i.e. CD1474, CD1524 and CD2845) likely to be involved in protection against oxidative stress in anaerobic bacteria<sup>35</sup> were identified in the putative exosporium proteome (Table 2). Interestingly, CD2845 has not been previously identified in the spore proteome<sup>19</sup>

nor in the spore coat proteome<sup>20</sup> of *C. difficile* 630 spores, and has been demonstrated to be significantly upregulated during *in vivo* sporulation in a CDI mouse model<sup>33</sup>. Further validation of their location in the exosporium layer is needed.

Polypeptides for all three subunits of a ferredoxin/ flavodoxin oxidoreductase homologue (i.e. alpha, beta and gamma subunits) were found in the exosporium extracts (Table 2). Ferredoxin is an electron acceptor for numerous low-potential oxidation-reduction reactions, including pyruvate:ferredoxin oxidoreductase<sup>36, 37</sup>. Expression of all three proteins is upregulated during *in vivo* sporulation in a CDI mouse model<sup>33</sup>, suggesting that these proteins might play some role in the resistance to oxidative stress in the gut<sup>38, 39</sup>.

These rubrerythrins and oxidoreductases were previously reported in the proteomic study of the spore coat of *C. difficile* 630 spores treated with proteases during purification<sup>20</sup> which coupled with the presence of their peptides in the exosporium-like layer, suggests that they are present in both layers of *C. difficile* 630 spores.

**3. Exosporium proteins possibly involved in pathogenesis.**—Some interesting proteins identified in the exosporium proteome of *C. difficile* 630 spores (Table 2 and S1) include those with high immunogenicity and present on the surface of *B. anthracis* spores<sup>28, 29</sup>. Two such proteins are the cytosolic Elongation Factor Tu (EF-Tu) and  $\alpha$ -enolase (Table 2 and S1), which perhaps during the assembly of the exosporium-like layer of *C. difficile* 630 spores, become encased in the exosporium layers during its assembly. The surface location of these proteins on the exosporium layer of *B. anthracis* spores<sup>28</sup> seems to be important during *B. anthracis* infection, acting as plasmin(ogen)-specific spore receptors that aid in the evasion *B. anthracis* spores from C3b-dependent innate immunity<sup>40</sup>. Peptides of the chaperonin protein, DnaK and glyceraldehyde-3-phosphate dehydrogenase (GADPH) were also identified in the spore exosporium proteome (Table 2), and have also been implicated in pathogenesis in other bacterial species<sup>28, 41</sup>. One last protein found in the exosporium proteome is the ATP-dependent Clp protease proteolytic subunit 1 (Table 2), which in several other bacteria has been shown to be required for survival to environmental stress<sup>28</sup>. Collectively, these results have identified several proteins located in the exosporium-like layer of *C. difficile* 630 spores and validation would be required to determine their predicted roles in pathogenesis.

**4. Uncharacterized exosporium proteins.**—Peptides from a number of uncharacterized proteins whose expression were shown to be under the control of sporulation specific sigma factors<sup>13, 32</sup>, and significantly upregulated during *in vivo* sporulation in a CDI mouse model<sup>33</sup>, were identified in the exosporium extracts of *C. difficile* 630 spores (Table 2). These candidates for exosporium proteins include the products of *CD0311*, *CD0654*, *CD2375*, *CD2434*, *CD2752*, *CD3522* and *CD3652* genes (Table 2). In addition, polypeptides of the products of *CD1880*, *CD2245A* and *CD3613* genes which are under the control of sporulation specific sigma factors (Table 2) were also present in the exosporium proteome. Their roles in *C. difficile* spore biology remain unclear. Polypeptides of protein CD3613 (Table 2), that belongs to the Stay green family of proteins involved in chlorophyll degradation, and of the putative protease CD3652 that belongs to the family of thermolysin-like peptidases that includes several zinc-dependent metallopeptidases, were

detected during this study. Notably, both of these proteins are highly expressed during *in vivo* sporulation in a mouse model<sup>33</sup> and might play some role during CDI infection.

**5. Cytosolic proteins.**—Despite our effort to thoroughly wash *C. difficile* 630 spores to remove cytosolic contaminants with treatments that would not affect the exosporium integrity (i.e. distilled water and NaCl), we identified a number of cytosolic proteins such as ribosomal proteins, proteins involved in amino acid biosynthesis, cellular processes, central and intermediary metabolism, energy metabolism and transport of proteins were also detected in the exosporium proteome (Fig. 3 and Table S1). Indeed, we and others<sup>20, 42</sup> believe that during the assembly of the exosporium, these proteins might be trapped during the polymerization of protein complexes, and represents the likely composition of the exosporium-like layer of *C. difficile* 630 spores found in the infective environment. The presence of cytosolic-proteins in the exosporium of *B. anthracis* spores also remain attached after extensive washes<sup>20, 28, 42</sup>. Thus, unlike the spore coat layer, which is mainly free of cytosolic proteins<sup>20</sup>, the exosporium layer, seems to have many cytosolic proteins that may be within the thick electron-dense layer removed by sonication or a trypsin digestion of the spore.

#### Validation of exosporium localization of selected proteins.

To validate our results, we used spores expressing Flag-tagged proteins identified in the exosporium proteome of *C. difficile* 630 spores, and selectively removed the exosporium layer using an exosporium-removal treatment based on proteinase K as previously described<sup>18</sup>. Nine proteins were selected based on the following criteria: i) several spore proteins whose localization to the spore coat and/or exosporium have not been defined (i.e. CotA, CotB, BclA2 and BclA3); ii) a putative novel exosporium morphogenetic protein (i.e. CdeM) (Paredes-Sabja unpublished work); iii) and two uncharacterized proteins (i.e. CdeA and CdeB). To demonstrate that this method removes exosporium proteins, we selected a *C. difficile* exosporium marker, CdeC, which is essential for the assembly of the exosporium layer of *C. difficile* R20291 spores<sup>9</sup>. Immunofluorescence analysis of untreated spores of strain 630 carrying CdeC<sub>630</sub>-FLAG fusion reveals that, as expected<sup>9</sup>, CdeC<sub>630</sub> is accessible to anti-FLAG antibodies, similarly as in R20291 spores (Fig. S2A). Removal of the exosporium layer by proteinase K-treatment abolished the immunofluorescence signal of CdeC-FLAG fusion in both 630 and R20291 spores (Fig. S2A). However, since antibodies do not penetrate the spore coat located below the exosporium layer, possible spore coat-localized CdeC cannot be ruled out. Therefore, to confirm that CdeC is exosporium-located, we used SDS-PAGE loading buffer to remove the spore coat and exosporium fractions of untreated spores or only the spore coat fraction of proteinase K-treated (removes exosporium) spores from spores of strains 630(CdeC<sub>630</sub>-FLAG) and R20291(CdeC<sub>R20291</sub>-FLAG), and analyzed their removed fractions by western blotting. As expected, CdeC-FLAG fusion was detected in the spore coat and exosporium fractions of untreated spores of strains 630(CdeC<sub>630</sub>-FLAG) and R20291(CdeC<sub>R20291</sub>-FLAG), albeit signal was evidently more abundant in 630 spore coat and exosporium fractions (Fig. 4). No immunoreactive signal was observed by Western blot analysis in the spore coat fraction from proteinase K-treated spores of strains 630(CdeC<sub>630</sub>-FLAG) and R20291(CdeC<sub>R20291</sub>-FLAG), indicating that CdeC is located in the exosporium layer. The extent of exosporium removal can be

confirmed by measuring spore hydrophobicity which drops to nearly 2% upon complete exosporium removal<sup>18</sup>. The hydrophobicity of 630(CdeC<sub>630</sub>-Flag) and R20291(CdeC<sub>R20291</sub>-FLAG) dropped from nearly 58% to 2% upon proteinase K-treatment (data not shown), indicating complete exosporium removal. However, we cannot rule out the possibility that CdeC might be located between the basal layer of the exosporium and the spore coat surface, as some proteins from the latter layer, above the protease impermeable layer of the spore coat, might also be removed by proteinase K treatment. Collectively, these results indicate that this method is, at most, adequate to determine the spore coat and/or exosporium localization of selected proteins present in the exosporium proteome.

CotA and CotB are suggested to be spore coat proteins<sup>20, 31</sup>, although this has not been fully demonstrated<sup>11</sup>. To evaluate the relative spore coat/exosporium abundance of CotA and CotB, untreated and proteinase K-treated spores of strains 630(CotA-FLAG) and 630(CotB-FLAG) were analyzed by immunofluorescence and Western blot analysis. Both, CotA and CotB were accessible to anti-FLAG antibodies and signal was completely abolished by proteinase K-treatment (Fig. S3A and B). Western blot analysis demonstrate that upon removal of exosporium layer, levels of CotA and CotB in the spore coat fractions decrease by 30 and 45% relative to spore coat-exosporium fractions (untreated spores) (Fig. 5A and B). These results indicate that at least some CotA and CotB are surface exposed and accessible to antibodies, while most (65–70%) of CotA and CotB is inaccessible to anti-FLAG antibodies, proteinase K insensitive and located in the spore coat.

We have recently demonstrated that the *C. difficile* collagen-like BclA1 glycoprotein is located in the exosporium-like layer of *C. difficile* 630 spores<sup>12</sup>. However, whether BclA2 and BclA3 are also located in the exosporium, remains unclear. Immunofluorescence analysis of untreated and proteinase K-treated spores of strains 630(BclA1-FLAG), 630(BclA2-FLAG) and 630(BclA3-FLAG) demonstrate that all three BclA are present in the spore surface and are absent in proteinase K-treated spores (Fig. S3C, D and E). To evaluate if any of the BclA proteins are also present in the spore coat, fractions of untreated and treated spores were analyzed by Western blotting. We found that all three BclA proteins are present as a 48-kDa immunoreactive species, which is smaller than their predicted molecular masses (i.e. predicted molecular mass of BclA1, BclA2 and BclA3 are 68-, 49- and 58-kDa, respectively), suggesting posttranslational cleavage and glycosylations<sup>43</sup>. Notably, BclA1 and BclA3 form stable high molecular mass complex of nearly 110–120-kDa (Fig. 5C, D and E) suggesting that similarly as with *B. anthracis* BclA<sup>44</sup>, *C. difficile* BclA1 and BclA3 might be forming stable dimers and/or trimers. Removal of the exosporium layer demonstrated that the majority (> 95 %) of BclA1 and BclA3 specific 44- and 110–120-kDa species are present in the exosporium fraction (Fig. 5C and E). Nearly 85 % of BclA2 was found to be present in the exosporium layer. Overall, these results indicate that BclA1, BclA2 and BclA3 are mostly exosporium proteins, where at least BclA1 and BclA3 form stable high molecular mass complex.

A recent study demonstrated that CD1581 is the most upregulated gene during sporulation of *C. difficile* strain 630 *in vivo*<sup>33</sup>. Genetic studies have demonstrated that CD1581 encodes an essential *C. difficile* exosporium morphogenetic protein termed CdeM (Paredes-Sabja unpublished work) that is found in the exosporium layer (Table 1); however, it is unclear if it

is mainly present in the exosporium layer. Immunofluorescence of untreated 630(CdeM-FLAG) spores demonstrated that CdeM is located in the spore surface and accessible to antibodies (Fig. S3F). No immunofluorescence signal was detectable upon treatment with proteinase K (Fig. S3F), suggesting that CdeM is located in the exosporium-like layer. Western blot analysis demonstrated that CdeM is present as a 46-kDa immunoreactive species, suggesting that the 19.1 kDa spore morphogenetic protein is forming stable complexes with other spore proteins. Removal of the exosporium-like layer decreased the presence of CdeM by nearly 95% (Fig. 5F) indicating that CdeM is mostly localized in the exosporium-like layer of *C. difficile* 630 spores.

Two additional uncharacterized proteins identified in the exosporium proteome were selected to confirm their localization into the exosporium layer: i) a 11.3-kDa uncharacterized protein, encoded by CD2375 and named CdeA, which is present in the spore proteome<sup>19</sup> and its expression decreases by 1.4-fold in a *sigK* mutant background<sup>13</sup>; ii) a 25.5-kDa uncharacterized protein, encoded by CD2752 and named CdeB, that has not been detected in previous proteome studies<sup>19, 20</sup>, but that its expression increases 3.3-fold during *in vivo* sporulation<sup>33</sup>. Immunofluorescence analysis of 630(CdeA-FLAG) and 630(CdeB-FLAG) spores demonstrates that both CdeA and CdeB are spore surface proteins removed by proteinase K (Fig. S3G and H), suggesting that they are located in the exosporium layer. Analysis of spore coat and exosporium fractions of untreated 630(CdeA-FLAG) and 630(CdeB-FLAG) spores by Western blot shows that CdeA-FLAG is present as 19- and 47-kDa species, while CdeB-FLAG is forming a stable 45-kDa molecular complex (Fig. 5G and H). Removal of the exosporium layer with proteinase K demonstrated that 99% and 90% of CdeA's 19-kDa and 47-kDa species, respectively, are present in the exosporium-like layer (Fig. 5G). Similarly, nearly 99% of CdeB was found to be located in the exosporium-like layer (Fig. 5H). These results indicate that CdeA and CdeB are exosporium proteins. Further validation of the remaining uncharacterized proteins (Table 1 and 2) is required to determine the precise composition of the exosporium-like layer of *C. difficile* 630 spores.

### Concluding remarks.

In conclusion, this study offers a strategy to identify proteins located in the exosporium-like layer of *C. difficile* 630 spores and complements previous proteomic studies of *C. difficile* 630 spores<sup>19, 20</sup>, since it defines the proteome of the outermost exosporium-like layer of *C. difficile* 630 spores. Many cytosolic proteins were detected, similarly as for *B. anthracis* studies<sup>28</sup>, indicating that the exosporium layer of *C. difficile* spores is a complex layer that may entrap many cytosolic proteins during its assembly; or alternatively, that these contaminants are derived from vegetative contaminants that could not be removed by the procedures to remove loosely-bound proteins. Several proteins involved in protection against environmental stress as well as putative virulence factors that might interact with complement or plasminogen were identified which may control the fate of *C. difficile* spores in the host. Importantly, a list of uncharacterized putative exosporium proteins is provided. We demonstrate that previously characterized spore coat proteins (i.e. CotA and CotB) are also present in the exosporium layer, while the collagen-like glycoproteins (i.e. BclA1, BclA2 and BclA3) as well as a novel spore morphogenetic protein (i.e. CdeM) and two

uncharacterized signature proteins (i.e. CdeA and CdeB) are exosporium proteins. Further on-going work to define the location and function of the remaining uncharacterized exosporium proteins (Table 1 and 2) will allow us to establish the composition of the exosporium layer of *C. difficile* spores. This work provides new protein targets for the diagnosis and/or therapeutics that may contribute to combat *C. difficile* infections.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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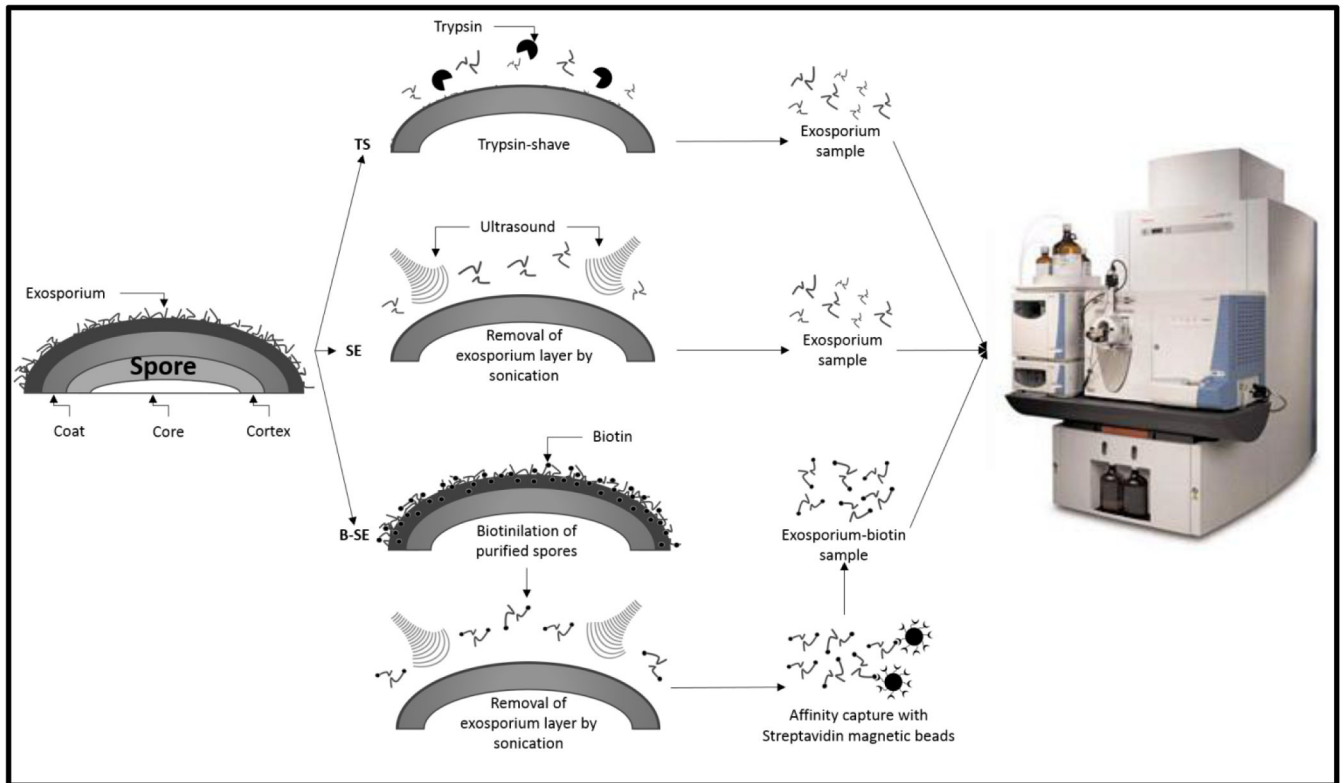
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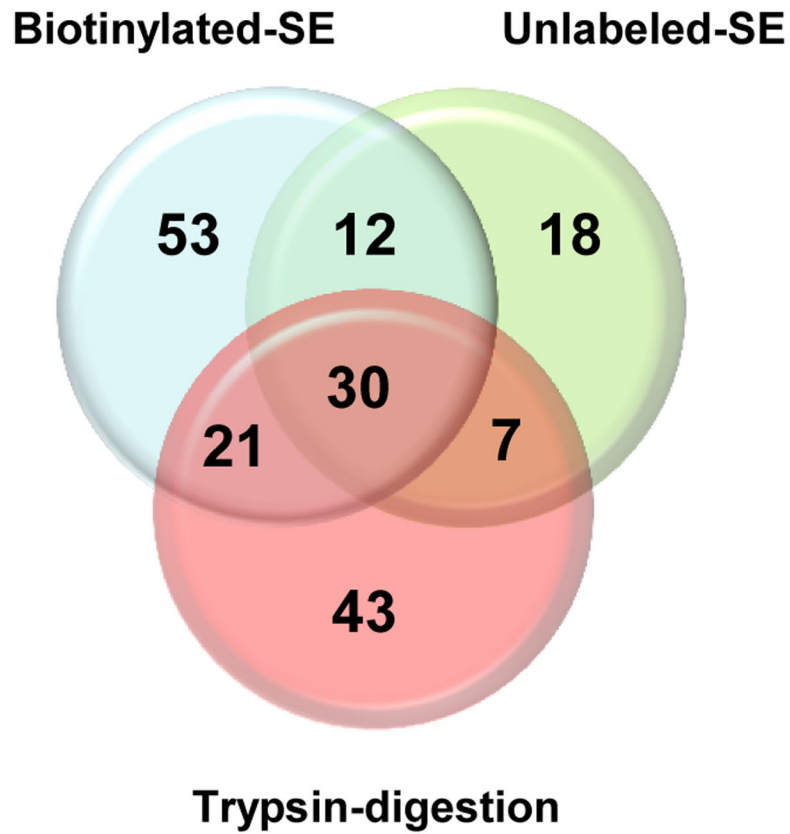
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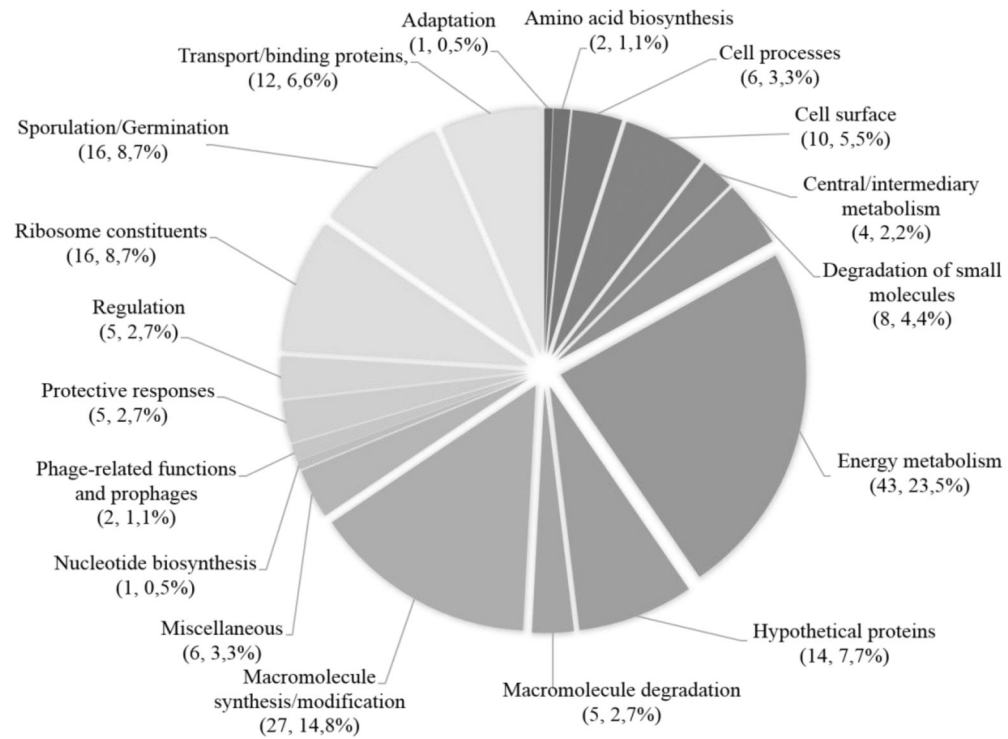
**Figure 1. Gel-free strategy to remove and identify exosporium-like layer proteins of *C. difficile* 630 spores.**

Experimental gel-free strategy to determine the proteome of the exosporium-like layer of *C. difficile* 630 spores. i) *C. difficile* spores were trypsin-digested (TS) for 18 h at room temperature and peptides analyzed by MS/MS as described in Methods section: ii) The exosporium-like layer of *C. difficile* 630 spores was removed by sonication, digested with trypsin and peptides analyzed by MS/MS; iii) *C. difficile* 630 spore surface proteins were biotin-labeled prior to sonication, and biotin-labeled exosporium extracts were captured by Streptavidin-magnetic beads and digested with trypsin and peptides were identified by MS/MS. All experiments were done with two biological replicates.



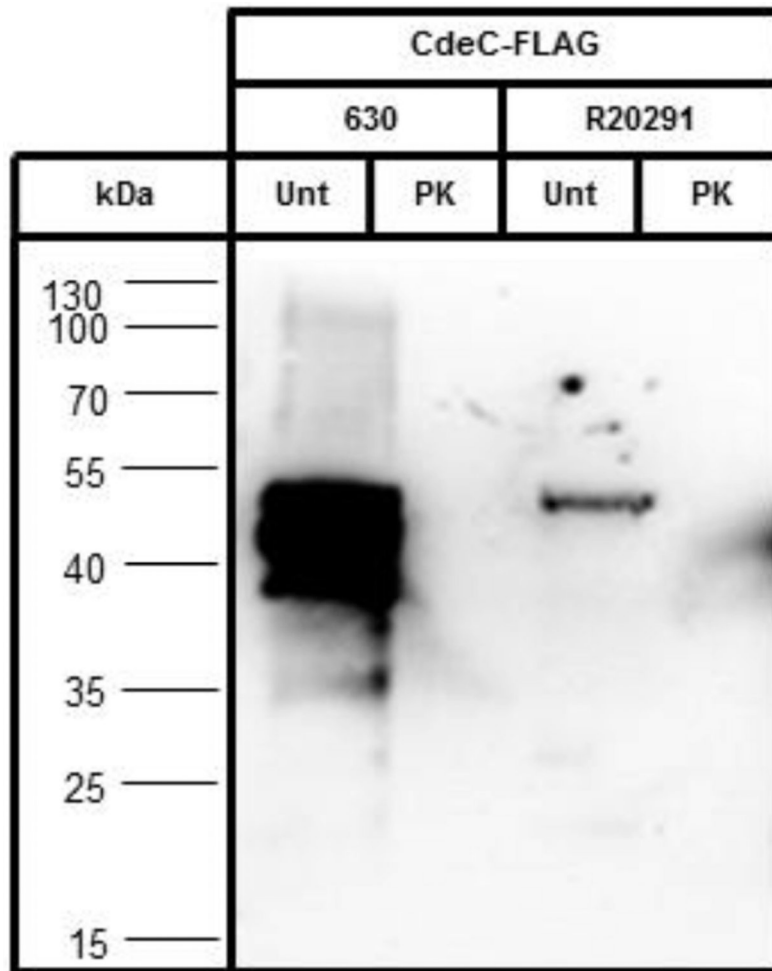
**Figure 2. Venn diagram of conserved superfamily domains in the identified spore exosporium proteins from *C. difficile* 630 spores.**

A total of 183 superfamily domains were identified in the proteins extracted from the exosporium-like layer of *C. difficile* 630 spores using three gel-free experimental strategies: Biotinylated sonicated exosporium (Biotinylated-SE); Unlabeled sonicated exosporium (Unlabeled-SE); and trypsin digested spores (Trypsin-Digestion). Numbers corresponds to the number of superfamily domains identified.



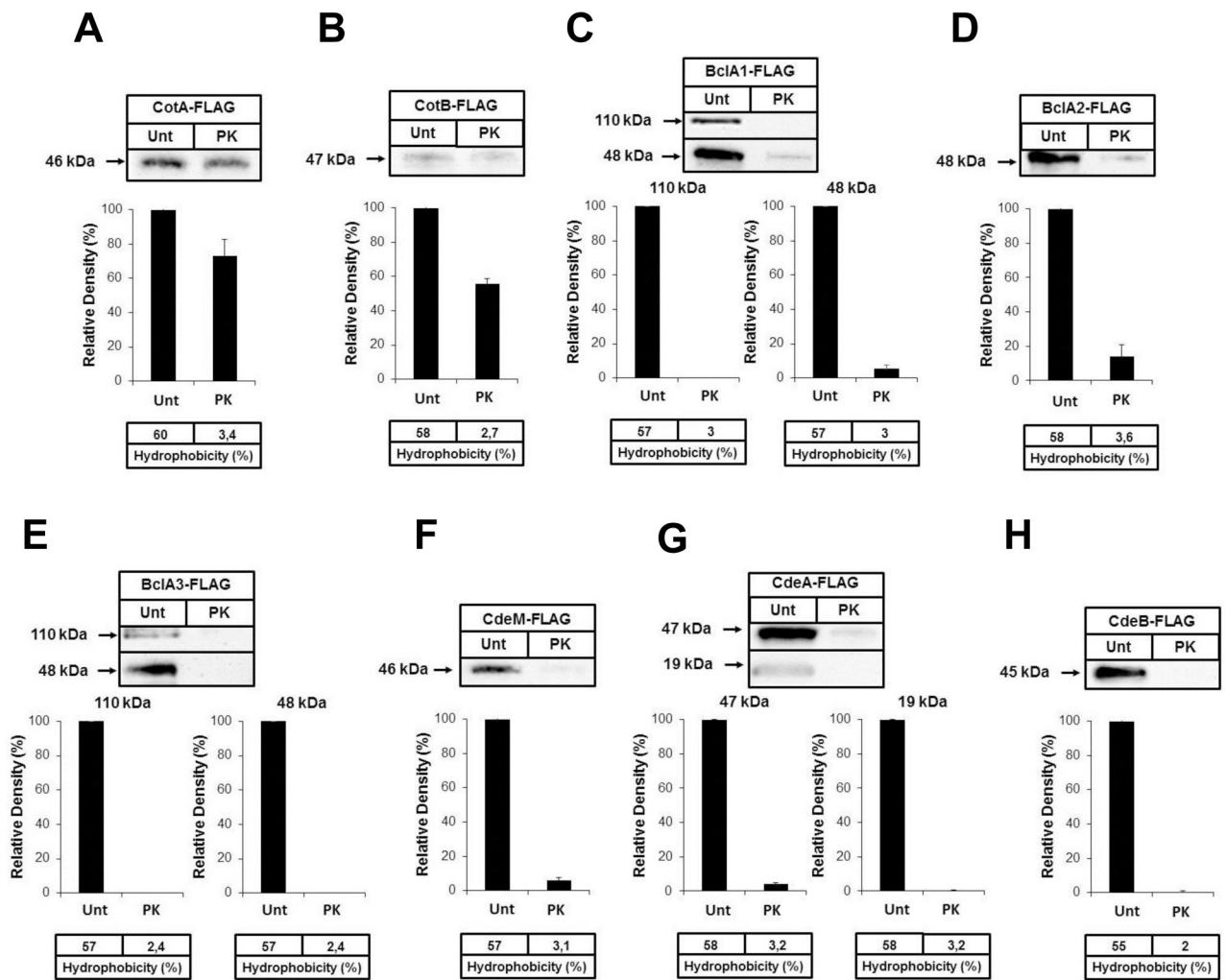
**Fig. 3. Representation of the distribution and abundance by functional classes of the exosporium proteins of *C. difficile* spores.**

Shown is the percentage of each functional class in the exosporium of *C. difficile* 630 spores (Functional classes are described in reference<sup>27</sup>). The elevated number of energy metabolism and cytosolic proteins identified in this spore layer are most likely vegetative cell contaminants entrapped during the assembly of the exosporium layer, and therefore, it is likely unlikely that these proteins are genuine exosporium proteins.



**Fig. 4. The exosporium morphogenetic protein, CdeC, localized uniquely to the exosporium layer of *C. difficile* 630 and R20291 spores.**

Spore coat and exosporium extracts of untreated (Unt) and spore coat extracts of proteinase K-treated (PK) spores ( $4 \times 10^7$ ) were fractionated on SDS-PAGE, transferred to nitrocellulose membrane and analyzed by western blot using anti-FLAG specific antibody as described in Material and Methods. Results are representative of three independent experiments.



**Fig. 5. Effect of removal of the exosporium layer on the relative abundance of selected outer surface spore proteins.**

The spore coat and exosporium from untreated spores (Unt) and spore coat from proteinase K-treated (PK) spores of strains: A) 630(CotA-FLAG); B) 630(CotB-FLAG), C) 630(BclA1-FLAG); D) 630(BclA2-FLAG); E) 630(BclA3-FLAG); F) 630(CdeM-FLAG); G) 630(CdeA-FLAG); H) 630(CdeA-FLAG) were extracted with SDS-PAGE loading buffer, electrophoresed analyzed by Western blot analysis as described in Material and Methods. All experiments were done three independent times.

TABLE 1.

Characterized exosporium proteins of *Clostridium difficile* 630 spores.

Protein <sup>a</sup> (Gene identification)	N° of unique peptides <sup>b</sup>	Percentage coverage <sup>c</sup>	MW <sup>d</sup> /pI	Proteome		Transcriptome	
				Spore <sup>e</sup>	Spore coat/exo <sup>f</sup>	<i>in vitro</i> <sup>g</sup>	<i>in vivo</i> <sup>h</sup>
<b>Exosporium proteins</b>							
BclA1 (CD0332; <i>bclA1</i> )	5	6.6	68.0/5.3	✓	✓	σ <sup>K</sup>	✓
CdeC (CD1067; <i>cdeC</i> )	19	41	44.7/4.8	✓	✓	σ <sup>K</sup>	✓
Peroxiredoxin chitinase (CD1433, <i>cotE</i> )	14	23	81.1/5.3	✓	✓	σ <sup>K</sup>	✓
CotB (CD1511, <i>cotB</i> )	4	16	34.9/4.8	✓	✓	σ <sup>E</sup>	✓
Putative uncharacterized protein (CD1581, <i>cdeM</i> )	10	51	19.1/5.3	✓	✓	σ <sup>K</sup>	✓
CotA (CD1613, <i>cotA</i> )	4	14	34.0/4.6	✓	✓	σ <sup>K</sup>	✓
CotD (CD2401, <i>cotD</i> )	1	9.5	21.4/4,8	✓	✓	σ <sup>K</sup>	✓

<sup>a</sup>Proteins identified by either of the three approaches.

<sup>b</sup>Peptides certain by integrating the three approaches.

<sup>c</sup>Represents the percentage of coverage by integrating the peptide sequences of the three approaches.

<sup>d</sup>MW, molecular weight.

<sup>e</sup>Protein present in the spore proteome by Lawley *et al.* (2009).

<sup>f</sup>Protein present in the spore coat proteome by Abhyankar *et al.* (2013).

<sup>g</sup>RNA polymerase sigma factors that governs the expression as described Fimlaid *et al.* (2013) and Saujet *et al.* (2013).

<sup>h</sup>Expression in pathogenesis *in vivo* as described Janoir *et al.* (2013).

TABLE 2.

Uncharacterized exosporium proteins of *Clostridium difficile* 630 spores<sup>a</sup>.

Protein <sup>a</sup> (Gene identification)	N° of unique peptides <sup>b</sup>	Percentage coverage <sup>c</sup>	MW <sup>d</sup> /pI	Proteome		Transcriptome	
				Spore <sup>e</sup>	Spore coat/exo <sup>f</sup>	in vitro <sup>g</sup>	in vivo <sup>h</sup>
<b>Proteins likely to play a role in spore resistance</b>							
Putative ferredoxin/ferredoxin oxidoreductase, alpha subunit (CD0116)	4	19	39.1/5.4	-	✓	-	✓
Putative ferredoxin/ferredoxin oxidoreductase, beta subunit (CD0117)	5	36	26.7/6.9	-	✓	-	✓
Putative ferredoxin/ferredoxin oxidoreductase, gamma subunit (CD0118)	2	20	19.8/5.1	-	-	-	✓
Putative rubrerythrin protein (CD1474)	4	27	20.0/5.5	✓	-	-	-
Putative rubrerythrin protein (CD1524)	4	27	20.0/5.6	✓	✓	-	-
Rubrerythrin (CD2845, <i>rbr</i> )	2	15	22.4/6.5	✓	✓	σ <sup>G</sup>	✓
<b>Proteins possibility involved in pathogenicity</b>							
Elongation factor Tu (CD0071, <i>tuf1</i> )	12	43	44.0/5.1	✓	✓	-	-
Chaperonin 60 kDa (CD0194, <i>groL</i> )	1	2.2	57.7/4.8	✓	✓	-	-
Chaperone protein (CD2461, <i>dnaK</i> )	3	7.8	66.4/4.8	✓	-	-	✓
Enolase (CD3170, <i>eno</i> )	3	11	46.1/4.7	✓	✓	-	✓
Glyceraldehyde-3-phosphate dehydrogenase (CD3174, <i>gapA</i> )	6	24	36.0/6.0	✓	✓	-	-
ATP-dependent Clp protease proteolytic subunit 1 (CD3305, <i>clpP</i> )	1	3.6	21.3/5.3	✓	✓	-	-
<b>Other exosporium proteins</b>							
Putative uncharacterized protein (CD0311)	1	7.1	31.4/4.6	-	-	σ <sup>E</sup>	✓
Putative cation efflux protein (CD0902)	1	3.5	32.4/7.0	-	-	σ <sup>K</sup>	✓
Putative ATP-dependent protease (CD0564)	3	5.2	85.4/5.2	-	-	σ <sup>K</sup>	✓
Putative ferrous ion transport protein (CD1517, <i>feoB</i> )	3	6.4	78.2/6.6	-	-	σ <sup>K</sup>	-
Putative ferrous ion transport protein (CD1518, <i>feoA</i> )	1	12	8.30/10	-	-	σ <sup>K</sup>	✓
Putative uncharacterized protein (CD1880)	1	23	12.8/5.7	-	-	σ <sup>G</sup>	-
Putative uncharacterized protein (CD2245A)	1	10	7.30/9.7	✓	-	σ <sup>G</sup>	-
Putative uncharacterized protein (CD2375, <i>cdeA</i> )	2	37	11.3/5.6	✓	-	σ <sup>K</sup>	✓
Putative uncharacterized protein (CD2434)	1	9.5	22.2/4.9	✓	-	-	✓
Putative uncharacterized protein (CD2752, <i>cdeB</i> )	2	9.1	25.5/5.2	-	-	-	✓
Putative uncharacterized protein (CD3522)	4	12	50.4/4.5	✓	✓	σ <sup>E</sup>	✓

Protein <sup>a</sup> (Gene identification)	N° of unique peptides <sup>b</sup>	Percentage coverage <sup>c</sup>	MW <sup>d</sup> /pI	Proteome		Transcriptome	
				Spore <sup>e</sup>	Spore coat/exo <sup>f</sup>	<i>in vitro</i> <sup>g</sup>	<i>in vivo</i> <sup>h</sup>
Putative uncharacterized protein (CD3613)	1	8.7	50.4/4.5	✓	✓	σ <sup>K</sup>	-
Putative peptidase, M1 family (CD3652)	3	9.6	54.6/5.7	✓	✓	σ <sup>E</sup>	✓

<sup>a</sup>Proteins identified by either of the three approaches.

<sup>b</sup>Peptides certain by integrating the three approaches.

<sup>c</sup>Represents the percentage of coverage by integrating the peptide sequences of the three approaches.

<sup>d</sup>MW, molecular weight.

<sup>e</sup>Protein present in the spore proteome by Lawley *et al.* (2009).

<sup>f</sup>Protein present in the spore coat proteome by Abhyanakr *et al.* (2013).

<sup>g</sup>RNA polymerase that governs the expression as described Fimlaid *et al.* (2013) and Saujet *et al.* (2013).

<sup>h</sup>Expression in pathogenesis *in vivo* as described Janoir *et al.* (2013).