

# The *Clostridium difficile* Exosporium Cysteine (CdeC)-Rich Protein Is Required for Exosporium Morphogenesis and Coat Assembly

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*Clostridium difficile* is an important nosocomial pathogen that has become a major cause of antibiotic-associated diarrhea. There is a general consensus that *C. difficile* spores play an important role in *C. difficile* pathogenesis, contributing to infection, persistence, and transmission. Evidence has demonstrated that *C. difficile* spores have an outermost layer, termed the exosporium, that plays some role in adherence to intestinal epithelial cells. Recently, the protein encoded by *CD1067* was shown to be present in trypsin-exosporium extracts of *C. difficile* 630 spores. In this study, we renamed the *CD1067* protein *Clostridium difficile* exosporium cysteine-rich protein (CdeC) and characterized its role in the structure and properties of *C. difficile* spores. CdeC is expressed under sporulation conditions and localizes to the *C. difficile* spore. Through the construction of an  $\Delta$ cdeC isogenic knockout mutant derivative of *C. difficile* strain R20291, we demonstrated that (i) the distinctive nap layer is largely missing in  $\Delta$ cdeC spores; (ii) CdeC is localized in the exosporium-like layer and is accessible to IgGs; (iii)  $\Delta$ cdeC spores were more sensitive to lysozyme, ethanol, and heat treatment than wild-type spores; and (iv) despite the almost complete absence of the exosporium layer,  $\Delta$ cdeC spores adhered at higher levels than wild-type spores to intestinal epithelium cell lines (i.e., HT-29 and Caco-2 cells). Collectively, these results indicate that CdeC is essential for exosporium morphogenesis and the correct assembly of the spore coat of *C. difficile*.

*Clostridium difficile* is an anaerobic, Gram-positive, endospore-forming bacterium that has established itself as an important nosocomial pathogen (1). Two toxins, the TcdA enterotoxin and the TcdB cytotoxin (2), are responsible for the clinical manifestations of *Clostridium difficile* infections (CDI), which include a massive inflammatory response, recruitment of phagocytic cells, and pseudomembrane formation (2). The severity of CDI may vary from mild diarrhea to pseudomembranous colitis and toxic megacolon, with a death rate that can reach 5% of the patients with a first episode of CDI (3). Unlike other nosocomial infections, CDI has a high level of recurrence which can reach 20% after a first episode and increase to up to 40% and 60% after a second and third recurrence, respectively (1). During the infection cycle, *C. difficile* begins a sporulation process, where at least part of the spores are believed to remain adhered to the colonic surfaces and the rest are released to the environment through the feces. Indeed, studies in a mouse model system have demonstrated that sporulation-deficient strains are incapable of producing recurrence and persistence of *C. difficile* in the infected mouse and transmission to neighboring mice (4), indicating that spore formation during CDI is essential for persistence and transmission. This is consistent with the fact that once antibiotic treatment for CDI has been discontinued, the majority of the patients continue shedding *C. difficile* spores for up to 2 weeks (5), highlighting the role that *C. difficile* spores play in *C. difficile* pathogenesis (1, 3).

The ultrastructure of bacterial spores in *Bacillus* and *Clostridium* genera is highly conserved (6–8). In some species such as *Bacillus anthracis* and *Bacillus cereus*, the outermost layers are formed by the exosporium, which is composed of at least 21 protein species forming a paracrystalline basal layer and a hair-like nap composed mainly of collagen-like proteins, such as BclA and BclB (6, 9, 10). The components of the spore coat and exosporium layers are synthesized in the mother cell and subsequently assem-

bled on the developing forespore (6). Most exosporium-specific proteins are regulated by the RNA polymerase bearing the sigma factor K,  $\sigma^K$  (6). SDS-PAGE analyses indicate that the exosporium layer of spores of *B. anthracis* is composed of a prominent band of >400 kDa formed by the immunodominant BclA and other exosporium proteins which are forming a stable high-molecular-mass complex (11–14). Besides BclA and BclB, other proteins that have been found to be structural components of spores of *B. anthracis* and *B. cereus* spores are BxpB, BspB or ExsFA, BxpC, ExsC, ExsD, ExsE, ExsF, and ExsG (13–16). However, bioinformatic analysis has shown that these proteins are absent in other species that have an exosporium layer (i.e., *C. difficile*) (6). Below the exosporium is the spore coat, where several morphogenetic factors essential for the proper assembly of the coat of *Bacillus subtilis*, *B. anthracis*, and *B. cereus* spores include ExsA (SafA orthologue), CotE, ExsB (CotG analogue), ExsY (CotZ orthologue), CotB, CotY, and ExsK (a cysteine-rich protein related to CotX) (10, 11, 14). However, the ultrastructure of *C. difficile* 630's spore exosporium layer is significantly different from that of *B. anthracis* spores (7, 8, 17) and bioinformatic and proteomic analyses (6, 18) indicate that the exosporium proteins of *B. anthracis* and *B. cereus* are absent in *C. difficile*, suggesting that the exosporium of *C. difficile* spores is made of proteins that are highly divergent.

From our proteomic work, we have identified trypsin-digested

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polypeptides of CD1067 at the surface of *C. difficile* spores (K. Escobar-Cortés, F. Díaz-Gonzalez, T. Shin-Chen, C.S. Maier, M.R. Sarker, and D. Paredes-Sabja, submitted for publication). Trypsin uniquely removes the majority of the exosporium layer, suggesting that CdeC might be an exosporium protein (17). In this study, we renamed the CD1067 protein *Clostridium difficile* exosporium cysteine-rich protein (CdeC) and characterized its role in spore structure and properties. We have demonstrated through fluorescence microscopy that CdeC is expressed in the mother cell compartment during sporulation and later localizes to the surface of *C. difficile* spores, specifically, to the exosporium layer. Through the construction of a  $\Delta cdeC$  knockout mutant, we showed that CdeC is required for the correct assembly of the spore coat and is essential for the assembly of the exosporium layer. Through immunofluorescence and Western blot analyses performed with anti-CdeC antibodies, we demonstrated that CdeC is localized in the exosporium layer accessible to antibodies. *C. difficile*  $\Delta cdeC$  spores were more sensitive to ethanol and heat treatments. *C. difficile*  $\Delta cdeC$  spores had a spore coat permeable to lysozyme and an increased adherence to intestinal epithelium cell lines. Collectively, these results contribute to our understanding of the biology of *C. difficile* spores.

## MATERIALS AND METHODS

**Bacterial and cell culture conditions.** The *C. difficile* R20291 strain used in this study belongs to ribotype 027, is positive for *tcdA* (*tcdA*<sup>+</sup>), *tcdB*<sup>+</sup>, *tcdC*<sup>+</sup>, and *cdtB*<sup>+</sup>, and lacks *ctdA* (19). *Escherichia coli* strains BL21  $\lambda$ (DE3)-RIL and HB101 harboring the conjugative plasmid pRK24 (Tra<sup>+</sup>, Mob<sup>+</sup>; *bla*, *tet*<sup>r</sup>) are described elsewhere (20–22). *C. difficile* was routinely grown under anaerobic conditions in a Bactron III-2 anaerobic chamber (Shel Lab) in 3.7% brain heart infusion broth supplemented with 0.5% yeast extract (BHIS) or on BHIS agar plates, and spore suspensions were prepared by plating a 1:500 dilution of an overnight culture onto 3% Trypticase soy–0.5% yeast extract (TY) agar plates and incubated for 5 days at 37°C under anaerobic conditions. Spores were harvested with ice-cold sterile distilled water and purified with 50% Nicodenz as previously described (23). Caco-2 cells were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) (HyClone), while HT-29 cells were grown in RPMI medium (HyClone). All tissue culture media were supplemented with 10% (vol/vol) fetal calf serum (HyClone), penicillin (100 U ml<sup>−1</sup>), and streptomycin (100  $\mu$ g ml<sup>−1</sup>).

**Construction of plasmids.** To construct a translational *cdeC*-tdTOMATO fusion, a ~3.0-kb BamHI-HindIII DNA fragment containing a *catP*-tdTOMATO fusion from plasmid pDP315 was cloned between BamHI and HindIII sites of pMTL82151, giving plasmid pDP318. Next, a DNA fragment (1,893 bp) containing 678 bp upstream of the start codon of *cdeC* and the entire *cdeC* open reading frame (ORF) with the exclusion of the stop codon of *cdeC* was PCR amplified from genomic DNA of strain R20291 with Phusion High Fidelity DNA polymerase (Thermo Scientific) using primer pair P113 (5'-GACCATATGGATGATGATGAGTTAACAGATGAAGGTG-3') and P114 (5'-GACGTCGACCTCTGTGGCAACTTGGCTTCCAC-3'); the forward primer had an NdeI site (underlined), and the reverse primer had an extra "C" (italic) and a SalI site (underlined) at the 5' end to give an in-frame fusion. The PCR fragment was cloned into NdeI and SalI sites of plasmid pDP315, yielding plasmid pDP324. The resulting plasmid was sequenced to confirm that no mutations were generated during PCR or cloning. Plasmid pDP324 was introduced by transformation into *E. coli* donor strain HB101 (pRK24), resulting in strain HB101 (pRK24, pDP324). To introduce plasmid pDP324 into the *C. difficile* 630 $\Delta$ ermB wild type, strain HB101 (pRK24, *cdeC*-tdTOMATO) was mated with *C. difficile* 630 $\Delta$ ermB, and transconjugants were selected as previously described (24). To analyze the expression of *cdeC*-tdTOMATO fusion in *C. difficile*, a 16-h BHIS culture of the *C.*

*difficile* 630 $\Delta$ ermB (pDP324) strain was plated at a dilution of 1:500 onto TY agar plates supplemented with 12  $\mu$ g/ml of thiamphenicol and incubated at 37°C under anaerobic conditions for 5 days. Sporulating cultures were harvested and analyzed by fluorescence microscopy using a BX53 Olympus fluorescence microscope (Olympus).

To label the C-terminal domain of *cdeC* with FLAG as a reporter tag, a DNA fragment (1,893 bp) containing 678 bp upstream of the start codon of *cdeC* and the entire *cdeC* ORF, with the exclusion of its stop codon, was PCR amplified from genomic DNA of strain R20291 with Phusion High Fidelity DNA polymerase (Thermo Scientific) using primer pair P113 (5'-GACCATATGGATGATGATGAGTTAACAGATGAAGGTG-3') and P165 (5'-GACAAGCTTTCATTTATCATCATCATCTTTATAATCGTTCGACTCTGTGGCAACTTGGCTTCCAC-3') (the forward primer had an NdeI site [underlined], and the reverse primer had a SalI site [underlined] followed by a FLAG-encoding sequence [bold], a stop codon [italic], and a HindIII site [underlined] at the 5' end). The PCR fragment was inserted into NdeI and HindIII sites of plasmid pMTL82151, giving plasmid pDP345. The resulting plasmid was sequenced to confirm that no mutations were generated during PCR or cloning. Plasmid pDP345 was introduced into HB101 (pRK24) and subsequently conjugated into strain *C. difficile* 630 $\Delta$ ermB. Sporulating cultures were obtained as described above and analyzed by Western blotting.

To label the C-terminal domain of *cdeC* with a 6 $\times$ His tag, a 1,212-bp sequence carrying *cdeC* was PCR amplified from R20291 genomic DNA using Phusion Hot Start High Fidelity DNA polymerase (Thermo Scientific), forward primer P66 (5'-GACCATATGCAAGATTATAAAAAAAAAA TAAAAGAAGATGATGAATCAGC-3'), and reverse primer P67 (5'-GACGGATCCGATCTGTGGCAACTTGGCTTCCAC-3'). The forward and reverse primers had NdeI and BamHI cleavage sites (underlined) at their 5' ends, respectively, and the reverse primer included GA (italic) to give an in-frame fusion between CdeC and the 6 $\times$ His tag. A ~1.2-kb NdeI-BamHI fragment was cloned into NdeI and BamHI sites of pET22b (EMB Biosciences), giving plasmid pDP339. The resulting plasmid was sequenced to confirm that no mutations were generated during PCR or cloning. Plasmid pDP339 was chemically transformed into *E. coli* BL21  $\lambda$ (DE3)-RIL in LB agar plates (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar), and transformants were selected with tetracycline (15  $\mu$ g/ml), chloramphenicol (50  $\mu$ g/ml), and ampicillin (150  $\mu$ g/ml). Recombinant CdeC-6 $\times$ His protein was overexpressed as previously described (M. Pizarro-Guajardo, V. Olguin-Araneda, J. Barra-Carrasco, C. Brito-Silva, M.R. Sarker, and D. Paredes-Sabja, submitted for publication).

To construct a CdeC-6 $\times$ His fusion, the entire *cdeC* ORF, excluding its stop codon, was amplified with forward primer P66 (5'-GACCATATGCAAGATTATAAAAAAAAAA TAAAAGAAGATGATGAATCAGC-3') and reverse primer P67 (5'-GACGGATCCGATCTGTGGCAACTTGGCTTCCAC-3'), where the forward and reverse primers have NdeI and BamHI restriction sites at their 5' ends (underlined). The reverse primer has two extra nucleotides (in bold) to give an in-frame fusion with the 6 $\times$ His tag of pET22b. The 1,215-bp PCR fragment was digested with NdeI and BamHI and cloned into the NdeI and BamHI sites of pET22b, giving plasmid pDP339. Plasmids were sequenced to confirm that no mutations were generated through PCR.

**Construction of *C. difficile* *cdeC* knockout mutant.** A derivative of *C. difficile* strain R20291 with an intron inserted into the *cdeC* gene was constructed as follows. To target the *L1.ltrB* intron to *cdeC*, the intron sequence in plasmid pJIR750ai was modified based on the sequences of the predicted insertion sites in the *cdeC* gene using the freely available algorithm in the ClosTron website ([www.clostron.com](http://www.clostron.com)). For optimal gene insertion, we picked the insertion in the antisense strand between positions 30 and 31 from the start codon (see Fig. 2A). Three short sequence elements from the intron RNA involved in base pairing with the DNA target sites (25) were modified by PCR, using *cdeC*-specific primers P68 (5'-AAAAAGCTTATAATTATCCTTATGATTCATCATCTGTGCGCCCAGATAGGGTG-3'), P69 (5'-CAGATTGTACAAATGTGGTGATA

ACAGATAAGTCATCATCTTAACTTACCTTTCTTTGT-3'), and P70 (5'-TGAACGCAAGTTTCTAATTCGGTTAATCATCGATAGAGGAAAGTGTCT-3') and the universal primer (CGAAATTAGAACTTGCGTTCAGTAAAC). The 353-bp *ClosTron cdeC* sequence was then cloned into sites HindIII and BsrGI in plasmid pMTL007C-E2, resulting in plasmid pDP306. Plasmid pDP306 was transformed into HB101 (pRK24) and subsequently transferred through conjugation to *C. difficile* strain R20291, and thiamphenicol-resistant colonies were selected. Next, overnight cultures of *C. difficile* R20291 (pDP306) were seeded onto BHIS agar plates supplemented with 20 µg/ml of lincomycin, and lincomycin-resistant colonies were further analyzed by PCR for mutant screening (see Fig. 2B).

To confirm that the putative mutants contained a single insertion of the *L1.ltrB* intron, genomic DNA was extracted using previously described protocols (26) and analyzed by Southern blotting using a probe against *ermB* and an alkaline phosphatase labeling kit (GE Healthcare) according to the manufacturer's instructions.

**Production of antibody.** The CdeC-6×His fusion proteins were produced in BL21λ(DE3)-RIL *E. coli* cells carrying plasmid pDP339. Fusion protein expression was induced by addition of 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) to 0.5 mM of an actively growing (optical density at 600 nm [OD<sub>600</sub>] of ~0.6) culture. Overnight cultures were lysed with insoluble buffer (20 mM Tris-HCl [pH 7.8], 500 mM NaCl, 5 mM imidazole, and 7.5 M urea), and insoluble 6×His-fusion proteins were purified by Ni<sup>2+</sup> affinity chromatography with Sepharose FastFlow (GE Healthcare). The purified protein was further purified by using 12% SDS-PAGE and stained with Coomassie brilliant blue, and the product corresponding to a band of the correct size was excised and used to immunize rats as previously described (27). Aliquots of antiserum were stored at -20°C until use. The antiserum recognized a 45-kDa polypeptide band in extracts made of *E. coli* cells expressing recombinant CdeC, indicating that at least some of the antibodies recognized CdeC. No band was detected with serum obtained prior to immunization of the rat (data not shown).

**Transmission electron microscopy (TEM).** To analyze the ultrastructure of spores of the *C. difficile* R20291 wild-type and Δ*cdeC* mutant strains, spores (~2 × 10<sup>8</sup>) were fixed with 3% glutaraldehyde-0.1 M cacodylate buffer (pH 7.2) overnight at 4°C and stained for 30 min with 1% tannic acid. Samples were further processed and embedded in spur resin as previously described (7). Thin sections obtained with a microtome were placed on glow discharge carbon-coated grids and doubly lead stained with 2% uranyl acetate and lead citrate. Grids were analyzed with a Philips Tecnai 12 BioTWIN electron microscope at the Electron Microscopy facility of the Pontificia Universidad Católica de Chile.

**Exosporium removal and spore extracts.** The exosporium of *C. difficile* spores can be completely removed by Sarkosyl and proteinase K treatment with no effects on the spore coat properties and ultrastructure (17). Briefly, ~10<sup>9</sup> spores were treated for 2 h at 37°C with 100 µl of 1% Sarkosyl-0.3 mg/ml proteinase K-25 mM Tris-HCl (pH 7.4) (SPK). SPK-treated spores were washed five times with sterile distilled water, and removal of the exosporium was confirmed by a hydrophobicity assay as described below and elsewhere (17). Since the exosporium layer is responsible for the hydrophobicity observed in *C. difficile* spores, those spores whose exosporium had been completely removed should have reduced hydrophobicity, meaning that less than 1% of the spores were found in the organic phase (17).

To extract the coat and exosporium layers from *C. difficile* spores for SDS-PAGE and Western blot analyses, 1 × 10<sup>8</sup> spores were resuspended in 100 µl of 2× SDS-PAGE sample loading buffer. Samples were boiled twice for 5 min and used immediately for analysis. Unlike the case with *Bacillus subtilis* spores, this method completely removes the coat and exosporium layers of *C. difficile* spores (17).

**Hydrophobicity assay.** To determine the hydrophobicity of spores of *C. difficile* R20291 wild-type and Δ*cdeC* mutant strains, untreated spores and spores treated for 2 h at 37°C with SPK were resuspended in distilled water to a final OD<sub>440</sub> of ~0.5 and mixed with hexadecane (Merck).

Adherence to hydrocarbon was measured by quantifying the drop in the OD<sub>440</sub> value of the aqueous solution (7).

**Immunofluorescence of *C. difficile* spores.** *C. difficile* R20291 wild-type and Δ*cdeC* spores were fixed with 3% paraformaldehyde (pH 7.4) for 20 min in poly-L-lysine-coated glass cover slides. Fixed spores were rinsed three times with phosphate-buffered saline (PBS) and blocked with 1% bovine serum albumin (BSA) for 30 min and further incubated overnight at 4°C with 1:50 of goat antiserum raised against *C. difficile* 630 spores (Pizarro-Guajardo et al., submitted) or with rat antiserum raised against CdeC. Next, covers containing fixed spores were incubated for 2 h at room temperature with 1:200 anti-goat IgG-Texas Red conjugate (Abcam) or 1:200 anti-rat Alexa 488 green conjugate (Invitrogen) in PBS-1% BSA and washed three times with PBS and once with distilled water. Dried samples (30 min at room temperature) were mounted with Dako fluorescence mounting medium (Dako North America) and sealed with nail polish. Samples were analyzed with a BX53 Olympus fluorescence microscope. Control experiments with goat and rat serum obtained prior to immunization yielded no fluorescence signal in R20291 and Δ*cdeC* spores (data not shown). The fluorescence intensity was quantified with ImageJ software (<http://rsbweb.nih.gov/ij/>).

**Western blot analysis.** Samples (10 µl) of SDS-PAGE loading buffer containing coat and exosporium extracts from 5 × 10<sup>7</sup> spores of *C. difficile* R20291 wild-type and Δ*cdeC* mutant strains were run 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). These Western blots were probed with a 1:1,000 dilution of goat antiserum raised against *C. difficile* 630Δ*ermB* spores (Pizarro-Guajardo et al., submitted) or a 1:250 dilution of rat antiserum raised against CdeC overnight at 4°C and then with a 1:10,000 dilution of donkey anti-goat or donkey anti-rat IgG-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 1 µl of a PageRuler Plus prestained protein ladder (Fermentas).

**Glycoprotein analysis.** After separation of the coat and exosporium extracts of *C. difficile* spores by 12% SDS-PAGE, glycoproteins were detected using Pro-Q Emerald 488 glycoprotein. A gel and blot stain kit (Molecular Probes, Invitrogen, OR) was used according to the manufacturer's instructions.

**Spore colony-forming efficiency.** To quantify the effect of a *cdeC* mutation on spore-forming efficiency, aliquots of *C. difficile* R20291 wild-type and Δ*cdeC* spores (1 × 10<sup>7</sup> spores/ml) were plated with or without heat activation (65°C, 20 min) onto BHIS agar plates supplemented with 0.1% sodium taurocholate (BHIS-ST) and incubated anaerobically at 37°C. After 36 h, colonies were counted. The total number of spores present in the suspension was quantified with a Neubauer counting chamber. Spore viability was calculated using the following formula: [(CFU ml<sup>-1</sup>)/(total spores ml<sup>-1</sup>)] × 100.

**DNA staining of *C. difficile* spores.** *C. difficile* spores (10<sup>6</sup>) were resuspended in 15 µl of distilled water containing 10 µg/ml of Hoechst stain (Sigma) and incubated at 37°C for 1 h. As a control, spores were incubated for 1 h at 37°C in PBS. Spores were washed three times by centrifugation (14,000 rpm for 5 min) with 30 µl of sterile distilled water, mounted on cover slides, and analyzed by phase-contrast and fluorescence microscopy using a BX53 Olympus fluorescence microscope.

**Spore resistance treatments.** Ethanol resistance of *C. difficile* R20291 wild-type and Δ*cdeC* spores was measured by resuspending 3 × 10<sup>6</sup> spores to a final volume of 30 µl of 50% ethanol. Spores were incubated with ethanol for 30 min at 37°C with shaking (200 rpm). Aliquots were plated onto BHIS-ST agar plates and incubated anaerobically for 36 h at 37°C. The effect of ethanol on spore viability was calculated by the following formula: survival = [Log(Nf) - Log(Ni)], where Nf represents counts of ethanol-treated spores and Ni represents counts of untreated spores.



Heat resistance of *C. difficile* spores was determined as previously described (28). Briefly,  $3 \times 10^6$  spores of the R20291 wild-type and  $\Delta cdeC$  mutant strains were resuspended in 30  $\mu$ l of PBS (pH 7.4) and heat treated at 75°C for various time periods (i.e., 1, 10, 20, 30, 45, and 60 min). Aliquots at appropriate dilutions were plated onto BHIS-ST agar plates and incubated anaerobically for 36 h at 37°C. As a control for non-heat-treated spores, an aliquot was plated onto BHIS-ST agar plates prior to the experiment and colonies were counted as described above.

Lysozyme resistance of *C. difficile* spores was measured by resuspending  $3 \times 10^6$  spores of the R20291 wild-type and  $\Delta cdeC$  strains in 30  $\mu$ l of PBS with 10, 250, and 1,000  $\mu$ g/ml of lysozyme and incubating the mixture for up to 5 h at 37°C with shaking (200 rpm). Germinated spores were visualized by phase-contrast microscopy. Spore viability was measured by plating aliquots onto BHIS-ST agar plates and incubating them anaerobically at 37°C for 36 h, after which colonies were counted. In some experiments, lysozyme-treated *C. difficile* R20291 wild-type and  $\Delta cdeC$  mutant spores were subsequently treated with 50% ethanol for 30 min at 37°C with shaking (200 rpm) and aliquots plated onto BHIS-ST agar plates and colonies counted after 36 h of incubation at 37°C under anaerobic conditions.

**Spore core wet density.** The spore core wet density was measured using protocols previously described (29). Briefly, *C. difficile* wild-type and  $\Delta cdeC$  spores were resuspended in 100  $\mu$ l of 30% Nycodenz (Sigma, St. Louis, MO), incubated for 60 min on ice, and loaded into the top of a 2-ml linear gradient of 45% to 50% Nycodenz in ultraclear tubes, and the tubes were centrifuged for 40 min at  $15,000 \times g$  for 40 min at 20°C. The density was calculated according to the following formula: density (g/ml) =  $3.242\eta - 3.323$ , where  $\eta$  = (% Nycodenz - 810.13)/607.75. The spore core water content was calculated according to the formula  $y = -0.00254x + 1.460$  (29), where  $y$  is the spore core wet density and  $x$  is the core water content in g per 100 g of wet protoplast (core). Measurements were done in duplicate.

**Assay of adherence of spores to intestinal epithelial Caco-2 and HT-29 cells.** Adherence of *C. difficile* spores to Caco-2 and HT-29 cells was measured as previously described (7). Briefly, epithelial cells were seeded ( $5 \times 10^5$  cells per well) onto 96-well plates and incubated until cells became confluent. In the case of HT-29 cells, confluent monolayers were immediately infected. For Caco-2, once monolayers reached confluence, they were further incubated for 2 and 8 days, respectively, obtaining undifferentiated and differentiated Caco-2 monolayers, respectively. Differentiation was assessed by immunofluorescence using sucrose-isomaltase as a marker for the appearance of microvilli (30), which appeared 8 days after monolayers of Caco-2 became confluent under our culture conditions. Before infection, monolayers of Caco-2 and HT-29 were washed three times with PBS, infected with 40  $\mu$ l of DMEM and RPMI medium, respectively, containing *C. difficile* R20291 wild-type and  $\Delta cdeC$  spores at a multiplicity of infection (MOI) of 10, and incubated for 1 and 3 h at 37°C in a 5% CO<sub>2</sub> atmosphere. To remove unbound spores, wells were washed three times with PBS. Caco-2 and HT-29 cells were then lysed with 95  $\mu$ l 0.06% Triton X-100 for 30 min at 37°C, and appropriate dilutions were plated onto BHIS-ST agar plates and incubated under anaerobic conditions at 37°C for 36 h. For total *C. difficile* spore determinations, spore-infected Caco-2 and HT-29 cells were directly lysed and plated onto BHIS-ST agar plates and incubated anaerobically at 37°C for 36 h. Numbers of CFU per ml were determined and percentages of adherence were calculated using the following formula: (final CFU ml<sup>-1</sup>/initial CFU ml<sup>-1</sup>)  $\times$  100. These experimental conditions do not trigger germination of *C. difficile* spores (31).

To evaluate if *C. difficile* spores remained in the surface of Caco-2 cells, we assessed internalization of *C. difficile* spores in monolayers of Caco-2 cells as previously described with minor modifications (32). Briefly, confluent monolayers of Caco-2 cells were infected at an MOI of 10 for 3 h at 37°C. Wells were rinsed twice with Dulbecco's PBS (DPBS) to remove any unbound spore and fixed for 15 min at room temperature with 200  $\mu$ l of freshly prepared 4% paraformaldehyde. Fixed Caco-2 cells were rinsed

twice with DPBS, and extracellular *C. difficile* spores were labeled with anti-spore goat serum diluted at 1:50 in 1% BSA-DPBS for 3 h at room temperature. Labeled spores were rinsed three times with DPBS, incubated for 2 h with a 1:200 dilution of anti-goat IgG conjugated with fluorescein isothiocyanate (FITC) (green) (Abcam), and rinsed three times with DPBS followed by a final rinse with distilled water. Samples were air dried, sealed with nail polish, and analyzed by fluorescence microscopy. Internalized spores were identified as bright-phase spores that were not labeled green, while extracellular or adherent spores were identified as bright-phase spores that were stained green by anti-goat IgG-FITC conjugate. For each experimental condition, at least 2,000 spores were analyzed. All experiments were performed at least three times.

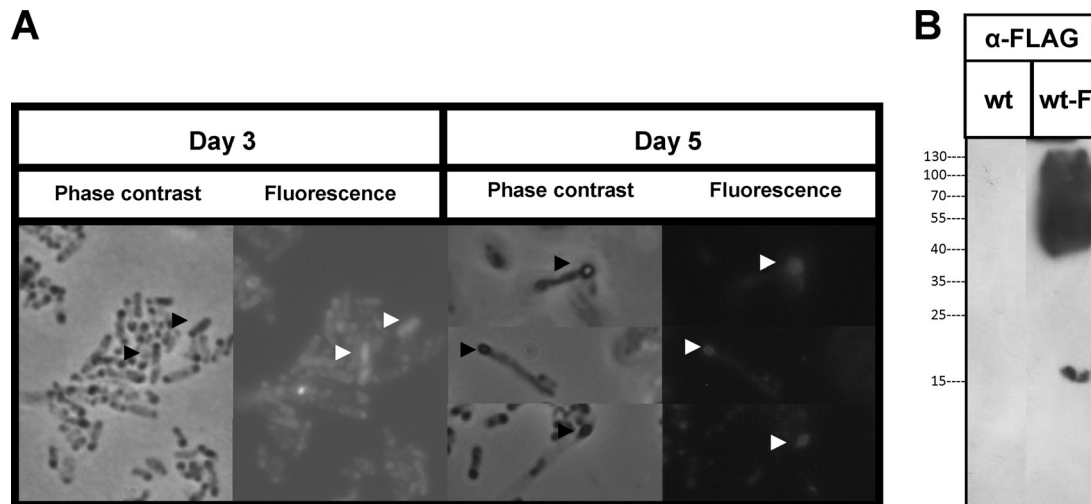
**Statistical analysis.** Student's *t* test was used for pairwise comparisons.

## RESULTS

**CD1067 is expressed in the mother cell compartment and localizes to the spore.** From recent proteomic work, we identified polypeptides encoded by *CD1067* in trypsin-digested extracts of the exosporium of *C. difficile* spores (K. Escobar-Cortés et al., submitted). Trypsin was recently shown to remove the majority of the exosporium layer, leaving a thin exosporium layer attached to the spore coat (17), which suggests that the *CD1067*-encoded protein might be an exosporium protein. *CD1067* is a 1.2-kb gene that encodes a 42-kDa protein, and the predicted amino acid sequence reveals an unusually high percentage of cysteine residues (i.e., 8% Cys). Therefore, we propose to rename this protein *Clostridium difficile* exosporium cysteine-rich protein (CdeC).

The promoter region of *cdeC* encodes consensus sequences for the sporulation-specific RNA polymerase sigma factors SigE and SigK, suggesting that it might be expressed during early and late stages of spore formation. To investigate if *cdeC* is expressed during sporulation and if CdeC localizes to the spore, we constructed a translational fusion of CdeC with tdTOMATO, the fluorescence protein, and evaluated its expression (Fig. 1A). Unlike the *B. subtilis* results, our sporulation assay demonstrated that *C. difficile* sporulation is asynchronous (Fig. 1A). We observed that expression of CdeC-tdTOMATO began in the mother cell compartment of the sporulating cell and localized in the spore (Fig. 1A). Control experiments performed with tdTOMATO alone under the control of a SigK-regulated promoter (i.e., *bclA1*) (Pizarro-Guajardo et al., submitted) did not produce red-fluorescent spores (data not shown). CdeC was also fused to the FLAG epitope, which has been previously used to localize spore proteins (33). Western blot analyses demonstrated that while wild-type spores had no band corresponding to immunoreactivity to anti-FLAG antibodies, a strong immunoreactive complex with a broad-range molecular mass of from ~40 to 120 kDa became detectable in extracts of *C. difficile* R20291 spores expressing a CdeC-FLAG fusion (Fig. 1B). Collectively, these results indicate that CdeC is expressed during sporulation and localizes to the spore as a high-molecular-mass complex.

**Construction of a *C. difficile* *cdeC* knockout mutant.** Therefore, to begin characterizing CdeC, we attempted to construct knockout mutants in *C. difficile* strains 630 $\Delta$ ermB and R20291 by redirecting the group II *L1.ltrB* intron into the antisense strand at position 30 (Fig. 2A). Although numerous attempts to inactivate *cdeC* in *C. difficile* strain 630 $\Delta$ ermB were made, we did not succeed (data not shown) and were able to obtain several knockout mutants only in strain R20291, as shown by PCR screening for insertions (Fig. 2B). Clones C2 and C5 were then analyzed by Southern



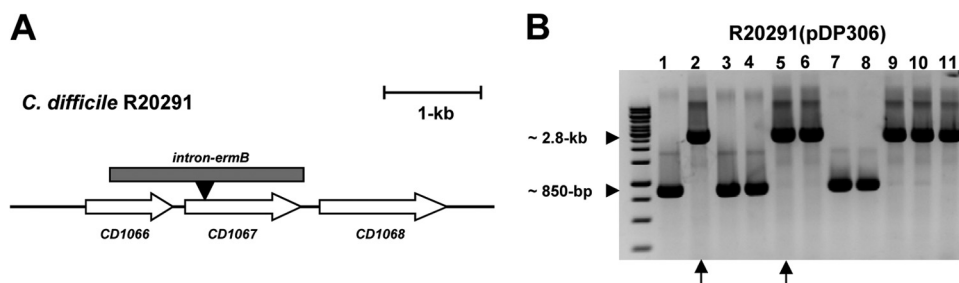
**FIG 1** Expression of CdeC-tdTOMATO and CdeC-FLAG fusions during sporulation of *C. difficile*. (A) TY sporulation cultures of the *C. difficile* 630ΔermB(pDP322) mutant were harvested after 3 and 5 days of sporulation in TY agar plates. Sporulating cultures were air dried in glass cover slides, mounted, and analyzed by fluorescence microscopy as described in Materials and Methods. (B) Exosporium and coat extracts of spores of the *C. difficile* 630ΔermB wild-type (wt) and 630ΔermB(pDP345) strains expressing CdeC-FLAG (wt-F) were analyzed by Western blotting with anti-FLAG antibodies. Lanes carrying wt and wt-F belong to the same blot.

blotting to demonstrate that the insertion of the *L1.ltrB* observed by PCR analysis was unique to the *cdeC* ORF (data not shown). These clones were used for subsequent phenotypic characterization.

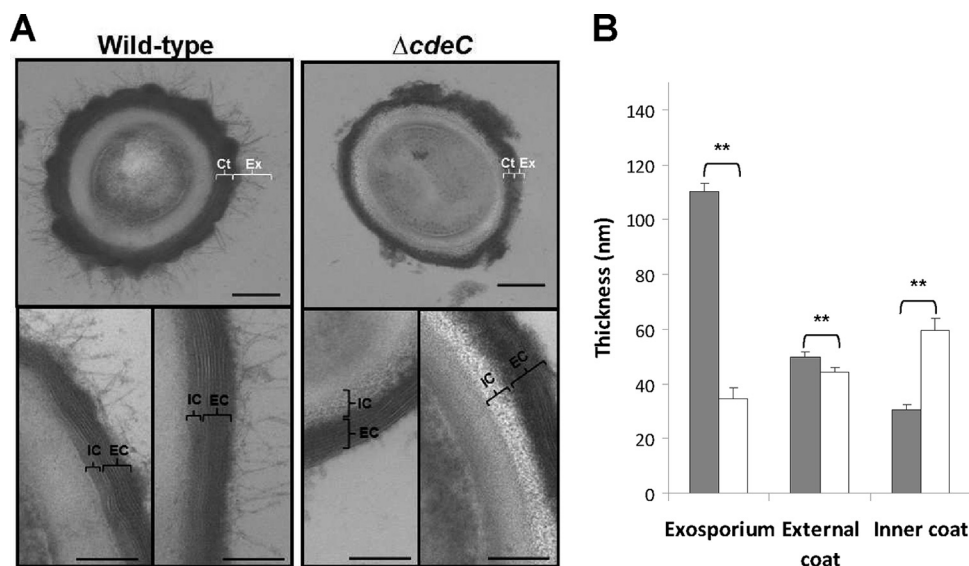
***cdeC* mutation affects the formation of the exosporium layer of *C. difficile* spores.** The sporulation rates of the *C. difficile* Δ*cdeC* and wild-type R20291 strains were similar, with comparable overall spore yields (data not shown). Unlike the case of the *B. anthracis* exosporium layer, *C. difficile* wild-type R20291 spores have an atypical exosporium layer that lacks the gap-intercoat space observed in several *Bacillus* species. Although the *C. difficile* R20291 exosporium has a hair-like nap similar to that observed in *B. anthracis* and *B. cereus* (6), *C. difficile* R20291's nap seems scruffier and less ordered (Fig. 3A). In contrast, the spore coat of *C. difficile* R20291 has laminations similar to those previously shown in *C. difficile* 630 spores and other *Bacillus* spores (6, 17). Strikingly, spores of strain Δ*cdeC* lacked most of the exosporium layer (Fig. 3A), a phenotype that was observed in more than 10 Δ*cdeC* spores analyzed by TEM (data not shown). In some Δ*cdeC* spores, remnants of the exosporium layer remained attached to the spore coat (Fig. 3A). On average, the exosporium layer of the Δ*cdeC* spores was 3-fold thinner than that of wild-type spores (Fig. 3). The un-

derlying coat of Δ*cdeC* spores seemed to possess ultrastructural features similar to those of the wild-type spore coat (Fig. 3). However, a closer examination of this layer revealed significant differences (Fig. 3). There was a small, though significant, decrease of ~13% in the thickness of the coat of Δ*cdeC* spores compared to that of the coat of wild-type spores (Fig. 3). Strikingly, the space between the spore coat and the peptidoglycan cortex, presumably the inner coat layer, was 2-fold greater in Δ*cdeC* spores than in wild-type spores (Fig. 3), suggesting that CdeC is required not only for the formation of the exosporium layer but also for the correct assembly of the spore coat.

**CdeC is located in the exosporium layer of *C. difficile* spores.** The morphological defects observed as described above suggest that CdeC might be an outer coat or exosporium protein. To define the location of CdeC, we prepared rat anti-CdeC serum and performed immunofluorescence analysis of untreated wild-type and Δ*cdeC* spores. Interestingly, while wild-type spores yielded significant fluorescence with rat anti-CdeC serum, no detectable fluorescence was observed in Δ*cdeC* spores (Fig. 4A). We have recently demonstrated that Sarkosyl and proteinase K (SPk) treatment removes the entire exosporium layer of *C. difficile* spores (17). Therefore, to evaluate if CdeC is located in the exosporium



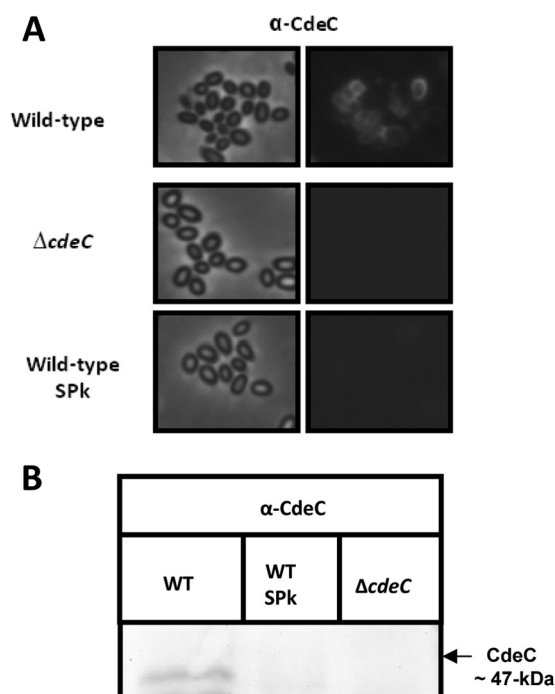
**FIG 2** Construction of a *C. difficile* Δ*cdeC* knockout mutant. (A) Genomic arrangement of *cdeC* and schematic representation of inactivation of *cdeC* in *C. difficile* R20291. (B) Screening of lincomycin resistance *C. difficile* colonies for ClosTron insertion by PCR. The numbers at the top are bacterial colony numbers. Colonies 2 and 5 (showing the 2.8-kb band) were used for subsequent characterization.



**FIG 3** *C. difficile*  $\Delta cdeC$  spores have a defective exosporium layer. (A) Thin sections of *C. difficile* wild-type and  $\Delta cdeC$  spores were analyzed by transmission electron microscopy as described in Materials and Methods. Ct, spore coat; Ex, exosporium. Scale bars are shown in each figure: the bars in the upper panels represent 200 nm, and the bars in the lower panels represent 100 nm. (B) The thicknesses of the exosporium and outer and inner coat layers of *C. difficile* wild-type (gray bars) and  $\Delta cdeC$  (white bars) spores were analyzed by transmission electron microscopy of at least 10 individual spores. IC, inner coat; OC, outer coat. Error bars denote standard errors of the means. Asterisks (\*\*) denote statistical difference at  $P < 0.001$ .

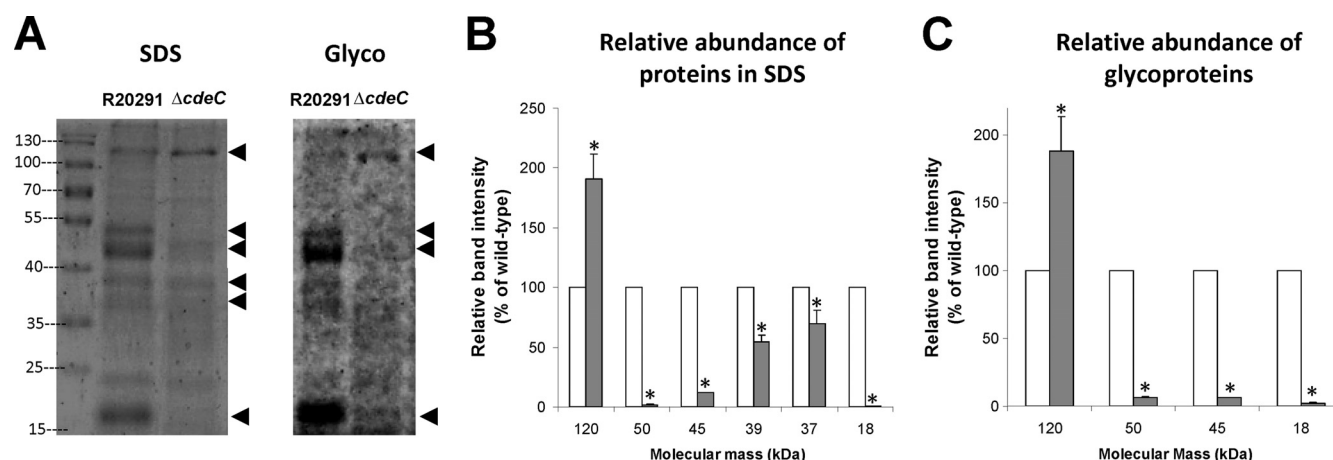
layer, SPk-treated wild-type spores were analyzed by immunofluorescence. Strikingly, SPk-treated wild-type spores yielded no fluorescence signal (Fig. 4A), indicating that CdeC is localized in the exosporium-like layer of *C. difficile* spores. To provide further evidence that CdeC is uniquely localized in the exosporium layer, coat and exosporium extracts of wild-type and  $\Delta cdeC$  spores were analyzed by Western blots. A ~47-kDa polypeptide band, with a mass slightly higher than the predicted (i.e., 42-kDa) molecular mass, was detected in the coat and exosporium extracts of wild-type but not of  $\Delta cdeC$  spores (Fig. 4B), suggesting that CdeC is located in the spore coat and exosporium extracts and that it might suffer some posttranslational modifications during spore assembly. The exosporium location of CdeC was confirmed by analyzing the coat extract of SPk-treated wild-type spores (i.e., lacking the exosporium layer), where, as with the  $\Delta cdeC$  strain, no immunoreactive band was detected (Fig. 4B). Collectively, these results provide evidence that CdeC is localized in the exosporium-like layer of *C. difficile* spores.

**A *cdeC* mutation affects the abundance of major protein species and glycoproteins in the spore surface.** The lack of the exosporium layer in  $\Delta cdeC$  spores suggests that the protein profile of  $\Delta cdeC$  spores might differ from that of wild-type spores. SDS-PAGE analyses of spore coat and exosporium extracts showed that there were several major protein bands that were absent or were present but at reduced levels in  $\Delta cdeC$  spores (Fig. 5A). Densitometry analyses of the major polypeptides observed in extracts of wild-type spores demonstrated that the levels of the ~18-, 37-, 39-, 45-, and 50-kDa proteins were reduced by 99%, 31%, 45%, 89%, and 98%, respectively, in extracts of  $\Delta cdeC$  spores (Fig. 5A and B). Interestingly, the level of the ~120-kDa polypeptide in extracts of  $\Delta cdeC$  spores increased by 190% relative to the levels found in the extracts of wild-type spores (Fig. 5B). These results indicate that CdeC is required for the presence of the polypeptides of 18, 37, 39, 45, and 50 kDa in the spore coat and exosporium



**FIG 4** CdeC is a spore surface protein localized in the exosporium layer of *C. difficile* spores. (A) The surface accessibility of CdeC on *C. difficile* R20291 untreated wild-type spores (Wild-type), Sarkosyl- and proteinase k (SPk)-treated wild-type spores (Wild-type SPk), and *C. difficile* untreated  $\Delta cdeC$  spores ( $\Delta cdeC$ ) was analyzed by immunofluorescence with rat anti-CdeC serum as described in Materials and Methods. (B) Western blot analysis of coat and exosporium extracts of  $5 \times 10^7$  *C. difficile* R20291 wild-type untreated (Wild-type) and Sarkosyl- and proteinase k (SPk)-treated (Wild-type SPk) spores and *C. difficile*  $\Delta cdeC$  spores ( $\Delta cdeC$ ). The spores were run on an SDS-PAGE gel, transferred onto a nitrocellulose membrane, and analyzed by Western blotting with rat anti-CdeC serum. A black arrow highlights the immunoreactive CdeC.





**FIG 5** Protein analysis of *C. difficile*  $\Delta cdeC$  spores. (A) Coat and exosporium extracts of  $7.5 \times 10^7$  spores of *C. difficile* wild-type and  $\Delta cdeC$  strains were electrophoresed and stained with Coomassie brilliant blue and analyzed for the presence of glycoproteins (Glyco) with the Pro-Q Emerald 488 glycoprotein gel and blot stain (Invitrogen). Black arrowheads highlight the major protein bands and glycoprotein polypeptides of *C. difficile* spores. (B and C) Densitometry analysis of the relative amounts of major protein bands in an SDS-PAGE gel (B) and of glycoprotein (C) of  $\Delta cdeC$  spores was performed with ImageJ, and the results are expressed relative to those determined with wild-type spores. White bars, wild-type spores; gray bars,  $\Delta cdeC$  spores. Asterisks denote statistical difference at  $P < 0.05$ .

extracts or for proper chemical extraction of these proteins in the misassembled coat and exosporium.

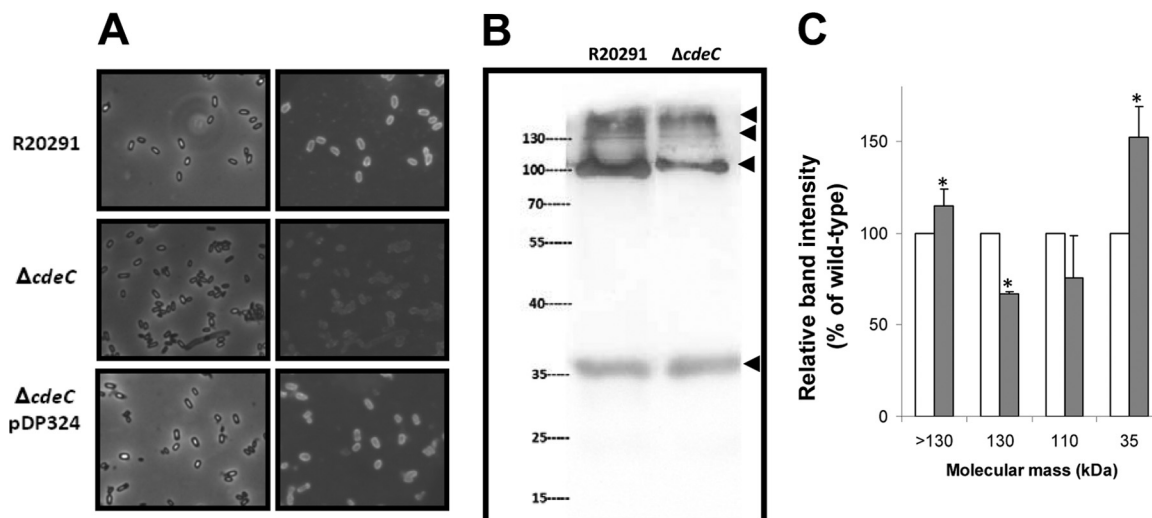
The major component of the exosporium layer of *B. anthracis* spores, the BclA protein, is highly glycosylated (9, 12). Recently, the exosporium of *C. difficile* strain 630 spores has also been shown to have glycosylated proteins of ~49 and 54 kDa (Pizarro-Guajardo et al., submitted). Analysis of glycoproteins in coat and exosporium extracts of *C. difficile* R20291 spores showed the presence of four glycosylated protein bands of ~18, 45, 50, and 120 kDa (Fig. 5A). Strikingly, the levels of the ~18-, 45-, and 50-kDa glycosylated proteins found in the coat and exosporium extracts of  $\Delta cdeC$  spores decreased by 96% (Fig. 5A and C). In contrast, the glycosylated protein band of ~120 kDa increased to 190% in the extracts of  $\Delta cdeC$  spores (Fig. 5A and C). These results indicate that CdeC is required for proper localization of the glycosylated proteins of 18, 45, and 50 kDa but not of 120 kDa to the exosporium of *C. difficile* spores or perhaps that its absence affects the ability to chemically extract these proteins from the misassembled coat and exosporium.

**Effect of a *cdeC* mutation on the levels of the immunodominant protein(s).** Previous work demonstrated that enzymatic removal of the exosporium layers leads to the removal of the immunodominant proteins detectable by immunofluorescence (Pizarro-Guajardo et al., submitted). Therefore, the absence of most of the exosporium layer in  $\Delta cdeC$  spores suggests that CdeC might also affect the abundance of the immunodominant proteins. Analysis by immunofluorescence with anti-spore antiserum, which detects only epitopes that are physically accessible to IgGs, demonstrated that  $\Delta cdeC$  spores had a lower fluorescence intensity than wild-type spores (Fig. 6A). The intensity of  $\Delta cdeC$  spores was estimated to be ~60% lower than that of wild-type spores (Fig. 6B). Control samples of spores incubated only with secondary antibody yielded no fluorescence signal (data not shown). These results suggest that lack of CdeC affects the abundance of exosporium-located immunodominant proteins in the misassembled coat and exosporium.

In *C. difficile* strain 630 spores, the immunodominant proteins

are also present in the exosporium-like layer. However, in spores with no exosporium-like layer (i.e., after removal of the layer with SPk treatment), although no immunofluorescence of immunodominant proteins is detected, they still retain much of the immunodominant proteins in their spore coat, where they are not accessible to IgGs (Pizarro-Guajardo et al., submitted). In accordance with the reasoning that this might also be the case in  $\Delta cdeC$  spores, the relative abundances of the immunodominant proteins of extracts of wild-type and  $\Delta cdeC$  spores were analyzed by Western blotting. Results of the experiment performed with extracts of R20291 spores showed the presence of four major immunodominant proteins with estimated molecular masses of 35-, 110-, 130-, and >130 kDa (Fig. 6C). In contrast to the low immunofluorescence of the immunodominant proteins observed in  $\Delta cdeC$  spores, Western blot results showed that the extracts of  $\Delta cdeC$  spores, mainly composed of the spore coat, retained a significant amount of immunodominant proteins, albeit the levels in the extracts from some species were slightly lower than those seen with wild-type spores (Fig. 6B). This indicates that the coat of  $\Delta cdeC$  spores retained much of these immunodominant proteins. The level of the 130-kDa immunodominant protein in  $\Delta cdeC$  spore coat extracts was 67% of that in the wild type, while levels of the immunodominant proteins of 35 and >130 kDa significantly increased in  $\Delta cdeC$  spore extracts compared to wild-type levels (Fig. 6B and C), suggesting that at least the 130-kDa protein might be involved in the immunofluorescence observed in wild-type spores. Collectively, these results suggest that the immunodominant proteins are more abundant in the spore coat and that the fluorescence observed in the surface of wild-type spores is due to the 130-kDa immunodominant protein.

**CdeC is not an immunodominant spore protein.** The fact that CdeC was found to be located in the surface, specifically, in the exosporium layer of *C. difficile* spores, and that its absence substantially affected the exosporium layer and the spore coat suggests that CdeC could be an immunodominant protein. A concentration gradient of partially purified rCdeC was electrophoresed and analyzed by Western blotting with spore goat antiserum.



**FIG 6** Effect of a *cdeC* mutation on the abundance of immunodominant proteins of *C. difficile* spores. The accessibility of antibodies against immunodominant proteins in *C. difficile* wild-type,  $\Delta cdeC$ , and  $\Delta cdeC$ (pDP324) (*cdeC* mutant complemented with wild-type *cdeC*) spores was measured by fluorescence microscopy as described in Materials and Methods. (A) Fluorescence micrographs of wild-type,  $\Delta cdeC$ , and  $\Delta cdeC$ (pDP324) spores were analyzed by ImageJ, and the relative fluorescence intensities of at least 200 spores were quantified and expressed as arbitrary units. (B) Coat and exosporium extracts of *C. difficile* wild-type and  $\Delta cdeC$  spores were run on SDS-PAGE gel, transferred onto nitrocellulose membrane, and analyzed by Western blotting with goat anti-spore serum. Black arrowheads highlight the immunoreactive bands. (C) Densitometry analysis of the relative amount of immunodominant protein of  $\Delta cdeC$  spores was performed with ImageJ, and data are expressed relative to wild-type spore data. White bars, wild-type spores; gray bars,  $\Delta cdeC$  spores. Asterisks denote statistical difference at  $P < 0.05$ .

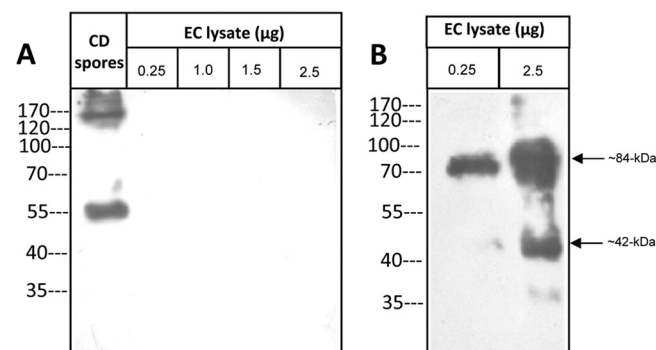
While antiserum reacted only with immunodominant proteins present in the extracts of R20291 spores (Fig. 7A), no immunoreactive band was detected in the gradient of *E. coli* lysate expressing rCdeC (Fig. 7A). However, rCdeC was readily detectable with anti-6×His antibodies as a 42-kDa monomer and a 84-kDa dimer (Fig. 7B). The fact that CdeC is present in the spore surface and that no IgGs were raised against CdeC in the anti-*C. difficile* spore serum suggests that it has poor antigenic properties.

**The *cdeC* mutation does not affect colony-forming efficiency and hydrophobicity of *C. difficile* spores.** Since a lack of CdeC produced spores with a defective coat and exosporium, we evaluated if these defects affected the colony-forming ability of  $\Delta cdeC$  spores. R20291 and  $\Delta cdeC$  spores, with or without heat activation,

were plated onto BHIS-ST agar plates and colonies counted. No significant difference between R20291 and  $\Delta cdeC$  spores in colony-forming ability was detected with or without heat activation (data not shown). The colony-forming efficiencies of mutant and parental spores remained similarly low (i.e., ~2%) (data not shown).

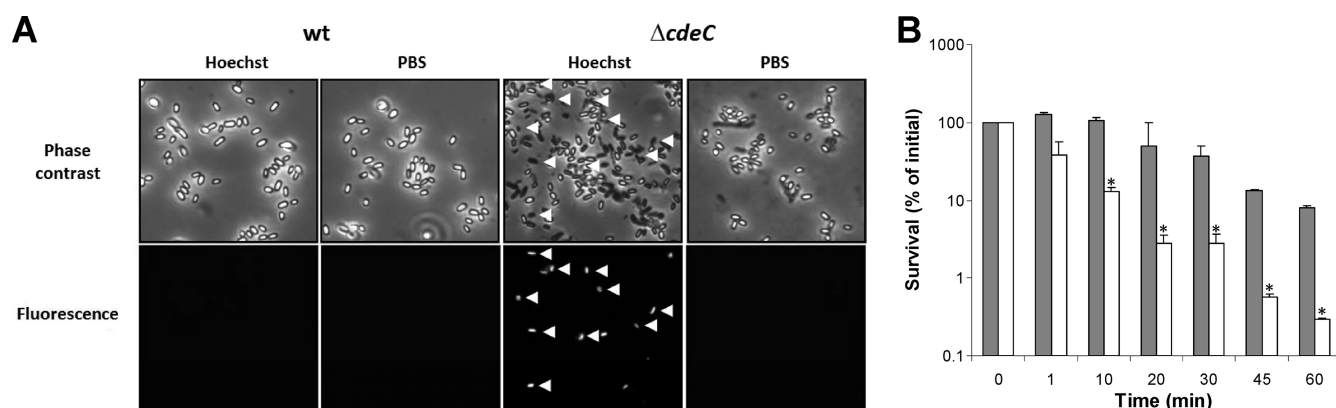
Recently, we demonstrated that the exosporium of *C. difficile* spores is responsible for the hydrophobicity of the surface of *C. difficile* spores (17). In fact, the progressive removal of the exosporium layer of *C. difficile* spores produced a progressive decrease in the spore's surface hydrophobicity; thus, hydrophobicity can be used as a marker for exosporium removal (17). Consequently, the lack of the majority of the exosporium layer in  $\Delta cdeC$  spores suggested that CdeC might affect the *C. difficile* spore's hydrophobicity. Strikingly, there was no significant difference between wild-type (i.e., 67%) and mutant (i.e., 66%) spores in the percentages that were in the organic phase (data not shown), suggesting that the *cdeC* mutation did not affect the *C. difficile* spore's hydrophobicity. Since SPk treatment removes the exosporium layer of *C. difficile* spores (17), we evaluated if the wild-type levels of hydrophobicity of  $\Delta cdeC$  spores were due to the remnants of the exosporium observed on  $\Delta cdeC$  spores. Interestingly, SPk-treated spores of both wild-type and  $\Delta cdeC$  strains had hydrophobicities of ~1% (data not shown), indicating that, as expected, the exosporium was completely removed by Sarkosyl and proteinase K treatment (17) and that the remnants of the exosporium layer present in  $\Delta cdeC$  spores observed as described above (Fig. 3A) are responsible for the wild-type levels of hydrophobicity observed in untreated  $\Delta cdeC$  spores.

**Effect of the absence of CdeC on resistance of *C. difficile* spores to ethanol and heat.** The defective spore coat structure observed in  $\Delta cdeC$  spores suggested that these spores might have reduced resistance properties. A major factor in ethanol resistance



**FIG 7** CdeC is not an immunogenic protein of *C. difficile* spores. Aliquots of exosporium and coat spore extracts (CD spores) and lysates of an overexpression culture of *E. coli* BL21 (DE3)pDP338 (EC lysate) were separated by SDS-PAGE, proteins were transferred to a nitrocellulose membrane, and immunodominant proteins were detected with anti-spore antibodies (A) or anti-6×His antibodies (B) as described in Materials and Methods. The ~42-kDa black arrow denotes the molecular mass of recombinant CdeC.





**FIG 8** CdeC is involved in *C. difficile* spore inner membrane permeability and resistance to heat treatment. (A) *C. difficile* wild-type (wt) and  $\Delta cdeC$  spores were incubated with 10  $\mu$ g/ml of Hoechst stain–PBS (Hoechst) for 1 h at 37°C and analyzed by fluorescence microscopy as described in Materials and Methods. As a control, spores were incubated in PBS alone (PBS) for 1 h at 37°C. (B) Heat resistance of *C. difficile* wild-type (gray bars) and  $\Delta cdeC$  (white bars) spores was measured by heat treating aliquots at 75°C for various times, and survivors were enumerated as described in Materials and Methods. Data represent the averages of the results of three independent experiments, and error bars represent standard errors of the means. Asterisks indicate statistical difference at  $P < 0.05$ .

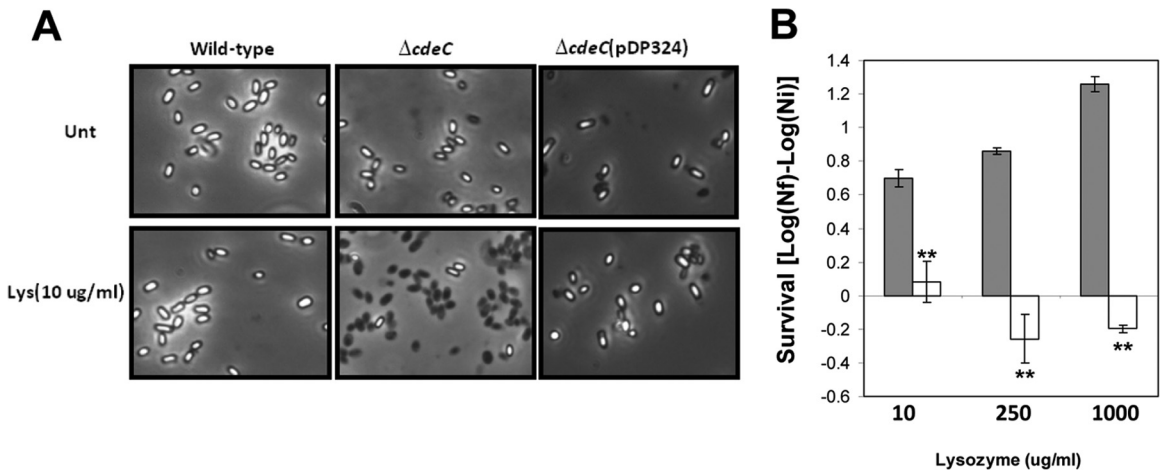
of bacterial spores is the spore's inner membrane, which acts as an impermeable barrier preventing the entry of ethanol into the spore core and denaturing proteins (34). Therefore, the permeability barrier of the inner membrane of *C. difficile* R20291 wild-type and  $\Delta cdeC$  spores was measured by treating spores of both strains with 50% ethanol. Strikingly, a significant increase in the viability of ethanol-treated wild-type spores was observed, with an overall increase in viability of 0.35 log cycles compared to untreated wild-type spores (data not shown). However, an inactivation of 0.2 log cycle was observed when  $\Delta cdeC$  spores were treated with ethanol (data not shown), indicating that the inner membrane of a fraction of  $\Delta cdeC$  spores is permeable to ethanol. Since the inner membrane of dormant spores is formed by a bilayer of immobile phospholipids (35) and is impermeable to DNA-staining dyes (34), we used Hoechst DNA-staining dye to evaluate if the inner membranes of  $\Delta cdeC$  spores might have different permeability properties. When wild-type spores were incubated in PBS for 1 h of Hoechst staining, no DNA staining was observed (Fig. 8A). Of a total of 400 wild-type spores analyzed, 92% remained in the bright phase, 6% changed to the gray phase (indicative of partial germination corresponding to stage I), and only 2% changed to the black phase (indicative of full germination corresponding to stage II) (Fig. 8A and data not shown). Further incubation of wild-type spores with Hoechst stain for 5 h did not produce any detectable staining or germination of wild-type spores (data not shown). No germination was observed when *C. difficile* wild-type spores were assayed for germination in buffer alone (i.e., PBS) (Fig. 8A). Strikingly, the spore core of ~23% of  $\Delta cdeC$  spores incubated with Hoechst stain became fluorescent (Fig. 8A). The most notable fact was that Hoechst stain induced significant germination of  $\Delta cdeC$  spores (Fig. 8A). Phase-contrast and fluorescence microscopy analyses of ~400  $\Delta cdeC$  spores demonstrated that after 1 h of incubation with Hoechst stain, ~38% remained in the bright phase, ~36% changed to the gray phase, and 26% changed to the dark phase (Fig. 8A and data not shown). Interestingly, Hoechst stained the spore core of the majority of  $\Delta cdeC$  spores that changed to dark phase (i.e., of 105 analyzed dark-phase  $\Delta cdeC$  spores, 92, equivalent to ~88%, of the dark-phase spores had their spore cores stained), indicating that

Hoechst stain might first trigger germination of some  $\Delta cdeC$  spores and then stain the spore core of most of the germinated  $\Delta cdeC$  spores, presumably because their spore inner membrane becomes more permeable to the diffusion of Hoechst. Collectively, these results indicate that the inner membrane of  $\Delta cdeC$  spores has increased permeability to organic solvents and DNA-staining dyes.

To gain more information on the effect of CdeC on *C. difficile* spore resistance, we measured the heat resistance of wild-type and  $\Delta cdeC$  spores at 75°C. Upon heat treatment of wild-type spores, ~50% and 92% of the spores were inactivated after 30 and 60 min, respectively (Fig. 8B). In contrast,  $\Delta cdeC$  spores were more sensitive to heat than wild-type spores; after 30 and 60 min of incubation at 75°C, only 2.8% and 0.3% of  $\Delta cdeC$  spores remained viable, respectively (Fig. 8B). These results indicate that CdeC is also involved in wet-heat resistance of *C. difficile* spores.

The increase in inner membrane permeability and higher heat sensitivity of  $\Delta cdeC$  spores suggest that these spores might have a core with a wet density different from that of the wild-type spore core. Strikingly, while the core wet density and water content of wild-type spores were 1.264 g/ml and 77 g per 100 g of wet protoplast (core), the  $\Delta cdeC$  spores had a core wet density and water content of 1.251 g/ml and 82 g per 100 g of wet protoplast (core), respectively. These results suggest that part of the properties of lower resistance to ethanol and heat observed in  $\Delta cdeC$  spores is due to a higher water content in their spore core.

**Absence of CdeC increases spore coat permeability.** The coat of *C. difficile* spores acts as an impermeable barrier against enzymes with molecular masses of  $> \sim 14$  kDa, such as lysozyme, proteinase K, and trypsin (17). Therefore, given the defects observed on the coat of  $\Delta cdeC$  spores, we evaluated if the absence of CdeC might affect the permeability properties of the spore coat. Consequently, when wild-type spores were treated with 10  $\mu$ g/ml of lysozyme for 5 h at 37°C, only ~4% of the spores changed to the dark phase (Fig. 9A). In contrast, under similar treatment conditions, ~95% of  $\Delta cdeC$  spores changed to the dark phase (Fig. 9A). Similar results were observed when wild-type and  $\Delta cdeC$  spores were treated with 250  $\mu$ g/ml or 1 mg/ml of lysozyme (data not shown). Lysozyme treatment increased the colony-forming effi-



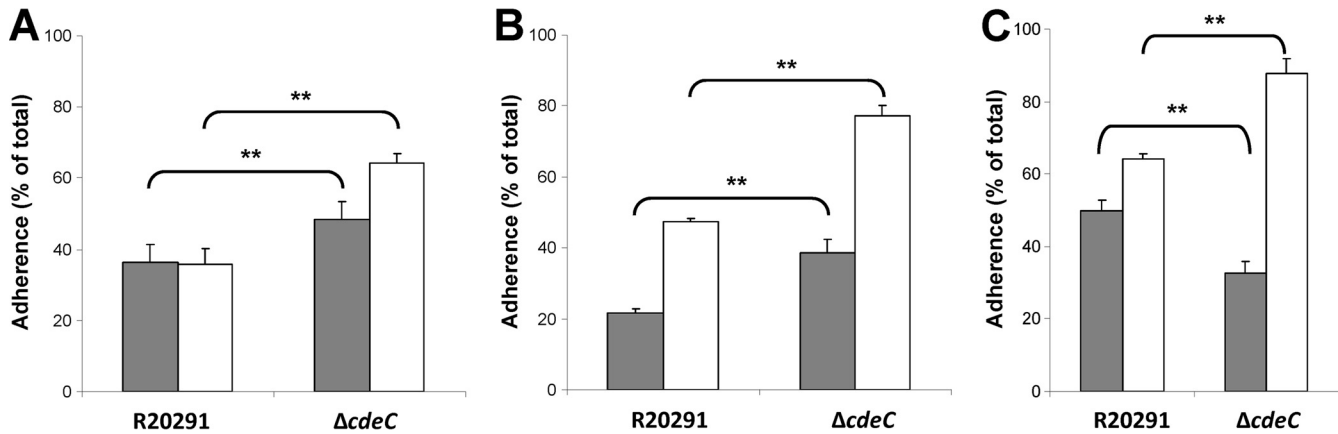
**FIG 9** *C. difficile*  $\Delta cdeC$  spores are susceptible to lysozyme. (A) Representative phase-contrast micrographs of *C. difficile* wild-type,  $\Delta cdeC$ , and  $\Delta cdeC(pDP324)$  ( $\Delta cdeC$  mutant complemented with wild-type *cdeC*) spores treated for 5 h at 37°C with PBS (Unt) or 10  $\mu$ g/ml of lysozyme–PBS [Lys (10  $\mu$ g/ml)]. (B) Resistance of *C. difficile* wild-type (gray bars) and  $\Delta cdeC$  (white bars) spores to lysozyme was measured by treating *C. difficile* spores for 5 h at 37°C with 10, 250, and 1,000  $\mu$ g/ml of lysozyme. Aliquots of lysozyme-treated spores were plated onto BHIS-ST agar plates and incubated anaerobically for 36 h at 37°C and survivors counted. Data represent the averages of the results of three independent experiments, and error bars represent standard errors of the means. Asterisks indicate statistical difference at  $P < 0.05$ .

ciency of wild-type spores, and yet it produced a significant decrease in the viability of  $\Delta cdeC$  spores at concentrations of  $>250$   $\mu$ g/ml (Fig. 9B). Collectively, these results indicate that (i) in wild-type spores, lysozyme facilitates the colony-forming ability in spores triggered to germinate via nutrients on BHIS-ST agar plates, and (ii) in  $\Delta cdeC$  spores, the absence of CdeC renders spore coats permeable to lysozyme.

**CdeC affects adherence of *C. difficile* spores to HT29 and Caco-2 cells.** To gain more insight into a possible biological role of CdeC in *C. difficile* spore adherence, we evaluated the adherence of wild-type and  $\Delta cdeC$  spores to undifferentiated monolayers of HT-29 cells and to 2- and 8-day-old monolayers of Caco-2 cells. First, undifferentiated monolayers of HT-29 cells were infected for 1 and 3 h with wild-type and  $\Delta cdeC$  spores. We observed that  $\Delta cdeC$  spores adhered to HT-29 cells at significantly ( $P < 0.05$ ) higher levels than wild-type spores after either 1 or 3 h of infection (Fig. 10A). As with the HT-29 mono-

layers, after 1 and 3 h of infection of an 2-day-old monolayer of Caco-2 cells,  $\Delta cdeC$  spores adhered at significantly higher levels than wild-type spores (Fig. 10B). However, when 8-day-old monolayers of differentiated Caco-2 cells were infected with wild-type and  $\Delta cdeC$  spores, the adherence of  $\Delta cdeC$  spores was lower and higher than that of wild-type spores after 1 and 3 h of infection, respectively (Fig. 10C). No significant (i.e.,  $\sim 0.5\%$ ) internalization of either wild-type or  $\Delta cdeC$  spores in HT-29 and Caco-2 cells was observed after 5 h of infection at 37°C, indicating that *C. difficile* spores remained at the surface of epithelial cells. These results indicate that the defects on the exosporium layer due to the absence of CdeC increase the levels of adherence of  $\Delta cdeC$  spores to undifferentiated and differentiated intestinal epithelial cells.

**Complementation of *C. difficile*  $\Delta cdeC$  with wild-type *cdeC*.** To provide the final evidence that CdeC plays a role in *C. difficile* spore coat and exosporium assembly, we complemented the *C.*



**FIG 10** Adherence of *C. difficile* spores to monolayers of intestinal epithelium cell lines HT-29 and Caco-2. Monolayers of HT-29 (A), undifferentiated 2-day-old Caco-2 (B), and differentiated 8-day-old Caco-2 (C) cells were infected at an MOI of 10 with *C. difficile* wild-type and  $\Delta cdeC$  spores for 1 h (gray bars) and 3 h (white bars) at 37°C. Unbound spores were removed by rinsing, and adherence was quantified as described in Materials and Methods. Data represent the averages of the results of three independent experiments, and error bars represent standard errors of the means. Asterisks indicate statistical difference at  $P < 0.05$ .

*difficile*  $\Delta cdeC$  mutant strain with wild-type *cdeC*. The immunofluorescence of the immunodominant exosporium proteins was restored to 78% of wild-type levels (Fig. 6A and B). The spore coat permeability barrier was fully restored, as determined by permeability to lysozyme, where, after 5 h of incubation with 10  $\mu$ g/ml, 250  $\mu$ g/ml, and 1 mg/ml of lysozyme at 37°C, in all cases >97% of  $\Delta cdeC$ (pDP324) ( $\Delta cdeC$  mutant complemented with wild-type *cdeC*) spores remained in the bright phase, indicating that lysozyme resistance was restored to wild-type levels (Fig. 9A and data not shown). Collectively, these results suggest that the CdeC is indeed responsible for the phenotypes observed above.

## DISCUSSION

*C. difficile* spores are well known to be important contributors to the pathogenesis of CDI (1, 3). Indeed, the *C. difficile*  $\Delta spo0A$  strain, incapable of forming spores, was unable to persist in a murine model (4). It has been suggested that persistence of *C. difficile* in the host might be mediated through the adherence of *C. difficile* spores to the host's intestinal epithelium (7) and by surviving attacks of phagocytic cells (36). However, there is little knowledge of the proteins that form part of the outermost layers of *C. difficile* spores and that might play a role in persistence (37). To date, the only protein that has been shown to belong exclusively to the exosporium is the collagen-like BclA1 protein (Pizarro-Guajardo et al., submitted). Other proteins identified to be located at the surface of proteinase K-treated spores (i.e., spores that lack the exosporium layer) are five proteins (i.e., CotA, CotB, CotCB, CotD, and CotE) (37), where only CotA is essential for the assembly of the coat and exosporium of *C. difficile* spores (38). In this context, this study has made several important contributions to the understanding of the formation of the outermost layers of *C. difficile* spores.

The most notable conclusions offered by this work are that CdeC is required for the correct assembly of the exosporium layer and spore coat and that it is localized in the exosporium layer. These findings are strongly supported by the almost complete absence of the exosporium layer observed in the ultrastructure of  $\Delta cdeC$  spores and by the difference in the thicknesses of the inner and outer spore coats (i.e., the inner coat and external coats of  $\Delta cdeC$  spores being thicker and thinner than those of wild-type spores, respectively). Unlike spores of *C. difficile* strain 630, *C. difficile* strain R20291 spores have an exosporium-like structure with a hair-like nap structure similar to that of *B. anthracis* spores (6), and yet there is no clear gap between the spore coat and the nap-like projections as is the case with the *B. anthracis* exosporium layer. The precise composition of the *C. difficile* hair-like nap remains unclear. This hair-like nap was almost completely absent in  $\Delta cdeC$  spores, and the fact that only a few spores (i.e., 2 of 10 examined) analyzed by TEM had remnants of these "hair-like" structures suggests that CdeC might be a structural component required for the proper attachment of the hairs that extend from the exosporium layer. Indeed, immunofluorescence and Western blot results obtained with untreated and SPk-treated spores using anti-CdeC antibodies indicate that CdeC is located in the spore surface, specifically, in the exosporium-like layer. It is tempting to speculate that CdeC might be part of the basal layer to which these hair-like structures might be attached, and yet the precise mechanism through which this would occur is unclear. These defects were also manifested in the protein profile of  $\Delta cdeC$  spore extracts observed by SDS-PAGE, where  $\Delta cdeC$  spore extracts lacked the

18-, 45-, and 50-kDa polypeptide bands. The molecular masses of at least the 45- and 50-kDa protein species are likely to correspond to the CotB and CotA proteins previously shown to be localized to the spore surface of *C. difficile* spores (37), suggesting that these could be exosporium proteins. It was most interesting that there was a significant decrease in the levels of immunodominant proteins detected by immunofluorescence but that when  $\Delta cdeC$  spore coat extracts were analyzed by Western blotting, only the 130-kDa polypeptide species showed a decrease of 33%. CdeC also affected the proper localization of the main glycosylated proteins, suggesting that these may also be localized in the exosporium layer. The relevance of the structural defects became evident upon challenging spores with lysozyme, which triggered germination of  $\Delta cdeC$  but not of wild-type spores, indicating that the coat of  $\Delta cdeC$  spores lost its impermeable barrier to molecules of >14 kDa and supporting the scenario that CdeC might be involved in spore coat assembly. These findings strengthen the scenario that CdeC might be localized in the basal layer of the exosporium, directly interacting with the spore coat. However, it seems as if the role that CdeC plays in coat assembly is more an auxiliary one given the fact that the gross features of the coat in  $\Delta cdeC$  spores resemble those of wild-type spores and yet show small structural differences such as in the thickness of the inner and outer coat layers.

Another relevant conclusion is that CdeC plays a role in spore resistance properties. The absence of CdeC also affected  $\Delta cdeC$  spore resistance to heat and increased the permeability of the spore inner membrane to ethanol, though it was notable that a significant increase in the viability of ethanol-treated wild-type spores was observed. This is consistent with the observation that the spore core water content of  $\Delta cdeC$  spores was higher than that seen with wild-type spores. A noteworthy observation was the ability of the DNA-staining dye, Hoechst, to trigger germination of  $\Delta cdeC$  spores to stage I and stain the core of a fraction of those spores that progressed to stage II of germination, suggesting that once Hoechst triggers germination it stains the spore DNA. The precise mechanism of Hoechst-triggered germination is unclear, and yet it might directly modulate the germinant receptor(s) and/or the channel(s) that releases DPA, which are known to be localized in the spore's inner membrane and to trigger germination if stimulated in a manner independent of the presence of the germinant receptor(s) (39).

The roles of CdeC in spore resistance properties, particularly in spore permeability, are in concordance with its early expression during sporulation. CdeC-tdTOMATO fusion was detected in the mother cell compartment well before the formation of the prespore, which is consistent with the presence of the predicted  $\sigma^E$  consensus sequence found in the promoter region and also suggests that it has a potential role in early forespore development as is the case for many proteins under the control of  $\sigma^E$  in *B. subtilis* (40). Indeed,  $\sigma^E$ -regulated spore-specific proteins have been shown to be involved in the formation of the lower layers of the spore coat that play some role in resistance to several DNA-damaging chemicals and heat (41). Expression during late stages of spore maturation, presumably under the control of  $\sigma^K$  and consistent with the predicted  $\sigma^K$  consensus sequence, allowed the maintenance of high expression levels and localization of the CdeC-tdTOMATO fusion to the spore, confirming that CdeC is a spore protein. It is unclear how CdeC contributes to the formation of the spore coat and to the assembly of the exosporium, but the fact that it is a cysteine-rich protein (i.e., ~8% of total amino acid



residues are cysteine) is consistent with the polymerization observed in the extracts of *C. difficile* spores expressing a CdeC-FLAG fusion. The only cysteine-rich protein identified in the exosporium of *B. anthracis* spores is the ~10-kDa ExsK protein, which has ~11% amino acid residues (42). This protein localizes in the exosporium surface as well underneath the exosporium layer (42), but potential defects on the spore coat were not analyzed in that study. In *B. cereus*, the cysteine-rich protein ExsY and its paralogue have been shown to be involved in the assembly of the exosporium and spore coat (43). *B. cereus* spores of strains with a mutation(s) in *exsY* and *cotY* are permeable to lysozyme and less resistant to organic solvents (43), in similarity to the *C. difficile*  $\Delta$ cdeC spores characterized in this study.

One minor observation provided by this work is that  $\Delta$ cdeC spores had an increased adherence to intestinal epithelium cell lines. Since  $\Delta$ cdeC spores lacked most of the exosporium layer, it is likely that the entire exosporium layer *per se* decreases interactions with the epithelial cells, although the possibility cannot be ruled out that the specific exosporium proteins play a role in reduction of adherence, and the identity of these proteins is a matter of current research.

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