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Immunogenicity and Protective Efficacy of *Clostridium difficile* Spore Proteins

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Abstract

Clostridium difficile is a spore-forming, anaerobic, Gram-positive organism that is the leading cause of antibiotic-associated infectious diarrhea, commonly known as *C. difficile* infection (CDI). *C. difficile* spores play an important role in the pathogenesis of CDI. Spore proteins, especially those that are surface-bound may play an essential role in the germination, colonization and persistence of *C. difficile* in the human gut. In our current study, we report the identification of two surface-bound spore proteins, CdeC and CdeM that may be utilized as immunization candidates against *C. difficile*. These spore proteins are immunogenic in mice and are able to protect mice against challenge with *C. difficile* UK1, a clinically-relevant 027/B1/NAP1 strain. These spore proteins are also able to afford high levels of protection against challenge with *C. difficile* 630 *erm* in golden Syrian hamsters. This unprecedented study shows the vaccination potential of *C. difficile* spore exosporium proteins.

Keywords

Clostridium difficile; Spores; Exosporium; CdeM; CdeC; BclA1; CotA; Vaccine

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INTRODUCTION

Clostridium difficile is a spore-forming, Gram-positive organism that is the leading cause of antibiotic associated infectious diarrhea in the U.S.A. and rivals methicillin-resistant *Staphylococcus aureus* (MRSA) as the most common hospital acquired infection [1]. The widespread use of broad-spectrum antibiotics and the emergence of a hypervirulent strain of *C. difficile* have changed the epidemiology of *C. difficile* infection (CDI) [2]. *C. difficile* strains are able to cause symptomatic disease due to the expression of two toxins-toxins A (TcdA) and B (TcdB) [3]. Given the importance of the hosts' humoral response to toxins in the outcome of CDI, vaccines against these toxins are currently under development [4–8].

C. difficile can exist in two forms: an active, disease-causing vegetative form that cannot survive in the environment due to its anaerobic nature, and an inactive spore form that is resistant to heat, drying, pressure and many commonly-used disinfectants [9]. These spores are dormant and, following ingestion, can germinate to its vegetative form, causing disease in a susceptible host [10]. Germination of spores occurs as an early event during the life cycle of *C. difficile* in the host [11]. Blocking the germination stage would prevent downstream events, including the production of TcdA and TcdB, as well as the binary toxin in some strains [12]. Therefore, the development of vaccines against spores or spore-specific proteins expressed before the germination stage may be an effective strategy to halt the growth of *C. difficile* and thus prevent CDI.

The *C. difficile* spore proteome consists of more than 300 proteins, almost half of which have no known homologs or predicted function in *C. difficile*, but are abundantly present on the spore [13]. Recent large-scale spore proteomic studies have led to the identification of at least 54 spore-associated proteins in *C. difficile* 630, of which 24 have been previously identified [14, 15]. These proteins are exclusively present in the two outer most layers of the spore: the exosporium and the coat [14, 15]. These proteins can be grouped according to their possible function and position on the spore into the following categories: 1) proteins involved in spore coat morphogenesis, such as the coat protein, CotA; 2) spore coat proteins involved in spore resistance, such as CotG, and other proteins that are predominantly hydrolases, oxireductases and catalases which are needed for resistance against oxidative stress; 3) spore proteins needed for germination, such as spore lytic enzyme, SleC; and 4) exosporium proteins, such as BclA1, CdeM and CdeC.

Studies investigating the active vaccination potential of spore-specific targets such as lipoteichoic acid (LTA), suggest that such proteins are critical for the initial stages of colonization and are immunogenic in mice [16]. We hypothesized that active immunization of susceptible individuals using *C. difficile* spore proteins could provide both a preventive and a therapeutic vaccine strategy. For example, elderly patients who get admitted to hospitals who may require antibiotics, patients in long term care facilities, such as nursing homes and hospices, patients with chronic illnesses (*e.g.* chronic obstructive pulmonary disease (COPD), renal failure, diabetes) that frequently lead to hospital admission with infections would be suitable candidates for vaccination [17].

However, the hosts' humoral immune responses in the form of protective circulating antibodies to these spore proteins of *C. difficile* is an under-investigated area of *C. difficile* pathogenesis. Indirect evidence from studying *C. difficile* colonization during infancy shows that breast-fed babies are colonized with *C. difficile* spores four times less than formula-fed babies, suggesting a protective role of maternal immunoglobulin against *C. difficile* colonization [18]. In our current study, we studied the immunization potential of five spore-associated proteins.

CdeC (CD1067) is a cysteine-rich spore exosporium protein and is one of the most abundant spore proteins in *C. difficile* [13, 15, 19]. Absence of CdeC leads to increased spore coat permeability, suggesting that CdeC may form a protective structure around the spore coat [19]. The N-terminus of CdeC shows 52% similarity to *C. trachomatis* OmcA and an ortholog of CdeC has been identified in *C. bartlettii*, suggesting a common function across clostridial species [13].

CdeM (CD1581) is a small cysteine-rich spore exosporium protein with no known homology to any protein [15, 20]. This spore protein is abundant in the exosporium of the spore [15]. CdeM is the most upregulated gene during sporulation and is highly expressed *in vivo* throughout infection in an axenic mouse model [20]. Results from a competition assay with the wild-type strain and an isogenic mutant of CdeM suggest that CdeM might play a role in colonization and persistence of *C. difficile* in the mouse model of CDI [20]. Additionally, CdeM is easily accessible to antibodies and is unique to *C. difficile* [21].

Collagen-like exosporium protein BclA1 (CD0332) has been identified in the exosporium layer of *C. difficile* [22]. Whether BclA1 plays a role in the pathogenesis of *C. difficile* is unknown [23, 24].

SleC (CD0551) is a spore cortex lytic enzyme. SleC is a multi-domained protein with a C-terminal peptidoglycan binding domain and an N-terminal exo-acting lytic transglycosylase catalytic domain [25]. SleC is essential for the germination of spores into its vegetative form [26].

CotA (CD1613) is a spore coat protein, although recent proteomics studies suggest that CotA may be present on the exosporium as well [15]. *C. difficile* *cotA* mutant strains fail to assemble an electron-dense outer layer and are sensitive to ethanol and lysozyme, thus suggesting that CotA is necessary for the assembly of the outer layer of the spore coat [23].

Here we report that CdeC and CdeM are immunogenic in mice and are able to protect mice from challenge with *C. difficile* strain UK1, a clinically-relevant 027/B1/NAP1 isolate. These spore proteins are also able to confer high levels of protection against challenge with *C. difficile* strain 630 *erm* in golden Syrian hamsters. This study shows, as a proof of concept, the immunization potential of spore proteins expressed in *C. difficile*, and should be evaluated for further development.

MATERIALS AND METHODS

Bacterial strains

Escherichia coli DH5 α and *E.coli* BL21DE3 * (Life technologies, Carlsbad, CA) were used for cloning and recombinant protein purification. For the bacterial challenge experiments in mice, *C. difficile* strain UK1, a North American pulsed-field gel electrophoresis type 1(NAP1)/BI/polymerase chain reaction (PCR) ribotype 027 (027) strain isolated during the Stoke-Mandeville Hospital outbreak, kindly provided by Dr. Gerding, was used. The oral challenge dose of 10⁶ CFU of *C. difficile* strain UK1 was calculated as previously described [27]. At this dose, severe CDI would develop 4 to 6 days post UK1 challenge in a substantial majority of antibiotic-treated mice.

An oral dose of 500 CFU of *C. difficile* strain 630 *erm* (ribotype 012) was used for the bacterial challenge experiments in hamsters. At this dose, severe CDI would develop 2 to 3 days post *C. difficile* strain 630 *erm* challenge in a substantial majority of antibiotic-treated hamsters. *C. difficile* 630 *erm* is a spontaneous erythromycin-sensitive derivative of the reference strain 630 obtained by serial passaging in antibiotic-free media [28]. It is a widely used as a challenge strain in *C.difficile* hamster models of infection [24, 29, 30]. *C. difficile* was propagated and spores prepared as described previously [27, 31].

In silico analysis of potential spore vaccine targets

Analysis of sequence diversity at the protein level between *C. difficile* strains of different ribotypes using BLAST (<http://blast.ncbi.nlm.nih.gov>) was performed to identify spore protein targets with high levels of identity at the protein level (greater than 98%).

Analysis of predicted glycosylation sites was performed using an online tool, GlycoPP (<http://www.imtech.res.in/raghava/glycopp/index.html>).

Protein expression and purification

The full-length protein sequences for CdeC (CD1067; NCBI GeneID 4915202), BclA1 (CD0332; NCBI GeneID 4915988), SleC (CD0551; NCBI GeneID 4916686), CotA (CD1613; NCBI GeneID 4913945), CdeM (CD1581; NCBI GeneID 4913041) were obtained from *C. difficile* strain 630 (ATCC BAA-1382). The corresponding codon-optimized nucleotides were commercially synthesized by Blue Heron Biotechnologies, Bothell, WA.

The nucleotides were cloned into the bacterial expression vector, pET19b (EMD Millipore, Billerica, MA) that contains a histidine (His) tag to facilitate purification. *E. coli* cultures were grown in Luria Bertani (LB) medium containing ampicillin (100 μ g/ml) at 37°C with aeration and induced with 0.1mM IPTG (isopropyl-beta-D-thiogalactopyranoside) for 3 hours (Sigma Aldrich, St. Louis, MO). The expressed proteins were purified using Talon metal affinity resin according to manufacturer's specifications (Clontech Laboratories Inc., Mountain View, CA). Recombinant protein purity was analyzed by SDS-PAGE gel electrophoresis, followed by Coomassie Blue staining. The identities of the His-tagged proteins were confirmed by Western blot analyses with monoclonal anti-His antibody (Life

Technologies). Endotoxin was removed by using Endotrap Blue columns according to manufacturer's specifications (Hyglos GmbH, Bernried, Germany).

For large-scale immunogenicity and protective efficacy studies in mice and hamsters, endotoxin-free CdeC and CdeM protein were commercially purified by Ascentgene, Rockville, MD.

Immunization regimen in mice

All animal work was approved by the Institutional Animal Care and Use Committee at the Rockefeller University, New York.

The first study was designed to investigate the immunogenicity and protective efficacy of all the recombinant spore proteins. To study the immunogenicity and protective efficacy of recombinant CdeC, BclA1 and SleC, we intraperitoneally (i.p.) immunized three cohorts of 4 female, 8- to 10-week-old, C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME). To investigate the immunogenicity and protective efficacy of CotA and CdeM, we immunized two cohorts of 10 female, 8- to 10-week-old, C57BL/6 mice. Each cohort was i.p. immunized with 25 µg of recombinant spore protein adjuvanted with 1:1 by volume of Inject Alum adjuvant according to manufacturer's specifications (Life Technologies). Inject Alum contains an aqueous solution of aluminum hydroxide (40 mg/mL) and magnesium hydroxide (40 mg/mL) plus inactive stabilizers. 4–10 female, 8- to 10-week-old, C57BL/6 mice were immunized with saline mixed with 1:1 by volume of Inject Alum as unimmunized control. All cohorts were immunized on days 0, 14 and 28. Serum samples were collected, processed, and stored from mice on days 0 (pre-immunized), 14, 28 and 42, as previously described [32]. All cohorts of mice were challenged two weeks after the last immunization, as described below.

In the dose escalation studies for CdeC, four cohorts of 8 to 10 female, age-matched 8- to 10-week-old, C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were i.p. immunized on days 0, 14 and 28 (three immunizations) or on days 0, and 14 (two immunizations). Cohorts of mice were i.p. immunized with 5 µg or 25 µg of CdeC, mixed with 1:1 by volume of Inject Alum adjuvant. Control mice were immunized with saline mixed with 1:1 by volume of Inject Alum adjuvant.

In the dose escalation studies for CdeM, four cohorts of 8 female, age-matched 8- to 10-week-old C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were i.p. immunized on days 0, 14 and 28 (three immunizations) or on days 0, and 14 (two immunizations). Cohorts of mice were i.p. immunized with 5 µg (received 3 immunizations) or 25 µg of CdeC (two cohorts, received two or three immunizations) with 1:1 by volume of Inject Alum adjuvant. Control mice were i.p. immunized with saline mixed with 1:1 by volume of Inject Alum adjuvant.

Serum samples were collected, processed, and stored from mice 24 hours before challenge and two weeks post challenge from surviving mice. All cohorts of mice were challenged two weeks post last immunization, as described below.

C. difficile infection model in mice

All cohorts of C57BL/6 mice were challenged two weeks after the last immunization. Following antibiotic treatment, which included orally administered antibiotic cocktails (kanamycin 40 mg/kg, gentamicin 3.5 mg/kg, colistin 4.2 mg/kg, metronidazole 21.5 mg/kg and vancomycin 4.5 mg/kg) in the drinking water for five days, mice were i.p. administered clindamycin (10 mg/kg) 24 hours before *C. difficile* challenge, as described previously [33]. Mice were challenged with a dose of 10^6 CFU of *C. difficile* strain UK1, and monitored daily for 14 days for changes in weight, diarrhea, mortality and morbidity. As per Rockefeller University's IACUC protocol, mice were sacrificed when the following conditions were observed: rapid or progressive weight loss of greater than 25% of starting weight, a lack of responsiveness to manual stimulation, immobility, ruffled fur, hunched position, and/or signs of diarrhea, such as wet tail.

C. difficile immunization and infection model in hamsters

All animal work was approved by the Institutional Animal Care and Use Committee at the Rockefeller University, New York.

5–6 week old male Golden Syrian hamsters (*Mesocricetus auratus*) were obtained from Harlan Laboratories (Indinapolis, IN) and were housed in sterile, individual ventilated cages. To assess the protective efficacy of recombinant spore protein CdeC, cohorts of hamsters (n=10) were i.p. immunized on days 0, 14 and 28 (three immunizations) and received 20 µg or 100 µg of recombinant CdeC adjuvanted with 1:1 by volume of Imject Alum adjuvant. One cohort of hamsters (n=4) was i.p. immunized on days 0 and 14 (two immunizations) and received 100 µg of recombinant CdeC adjuvanted with 1:1 by volume of Imject Alum. To assess the protective efficacy of recombinant spore protein CdeM, one cohort of hamsters (n=10) was i.p. immunized on days 0, 14 and 28 (three immunizations) and received 100 µg of recombinant CdeM adjuvanted with 1:1 by volume of Imject Alum. Control hamsters (n=8) were i.p. immunized with saline mixed with 1:1 by volume of Imject Alum. The low and high vaccine dose in the hamsters was adjusted for the weight difference between a mouse and a hamster, which is approximately 4 to 5-fold. Blood was collected, serum was processed, and stored from hamsters 1 week before challenge and two weeks post challenge from surviving hamsters. All hamsters were dosed orogastrically with clindamycin (30 mg kg^{-1}) and orally challenged 5 days later with 500 CFU of *C. difficile* strain 630 *erm*. Following oral challenge, hamsters were monitored every 8 hours for signs of disease progression for the duration of 14 days. As per Rockefeller University's IACUC protocol, hamsters that were moribund with weight loss greater than 20% from baseline weights, and showed signs of disease such as lethargy, ruffled fur, wet tail or diarrhea were euthanized.

Measurement of immune responses in mice and hamsters

To standardize and validate the enzyme-linked immunosorbent assay (ELISAs) for consistency, reproducibility and accuracy for the detection of immune responses to CdeC, BclA1, SleC, CotA and CdeM in mice and hamster serum, we completed a checkerboard dilution series with various concentrations of mouse and hamster serum [34]. We coated plates with 10 ng/well, 20 ng/well, 50 ng/well, 100 ng/well, 200 ng/well and 500 ng/well of CdeC, BclA1, SleC, CotA and CdeM in 50 mM carbonate buffer, pH 9.6. We blocked plates

with PBS-1% bovine serum albumin (BSA) (Sigma Aldrich). We diluted immune and naïve sera 1:10, 1:50, 1:250, 1:500, 1:1000, 1:2000, 1:5000, and 1:1:10000 in PBS containing 0.05% Tween 20 (PBS-T) and 0.1% BSA (Sigma Aldrich). We used a dilution of 1:500, 1:1000 and 1:2000 of goat anti-mouse immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP) and 1:2000, 1:4000, and 1:8000 of goat-anti-hamster IgG conjugated with HRP to detect bound antibodies (Southern Biotech, Birmingham, AL). We developed the plates with 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma Aldrich) and 0.03% H₂O₂ (Sigma Aldrich) and determined optical density using a Vmax microplate reader (Molecular Devices Corp, Sunnyvale, CA) at 405 nm kinetically for 5 min at 14-second intervals, as previously reported [35]. We used a kinetic ELISA where data is expressed as change in milli-optical density units over time (OD/min) [36, 37].

To detect antibody responses to the recombinant spore proteins CdeC, BclA1, SleC, CotA and CdeM, we coated plates with 100 ng/well of each of the purified recombinant spore protein in 50 mM carbonate buffer, pH 9. Briefly, we blocked plates with PBS-1% BSA. We diluted mice sera 1:1,000 in PBS containing 0.05% Tween 20 (PBS-T), 0.1% BSA and used a dilution of 1:1,000 of goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) to detect bound mice antibodies. We diluted hamster sera 1:1,000 in PBS containing 0.05% Tween 20 (PBS-T), 0.1% BSA and used a dilution of 1:8,000 of goat anti-hamster IgG conjugated with HRP to detect bound hamster antibodies. We developed the plates using kinetic ELISA as mentioned above.

Culture of *C. difficile* from mice fecal pellets

Fresh fecal pellets were collected, weighed and stored in PBS at -80°C from each mouse on days 3, 4 and 5 post challenge for the evaluation of *C. difficile* colonization. Spore counts of *C. difficile* were calculated by combining fecal samples of each mouse and plating serial dilutions on cycloserine-cefoxitin fructose agar plates with horse blood and taurocholate (CCFA-HT) (Anaerobe Systems, Morgan Hill, CA). Plates were incubated anaerobically for 48 hours following which grayish-white colonies were manually counted.

Statistical analysis

For normally distributed data, we used an unpaired Student *t* test analysis for comparison of means; for nonparametric data, we used the Mann-Whitney U test. We performed statistical analyses and plotted graphs using GraphPad Prism (GraphPad Software, San Diego, CA). A *p* value of less than 0.05 was considered to indicate statistical significance. Kaplan–Meier plots were used to analyze survival in the challenged mice and hamsters. Log rank (Mantel-Cox) test for pairwise comparisons was used to assess statistical significance between the cohorts.

RESULTS AND DISCUSSION

Identification of potential *C. difficile* spore protein vaccine candidates

Spore proteins that are present on the exosporium or the coat, with no known paralogs or functional redundancy, were chosen as vaccine candidates in our study. Many *C. difficile* spore proteins have multiple glycosylation sites that might hinder the recombinant protein

purification process in *E. coli*. Therefore, proteins with high numbers of glycosylation sites in their sequence were excluded from our study. *C. difficile* spore proteins which have a high level of identity between sequenced strains of different ribotypes were included in our study. Lastly, proteins with well-defined functional importance were included in our study. We identified 5 spore proteins that we tested for immunogenicity and protective efficacy in a murine model of CDI: CdeC, BclA1, SleC, CotA and CdeM (Table 1).

C. difficile outer surface spore proteins CdeC (45 kDa), BclA1 (68 kDa), SleC (47 kDa), CotA (34 kDa) and CdeM (19 kDa) were expressed from *E. coli* using the expression plasmid pET19B. Following removal of endotoxin to levels below 50 EU/ml, concentrations of the recombinant proteins varied between 0.5 mg/ml to 0.7 mg/ml.

Immunogenicity and protective efficacy of *C. difficile* spore proteins

A recent study found that *C. difficile* spore protein CdeC is not immunogenic [19]. To enhance the inherent immunogenic properties of this recombinant spore protein, as well as of SleC, BclA1, CdeM, and CotA, mice were immunized three times with 25 µg of each protein adjuvanted with Imject Alum. All five recombinant spore proteins were found to be immunogenic in mice (Figure 1). For recombinant spore proteins BclA1, SleC, CdeM and CotA, the immune responses in mice were significantly enhanced following a boost on day 14 ($p < 0.05$) (Figure 1a, b, d and e). A second boost on day 28 did not significantly enhance the immune response against BclA1 ($p = 0.574$), SleC ($p = 0.435$), CdeM ($p = 0.07$) or CotA ($p = 0.82$), suggesting a plateau effect. For CdeC, the second immunization did not significantly boost the immune response induced by the first immunization ($p = 0.106$); however, a third immunization was able to significantly enhance the immune response ($p < 0.05$) (Figure 1c).

To test whether the induced IgG responses in the serum following three immunizations are protective, we challenged all cohorts of immunized mice and unimmunized controls, two weeks post last immunization with a clinically relevant *C. difficile* strain, UK1 (B1/NAP1/027). 100% of mice immunized with CdeC survived with no signs of disease through the end of the study and were euthanized (2 weeks post challenge) ($p < 0.05$, compared to control mice) (Figure 2a). Day 6 was the earliest time point when diarrhea or death was observed in a mouse. By day 10 all of the BclA1, SleC and control mice were sacrificed due to the presence of diarrhea, weight loss or signs of moribundity. There was no statistically significant difference between the survival of mice receiving SleC, BclA1 and the control cohort.

In our current study, recombinantly-produced BclA1 failed to protect mice against *C. difficile* UK1. The ORF encoding BclA1 has an early stop codon at the N-terminal domain in most *C. difficile* ribotype 027 strains, including UK1, leading to the expression of a truncated 6 kDa protein [15, 24, 38]. Given the importance of B1/NAP1/027 strains in the epidemiology of CDI, vaccine targets that are not capable of providing protection against such strains may not be ideal candidates for development.

Recombinantly-produced SleC also failed to protect mice against bacterial challenge. Recent studies using ClosTron technology has shown that while germination of spores was slowed

down in the absence of SleC, a small percentage of the *sleC* mutants were able to germinate and were viable [25]. Therefore, it is possible that although immunization with SleC may have been sufficient to block the germination of all the challenge spores via a SleC-mediated pathway, an alternate pathway may have allowed germination. Since SleC is embedded within the spore coat, antibodies generated by immunization may not be able to access SleC, thus making SleC an inadequate target for vaccination.

The cohort of mice that were immunized with 25 µg of CotA had a protective efficacy of 60% ($p < 0.05$ compared to control mice). 2 mice (20%) died on day 6 and day 8 following challenge. Control mice succumbed to CDI between days 6 and 8 post-challenge. CotA was able to provide partial protection against *C. difficile* UK1 in mice. CotA was shown previously to be essential for the proper assembly of the coat and the exosporium layers of spores [23]. One possible explanation for the partial protection we observed in mice is that not all spores express CotA and a small subpopulation of *C. difficile* spores (1–5%) may have proceeded to germination and caused CDI in these immunized and challenged mice [23]. Therefore, if CotA is not expressed on the surface of all the spores, immunization with CotA may not be sufficient to protect against *C. difficile* challenge.

The cohort of mice that were immunized with 25 µg of CdeM had a protective efficacy of 90%, with one out of 10 mice succumbing to challenge by *C. difficile* UK1 on day 8 ($p < 0.05$ compared to control mice) (Figure 2b). No statistically significant difference was found between the survival of the two cohorts that were immunized with CdeM and CotA ($p = 0.1385$).

To test whether immunization with recombinant spore proteins CdeC, BclA1, SleC, CotA and CdeM would have an effect on the levels of spore shedding in *C. difficile*-challenged mice, we compared the levels of spores present in the fecal pellets of immunized and control mice over a period of 3 days, starting from day 3. Cohorts of mice immunized with recombinant spore proteins CdeC, CdeM and SleC shed similar levels of *C. difficile* in their stool ($p = \text{NS}$), and these levels were significantly lower compared to control mice for up to day 5 post challenge ($p < 0.05$) (Figure 2c). There was no difference in the levels of spores shed in the stool of cohorts of mice immunized with CotA ($p = 0.064$) and BclA1 ($p = 0.346$) compared to the control group ($p = \text{NS}$). Studies have shown that *C. difficile* spores rapidly transit through the gastrointestinal tract of mice and can be found in the feces of mice as early as 4 days post-infection, with the numbers significantly waning after day 7 [39]. Additionally, given that the control animals succumbed to CDI from day 6 post-challenge, we restricted our study to days 3 to 5 post challenge for comparison between cohorts.

Effect of dose-response of CdeC and CdeM in a mouse model of CDI

To further study the protective efficacy of CdeC and CdeM against *C. difficile* UK1 challenge, we studied the effect of dose response (either 5 µg or 25 µg) and varied the number of immunizations (two or three) given to mice. For the dose response experiments, we used commercially produced CdeC and CdeM protein. The presence of aggregates in the form of dimers and higher multiples of the desired products were seen in the preparations of CdeC and CdeM by SDS-page gel electrophoresis and Western Blot, and is consistent with previously published data (Figure 3) [15, 19].

The cohort of mice that was immunized three times with 25 µg of CdeC had the highest protective efficacy of (100%), with 9 out of 9 mice surviving challenge by *C. difficile* UK1 (Figure 4a). The cohort of mice that was immunized with 25 µg of CdeC and a total of two doses had a protective efficacy of 90% with one mouse dying on day 7 following challenge. Cohorts of mice that were immunized with 5 µg of CdeC as either two or three doses had 70% protective efficacy. No statistically significant difference was found between the survival of the CdeC-immunized cohorts, whereas the percent survival for all CdeC-immunized cohorts was significantly different from the control unimmunized cohort ($p < 0.001$). Protective efficacy of the recombinant CdeC vaccine also correlated with weight loss between the CdeC-immunized cohorts compared to the unimmunized control mice, which had significant weight loss (Figure 4b). On day 7, all control mice (infected, but unimmunized) were either dead or euthanized due to presence of diarrhea, and weights were no longer measured for comparison.

To correlate protection in mice with level of antibodies present in the blood, we analyzed the anti-CdeC IgG in the serum of mice 24 hours before challenge. Mice that were immunized with CdeC, regardless of dose and number of immunization, were able to mount a statistically significant anti-CdeC IgG response compared to the unimmunized cohort ($p < 0.01$) (Figure 4c). There was no statistically significant difference in the levels of anti-CdeC IgG induced in the two cohorts that were immunized with 25 µg of CdeC ($p = 0.456$). There was a statistically significant difference in the levels of anti-CdeC IgG induced in mice that were immunized three times with 25 µg of CdeC compared to the two cohorts that were immunized with 5 µg of CdeC.

Mice in all cohorts that succumbed to challenge had lower levels of circulating anti-CdeC IgG in their serum at the time of challenge compared to protected mice ($p < 0.001$) (Figure 4d). We also analyzed the anti-CdeC IgG in the serum of surviving mice two weeks post challenge. The *C. difficile* challenge significantly boosted the anti-CdeC IgG response in the serum of the cohort of mice that received 25 µg of CdeC three times, compared to the other immunized survivors ($p < 0.05$) (Figure 4e). We also analyzed circulating anti-toxin IgG in the surviving mice but were unable to detect any using a toxin-specific kinetic ELISA.

Therefore, protection from CDI and death in immunized mice correlated with a dose-response and the number of immunizations received, as well as circulating antibody titers at the time of challenge. The induced anti-CdeC IgG response was protective in 100% of the mice following heterologous challenge with a clinically relevant *C. difficile* strain, UK1 (027/B1/NAP1 strain) in two separate challenge experiments in mice that were immunized with three doses of 25 µg of CdeC.

For CdeM, the cohort of mice that was immunized with 25 µg of CdeM and a total of three doses had the highest protective efficacy of 100%, with 8 out of 8 mice surviving challenge by *C. difficile* UK1 ($p < 0.001$, compared to the control cohort) (Figure 5a). The cohort of mice that was immunized with 25 µg of CdeM and a total of two doses had a protective efficacy of 75% with two mice dying on day 4 following challenge ($p < 0.05$, compared to the control cohort). Cohorts of mice that were immunized with three doses of 5 µg of CdeM had a 50% protective efficacy, with two mice dying on day 4 and day 7 ($p = 0.14$ compared

to the control cohort). The control unimmunized mice succumbed to CDI between days 4 and 9. There was no statistically significant difference in the percent survival between the two cohorts that were immunized with 25 µg of CdeM. The percent survival of the cohort that was immunized with 5 µg of CdeM was significantly lower than the cohort that was immunized with three doses of 25 µg of CdeM ($p < 0.05$). Control unimmunized mice had significant weight loss compared to the immunized cohorts that went back to their baseline weights by the end of the study (Figure 5b). On day 9, the last control mice (infected, but unimmunized) died and weights were no longer measured for comparison.

Mice that were immunized with CdeM had similar levels of circulating serum anti-CdeM IgG response regardless of dose and number of immunization at the time of challenge (Figure 5c). Mice in all cohorts that succumbed to challenge had similar levels of anti-CdeM IgG in their serum compared to protected mice ($p = 0.684$) (Figure 5d). We analyzed the levels of anti-CdeM IgG in the serum of surviving mice two weeks post challenge. The cohort of mice that received three doses of 25 µg of CdeM was able to mount a significantly stronger immune response to CdeM following challenge with *C. difficile* spores, compared to the cohort of mice that received two doses of 25 µg of CdeM, ($p < 0.05$), although this response was not significantly different from the cohort that received three doses of 5 µg of CdeM ($p = 0.54$) (Figure 5e). As with CdeC, we were unable to detect anti-toxin IgG in the serum of surviving mice.

The induced anti-CdeM IgG response was protective in 90–100% of the mice following heterologous challenge with a clinically relevant *C. difficile* strain, UK1 (027/B1/NAP1 strain) in two separate challenge experiments in mice that were immunized with three doses of 25 µg of CdeM. The cohort of mice that received three doses of 25 µg of CdeM had significantly greater percent survival compared to the cohort of mice that received three doses of 5 µg of CdeM. Unlike CdeC, there was no correlation between the levels of circulating anti-CdeM antibody titer at the time of challenge to protection from CDI and death. All cohorts of immunized mice were able to induce similar levels of anti-CdeM IgG.

Protective efficacy of CdeC and CdeM in a hamster model of CDI

We confirmed our findings from the protective efficacy experiments in mice with protective efficacy experiments in golden Syrian hamsters. Hamsters were immunized with a high dose of 100 µg or a low dose of 20 µg of CdeC as shown in Figure 6. We observed that 7 out of 8 animals (87%) in the control group developed diarrhea (wet tail) and were euthanized within 2–6 days after challenge (Figure 7a). 9 out of 10 hamsters (90%) in the cohort that received three immunizations with 100 µg of CdeC survived, with one hamster dying on day 4 post challenged. All 4 hamsters (100%) that received two immunizations with 100 µg of CdeC survived. In the low dose cohort, hamsters receiving 20 µg given three times, 7 out of 10 hamsters (70%) survived, with the hamsters succumbing to CDI on days 3, 5 and 6 following challenge. Mantel–Cox analysis revealed that there are statistically significant differences in the survival between all of the vaccinated cohorts and the controls ($p < 0.05$), and that there are no statistically significant differences between the high dose vaccinated cohorts and the low dose cohort. Of a total of 24 immunized hamsters, four succumbed to CDI following challenge, thus affording a protective efficacy of 83% against *C. difficile*

strain 630 *erm*. Although the numbers of hamsters are small (n=4) in the cohort that received two doses of 100 µg of CdeC, our results suggest that a simple prime-boost regimen might be sufficient to induce high levels of protection. We also measured the level of circulating anti-CdeC IgG in the serum of hamsters one week before challenge (Figure 7b) and two weeks after challenge (Figure 7c). All cohorts of hamsters had measurable and comparable levels of circulating anti-CdeC IgG in their serum. We were unable to detect any anti-toxin antibodies in the serum of surviving hamsters. If CdeC-based immunization can induce high-level protection against challenge with *C. difficile* spores, while significantly improving on the number of immunizations and the time interval to protection (two weeks) compared to current vaccines in development, then a CdeC-based vaccine would be clinically and commercially feasible for patients susceptible to *C. difficile* in a public health setting. Additional protective efficacy studies with *C. difficile* strains of different ribotypes will be undertaken to confirm our initial results.

To evaluate the protective efficacy of recombinant *C. difficile* spore protein CdeM in a hamster model of CDI, we immunized 10 hamsters with 100 µg of recombinant CdeM, adjuvanted with alum (Figure 8a). Following challenge with *C. difficile* 630 *erm*, 8 out of 10 hamsters (80%) survived, with one hamster each succumbing to CDI on days 2 and 4 following challenge (p<0.05). We measured the level of circulating anti-CdeM IgG in the serum of hamsters one week before challenge and compared the levels to circulating anti-CdeM IgG in the serum of surviving hamsters two weeks after challenge and found lower levels of anti-CdeM IgG post challenge (p<0.05) (Figure 8b). In the hamster protective efficacy experiments, we tested only one dose (100 µg high dose, given three times) based on our initial mice experiments in which the high dose of CdeM given three times afforded the highest levels of protection. 80% of the immunized hamsters survived challenge with *C. difficile* strain 630 *erm*. We were unable to detect any anti-toxin antibodies in the serum of surviving hamsters. Whether a simple prime-boost regimen using CdeM might be sufficient to induce high levels of protection was not addressed in our current study.

The life-cycle of *C. difficile* consists of a dormant inactive spore phase and a disease-causing vegetative stage during which toxins and other virulence factors are expressed [40]. Spores are the infectious units of *C. difficile*. Patients with CDI excrete around 1×10^7 spores per gram of feces [41]. These highly infectious spores can remain in the dormant state in the hospital environment for longer than six months and, following ingestion, can cause CDI in a susceptible host.

Most, if not all, current vaccine and monoclonal antibody development efforts are focused on the neutralization of the toxins that are expressed in the vegetative stage of the lifecycle of the spore [7, 8, 42]. The innovation of our research lies in the use of the immunogenic properties of *C. difficile* spore exosporium proteins to develop targets for active vaccination and represents a significant advantage over other immunization strategies currently in development. We have identified two exosporium proteins, CdeC and CdeM, that afford high levels of protection in two animal models of CDI. Further studies are currently ongoing to delineate the mechanism of protection afforded by the induced responses to CdeC and CdeM. Our approach of targeting spores can be used for dual purposes. First, active vaccination can be used for prophylaxis in high-risk patients, thus preventing primary CDI

in these patients. Second, our approach could be used to treat patients with recurrent and refractory CDI, which amounts to a third or more of all CDI patients. Recurrent CDI may be due to persistent spores present in the colon from the initial infection and recent findings suggest that *C. difficile* spores are able to subvert the hosts immune system such as macrophage-mediated killing and is able to persist in the colonic environment [43, 44]. Active or passive vaccination of recurrent patients may lead to the elimination of recalcitrant spores, thus preventing recurrence.

There is a clear and clinically unmet need for novel non-antimicrobial approaches against *C. difficile* that can potentially stop the growth of the CDI epidemic. This unprecedented study highlights the vaccination potential, either active or passive, of spore proteins expressed in *C. difficile*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights for review

- *C. difficile* spore proteins play an important role in the disease pathogenesis of *C. difficile* infection (CDI).
- Exosporium spore proteins, that are present on the outermost surface of the spores, may be potential targets for vaccination.
- Exosporium proteins CdeC and CdeM are immunogenic and protective in mice and hamster models of *C. difficile* infection and therefore show the vaccination potential of *C. difficile* spore exosporium proteins.

Figure 1.

Immunogenicity of purified recombinant *C. difficile* spore proteins in mice. Serum anti-BclA1 IgG (a), anti-SleC IgG (b), anti-CdeC IgG (c), anti-CdeM IgG (d) and anti-CotA IgG (e) responses in mice immunized on days 0, 14 and 28 in serum collected on days 0 (prebleed or pb), 14, 28 and 42. Cohorts of mice received three immunizations of 25 μ g of recombinant spore proteins adjuvanted with Imject Alum. Results were determined by kinetic ELISA and are reported as optical density (OD) units; the geometric mean plus standard error of the mean for each cohort is shown. * denotes statistical significance ($p < 0.05$), using an unpaired Student *t* test analysis for comparison of means; for nonparametric data, we used the Mann-Whitney U test.

Figure 2.

Survival in control and vaccinated C57BL/6 mice following orogastric challenge with 10^6 cfu of *C. difficile* strain UK1. Cohorts of mice received three immunizations of 25 μ g of BclA1, SleC, CdeC (a), CdeM and CotA (b) adjuvanted with Imject Alum. Control mice were immunized with saline and Imject Alum. Mice were immunized on day 0, 14 and 28 and challenged two weeks after the last immunization following antibiotic treatment. * denotes statistical significance ($p < 0.05$) compared to the control cohort. Mantel-Cox test for pairwise comparisons was used to assess statistical significance between the cohorts. (c) *C. difficile* shedding in mouse fecal samples from immunized and control mice following orogastric challenge with 10^6 cfu of *C. difficile* strain UK1. Results denote fecal shedding of *C. difficile* reported as CFU/gram (the geometric mean plus standard error of the mean) from fecal pellets. * denotes statistical significance ($p < 0.05$), using an unpaired Student *t* test analysis for comparison of means.

Figure 3. Analysis of purified recombinant *C. difficile* spore protein CdeC (a and b) and CdeM (c and d) analyzed by SDS-PAGE on a gel stained with Coomassie blue (a and c) and by Western Blot (b and d). Molecular weight markers (M) and multimeric forms of the recombinant protein are shown.

Figure 4.

Protective efficacy of CdeC in mice. a. Survival in control and vaccinated C57BL/6 mice following orogastric challenge with 10^6 cfu of *C. difficile* UK1. Cohorts of mice received either 5 μ g or 25 μ g total of CdeC adjuvanted with Imject Alum. Control mice were immunized with saline and Imject Alum. Mice were immunized on day 0, 14 and 28 (three immunizations) or on days 0 and 14 (two immunizations) and challenged two weeks after the last immunization following antibiotic treatment. * denotes statistical significance ($p < 0.05$) compared to the control mice. Mantel-Cox test for pairwise comparisons was used to assess statistical significance between the cohorts.

b. Percent weight change of surviving mice compared to prechallenge (baseline weight), day 2 (all mice are alive in all cohorts), day 4, and day 7 following orogastric challenge with 10^6 CFU *C. difficile* UK1. Results are reported as the geometric mean plus standard error of the mean for each cohort for each day. All control mice succumb to CDI at day 7. * denotes statistical significance ($P < 0.05$), using an unpaired Student *t* test analysis for comparison of means; when compared to the control cohort for each time point.

c. Anti-CdeC IgG responses in serum of immunized and control mice 1 day before challenge. Results were determined by kinetic ELISA and are reported as OD/min; the geometric mean plus standard error of the mean for each cohort is shown. * denotes statistical significance ($p < 0.05$), using an unpaired Student *t* test analysis for comparison of means.

d. Comparison of anti-CdeC IgG responses in serum of protected and unprotected mice 1 day before challenge. Results were determined by kinetic ELISA and are reported as OD/min; the geometric mean plus standard error of the mean for each cohort is shown. * denotes statistical significance ($p < 0.05$), using an unpaired Student *t* test analysis for comparison of means.

e. Anti-CdeC IgG responses in serum of surviving mice 2 weeks post challenge. Results were determined by kinetic ELISA and are reported as OD/min; the geometric mean plus

standard error of the mean for each cohort is shown. * denotes statistical significance ($p < 0.05$), using an unpaired Student t test analysis for comparison of means.

Figure 5.

Protective efficacy of CdeM in mice. a. Survival in control and vaccinated C57BL/6 mice following orogastric challenge with 10^6 cfu of *C. difficile* UK1. Cohorts of mice received either 5 µg or 25 µg total of CdeM adjuvanted with Imject Alum. Control mice were immunized with saline and Imject Alum. Mice were immunized on day 0, 14 and 28 (three immunizations) or on days 0 and 14 (two immunizations) and challenged two weeks after the last immunization following antibiotic treatment. * denotes statistical significance ($p < 0.05$) compared to the control mice. Mantel-Cox test for pairwise comparisons was used to assess statistical significance between the cohorts.

b. Percent weight change of surviving mice compared to prechallenge (baseline weight), day 2 (all mice are alive in all cohorts), day 4, and day 7 following orogastric challenge with 10^6 CFU *C. difficile* UK1. Results are reported as the geometric mean plus standard error of the mean for each cohort for each day. All control mice succumb to CDI at day 9. * denotes statistical significance ($P < 0.05$), using an unpaired Student *t* test analysis for comparison of means; when compared to the control cohort for each time point.

c. Anti-CdeM IgG responses in serum of immunized and control mice 1 day before challenge. Results were determined by kinetic ELISA and are reported as OD/min; the geometric mean plus standard error of the mean for each cohort is shown.

d. Comparison of anti-CdeM IgG responses in serum of protected and unprotected mice 1 day before challenge. Results were determined by kinetic ELISA and are reported as OD/min; the geometric mean plus standard error of the mean for each cohort is shown.

e. Anti-CdeM IgG responses in serum of surviving mice 2 weeks post challenge. Results were determined by kinetic ELISA and are reported as OD/min; the geometric mean plus standard error of the mean for each cohort is shown. * denotes statistical significance ($p < 0.05$), using an unpaired Student *t* test analysis for comparison of means.

Figure 6.
Experimental design of immunization and protective efficacy experiments in male Golden Syrian hamsters.

Figure 7.

Protective efficacy of CdeC in hamsters a. Survival in control and vaccinated golden Syrian hamsters following orogastric challenge with 500 CFU of *C. difficile* 630 *erm*. Cohorts of hamsters received 2 or 3 immunizations of 20 μ g or 100 μ g of CdeC adjuvanted with Imject Alum. Control hamsters were immunized with saline and Imject Alum. * denotes statistical significance ($p < 0.05$) compared to the control mice. Mantel-Cox test for pairwise comparisons was used to assess statistical significance between the cohorts.

b. Anti-CdeC IgG responses in serum of vaccinated and control hamsters one week before challenge. Results were determined by kinetic ELISA and are reported as OD/min; the geometric mean plus standard error of the mean for each cohort is shown

c. Anti-CdeC IgG responses in serum of surviving hamsters two weeks after challenge. Results were determined by kinetic ELISA and are reported as OD/min; the geometric mean plus standard error of the mean for each cohort is shown.

Figure 8.

Protective efficacy of CdeM in hamsters a. Survival in control and vaccinated golden Syrian hamsters following orogastric challenge with 500 CFU of *C. difficile* 630 *erm*. Hamsters received 3 immunizations of 100 μ g of CdeM adjuvanted with Imject Alum. Control hamsters were immunized with saline and Imject Alum. * denotes statistical significance ($p<0.05$) compared to the control mice. Mantel-Cox test for pairwise comparisons was used to assess statistical significance between the cohorts.

b. Anti-CdeM IgG responses in serum of vaccinated and control hamsters one week before challenge and in surviving hamsters two weeks after challenge. Results were determined by kinetic ELISA and are reported as OD/min; the geometric mean plus standard error of the mean for each cohort is shown. * denotes statistical significance ($p<0.05$), using an unpaired Student *t* test analysis for comparison of means.

Table 1

Spore Proteins as Targets for Immunization

Gene Name	NCBI Gene ID	Molecular Weight (kDa)	Predicted Glycosylation		Percent Identity (blastp)	Protein description	Reference
			N-linked	O-linked			
CD1067 CdeC	4915202	45	1	3	96	Exosporium protein essential for exosporium morphogenesis	13,15,19
CD0332 BclA1	4915988	68	2	1	73	Exosporium collagen like-glycoprotein	22,23,24
CD0551 SleC	4916686	47	2	7	97	Spore cortex lytic enzyme with lytic transglycosylase activity	25,26
CD1613 CotA	4913945	34	0	0	98	Present on the coat and/or the exosporium	15,23
CD1581 CdeM	4913041	19	1	1	83	Hypothetical exosporium protein	15,20,21