Anaerobe 37 (2016) 85–95

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Immunogenicity and protective efficacy of *Clostridium difficile* spore proteins

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ARTICLE INFO

Article history:
Received 31 July 2015
Received in revised form
28 November 2015
Accepted 3 December 2015
Available online 11 December 2015

Keywords:
Clostridium difficile
Spores
Exosporium
CdeM
CdeC
BclA1
CotA
Vaccine

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* 630Δ*erm* in golden Syrian hamsters. This unprecedented study shows the vaccination potential of *C. difficile* spore exosporium proteins.

1. 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

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http://dx.doi.org/10.1016/j.anaerobe.2015.12.001
1075-9964/

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 *Clostridium trachomatis* OmcA and an ortholog of CdeC has been identified in *Clostridium bartletii*, 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 630Δ*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.

2. Materials and methods

2.1. Bacterial strains

Escherichia coli DH5α 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 10^6 CFU of *C. difficile* strain UK1 was calculated as previously described [27]. At this dose, severe CDI would develop 4–6 days post UK1 challenge in a substantial majority of antibiotic-treated mice.

An oral dose of 500 CFU of *C. difficile* strain 630Δ*erm* (ribotype 012) was used for the bacterial challenge experiments in hamsters. At this dose, severe CDI would develop 2–3 days post *C. difficile* strain 630Δ*erm* challenge in a substantial majority of antibiotic-treated hamsters. *C. difficile* 630Δ*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].

2.2. 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).

2.3. 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 μg/ml) at 37 °C with aeration and induced with 0.1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) for 3 h (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).