INFECTION AND IMMUNITY, June 2011, p. 2295–2302 Vol. 79, No. 6
0019-9567/11/$12.00 doi:10.1128/IAI.00130-11
Copyright © 2011, American Society for Microbiology. All Rights Reserved.

Immunization with *Bacillus* Spores Expressing Toxin A Peptide Repeats Protects against Infection with *Clostridium difficile* Strains Producing Toxins A and B▽†

Patima Permpoonpattana,[1]‡ Huynh A. Hong,[1]‡ Jutarop Phetcharaburanin,[1] Jen-Min Huang,[1] Jenny Cook,[1] Neil F. Fairweather,[2] and Simon M. Cutting[1]*

School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, TW20 0EX United Kingdom,[1] and Department of Life Sciences, Imperial College London, London, SW7 2AZ United Kingdom[2]

Received 8 February 2011/Returned for modification 2 March 2011/Accepted 30 March 2011

***Clostridium difficile* is a leading cause of nosocomial infection in the developed world. Two toxins, A and B, produced by most strains of *C. difficile* are implicated as virulence factors, yet only recently has the requirement of these for infection been investigated by genetic manipulation. Current vaccine strategies are focused mostly on parenteral delivery of toxoids. In this work, we have used bacterial spores (*Bacillus subtilis*) as a delivery vehicle to evaluate the carboxy-terminal repeat domains of toxins A and B as protective antigens. Our findings are important and show that oral immunization of the repeat domain of toxin A is sufficient to confer protection in a hamster model of infection designed to closely mimic the human course of infection. Importantly, neutralizing antibodies to the toxin A repeat domain were shown to be cross-reactive with the analogous domain of toxin B and, being of high avidity, provided protection against challenge with a *C. difficile* strain producing toxins A and B (A^+B^+). Thus, although many strains produce both toxins, antibodies to only toxin A can mediate protection. Animals vaccinated with recombinant spores were fully able to survive reinfection, a property that is particularly important for a disease with which patients are prone to relapse. We show that mucosal immunization, not parenteral delivery, is required to generate secretory IgA and that production of these neutralizing polymeric antibodies correlates with protection. This work demonstrates that an effective vaccine against *C. difficile* can be designed around two attributes, mucosal delivery and the repeat domain of toxin A.**

Clostridium difficile is the most common cause of nosocomial antibiotic-associated diarrhea in developed countries. Antibiotic therapy and disruption of the normal gastrointestinal (GI) microflora are the primary causes of *C. difficile*-associated disease (CDAD), and the presence of one or both of these factors is a prerequisite for colonization of the gut by this Gram-positive bacterium. Morbidity and mortality rates have been steadily increasing in recent years and probably result from the emergence of more virulent strains of *C. difficile* as well as the changing patterns of antibiotic usage. Recent estimates of CDAD in the United States suggest as many as 500,000 cases per year, with up to 20,000 deaths (32). CDAD is caused by the secretion of two toxins, toxin A (TcdA) and toxin B (TcdB), both of which are monoglucosyltransferases that are cytotoxic, enterotoxic, and proinflammatory (5). CDAD is particularly problematic to treat and contain because of the ability of the bacterium to form robust endospores that can persist and be easily transferred in a hospital environment. Currently, the only treatment for CDAD is the use of antibiotics such as vancomycin and metronidazole, possibly followed by surgery if the disease is serious and refractory to antimicrobial treatments. Recurrence of CDAD (i.e., diarrhea recurring within 30 days after the first treatment) is a particular challenge for which there is no standard, uniformly effective treatment.

Although *C. difficile* can naturally cause disease without toxin A, most clinically isolated *C. difficile* strains produce both toxin A and toxin B (A^+B^+) (28). Therefore, an effective vaccine to CDAD should target the two principal virulence factors, toxin A and toxin B, since high titers of antibodies against these toxins correlate well with protection in both hamsters and humans (1, 21, 26). Recent studies have shown that both toxins are important for disease and that recombinant, isogenic *C. difficile* strains that are A^-B^+ or A^+B^- are able to cause disease in the hamster model of infection (23). This work seemingly contradicts an earlier study suggesting that only toxin B is responsible for virulence (30) yet is supported by numerous other studies implicating both toxin A and toxin B in infection (7, 20, 29, 42). Both of the *tcdA* and *tcdB* genes, which encode toxin A and toxin B, respectively, carry limited identity at their C termini, where each carries an elaborate array of repeated domains (40). The C-terminal domain of *tcdA* has been shown to be involved in initial binding of the toxin to sensitive cells prior to its translocation across the endosomal membrane (17). Previous studies indicate that these repetitive domains may be suitable as antigens against CDAD. Some examples are, first, that toxin A cell binding repeats, and a monoclonal antibody (MAb) directed against them, prevented cytotoxicity (34). Second, a defined segment of repeats known as 14CDTA expressed in a recombinant *Salmonella* vaccine elicited local and systemic immunity and toxin A-neutralizing activity (44). Finally, human monoclonal antibodies directed

* Corresponding author. Mailing address: School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, TW20 0EX United Kingdom. Phone: 44-1784-443760. Fax: 44-1784-414224. E-mail: s.cutting@rhul.ac.uk.

‡ These authors contributed equally to this work.

† Supplemental material for this article may be found at http://iai.asm.org/.

▽ Published ahead of print on 11 April 2011.

against toxins A and B prevent *C. difficile*-induced mortality in hamsters (2) and reduced recurrence in humans (27).

The colon is the primary site of residency of germinated *C. difficile* spores, and luminal epithelial cells are targeted by the C-terminal regions of toxins A and B. High-avidity binding facilitates the subsequent internalization of the toxins via receptor-mediated endocytosis in clathrin-coated pits (41). Antibodies to toxin A have been shown to confer protection against *C. difficile* A^+B^+ strains, whether delivered mucosally (21) or parenterally (2, 20), although levels of protection are more complete if antibodies to both toxins are used. Such passive-immunization studies show that antibodies are the key effector molecule, and in the GI tract, polymeric secretory IgA (sIgA) may interfere with toxin binding. Despite this, current vaccination strategies are based mostly on parenteral delivery and inducing IgG, whose mechanistic action is far from clear (9). Recombinant bacterial vaccines expressing the toxin A binding domain have been shown to induce both mucosal-sIgA- and serum IgG-neutralizing antibodies following oral administration (43, 44), which prompted us to consider *Bacillus subtilis* spores as a delivery vehicle for *C. difficile* antigens.

Recombinant, heat-stable spores of *B. subtilis* have been used for mucosal delivery of heterologous antigens. In experiments using spores expressing antigens on their surface coats, they have been shown to protect mice immunized against tetanus toxin from *Clostridium tetani* (8) and *Clostridium perfringens* alpha toxin (13). In both cases, significant levels of local immunity (sIgA) were induced. Interestingly, spores appear to possess natural adjuvant properties, and coupled with their heat stability and existing use as probiotics (14), they are attractive vehicles with which to develop a vaccine to CDAD.

In the present study, *B. subtilis* spores expressing the cell-binding domains of toxin A and toxin B were evaluated for their ability to elicit neutralizing antibodies. Our findings show that antibodies to the toxin A domain are cross-reactive to the toxin B domain and can protect hamsters from challenge with spores of a toxigenic strain of *C. difficile*. Our work shows that oral immunization and the production of local immunity are the key attributes required for vaccination against CDAD.

MATERIALS AND METHODS

General methods. Methods for work with *B. subtilis* are described elsewhere (11). *B. subtilis* strain PY79 is a standard, prototrophic laboratory strain. *C. difficile* spores of strain 630 (*tcdA*$^+$ *tcdB*$^+$; lab stock) were prepared using an anaerobic incubator (Don Whitley, United Kingdom) for all manipulations as follows. A single colony was grown on BHIS (brain heart infusion supplemented with 0.1% L-cysteine and 5 mg/ml yeast extract) agar overnight at 37°C. One fresh single colony from the BHIS plate was inoculated in 10 ml of TGY medium (3% tryptic soy broth, 2% glucose, 1% yeast extract, 0.1% L-cysteine) and incubated at 37°C overnight. One milliliter of TGY culture was then subcultured into SMC broth [90 g peptone, 5 g proteose peptone, 1 g $(NH_4)_2SO_4$, 1.5 g Tris, 0.1% L-cysteine], incubated overnight, and then plated onto SMC agar and incubated for 7 days. Sporulation was confirmed by phase-contrast microscopy, and spore crops were harvested and purified as described elsewhere (25).

Recombinant *B. subtilis* vaccine strains. Detailed strain constructions are provided in the text in the supplemental material. *B. subtilis* merodiploid strains contained chimeric genes of *cotB* and/or *cotC* fused at their 3′ ends to the C-terminal domains of toxin A (A26-39; codons for Ser_{2388} to Pro_{2706} [6]) or toxin B (B15-24; codons for Glu_{2137} to Glu_{2366} [3]). The entire *cotC* gene was used for fusion to the toxin domains, while for *cotB*, a 3′ deletion was used so that the encoded CotB segment was 33 kDa. Genes were integrated into the chromosome by a stable double-crossover recombination and were placed in *trans* into the indigenous *cotB* or *cotC* gene.

PP059 (*thrC*::*cotB*-A26-39) encodes a fusion of A26-39 to the C terminus of CotB (predicted molecular mass, 69 kDa). The chimeric gene was carried at the *thrC* locus of *B. subtilis*.

PP052 (*amyE*::*cotC*-A26-39) encodes a fusion of A26-39 to the C terminus of the CotC (predicted molecular mass, 49 kDa). The chimeric gene was carried at the *amyE* locus of *B. subtilis*.

PP108 (*thrC*::*cotB*-A26-39 *amyE*::*cotC*-A26-39) was created by transforming competent cells of PP052 with chromosomal DNA of PP059, with selection for Erm^r, conferred by the *thrC*::*cotB*-A26-39 cassette.

PP132 (*thrC*::*cotB*-B15-24) encodes a fusion of B15-24 to the C terminus of CotB (predicted molecular mass, 60 kDa).

PP142 (*thrC*::*cotB*-B15-24 *amyE*::*cotC*-A26-39) was created by transforming competent cells of PP052 with chromosomal DNA of PP132 (*thrC*::*cotB*-B15-24), with selection for Erm^r, conferred by the *thrC*::*cotB*-B15-24 cassette.

Recombinant proteins. Recombinant A26-39 (36.6 kDa) and B15-24 (29.4 kDa) were produced in *Escherichia coli* BL21(DE3)(pLys) from a pET28b expression vector that separately carried the rA26-39 and rB15-24 open reading frames (ORFs) fused to a C-terminal polyhistidine tag. High levels of expression were obtained upon isopropyl-β-D-thiogalactopyranoside (IPTG) induction and purification of rA26-39 and rB15-24 by passage of the cell lysate through a HiTrap chelating high-performance (HP) column on a Pharmacia AKTA liquid chromatography system.

Protein quantification. Coat proteins were extracted from purified spore suspensions of PP108 and PP142 using two extraction procedures, SDS-dithiothreitol (DTT) and alkali extraction, as described elsewhere (8). SDS-DTT solubilizes both CotB and CotC, while alkali extraction preferentially solubilizes CotC. Quantification was made using a Bio-Rad Gel Doc imaging system.

Polyclonal antibodies. Polyclonal antibodies were raised in mice immunized by the intraperitoneal (i.p.) route with 2 μg of purified protein on days 1, 14, and 28. Dilutions used were 1:3,500 for anti-A26-39 and 1:1,500 for anti-B15-24.

Immunizations in mice. Animals used in this work were pathogen-free BALB/c mice (Charles River) for antibody production and for analysis of immune responses. In all cases, females, aged 6 to 8 weeks, were used. All animal procedures were performed under the Home Office project license PPL 70/6126. Mice were dosed orogastrically (o.g.) (0.2 ml) on days 0, 14, 35, and 57 with a dose of 5×10^{10} spores (PY79, PP108, or PP142). A naïve group of unimmunized animals was included in all experiments, as well as a group receiving a mixture of the rA29-36 (10 μg) and rB15-24 (10 μg) proteins. Intraperitoneal (i.p.) immunizations consisted of doses on days 0, 7, and 28 with the rA26-29 and rB15-24 proteins (10 μg of each).

Determination of mouse antibody titers by indirect ELISA. For analysis of responses, serum was taken on days −1, 34, and 56 (o.g. groups) or day 42 (i.p. groups), and feces were collected on days 21, 42, and 67 and kept at −80°C. Sample extractions were made at a one-fifth (wt/vol) dilution in extraction buffer (2% fetal calf serum [FCS], Dulbecco's modified Eagle's medium [DMEM] plus protease inhibitor cocktails, trypsin [0.1 mg/ml], leupeptin [1 μg/ml], benzamide [1 μg/ml], aprotinin [10 μg/ml], phenylmethylsulfonyl fluoride [1 mM], and EDTA [0.05 mg/ml]). Samples were gently shaken for 30 min at 4°C to disrupt solid material and then centrifuged (13,000 rpm for 15 min). Supernatants were filtered (0.45-μm pore size) before analysis. Antibodies from sera and feces were determined by indirect enzyme-linked immunosorbent assay (ELISA). Greiner 96-well plates (MaxiSorp) were coated with purified recombinant A26-29 (rA26-39) (10 μg/ml) or rB15-24 (50 μl/well) protein in phosphate-buffered saline (PBS) overnight at room temperature (RT). After the plates were blocked for 1 h at 30°C with 2% bovine serum albumin (BSA), 2-fold serially diluted samples were added, starting at a dilution of 1/50 in diluent buffer (0.01 M PBS [pH 7.4], 0.5% [wt/vol] bovine serum albumin [BSA], 5% [vol/vol] fetal bovine serum [FBS], 0.1% [vol/vol] Triton X-100, 0.5% [vol/vol] Tween 20). Replicate samples were used together with a negative control (preimmune serum). Plates were incubated for 2 h at RT before addition of appropriate horseradish peroxidase-conjugated anti-mouse antibodies in conjugate buffer (5% FBS [vol/vol], 1% BSA [wt/vol], 0.05% Tween 20 in 0.01 M PBS). Plates were incubated for 1 h at RT and then developed using tetramethyl benzidine (TMB) substrate (0.1 mg/ml 3.3′,5.5′-tetramethylbenzidine in 0.1 M sodium acetate buffer [pH 5.5] in distilled water). Reactions were stopped using 2 M H_2SO_4, and optical densities (ODs) were read at 450 nm. Dilution curves were created for each sample, and endpoint titers for each specific antibody were estimated at the maximum dilution of serum giving an absorbance reading of 0.1 U over the ODs of naïve samples.

Neutralization assays. The ability of the antibody samples to neutralize *C. difficile* toxins *in vitro* was determined as described recently (30), with some modifications. HT29 and VERO cells were grown in McCoy's 5A medium and Dulbecco's modified Eagle complete medium (10% [vol/vol] fetal calf serum and 1% [vol/vol] penicillin and streptomycin). Cells were cultured at 37°C with 5%

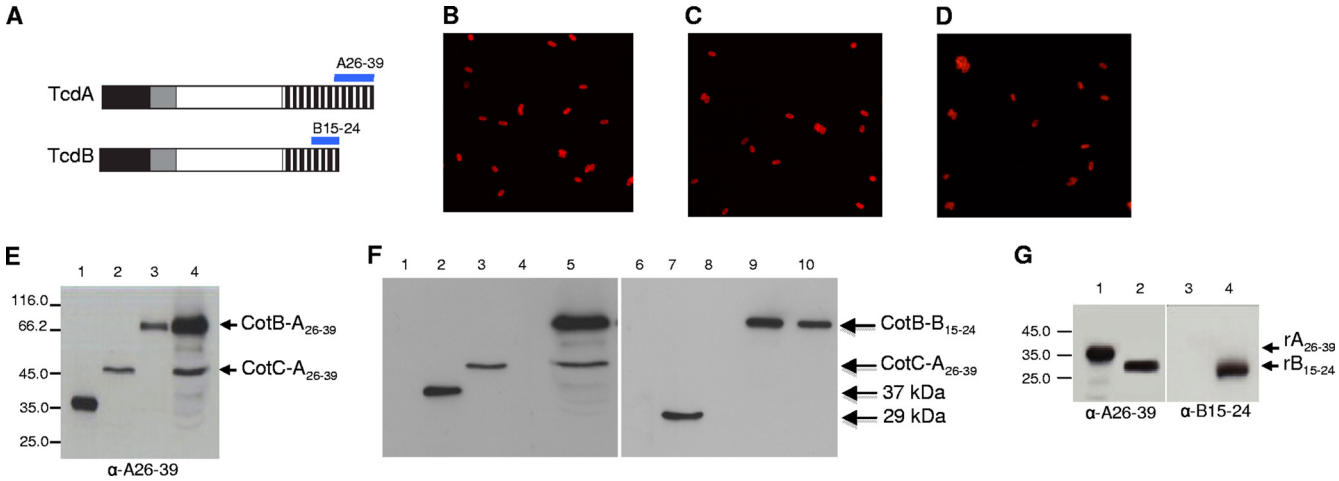

FIG. 1. Recombinant spores expressing *C. difficile* toxin domains. (A) The functional domains of toxins A and B are shown, including the glycosyltransferase (black), cysteine protease (gray), and translocation (white) domains and the repetitive sequences involved in cell binding (stripes). The regions cloned and expressed in *B. subtilis* spores are indicated. These are the A26-39 domain (formerly known as 14CDTA), which carries 14 repeat sequences and has been shown to be immunogenic and capable of inducing neutralizing antibodies in mice when delivered by a mucosal route (43), and the B15-24 domain, which carries 10 repeats and shares homology with A26-39 (see Fig. S2 in the supplemental material). (B to D) Surface display of A26-39 and B15-24, confirmed using confocal imaging of samples labeled with mouse anti-A26-39 or -B15-24 serum followed by an anti-mouse IgG-tetramethyl rhodamine isocyanate (TRITC) conjugate. Spores of PP108 were labeled with anti-A26-39 (B), and spores of PP142 were labeled with either anti-A26-39 (C) or anti-B15-24 (D). Nonrecombinant PY79 spores showed no labeling with either antiserum. Images were taken using a Nikon Eclipse fluorescence microscope equipped with a Bio-Rad Radiance 2100 laser scanning system (image size = 37 by 37 μm). (E) Expression of A26-39 on spores of PP108 (CotB-A_{26-39} CotC-A_{26-39}). Lane 4 shows PP108-extracted spore coat proteins probed with antibodies to rA26-39. Lanes 1 to 3 were also probed with anti-A26-39. Lane 1, rA26-39 protein; lane 2, coat proteins from PP052 (CotC-A_{26-39}); lane 3, coat proteins from PP059 (CotB-A_{26-39}). (F) Expression of A26-39 and B15-24 on spores of PP142 (CotB-B_{15-24} CotC-A_{26-39}) probed with anti-A26-39 (lanes 1 to 5) and anti-B15-24 antibodies (lanes 6 to 10). Lanes 1 and 6, nonrecombinant PY79 spore coat proteins; lane 2, rA26-39 protein (36.6 kDa); lanes 3 and 8, PP052 extracts (CotC-A_{26-39}); lanes 4 and 9, PP132 extracts (CotB-B_{15-24}); lanes 5 and 10, PP142 extracts; lane 7, rB15-24 protein (29.4 kDa). The positions of the 60-kDa CotB-B_{15-24} and 49-kDa CotC-A_{26-39} bands are shown. (G) Purified rA26-39 (lanes 1 and 3) (36.6-kDa) and rB15-24 (lanes 2 and 4) (29.4-kDa) polypeptides were probed with anti-A26-39 or anti-B15-24. Numbers at the left of the blots are molecular masses (in kilodaltons).

(vol/vol) CO_2 in air (100% humidity). HT29 and VERO cells were seeded in 96-well plates (0.5×10^4 cells/well and 2.5×10^4 cells/well, respectively). After 24 h, cells were washed twice in sterile PBS. Before the assay, toxins were incubated with serially diluted pooled serum or fecal samples (1:1, vol/vol) in medium containing 2% FCS and incubated for 1 h at 37°C before being added to the prewashed cell monolayer. Cells were evaluated at 24 h (HT29) and 48 h (VERO). Toxins used were toxins A and B obtained from the culture supernatant of strain 630 (toxin 630), which had been partially purified by ammonium sulfate precipitation (60%). The presence of toxins A and B was confirmed in the supernatant of 630 cultures by Western blotting (see Fig. S1 in the supplemental material). Toxin A and toxin B had been purified from cell supernatants. Appropriate toxin concentrations were determined by the highest dilutions causing 100% cytopathicity (i.e., cell rounding) (toxin 630 [120 ng/ml], toxin A [4.5 ng/ml], toxin B [6.2 pg/ml]). All assays were carried out in duplicate.

Hamster challenge. Groups of 6 to 10 female Golden Syrian hamsters (70 to 80 g; Charles River, United Kingdom), were immunized o.g. (0.2 ml) with either (i) recombinant (PP108, PP142) and nonrecombinant PY79 spores (5×10^{10} spores/dose/hamster) or (ii) a mixture of the proteins rA26-39 (10 μg) and rB15-24 (10 μg). One further group was dosed with the protein mixture delivered by the i.p. route (0.2 ml). Hamsters were dosed o.g. on days 0, 14, 35, and 57 and i.p. on days 0, 7, and 28. The challenges were performed as described by Goulding et al. (10), with some minor modifications. In brief, 14 days after the last dose, hamsters were transferred to individual sterile cages (including all food, bedding, and water) and treated with clindamycin (30 mg/kg of body weight). Twelve hours later, hamsters were o.g. infected with 100 *C. difficile* spores (strain 630). Twenty-four hours after challenge and every 2 days thereafter, hamsters were repeatedly transferred to new sterile cages. Hamsters were intensively monitored 38 to 60 h after challenge. Hamsters showing clear symptoms were killed and considered unprotected.

Statistics. The unpaired *t* test was used to compare between groups. A *P* value of >0.05 was considered nonsignificant.

RESULTS

Expression of the C-terminal domains of *C. difficile* toxin A and toxin B on *B. subtilis* spores. *B. subtilis* was engineered to express the A26-39 domain of toxin A (TcdA) and the B15-24 domain of toxin B (TcdB) on the outermost layer of the spore coat. Both A26-39 and B15-24 lie within the C-terminal repeat domains of each toxin and carry repetitive sequences (40) (Fig. 1A). Expression was achieved by fusing A26-39 and B15-24 to the C termini of the outer spore coat proteins CotB (43 kDa) and CotC (12 kDa), both of which have successfully been used for surface display and mucosal delivery of heterologous antigens (16, 31) (in the case of CotB, fusions were made to a C-terminally truncated version of CotB). Two different spore constructions were made: PP108 (*cotB*-A26-39 *cotC*-A26-39), which expressed A26-39 attached to both CotB and CotC, and PP142 (*cotB*-B15-24 *cotC*-A26-39), which coexpressed two chimeras, CotB-B_{15-24} and CotC-A_{26-39}. Surface expression of A26-39 and B15-24 on PP142 spores and A26-39 on PP108 spores was confirmed by confocal imaging of spores (Fig. 1B to D).

Antibodies to the C-terminal domain of toxin A cross-react with the reciprocal domain of toxin B. The coat proteins of PP108 and PP142 were extracted and probed with antibodies to confirm expression of the chimeric CotB and CotC proteins (Fig. 1E to F). PP108 spore coat protein extracts when probed with anti-A26-39 antibodies revealed bands of 69 kDa (CotB-

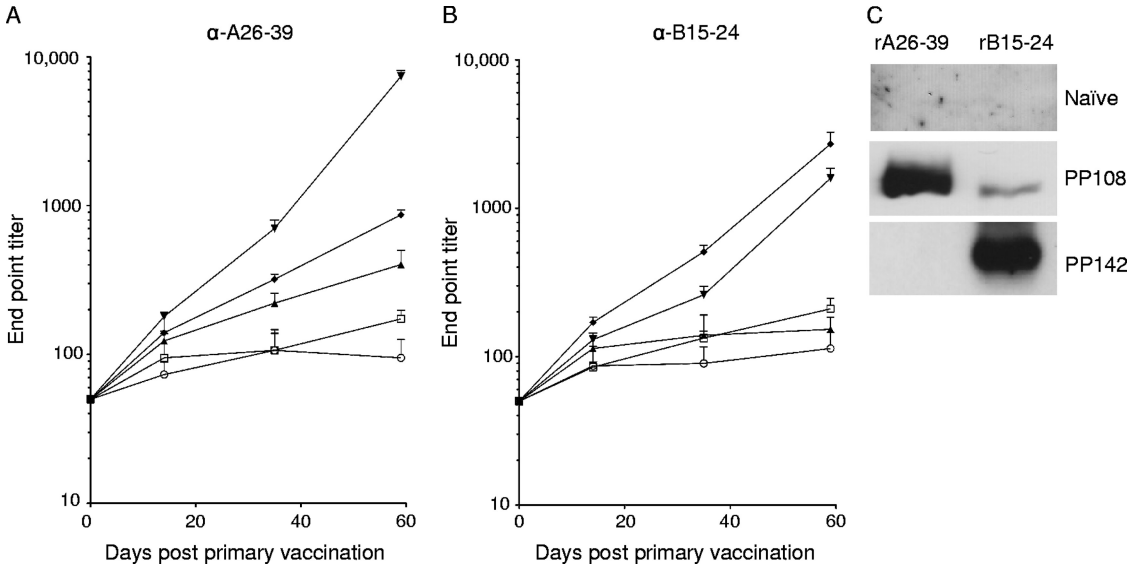

FIG. 2. IgG responses after orogastric immunization of mice with recombinant spores. Anti-A26-39-specific IgG (A) and anti-B15-24 IgG (B) responses are shown. Mice were immunized with spores of strain PP108 (CotB-A_{26-39} CotC-A_{26-39}) (▼), PP142 (CotB-B_{15-24} CotC-A_{26-39}) (◆), nonrecombinant PY79 (□), a mixture of the rA26-39 and rB15-24 recombinant proteins (▲) (10 μg of each) and naïve mice (○). (C) Western blots containing pooled sera from naïve and PP108- and PP142-immunized groups were used to probe rA26-39 proteins and rB15-24.

A_{26-39}) and 49 kDa (CotC-A_{26-39}). Their molecular masses were in agreement with the predicted sizes of the fusion proteins (Fig. 1E) and corresponded in size to proteins in extracts taken from spores carrying each chimera alone. Quantification revealed that PP108 spores carried 2.2×10^{-4} pg/spore of A26-39. When PP142 extracts were probed with anti-B15-24, one principal band of 60 kDa was detectable (Fig. 1F, lane 6), corresponding in size to CotB-B_{15-24}. Using anti-A26-39, CotC-A_{26-39} was observed as a 49-kDa species; a second band of 60 kDa was also observed (Fig. 1F, lane 3). We predict that this higher-molecular-mass species is CotB-B_{15-24} and could be explained by the fact that the C-terminal cell-binding domains of TcdA and TcdB share some sequence identity (30% identical residues [see Fig. S2 in the supplemental material]) and may, as shown elsewhere, carry related epitopes (40). In support of this, we demonstrated that anti-A26-39 antibodies could cross-react with both the purified-rA26-39 (36.6-kDa) and -rB15-24 (29.4-kDa) polypeptides, yet anti-B15-24 antibodies reacted only with rB15-24 (Fig. 1G). Protein expression was quantified, and each spore of PP142 was shown to contain 2.45×10^{-5} pg of B15-24 and 1.9×10^{-4} pg of A26-39. In additional work, we verified, using anti-CotB and anti-CotC polyclonal antibodies, the expected change in the molecular masses of the chimeric CotB and CotC spore coat proteins in PP108 and PP142 spores (see Fig. S3 in the supplemental material).

Oral delivery of the C-terminal domains of toxin A and toxin B displayed on spores induces systemic and mucosal antibodies. Immune responses were determined in mice dosed orogastrically (o.g.) with PP108 or PP142 spores. Control groups included naïve mice and groups dosed (o.g.) with nonrecombinant spores (PY79). In addition, we included one group dosed (o.g.) with a mixture of the rA26-39 (10-μg/dose) and rB15-24 (10-μg/dose) proteins using the same dosing regimen. Ten micrograms was chosen for each protein since this equaled or exceeded the dose of rA26-36 or rB15-24 delivered in one o.g. dose of PP108 (11 μg of A26-39) or PP142 (9.5 μg of A26-39 and 1.25 μg of B15-24) spores.

Specific antibodies (serum IgG and fecal IgA) against A26-39 and B15-24 were measured by indirect ELISA. Compared to control groups (naïve mice, mice dosed with PY79 spores, or mice receiving proteins alone), significant ($P < 0.01$) levels of A26-39 and B15-24 (cross-reacting) IgG were detected in the sera of animals dosed with PP108 (Fig. 2A and B). In mice dosed with PP142 spores, anti-A26-39 IgG responses were not significantly greater ($P > 0.05$) than in control groups, while anti-B15-24 IgG responses were significantly greater ($P < 0.01$) (Fig. 2B) and showed seroconversion. IgG isotypes were also determined (see Fig. S4 and S5 in the supplemental material), and significant ($P < 0.01$) levels of anti-A26-39 and anti-B15-24 (cross-reacting) IgG1 and IgG2a were found in the PP108 groups compared to the control groups. However, in the PP142 groups, significant levels ($P < 0.01$) of the IgG1 and IgG2a isotypes were found only against B15-24. Analysis of the IgG1/IgG2a ratios over time (see Fig. S6 in the supplemental material) showed a clear increase (3-fold) after the third dose, indicative of a Th2-biased immune response. Anti-spore IgG responses were also measured and found to be markedly low, with no responses substantially greater than those of the control groups (see Fig. S7 in the supplemental material). IgG present in the sera of PP108-immunized mice was found to bind in a Western blot to rA26-39 and rB15-24, while that present in PP142-dosed mice bound only to rB15-24 (Fig. 2C).

Secretory IgA (sIgA) was measured in fecal samples (Fig. 3), and anti-A26-39 responses were found to be particularly high (Fig. 3A), with clear seroconversion in animals dosed with PP108 spores ($P < 0.001$) but not with PP142 spores ($P > 0.05$). Anti-B15-24 sIgA responses were lower, but seroconversion was found in animals dosed with both PP108 and PP142 (Fig. 3B) ($P < 0.05$). The anti-B15-24 cross-reacting IgG and sIgA responses found in animals dosed with PP108 spores expressing only the A26-39

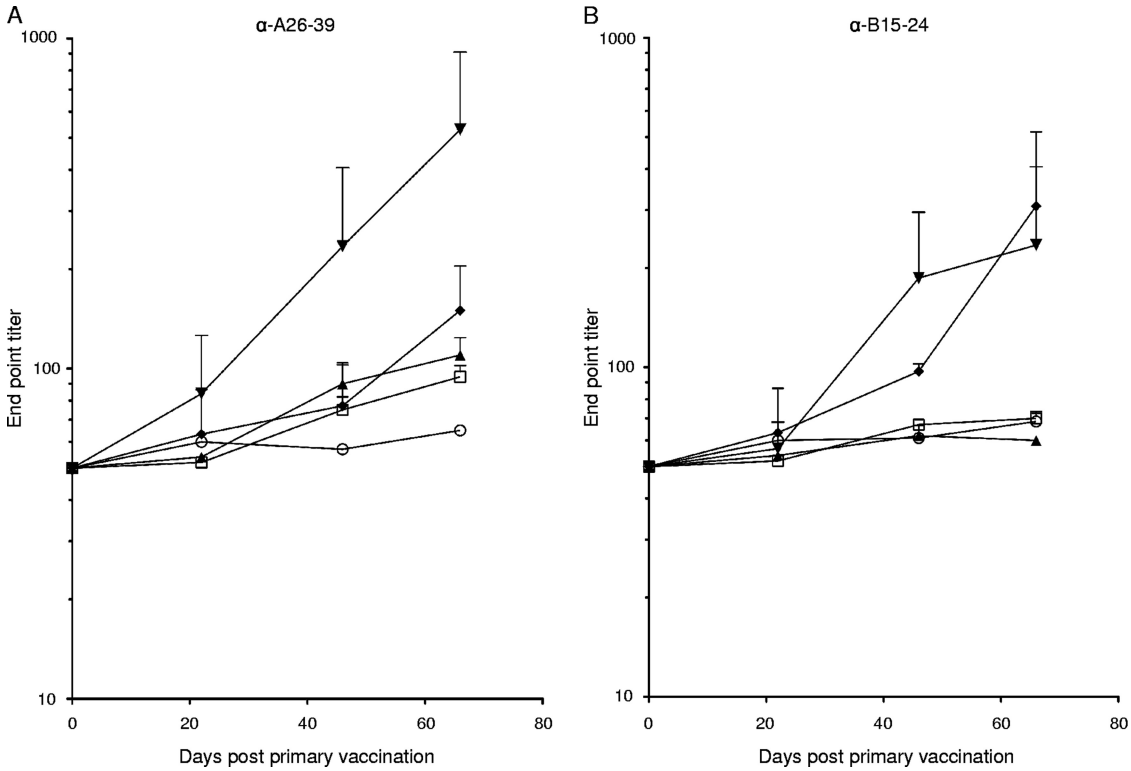

FIG. 3. Secretory IgA responses after orogastric immunization of mice with recombinant spores. Anti-A26-39-specific sIgA (A) and anti-B15-24-specific sIgA (B) responses. Mice were immunized with spores of strain PP108 (CotB-A_{26-39} CotC-A_{26-39}) (▼), PP142 (CotB-B_{15-24} CotC-A_{26-39}) (◆), nonrecombinant PY79 (□), and a mixture of the rA26-39 and rB15-24 recombinant proteins (▲) (10 μg of each). ○, naïve mice.

protein further support our finding that antibodies against the A26-39 domains are cross-reactive to B15-24.

***In vitro* neutralization of cytotoxicity.** Both toxin A and toxin B exhibit cytotoxicity on cultured cells, with HT29 cells being most sensitive to toxin A and VERO cells most sensitive to toxin B (30, 38). Serum (IgG) and mucosal (fecal sIgA) antibodies from mice were assessed for their ability to neutralize toxin A- and toxin B-mediated cytotoxicity using either purified toxins or partially purified supernatants from strain 630 (toxin 630) (Table 1). Cytotoxicity was confirmed by examination of cells over a 24- to 48-h period, with susceptible cells showing a rounded cell morphology (examples in Fig. S8 in the supplemental material).

Antibodies from PP108-immunized (o.g.) mice were found to neutralize both toxin A and toxin B when either HT29 or VERO cells were used, and higher titers were shown in HT29 cells (toxin A specific) than in VERO cells. In contrast, antibodies produced by PP142-immunized (o.g.) animals neutralized only toxin B. These results show, first, that in mice, o.g. delivery of recombinant spores expressing A26-39 or B15-24

TABLE 1. Neutralization of *in vitro* cytotoxicity and protection

Group	Route	Toxin neutralization titer[a]						Protection (%)[b]	
		Anti-toxin A		Anti-toxin B		Anti-toxins A and B[c]		1st	2nd
		IgG	sIgA	IgG	sIgA	IgG	sIgA		
Naïve		−	−	−	−	−	−	0	
PY79	o.g.	−	−	−	−	−	−	0	
rA26-39 + rB15-24	o.g.	−	−	−	−	−	−	0	
PP108	o.g.	++	+++	+	+	++	+++	75	100
PP142	o.g.	−	−	+	+	−	−	0	
rA26-39 + rB15-24	i.p.	+++	−	+	−	+++	−	25	0

[a] Neutralization titers against toxin A or toxin B were measured on HT29 (anti-toxin A) or VERO (anti-toxin B) cells. Endpoint titers were determined as the highest dilution of sample that prevented the cytopathic cell-rounding effect of the toxin in 100% of cells. Negative results are expressed as − when the endpoint titer dilution was <1/10 for serum samples and <1/50 (wt/vol) for fecal samples. Representative neutralization data are shown in Fig. S8 in the supplemental material and summarized as follows. For serum titers, + indicates a titer of 10 to 20, ++ indicates 40 to 80, and +++ indicates >160. For feces titers, + indicates a titer of 50 to 100, ++ indicates 100 to 200, and +++ indicates >200.

[b] Survival data are shown in Fig. 4. Animals surviving the first challenge were then rechallenged (2nd).

[c] Neutralization against toxins partially purified from *C. difficile* strain 630, measured using HT29 cells.

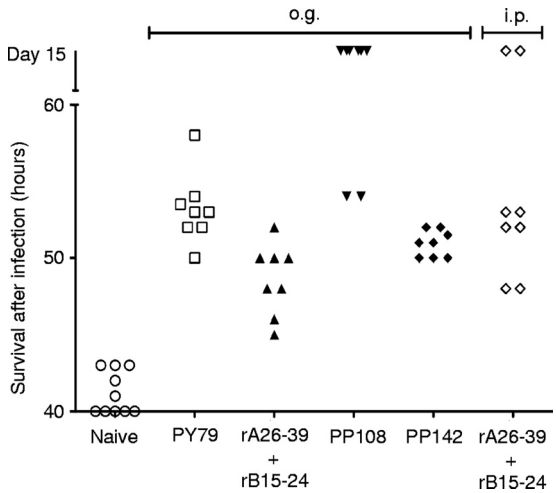

FIG. 4. Protection in Golden Syrian hamsters. Hamsters were given four o.g. doses (days 0, 14, 35, and 57) of recombinant spores (PP108 or PP142) and then challenged with *C. difficile* 630 (A^+B^+). Control groups included naïve animals, a group dosed with nonrecombinant PY79 spores, and, finally, a group receiving a mixture of the rA26-39 and rB14-24 proteins (10 μg each). Further groups were hamsters dosed parenterally (i.p.) with rA26-39 plus rB14-24 (10 μg each). Colonization of hamsters is presented as time from inoculation to signs of first symptoms, when animals were killed. Animals showing no symptoms after 14 days were considered protected (i.e., the PP108 group). For animals showing signs of infection, the mean times (±standard deviations) to visible symptoms were, for naïve hamsters, 40.9 h (±1.7 h), for PY79 hamsters, 53.2 h (±2.3 h), for PP108 hamsters, 55.6 h (±1.7 h), for PP142 hamsters, 50.9 h (±0.9 h), and for hamsters dosed o.g. with the protein mixture and for hamsters in the i.p. group, 51.8 h (±2.4 h) and 48.6 h (± 2.3), respectively.

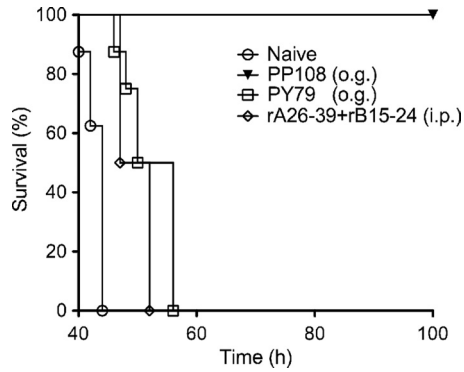

FIG. 5. Protection against reinfection. Hamsters immunized orally with PP108 spores or parenterally with recombinant proteins that had survived challenge with *C. difficile* 630 (as shown in Fig. 4) were then rechallenged with *C. difficile* 630 spores 16 days after the end of the first challenge. Kaplan-Meier survival estimates are shown. Results are also shown for control groups of naïve hamsters and animals dosed with nonrecombinant PY79 spores (dosed on days 0, 14, 35, and 57).

can generate systemic and mucosal neutralizing antibodies. Second, using PP108 spores expressing only A26-39, neutralizing antibodies to toxin A as well as toxin B could be elicited.

Protection in hamsters. The hamster model of *C. difficile* infection most closely resembles the human disease and is the best indicator of protective immunity (10, 33). We dosed hamsters o.g. with PP108 and PP142 spores, treated them with clindamycin, and then challenged them with 100 spores of *C. difficile* strain 630 (Fig. 4). Control groups included hamsters dosed with nonrecombinant PY79 spores and animals dosed with a mixture of the rA26-39 and rB15-24 proteins (10 μg of each). Spores of *C. difficile* were used instead of live cells since, as shown recently, they better mimic the natural infection process (10) as well as minimize the difficulties of administering live anaerobic bacteria. All naïve animals (10/10) were susceptible to *C. difficile* and showed symptoms of disease after just 40 to 42 h, in close agreement with a previous study (10). Interestingly, most of the animals dosed with PY79 spores showed delayed symptoms, as did those dosed with a mixture of the rA26-39 and rB15-24 proteins, but in both cases, all animals succumbed. Hamsters dosed with PP142 spores showed no protection, with every animal displaying symptoms. PP108-immunized animals, however, showed protection, with six out of eight animals surviving the duration of the experiment.

Protection against reinfection. All surviving hamsters that showed no symptoms of *C. difficile* infection were then rechallenged 16 days after the first challenge using clindamycin to induce *C. difficile* colonization. Control groups included unimmunized (naïve) hamsters and animals dosed o.g. with nonrecombinant PY79 spores. We found that only PP108 hamsters were 100% protected to *C. difficile* challenge and showed no symptoms of infection (Fig. 5). These results show that o.g. immunization of hamsters with the A26-39 domain of toxin A displayed on the surfaces of *B. subtilis* spores is sufficient to confer protection against a strain of *C. difficile* that produces both toxin A and toxin B. Moreover, protected animals were fully resistant to reinfection.

Parenteral immunization of the cell-binding domains of toxin A and toxin B. As a comparator of oral-versus-parenteral delivery, mice were dosed (intraperitoneally [i.p.]) with a mixture of rA26-39 (10 μg/dose) and rB15-24 (10 μg/dose). High ELISA titers of IgG antibodies specific to A26-39 (34,161 ± 9,838) and B15-24 (21,475 ± 7,152) were found 2 weeks after the last dose (day 45), significantly greater than titers in naïve animals ($P < 0.001$) and higher than in serum samples from PP108-immunized mice ($P < 0.001$). In contrast, no significant ($P > 0.05$) levels of sIgA were detectable in feces. Serum antibodies were shown to be able to neutralize the cytotoxic effects of both toxin A and toxin B (Table 1). In the hamsters dosed i.p. with the recombinant proteins, 25% protection was achieved (Fig. 4), but animals failed to survive reinfection with *C. difficile* (Fig. 5).

DISCUSSION

Our use of *B. subtilis* spores as a vehicle for *C. difficile* vaccination demonstrated a number of important findings that will be invaluable to the design of an effective *C. difficile* vaccine: first, that only toxin A antibodies are required for protection, second, that mucosal immunity is very important, and, finally, that vaccination provides protection against relapse. We discuss each of these in turn.

(i) Toxin A antibodies and protection. Expression of the toxin A (A26-39) and toxin B (B15-24) C-terminal domains on *B. subtilis* spores was shown to generate high titers of specific IgG and sIgA antibodies. Using spores (PP108) displaying only the toxin A domain, A26-39, serum and fecal antibodies were

found to be cross-reactive to B15-24. Moreover, they neutralized both toxin A and toxin B *in vitro*, and hamsters immunized with A26-39 were protected against *C. difficile* disease. Further evidence of the cross-reactivity of toxin A antibodies came from Western blotting, which showed that anti-A26-39 antibodies could recognize B15-24. The repeat domains of both toxins share limited sequence identity, which could explain this cross-reactivity. In particular, the sequences YFAPANT, MQ IGVF, AAT, and YYF are conserved between the toxin A and B repeat used here, and together they may contain epitopes responsible for the observed cross-reactivity.

We infer that antibodies to A26-39 are of higher avidity than those to B15-24, since in mice, ELISA antibody titers to A26-39 and B15-24 were equivalent. PP142 spores present both toxin A and toxin B antigens, so why do these spores not provide protection against disease after administration? Although B15-24-neutralizing antibodies were produced, no neutralizing antibodies to A26-39 were detected. This implies that, for protection, antibodies to toxin A are of greater importance than those to toxin B. PP142 spores do express the A26-39 domain, yet no neutralization of toxin A was observed. To account for this, we predict that insufficient A26-29 was expressed, since PP142 spores carried nine times less A26-39 than PP108 spores. Of course, we cannot completely rule out the potential contribution of anti-B15-24 antibodies in protection; rather, our work shows that anti-A26-39 responses are sufficient. It is possible that our prototype vaccines simply do not elicit sufficiently high toxin B-neutralizing titers, possibly due to B15-24 being displayed in a partially denatured form that impairs the generation of appropriate neutralizing antibodies. This may reflect the action of its fusion partner, the spore coat protein CotB, although this has been used successfully previously (8, 13). Full protection to challenge using PP108 spores might be achieved either by changing the dosing regimen or by increasing the dose of heterologous protein expression (whether of A26-39 and/or of B15-24).

This work agrees with a number of studies linking protection against *C. difficile* infection with toxin A, for example, a passive immunization study using oral delivery of anti-toxin A antibodies (21) and studies showing that there is strong association between serum antibody responses to toxin A and protection against *C. difficile* in humans (1, 22, 24). Of particular note is the study of Kim et al. (20), where hamsters immunized (subcutaneously) with *C. difficile* toxoid A were fully (100%) protected but hamsters immunized with toxoid B demonstrated no protection. Our work does show, however, that a vaccine expressing only A26-39 should be protective to all known naturally virulent strains of *C. difficile* (A^+B^+ and A^-B^+). However, a challenge experiment using an A^-B^+ strain is required for confirmation; this finding is important for the design of future *C. difficile* vaccines.

(ii) Mucosal immunity. Considerable effort has been directed toward parenteral vaccines to *C. difficile* and the generation of systemic responses. However, far less attention has been spent on examining the role of local immunity, despite a number of reports demonstrating the importance of sIgA. First, a correlation between neutralizing sIgA and protection in hamsters has been reported using mucosal delivery of toxoids, with 100% protection by intranasal delivery and 40% protection by o.g. delivery (39). Second, and most importantly, several studies demonstrate unequivocally that sIgA both is capable of neutralizing toxin A and is superior to IgG and monomeric serum, IgA (19, 35).

Oral delivery of A26-39 using PP108 spores promoted high titers of fecal sIgA, with levels of toxin A- and toxin B (cross-reacting)-neutralizing sIgA correlating with protection. In contrast, i.p. delivery of both the rA26-39 and rB15-24 proteins produced high titers of serum-neutralizing IgG to toxin A and toxin B, no localized immune responses (sIgA), and lower levels of protection. In another study, however, Torres et al. (39) used parenteral delivery (including i.p.) of toxoids A and B and demonstrated 100% protection in hamsters. A number of factors might account for why we did not observe complete protection when using the recombinant proteins. For example, in the Torres et al. study, the complete toxoids were administered together with an adjuvant and a different virulent strain of *C. difficile* was used for challenge (39).

(iii) Relapse. PP108-immunized hamsters that survived *C. difficile* challenge were fully protected against rechallenge, a phenomenon particularly important for treatment of CDAD when patients succumb to relapse. Up to 20% of patients with CDAD 2 to 8 weeks after discontinuation of antibiotic therapy (metronidazole or vancomycin) relapse, and a further 30% of these patients may do so again after a second course of therapy (4, 18). Antibiotic treatment may disrupt the normal human microflora and lead to overgrowth of toxigenic strains of *C. difficile*, a condition favoring relapse. Vaccination then may provide the only rational treatment to control CDAD, so it is encouraging that we found no symptoms of disease in rechallenged animals. This is consistent with an earlier study that has shown that antibodies to toxins A and B, administered orally, can prevent relapse (21) and also with a study in which 75% protection against relapse was found after mucosal and parenteral delivery of toxoids A and B (39). In our studies, protection against relapse was found only in hamsters dosed with PP108 spores and not in animals that had been vaccinated with the recombinant proteins by a parenteral-dosing regimen. Interestingly, animals dosed parenterally all failed to generate significant sIgA responses, and it is possible that production of mucosal antibodies is important for protection from reinfection. Some probiotic formulations have been shown to significantly reduce the occurrence of relapses in double-blind controlled trials (12, 36). Potentially, live spores may share some attributes with these "probiotic" bacteria, and it is notable that *B. subtilis* is itself used extensively as a probiotic (14). *B. subtilis* spores can germinate and resporulate in the GI tract and possibly even transiently colonize (37). PP108 spores may therefore be able to promote a more extensive production of anti-A26-39 sIgA, sufficient to protect against reinfection. With regard to their potential probiotic attributes, it is noteworthy that even nonrecombinant PY79 spores, when they were administered orally to hamsters, provided a noticeable delay in the onset of symptoms, and it is possible that this phenomenon may reflect involvement of an innate immune response, such as interaction with Toll-like receptors. Alternatively, although the suggestion is highly speculative, we cannot rule out the possibility of sequestration of the toxin in an animal's GI tract, since *Bacillus* spores have been shown to be able to adsorb *C. difficile* toxin *in vitro* (15).

Finally, we have used *C. difficile* spores, in contrast to vegetative cells or toxins, as our challenge in the vaccine protection

experiments, which, as has been shown recently, best mimics the natural course of infection in humans (10). Our study confirms that in the hamster model of infection, there is a correlation between neutralizing antibodies and protection in hamsters, with HT29 cells providing a more precise *in vitro* indicator of protection.

We show that oral delivery of *B. subtilis* spores displaying high levels of toxin A repeats can confer to hamsters immunological protection from a lethal dose of *C. difficile* spores. The basis of the vaccination strategy is induction of secretory IgA, which shows cross-reactivity to toxin B. Such a vaccine would be particularly attractive to the end user, as it can be delivered orally, it is heat stable, and the vehicle is currently used as a probiotic.

ACKNOWLEDGMENT

We thank Gill Douce for invaluable advice regarding the challenge studies.

P.P. and J.P. were supported by a scholarship from the Royal Thai Government.

REFERENCES

1. **Aboudola, S., et al.** 2003. *Clostridium difficile* vaccine and serum immunoglobulin G antibody response to toxin A. Infect. Immun. **71:**1608–1610.
2. **Babcock, G. J., et al.** 2006. Human monoclonal antibodies directed against toxins A and B prevent *Clostridium difficile*-induced mortality in hamsters. Infect. Immun. **74:**6339–6347.
3. **Barroso, L. A., S. Z. Wang, C. J. Phelps, J. L. Johnson, and T. D. Wilkins.** 1990. Nucleotide sequence of *Clostridium difficile* toxin B gene. Nucleic Acids Res. **18:**4004.
4. **Buggy, B. P., R. Fekety, and J. Silva, Jr.** 1987. Therapy of relapsing *Clostridium difficile*-associated diarrhea and colitis with the combination of vancomycin and rifampin. J. Clin. Gastroenterol. **9:**155–159.
5. **Carter, G. P., J. I. Rood, and D. Lyras.** 2010. The role of toxin A and toxin B in *Clostridium difficile*-associated disease: past and present perspectives. Gut Microbes **1:**58–64.
6. **Dove, C. H., et al.** 1990. Molecular characterization of the *Clostridium difficile* toxin A gene. Infect. Immun. **58:**480–488.
7. **Du, T., and M. J. Alfa.** 2004. Translocation of *Clostridium difficile* toxin B across polarized Caco-2 cell monolayers is enhanced by toxin A. Can. J. Infect. Dis. **15:**83–88.
8. **Duc, L. H., H. A. Hong, N. Fairweather, E. Ricca, and S. M. Cutting.** 2003. Bacterial spores as vaccine vehicles. Infect. Immun. **71:**2810–2818.
9. **Giannasca, P. J., and M. Warny.** 2004. Active and passive immunization against *Clostridium difficile* diarrhea and colitis. Vaccine **22:**848–856.
10. **Goulding, D., et al.** 2009. Distinctive profiles of infection and pathology in hamsters infected with *Clostridium difficile* strains 630 and B1. Infect. Immun. **77:**5478–5485.
11. **Harwood, C. R., and S. M. Cutting.** 1990. Molecular biological methods for *Bacillus.* John Wiley & Sons Ltd., Chichester, England.
12. **Hickson, M., et al.** 2007. Use of probiotic *Lactobacillus* preparation to prevent diarrhoea associated with antibiotics: randomised double blind placebo controlled trial. BMJ **335:**80.
13. **Hoang, T. H., H. A. Hong, G. C. Clark, R. W. Titball, and S. M. Cutting.** 2008. Recombinant *Bacillus subtilis* expressing the *Clostridium perfringens* alpha toxoid is a candidate orally delivered vaccine against necrotic enteritis. Infect. Immun. **76:**5257–5265.
14. **Hong, H. A., H. le Duc, and S. M. Cutting.** 2005. The use of bacterial spore formers as probiotics. FEMS Microbiol. Rev. **29:**813–835.
15. **Huang, J. M., et al.** 2010. Mucosal delivery of antigens using adsorption to bacterial spores. Vaccine **28:**1021–1030.
16. **Isticato, R., et al.** 2001. Surface display of recombinant proteins on *Bacillus subtilis* spores. J. Bacteriol. **183:**6294–6301.
17. **Jank, T., T. Giesemann, and K. Aktories.** 2007. Rho-glucosylating *Clostridium difficile* toxins A and B: new insights into structure and function. Glycobiology **17:**15R–22R.
18. **Jobe, B. A., A. Grasley, K. E. Deveney, C. W. Deveney, and B. C. Sheppard.** 1995. *Clostridium difficile* colitis: an increasing hospital-acquired illness. Am. J. Surg. **169:**480–483.
19. **Johnson, S., W. D. Sypura, D. N. Gerding, S. L. Ewing, and E. N. Janoff.** 1995. Selective neutralization of a bacterial enterotoxin by serum immunoglobulin A in response to mucosal disease. Infect. Immun. **63:**3166–3173.
20. **Kim, P. H., J. P. Iaconis, and R. D. Rolfe.** 1987. Immunization of adult hamsters against *Clostridium difficile*-associated ileocecitis and transfer of protection to infant hamsters. Infect. Immun. **55:**2984–2992.
21. **Kink, J. A., and J. A. Williams.** 1998. Antibodies to recombinant *Clostridium difficile* toxins A and B are an effective treatment and prevent relapse of *C. difficile*-associated disease in a hamster model of infection. Infect. Immun. **66:**2018–2025.
22. **Kotloff, K. L., et al.** 2001. Safety and immunogenicity of increasing doses of a *Clostridium difficile* toxoid vaccine administered to healthy adults. Infect. Immun. **69:**988–995.
23. **Kuehne, S. A., et al.** 2010. The role of toxin A and toxin B in *Clostridium difficile* infection. Nature **467:**711–713.
24. **Kyne, L., M. Warny, A. Qamar, and C. P. Kelly.** 2000. Asymptomatic carriage of *Clostridium difficile* and serum levels of IgG antibody against toxin A. N. Engl. J. Med. **342:**390–397.
25. **Lawley, T. D., et al.** 2009. Proteomic and genomic characterization of highly infectious *Clostridium difficile* 630 spores. J. Bacteriol. **191:**5377–5386.
26. **Leav, B. A., et al.** 2010. Serum anti-toxin B antibody correlates with protection from recurrent *Clostridium difficile* infection (CDI). Vaccine **28:**965–969.
27. **Lowy, I., et al.** 2010. Treatment with monoclonal antibodies against *Clostridium difficile* toxins. N. Engl. J. Med. **362:**197–205.
28. **Lyerly, D. M., H. C. Krivan, and T. D. Wilkins.** 1988. *Clostridium difficile*: its disease and toxins. Clin. Microbiol. Rev. **1:**1–18.
29. **Lyerly, D. M., K. E. Saum, D. K. MacDonald, and T. D. Wilkins.** 1985. Effects of *Clostridium difficile* toxins given intragastrically to animals. Infect. Immun. **47:**349–352.
30. **Lyras, D., et al.** 2009. Toxin B is essential for virulence of *Clostridium difficile*. Nature **458:**1176–1179.
31. **Mauriello, E. M., et al.** 2004. Display of heterologous antigens on the *Bacillus subtilis* spore coat using CotC as a fusion partner. Vaccine **22:**1177–1187.
32. **Rupnik, M., M. H. Wilcox, and D. N. Gerding.** 2009. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. Nat. Rev. Microbiol. **7:**526–536.
33. **Sambol, S. P., J. K. Tang, M. M. Merrigan, S. Johnson, and D. N. Gerding.** 2001. Infection of hamsters with epidemiologically important strains of *Clostridium difficile.* J. Infect. Dis. **183:**1760–1766.
34. **Sauerborn, M., P. Leukel, and C. von Eichel-Streiber.** 1997. The C-terminal ligand-binding domain of *Clostridium difficile* toxin A (TcdA) abrogates TcdA-specific binding to cells and prevents mouse lethality. FEMS Microbiol. Lett. **155:**45–54.
35. **Stubbe, H., J. Berdoz, J. P. Kraehenbuhl, and B. Corthesy.** 2000. Polymeric IgA is superior to monomeric IgA and IgG carrying the same variable domain in preventing *Clostridium difficile* toxin A damaging of T84 monolayers. J. Immunol. **164:**1952–1960.
36. **Surawicz, C. M., et al.** 2000. The search for a better treatment for recurrent *Clostridium difficile* disease: use of high-dose vancomycin combined with *Saccharomyces boulardii.* Clin. Infect. Dis. **31:**1012–1017.
37. **Tam, N. K., et al.** 2006. The intestinal life cycle of *Bacillus subtilis* and close relatives. J. Bacteriol. **188:**2692–2700.
38. **Torres, J., M. Camorlinga-Ponce, and O. Munoz.** 1992. Sensitivity in culture of epithelial cells from rhesus monkey kidney and human colon carcinoma to toxins A and B from *Clostridium difficile*. Toxicon **30:**419–426.
39. **Torres, J. F., D. M. Lyerly, J. E. Hill, and T. P. Monath.** 1995. Evaluation of formalin-inactivated *Clostridium difficile* vaccines administered by parenteral and mucosal routes of immunization in hamsters. Infect. Immun. **63:**4619–4627.
40. **von Eichel-Streiber, C., R. Laufenberg-Feldmann, S. Sartingen, J. Schulze, and M. Sauerborn.** 1992. Comparative sequence analysis of the *Clostridium difficile* toxins A and B. Mol. Gen. Genet. **233:**260–268.
41. **von Eichel-Streiber, C., I. Warfolomeow, D. Knautz, M. Sauerborn, and U. Hadding.** 1991. Morphological changes in adherent cells induced by *Clostridium difficile* toxins. Biochem. Soc. Trans. **19:**1154–1160.
42. **Voth, D. E., and J. D. Ballard.** 2005. *Clostridium difficile* toxins: mechanism of action and role in disease. Clin. Microbiol. Rev. **18:**247–263.
43. **Ward, S. J., G. Douce, G. Dougan, and B. W. Wren.** 1999. Local and systemic neutralizing antibody responses induced by intranasal immunization with the nontoxic binding domain of toxin A from *Clostridium difficile*. Infect. Immun. **67:**5124–5132.
44. **Ward, S. J., G. Douce, D. Figueiredo, G. Dougan, and B. W. Wren.** 1999. Immunogenicity of a *Salmonella typhimurium aroA aroD* vaccine expressing a nontoxic domain of *Clostridium difficile* toxin A. Infect. Immun. **67:**2145–2152.

Editor: R. P. Morrison