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MICROBIAL IMMUNITY AND VACCINES

Mucosal Antibodies to the C Terminus of Toxin A Prevent Colonization of *Clostridium difficile*

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ABSTRACT Mucosal immunity is considered important for protection against *Clostridium difficile* infection (CDI). We show that in hamsters immunized with *Bacillus subtilis* spores expressing a carboxy-terminal segment ($TcdA_{26-39}$) of *C. difficile* toxin A, no colonization occurs in protected animals when challenged with *C. difficile* strain 630. In contrast, animals immunized with toxoids showed no protection and remained fully colonized. Along with neutralizing toxins, antibodies to $TcdA_{26-39}$ (but not to toxoids), whether raised to the recombinant protein or to $TcdA_{26-39}$ expressed on the *B. subtilis* spore surface, cross-react with a number of seemingly unrelated proteins expressed on the vegetative cell surface or spore coat of *C. difficile*. These include two dehydrogenases, AdhE1 and LdhA, as well as the CdeC protein that is present on the spore. Anti-$TcdA_{26-39}$ mucosal antibodies obtained following immunization with recombinant *B. subtilis* spores were able to reduce the adhesion of *C. difficile* to mucus-producing intestinal cells. This cross-reaction is intriguing yet important since it illustrates the importance of mucosal immunity for complete protection against CDI.

KEYWORDS *Clostridium difficile*, colonization, immune exclusion, mucosal immunity, oral vaccines

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Clostridium difficile infection (CDI) has emerged as a major public health burden and one of the leading causes of antibiotic-associated diarrhea. Symptoms of disease are typically mild diarrhea, but in nonimmunocompetent hospital patients, more severe, and potentially fatal, colitis and toxic megacolon can occur (1–3). Disease is presumed to result from antibiotic treatment that disrupts the intestinal microbiota, enabling resident spores of *C. difficile* to germinate and outgrow. Spores are either resident in low numbers or acquired from the environment, and dysbiosis provides a temporary environmental niche enabling *C. difficile* to proliferate and secrete two cytotoxins (toxins A and B), resulting in inflammation and tissue damage (3, 4). The spore form of *C. difficile* plays an important role in this disease, and in hospitals, spores persist due to shedding from infected patients (5, 6). In murine systems, the transmission and dissemination of *C. difficile* spores have been studied in detail, providing a number of important insights into human disease (7). First, while spores can persist in the gastrointestinal (GI) tract in a carrier state without symptoms, upon antibiotic treatment, the number of spores dramatically increases by over 6 logs in as little as 2 days. Second, spores shed in the feces are highly infectious and potentially fatal in mice with a compromised innate immune system. Antibiotic therapy is the most frequent

clinical treatment, but recurrence is both common (~20% of patients) and potentially life-threatening (1, 2).

Active vaccination is a dependable approach to providing long-term protection against primary infection as well as recurrence. Current vaccine approaches have focused on humoral immunity and neutralization of toxins A and B, which are considered the primary virulence factors (8, 9). Thus, vaccines currently in clinical evaluation use parenteral administration of toxoids (A and B) or defined segments of them (10). These vaccines generate antitoxin IgG, and preclinical studies show protection (11). While protection against symptoms of CDI would be expected, there is no report of any effect on bacterial colonization (12). This approach, then, is limited since it does not address the primary stages of colonization and infection. One of the goals of vaccination is to eradicate the pathogen, and for CDI, this point is salient since, as mentioned above, infection results in the shedding of significant numbers of spores into the environment. For hospitals, this presents a significant burden for control and containment, and in addition, there are other concerns. First, *C. difficile* is known to carry multiple antibiotic resistance elements, and although not yet documented, the potential for the acquisition and transfer of resistance genes seems likely (13–15). Second, animals, including livestock, provide a potent reservoir of *C. difficile*, and the transfer of animal-derived strains to humans is assumed to have occurred (16, 17). It is therefore likely that a vaccine focused only on the neutralization of toxins will have little impact on the continued evolution and dissemination of this pathogen.

Several lines of evidence show that colonization resistance improves protection against CDI. First, fecal microbiota transplantation (FMT), where extracts of feces are administered to patients via enema or nasogastric tube, restores colonization resistance and provides protection against recurrence (18). Second, nontoxigenic strains (e.g., strain NTCD-M3) of *C. difficile* orally administered to patients prevent recurrence (19–21). Although the underlying mechanisms are not known, it is assumed that nontoxigenic strains such as NTCD-M3 transiently colonize the GI tract and presumably outcompete, or exclude, the pathogenic strain.

An oral vaccine consisting of spores of *Bacillus subtilis* (named PP108) for the delivery of a defined segment of toxin A corresponding to the toxin cell-binding domain ($TcdA_{26-39}$) has been reported by us (22). These heat-stable spores were engineered to express $TcdA_{26-39}$ on their surface, and in immunized animals, they generated IgG as well as secretory IgA (SIgA), both of which were able to neutralize toxin A and toxin B. In hamsters, 75% protection against primary CDI was observed, and animals were fully protected against rechallenge (simulating recurrence).

In this study, we have characterized the *B. subtilis* spore vaccine in greater detail, showing that it prevents bacterial colonization in hamsters challenged with *C. difficile*. In contrast, animals immunized by injection with toxoids A and B remained fully colonized. We then demonstrated that antibodies against the $TcdA_{26-39}$ domain recognize a number of seemingly unrelated proteins found on vegetative cells and spores of *C. difficile*. We further show that anti-$TcdA_{26-39}$ mucosal IgA can reduce the adhesion of vegetative *C. difficile* cells to mucus-producing cells. This finding is important because, first, it defines the requirements for the design of efficacious vaccines and, second, it can, in part, provide a mechanism for colonization resistance.

RESULTS

Oral immunization with PP108 spores prevents colonization by *C. difficile*. Spores of *B. subtilis* strain PP108 were produced according to contract good manufacturing practice (cGMP) standards and used to dose hamsters either in the live form or following formaldehyde inactivation. The dosing strategy involved a combined sublingual-oral (s.o.) administration of four cycles, after which animals were treated with clindamycin to disrupt the intestinal microbiota before challenge (intragastrically [i.g.]) with 100 spores of *C. difficile* strain 630. Control groups included animals dosed s.o. with live *B. subtilis* PY79 spores (identical to PP108 but not presenting the $TcdA_{26-39}$ antigen), animals dosed intramuscularly (i.m.) with the recombinant $TcdA_{26-39}$

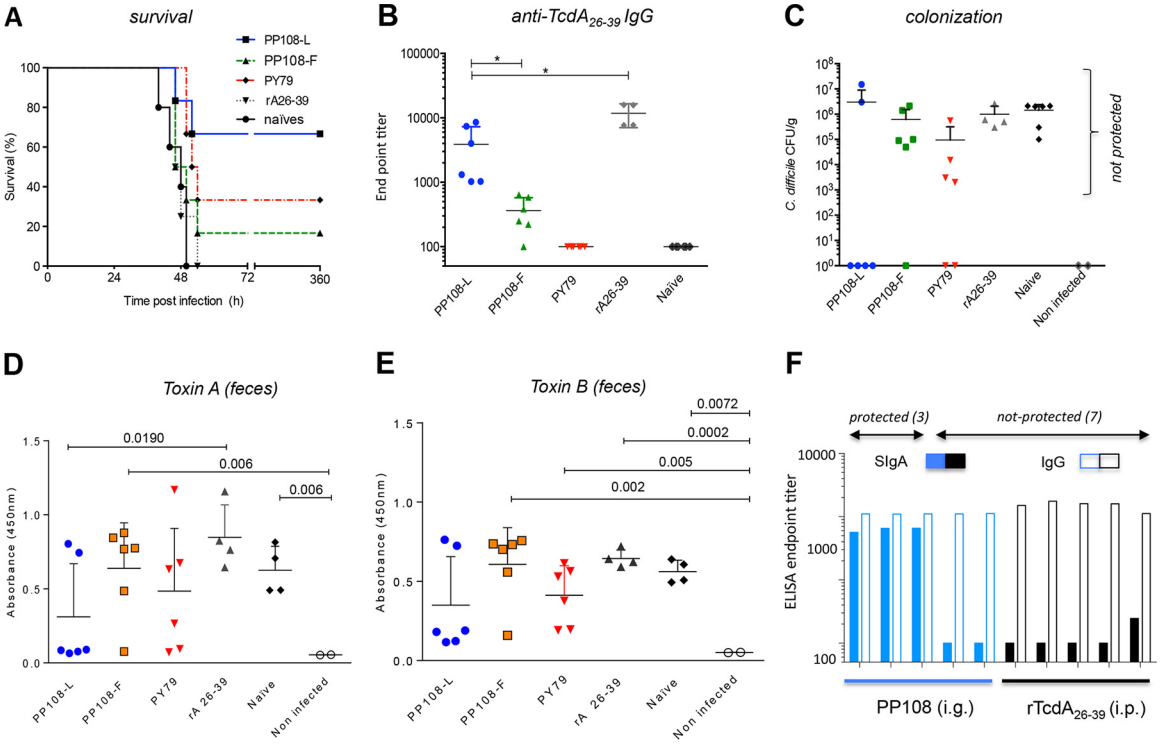

FIG 1 Mucosal vaccination with PP108 spores. Hamsters (6/group) were immunized by the s.o. route with live (PP108-L) or formalin-inactivated (PP108-F) PP108 spores, live PY79 spores, or the $rTcdA_{26-39}$ protein by injection (i.m.). (A) Kaplan-Meier survival estimates after oral challenge of these animals with 100 spores of *C. difficile* 630. (B) Anti-$TcdA_{26-39}$ IgG titers in individual animals from each group 2 days before challenge. *, $P < 0.05$. (C) Counts of 630 spores in feces at 24 h postchallenge in individual animals. (D and E) Levels of toxin A (D) and toxin B (E) detected in hamster feces from individual groups. A capture ELISA was used to determine toxin levels, and the presence of functional toxins was also confirmed by using a cell cytotoxicity assay to measure toxin A (HT29 cells) and toxin B (Vero cells) levels (data not shown). (F) Analysis of fecal IgA and serum IgG obtained from a separate hamster experiment. In this study, groups ($n = 5$) were orally dosed with PP108 spores or by injection (i.m.) with $rTcdA_{26-39}$, with 3/5 hamsters being protected in the PP108-dosed group. i.p., intraperitoneal.

($rTcdA_{26-39}$) protein, and naive animals. All naive animals and those parenterally immunized with $rTcdA_{26-39}$ ($P = 0.6497$) succumbed to CDI and died within 50 h (Fig. 1A). For animals dosed with live PP108 spores, 67% were protected ($P = 0.0115$). For animals dosed with formaldehyde-inactivated PP108 spores, 17% were protected ($P = 0.2528$), and for those dosed with live PY79 spores, 33% survived ($P = 0.0084$). Using s.o. delivery, we obtained similar levels of protection using non-cGMP PP108 spores in two independent trial experiments (data not shown).

In surviving animals, dosed 7 days later with clindamycin (simulating recurrence), there was an absence of colonization (see Fig. S1 in the supplemental material), in agreement with data from our previous study (22). Anti-$TcdA_{26-39}$ IgG responses in serum were measured (Fig. 1B), showing that although high IgG titers were induced in animals dosed orally with PP108 and parenterally with $rTcdA_{26-39}$, protection was restricted to animals immunized with PP108. The formaldehyde-inactivated PP108 spores gave low IgG titers, indicating that the inactivation method impaired the immunogenicity of the expressed antigen, correlating with the low level of protection observed. As would be expected, PY79-dosed animals and naive animals produced no IgG response. Counts of *C. difficile* spores (CFU) in feces at 24 h postchallenge showed that in all protected animals, no viable *C. difficile* bacteria were detectable, indicating complete pathogen clearance. In contrast, high counts were present in all nonprotected animals (Fig. 1C). The levels of toxin A and toxin B in feces of protected (no toxins) and nonprotected (toxins present) animals were determined (Fig. 1D and E) and confirmed by using cytotoxicity assays (data not shown). This analysis together with bacteriological assessments (Fig. 1C) demonstrated the absence of infection (toxins and CFU) in protected animals. Similarly, for protected animals in which recur-

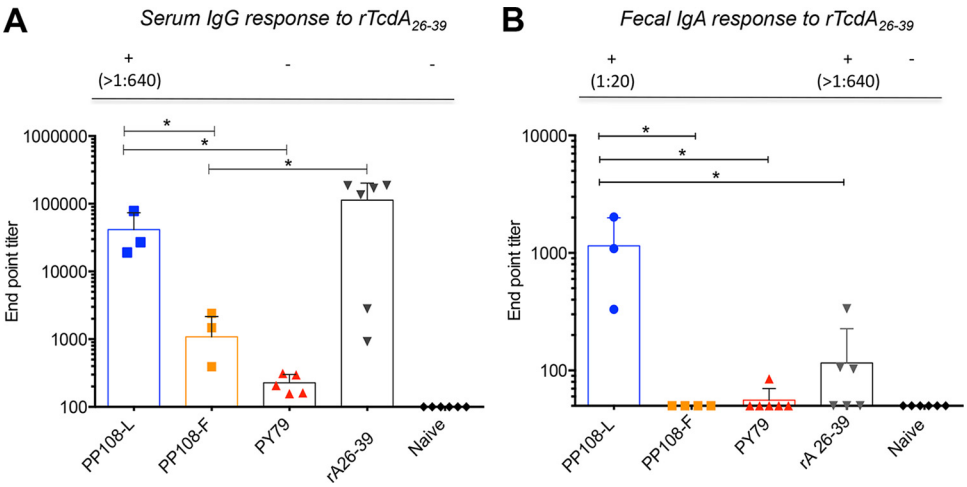

FIG 2 Immune responses in immunized CD-1 mice. Groups ($n = 6$) of CD-1 mice were immunized i.g. with live spores of PP108 (PP108-L), formaldehyde-inactivated PP108 spores (PP108-F), or live PY79 spores or parenterally (i.m.) with $rTcdA_{26-39}$ (5 μg/dose). Oral dosing was done by using 5×10^{10} CFU/dose on days 0, 1, 2, 14, 15, 16, 28, 29, 30, 55, 56, and 57. Parenteral dosing was done on days 0, 14, and 28. Fecal IgA was collected 10 to 15 days after the final immunization, serum IgG was collected 21 days after the final immunization (A), and anti-$TcdA_{26-39}$ titers were determined (B). *, $P < 0.05$. Neutralization endpoint titers are shown above the bars and were determined as described previously (22).

rence was induced, no toxins were present during days 3 to 15 following reinfection (Fig. S1B and S1C).

To measure SIgA levels in hamster feces, we used a goat anti-mouse secondary antibody to detect the heavy chain of IgA. Note that commercial secondary antibodies recognizing hamster SIgA are not currently available, but in our hands, anti-mouse IgA secondary antibody was suitable for the detection of fecal SIgA, as also reported previously (23). Using fecal samples taken 7 days after the final immunization, we were unable to detect SIgA. However, in a separate study, we were successful in detecting fecal IgA sampled 14 days following the final immunization (Fig. 1F). For simplicity, we show these data for animals dosed mucosally either with PP108 spores or by injection with $rTcdA_{26-39}$ (Fig. 1F) (regimens were the same as those used in the first study). In this study, 3/5 hamsters dosed with PP108 were protected and exhibited high levels of $TcdA_{26-39}$-specific SIgA as well as IgG. On the other hand, all animals dosed with $rTcdA_{26-39}$ and the two unprotected PP108-immunized animals showed no SIgA responses yet retained high levels of IgG. This demonstrates that protection against colonization best correlates with mucosal antibody responses.

To support this, we also immunized CD-1 mice with cGMP-compliant PP108 spores (s.o.) (live and inactivated) together with $rTcdA_{26-39}$ (parenteral). We then showed that vaccination with live PP108 spores induced neutralizing SIgA in feces as well as neutralizing serum IgG specific for $TcdA_{26-39}$, in contrast to injection of $rTcdA_{26-39}$, which induced neutralizing serum IgG only (Fig. 2).

First, this study demonstrates that protection against CDI correlates with a failure to colonize the host together with the production of mucosal antibodies rather than serum IgG. Second, spores of *B. subtilis* that do not express a *C. difficile* antigen can provide a low level of protection against CDI.

Parenteral vaccination with toxoids does not prevent colonization. Hamsters were given three i.m. injections of toxoids A and B (plus adjuvant) and, together with a naive group, then challenged (i.g.) with 100 spores of *C. difficile* strain 630. Compared to naive animals, symptoms of CDI were delayed, but all animals ultimately succumbed to infection (Fig. 3A) ($P = 0.0006$). In toxoid-immunized animals, serum IgG levels specific to toxins A and B were high (Fig. 3B), but counts of *C. difficile* spores in feces at 24 h postchallenge demonstrated that animals were fully colonized with *C. difficile* (Fig. 3C) at levels indistinguishable from those in naive animals. Consistent with these

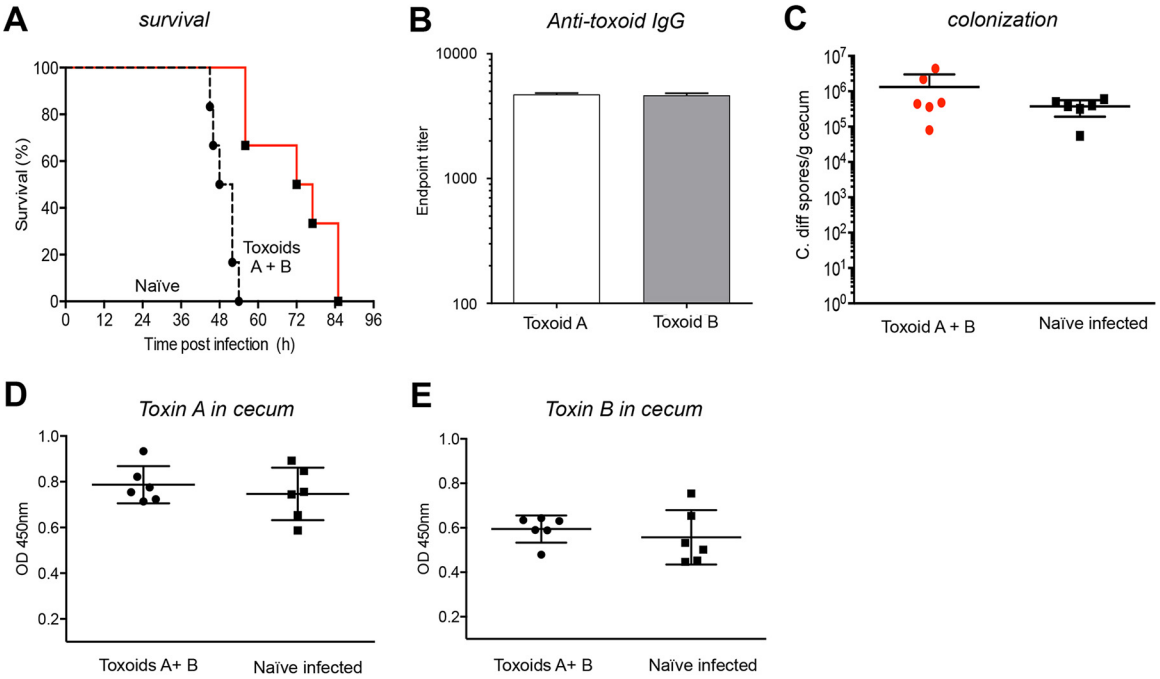

FIG 3 Parenteral vaccination with toxoids. Hamsters (6/group) were immunized with toxoids A and B using three injections (i.m.) and then challenged with 100 spores of *C. difficile* 630. (A) Survival of animals compared to the naive groups. (B) Anti-toxoid A and anti-toxoid B IgG in immunized groups 2 days before challenge. (C) Counts of 630 spores in feces at 24 h postchallenge in immunized and naive groups. This experiment was repeated once. (D and E) Levels of toxins A (D) and B (E) detected in cecal samples as determined by a capture ELISA, together with samples from nonimmunized animals (naive infected). Cell cytotoxicity assays were also used to confirm the presence of toxins.

observations, toxins A and B were readily detectable in cecum samples of sacrificed animals (Fig. 3D and E).

Antibodies to $TcdA_{26-39}$ cross-react with proteins associated with vegetative cells and spores. One explanation for how immunization with PP108 spores prevents colonization is by blocking the adhesion of the bacterium to the mucosa, a phenomenon associated with mucosal IgA and its ability to interfere with the adhesion of bacteria to the host (24). To accomplish this, we would have to reason that antibodies resulting from oral immunization of PP108 cross-react with vegetative cells or spores of *C. difficile*. Since nonrecombinant spores of PY79 were also able to generate some level of protection against CDI in the experiments described above, we wondered whether an antibody raised against *B. subtilis* spores might recognize *C. difficile* in either its spore or its vegetative cell form (Fig. 4). Rabbit IgG polyclonal antibodies (PAbs) raised against *B. subtilis* PY79 spores (confirmed in Fig. 4A, lane 4) exhibited no detectable cross-reactivity with vegetative cells of *C. difficile* 630 or a nontoxigenic isogenic mutant (Fig. 4A, lanes 3 and 4). However, two bands of about 14 and 30 kDa were detected in 630 spore coats (lane 5). Finally, antispore antibodies failed to recognize the r$TcdA_{26-39}$ protein (lane 1).

A second intriguing possibility was that antibodies to $TcdA_{26-39}$ were themselves able to recognize *C. difficile*. Using an enzyme-linked immunosorbent assay (ELISA), we showed that vegetative cells of 630 were recognized by $TcdA_{26-39}$-specific IgG raised in CD-1 mice by injection (Fig. 4B). Although toxins would be secreted, to confirm that these antibodies were recognizing proteins other than toxin A or toxin B, we showed a similar recognition of cells of an isogenic mutant carrying insertional mutations in the *tcdA* and *tcdB* genes (encoding toxins A and B) (Fig. 4B). Next, we used a panel of different antisera to probe either spores (Fig. 4C) or vegetative cells (Fig. 4D) of *C. difficile* 630 by an ELISA. Naive serum or antisera raised against *C. difficile* spores or vegetative cells (both by parenteral immunization) were used as negative or positive controls, respectively. Anti-PP108 and anti-$TcdA_{26-39}$ IgG recognized 630 spores

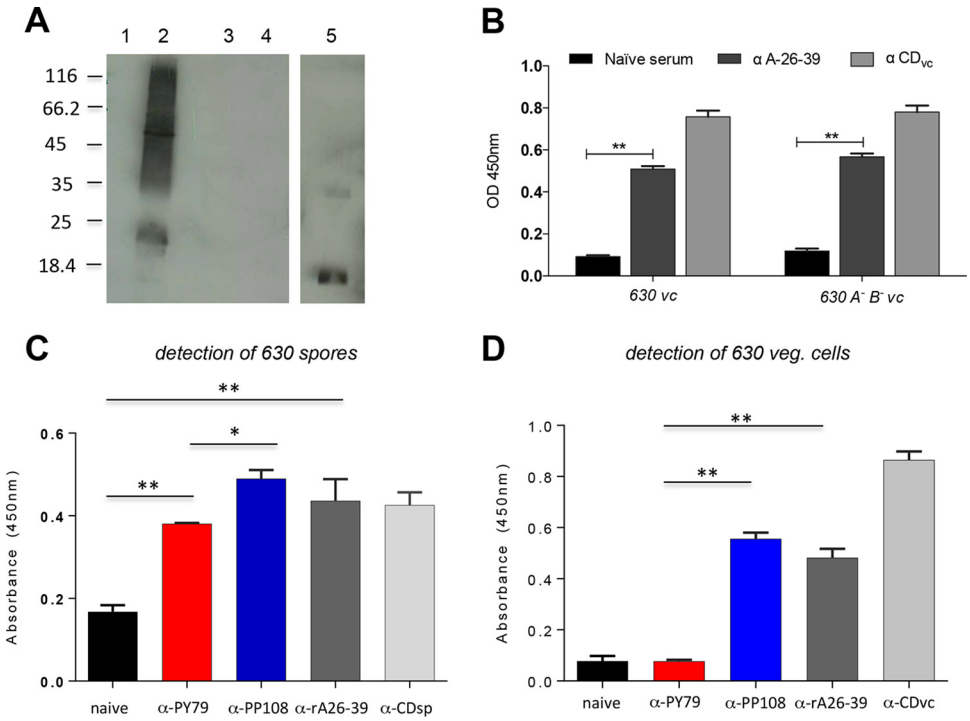

FIG 4 Anti-TcdA$_{26-39}$ antibodies recognize *C. difficile*. (A) Protein extracts were run on SDS-PAGE gels and probed with rabbit PAbs (1/6,000 dilution) raised against *B. subtilis* PY79 spores. Lane 1, rTcdA$_{26-39}$ protein (~0.7 to 1 μg); lane 2, spore coat proteins extracted from PY79 spores; lane 3, proteins extracted from *C. difficile* 630 vegetative cells; lane 4, extracts of vegetative cells of a toxin A-negative, toxin B-negative mutant; lane 5, spore coat extracts from 630 spores. (B) Cross-reaction to vegetative cells (vc) of 630 or an isogenic 630 mutant carrying insertions in the *tcdA* and *tcdB* genes (toxin A negative and toxin B negative) by an ELISA. Three murine antisera were used: naive, anti-TcdA$_{26-39}$, and anti-*C. difficile* 630. **, $P < 0.0018$. (C) Identification of cross-reactions of different murine antibodies to spores of *C. difficile* 630 by an ELISA. Wells of 96-well plates were coated with spores (~10^8 spores/well), and sera (all diluted 1/300) from immunized mice were used for detection by an ELISA. α-rA26-39, anti-TcdA$_{26-39}$; α-CDsp, anti-*C. difficile* 630 spores. **, $P = 0.008$; *, $P < 0.0112$. (D) Identification of cross-reactions of different murine antibodies to vegetative cells of *C. difficile* 630 by an ELISA. Wells of 96-well plates were coated with spores (~10^8 spores/well), and sera (all diluted 1/300) from immunized mice were used for detection by an ELISA. α-rA26-39, anti-TcdA$_{26-39}$; α-CDvc, anti-*C. difficile* 630 vegetative cells. **, $P < 0.002$.

(Fig. 4C) and vegetative cells (Fig. 4D) at levels significantly higher than those in naive animals and at levels similar to those of anti-630 spore or anti-630 vegetative cell PAbs. Using an ELISA, we observed that anti-PY79 spore PAbs also recognized 630 spores but not 630 vegetative cells, potentially correlating with data from our Western blot analysis described above (Fig. 4A). Taken together, data from this analysis show first that antibodies to TcdA$_{26-39}$, whether raised against the recombinant protein or when presented on *B. subtilis* spores (PP108), can recognize both spores and vegetative cells of *C. difficile* and second that antibodies to *B. subtilis* PY79 spores are also able to recognize *C. difficile* spores but not vegetative cells.

TcdA$_{26-39}$-specific antibodies recognize two dehydrogenases and an exosporial protein. Using PAbs raised in rabbits against recombinant TcdA$_{26-39}$, we probed extracts of 630 vegetative cells and proteins extracted from 630 spores by Western blotting (Fig. 5A). These antibodies recognized a high-molecular-mass protein (~100 kDa) present in vegetative cells (Fig. 5A, lane 3). To exclude the possibility that this protein was either toxin A or toxin B, we showed that the same band was detectable in an isogenic mutant insertionally inactivated in the *tcdA* and *tcdB* genes encoding toxins A and B, respectively (lane 4). Similarly, the band was also apparent in cells of an isogenic strain (Δ*sigK*) that is unable to form spores (lane 5) due to an insertion in the *sigK* gene encoding a sporulation-specific transcription factor (σ^K), confirming that the band originated from the vegetative cell and not from a contaminating spore. The same PAbs also recognized a number of bands in extracts of 630 spores running at ~100, 60,

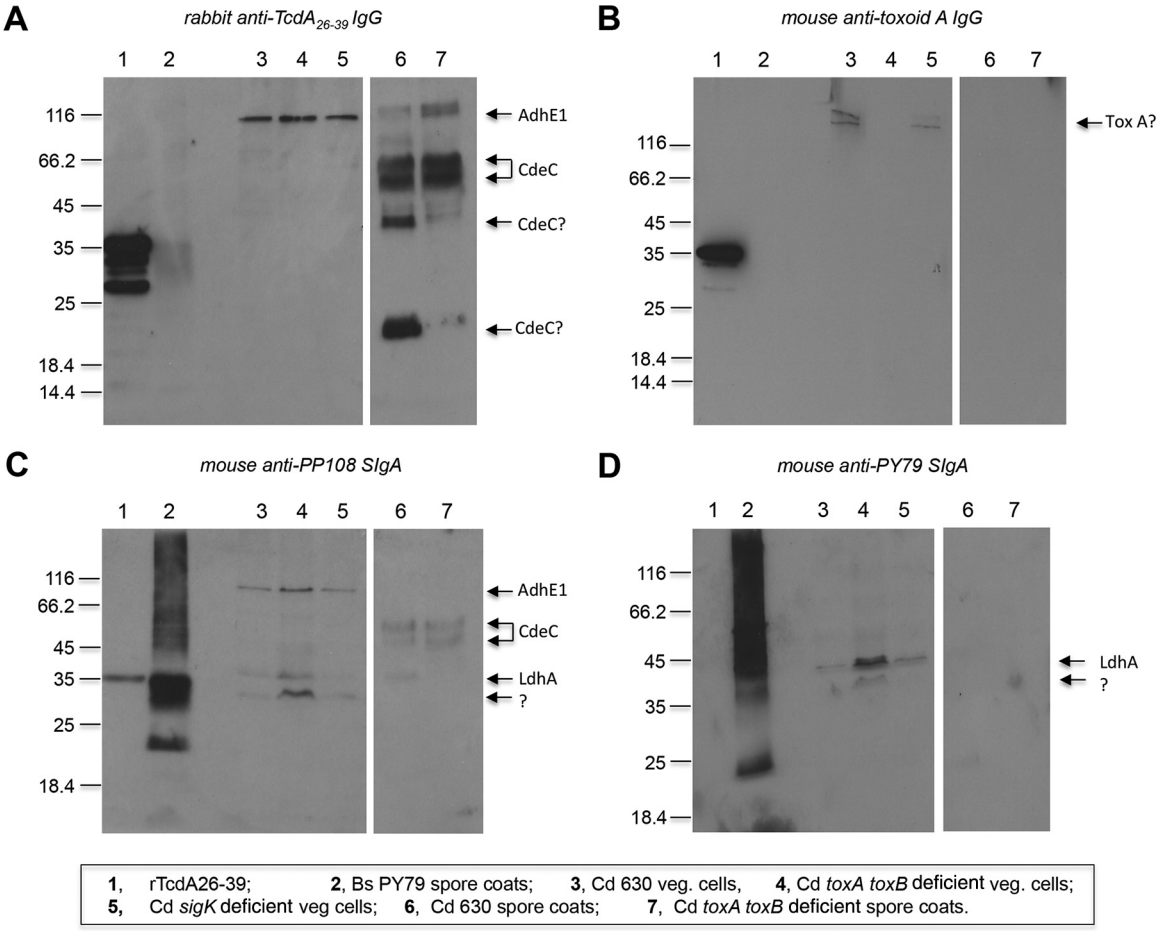

FIG 5 *C. difficile* proteins recognized by anti-TcdA$_{26-39}$. Shown are Western blots of proteins probed with rabbit anti-rTcdA$_{26-39}$ IgG (1/3,000 dilution) (A), mouse anti-toxoid A IgG (1/5,000 dilution) (B), mouse anti-PP108 IgA (1/200 dilution) (C), and mouse anti-PY79 IgA (1/200 dilution) (D). SDS-PAGE gels were loaded as follows: lane 1, rTcdA$_{26-39}$ protein (~1 μg); lane 2, extracts of *B. subtilis* (Bs) PY79 spore coats; lane 3, extracts of *C. difficile* (Cd) 630 vegetative cells; lane 4, extracts of vegetative cells of a 630 strain deficient in toxin A and toxin B; lane 5, extracts of vegetative cells of a 630 strain unable to produce the sporulation transcription factor σ^K; lane 6, spore coat protein extracts from 630 spores; lane 7, spore coat protein extracts from spores deficient in the production of toxin A and toxin B. Molecular mass markers (in kilodaltons) are shown, together with the identity of known proteins or proteins referred to in the text.

50, 40, and 20 kDa (Fig. 5A, lane 6). To exclude the possibility that the spore-associated proteins were toxin fragments, we probed extracts from spore coats of a toxin-deficient strain (lane 7). With the exception of the 40-kDa band (which might therefore be toxin derived), the same polypeptides were recognized. To identify proteins recognized by anti-TcdA$_{26-39}$ PAbs, we used mass spectrometry (MS) analysis (see Table S1 in the supplemental material). In both vegetative cells and spore extracts, the ~100-kDa band was identified as AdhE1 (aldehyde alcohol dehydrogenase), and the 50-kDa spore-specific species was identified as CdeC (25).

When we conducted the same analysis using a PAb to toxoid A, we failed to identify any of the above-described protein bands using the same extracts as those used for the anti-TcdA$_{26-39}$ blots, which were run in parallel (Fig. 5B). The integrity of the antibodies was confirmed by the recognition of rTcdA$_{26-39}$ (Fig. 5B, lane 1). High-molecular-mass bands (>140 kDa) were apparent in 630 vegetative cells (lane 3) and the Δ*sigK* mutant (lane 5) but were absent in the toxin-deficient mutant (lane 4), indicating that these bands corresponded to toxin A and possibly also toxin B.

These experiments were repeated with three independent MS analyses. We next examined mucosal antibodies raised in CD-1 mice that had been immunized (s.o.) with PP108 spores (Fig. 5C). As expected, fecal IgA reacted with rTcdA$_{26-39}$ (Fig. 5C, lane 1) and PY79 spore coat extracts (lane 2). Fecal IgA was also able to detect a 100-kDa

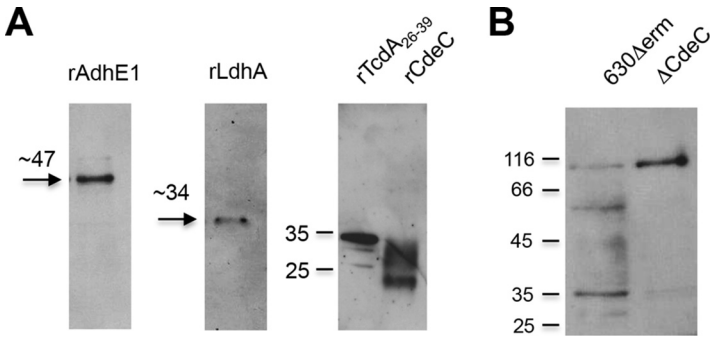

FIG 6 Verification of cross-reacting proteins. (A) Recombinant proteins identified by anti-TcdA_{26-39}. Approximately 1 μg of purified protein was run per lane and probed with antibodies at a dilution of 1/4,000. (B) Bands recognized in proteins extracted from spores of the wild type (630Δerm) and a Δ*cdeC* mutant probed with anti-TcdA_{26-39} (1/4,000 dilution). Molecular masses (in kilodaltons) are indicated.

species in *C. difficile* 630 vegetative cells (lanes 3 to 5), which was identified as AdhE1 by using MS. In addition, bands of 30 and 36 kDa were apparent. The upper band was identified as *R*-2-hydroxyisocaproate dehydrogenase (also referred to as lactate dehydrogenase [LdhA]). Analysis of spore coat extracts revealed a number of bands of 60, 50, and 40 kDa. MS analysis was used to confirm that these bands were indeed CdeC. Finally, we used fecal IgA obtained from mice orally immunized with PY79 spores (Fig. 2), and therefore devoid of TcdA_{26-39}, to probe extracts (Fig. 5D). These antibodies recognized coat protein extracts from PY79 spores (Fig. 5D, lane 2) but not r TcdA_{26-39} (lane 1). These antibodies also recognized cross-reacting bands of about 35 and 40 kDa in vegetative cell extracts (lanes 3 to 5), with the upper band corresponding to LdhA. The 100-kDa AdhE1 band was not detected by using these antibodies, confirming that this protein was uniquely recognized by anti-TcdA_{26-39} PAbs.

To confirm the identities of some of the principal proteins recognized by anti-TcdA_{26-39}, we used two approaches. First, we cloned and expressed the LdhA, AdhE1, and CdeC proteins in *Escherichia coli* as His-tagged polypeptides. For rLdhA, the full-length protein was expressed, while for rAdhE1 and rCdeC, sequences encoding N-terminal segments of each protein were cloned. Anti-TcdA_{26-39} PAbs were able to recognize each of these recombinant proteins as well as r TcdA_{26-39} (Fig. 6A). In the case of rAdhE1, a molecular mass of 47 kDa was detected because only the N-terminal half of AdhE1 was cloned and expressed, while for rCdeC, only a small N-terminal domain (~12 kDa) could be cloned. The appearance of multiple CdeC species of higher molecular masses is most probably due to extensive covalent cross-linking found in this cysteine-rich protein, as reported previously (25).

The anti-TcdA_{26-39} antibodies were raised to a recombinant His-tagged TcdA_{26-39} polypeptide, so it is formally possible that recognition arose due to antibodies recognizing the C-terminal polyhistidine moiety. Therefore, we used a more stringent approach and made ClosTron insertional mutants for each gene. Using Δ*cdeC* and 630Δerm spores, we probed coat proteins extracted from spores (Fig. 6B). In wild-type extracts, principal species of about 35, 50, and 100 kDa were observed, in agreement with data from previous studies showing that CdeC is present in multimeric forms in spores (25). In contrast, in the Δ*cdeC* mutant, with the exception of the 100-kDa species, these bands were absent, confirming that the bands of <100 kDa were indeed CdeC. The 100-kDa species that was recognized by anti-TcdA_{26-39} PAbs was identified as AdhE1 (26).

Antibodies to TcdA_{26-39} reduce adhesion of *C. difficile* to mucus-producing HT29-MTX cells. The human intestinal cell line HT29-MTX, derived from HT29, is a mucus-secreting cell line that contains differentiated goblet cells that secrete low levels of MUC2 mucins typically found in the small and large intestines (27). Cells grown for 14 days and confirmed to produce mucus by alcian blue staining were inoculated with vegetative cells or spores of strain 630 with or without adsorption with fecal IgA or

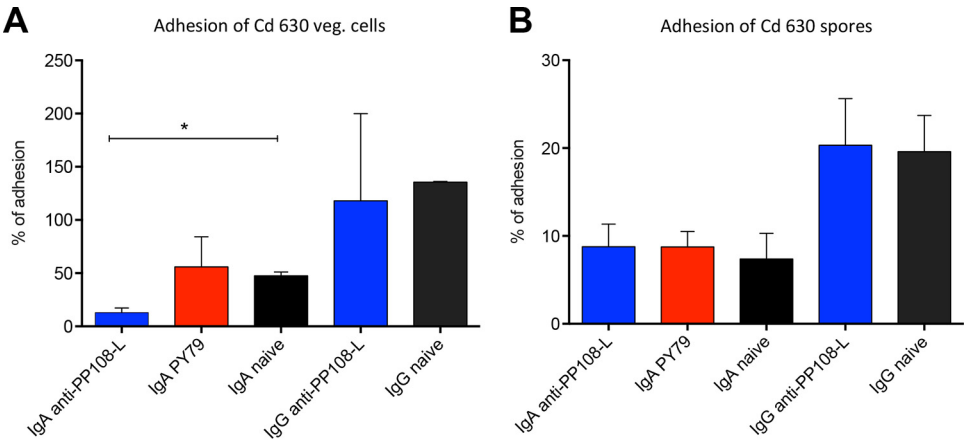

FIG 7 Mucosal antibodies to $TcdA_{26-39}$ reduce adhesion of vegetative cells to mucus-producing HT29-MTX cells. HT29-MTX cells were grown for 14 days, and the production of mucus was confirmed. Vegetative cells (A) or spores (B) of *C. difficile* 630 were pretreated with either serum IgG or fecal IgA (1/300 dilution) obtained from CD-1 mice immunized s.o. with live PP108 spores or live PY79 spores, as shown in Fig. 2. Adhesion to cells was expressed as a percentage of untreated cells or spores run in parallel. Adhesion of 100% equates to the number of cells or spores adhering to HT29-MTX cells without pretreatment and in parallel. The experiment was repeated two times, with similar findings. *, $P < 0.05$.

serum IgG obtained from CD-1 mice immunized (s.o.) with PP108 or PY79 spores (Fig. 7A). Naive IgA was capable of reducing adhesion, which is consistent with data from other studies showing that innate, nonspecific SIgA by itself has the ability to interfere with the attachment of cells to mucus (28). However, compared to naive cells, anti-PP108 fecal IgA significantly reduced the adhesion of vegetative cells to HT29-MTX cells. However, fecal IgA from animals immunized with PY79 spores did not show any reduction in adhesion compared to naive IgA. In contrast, anti-PP108 IgG showed no significant reduction in adhesion compared to naive IgG. When we conducted the same analysis on 630 spores, anti-PP108 IgA showed no effect on adhesion compared to anti-PY79 IgA or naive IgA, as was also the case with anti-PP108 IgG (Fig. 7B).

DISCUSSION

The oral PP108 vaccine utilizing bacterial spores for the display of a carboxy-terminal domain ($TcdA_{26-39}$) of toxin A was reported previously (22). The key findings, then, were that (i) oral delivery generated mucosal as well as systemic humoral neutralizing responses, but only mucosal antibodies correlated with protection; (ii) antibodies to the $TcdA_{26-39}$ domain cross-reacted with toxin B, presumably by virtue of shared homology between the C-terminal domains of toxins A and B; and (iii) immunization with nonrecombinant spores provided evidence of protection. Here, we show that the PP108 spore vaccine is also able to inhibit the colonization of hamsters with *C. difficile*. Colonization in this case was defined as the absence of toxins as well as viable spores in the feces. We assume that the complete protection observed here occurs because $TcdA_{26-39}$-specific IgA prevents the early stages of attachment of *C. difficile* vegetative cells to the mucosal epithelium. These initial stages of colonization are presumably critical to the infection process and prevent the subsequent establishment of the pathogen at the mucosal barrier, e.g., in the creation of biofilms (29). Therefore, this correlate of protection is particularly important and more meaningful than measurements of toxin levels alone.

Most intriguing was that in studies using the same hamster model of infection, parenteral immunization of toxoids A and B failed to confer protection, even though high levels of IgG were found in serum. It should be noted that, as far as we are aware, most vaccination studies using hamsters have used the VPI 10463 strain, since it is assumed to be highly toxigenic. Although not reported here, we also evaluated, in parallel, toxoid-immunized hamsters challenged with spores of VPI 10463 and obtained

only 25% protection. Our findings suggest that, at least in hamsters, 630 either is more infectious or produces more severe infection.

The single most important finding from this work is that antibodies to the $TcdA_{26-39}$ domain recognize not only $TcdA_{26-39}$ but also other peptide sequences. First, and as discovered previously (22), they recognize the C terminus of toxin B, and second, they react with a number of other proteins present in vegetative cells or spores of *C. difficile*, including AdhE1, LdhA, and CdeC. Intriguingly, we were unable to identify any meaningful homology in amino acid sequences between $TcdA_{26-39}$ and AdhE1, LdhA, and CdeC, although both AdhE1 and LdhA share some homology in their secondary structures (30). The ability of $TcdA_{26-39}$ antibodies to recognize additional *C. difficile* proteins could therefore provide immunized animals with the ability to recognize vegetative cells and spores of *C. difficile* during infection as well as neutralize toxins, explaining the complete absence of colonization in protected animals.

Mucosal antibodies consist primarily of SIgA, but opsonizing IgG could potentially also be present. SIgA is well known for its ability to interfere with the earliest steps of infection by blocking toxins and preventing the adhesion of pathogens to the mucosal epithelium (31). For example, SIgA has been shown to sterically hinder the interaction of cholera toxin with epithelial cells (32). The two dehydrogenases recognized by $TcdA_{26-39}$-specific antibodies, AdhE1 and LdhA, could also have potential roles in virulence. In *Listeria monocytogenes*, an aldehyde alcohol dehydrogenase is a housekeeping enzyme but is also a surface-exposed adhesin commonly referred to as LAP (*Listeria* adhesion protein) (33). In *C. difficile*, LdhA participates in a biosynthetic pathway, another member of which, HadA, has been found to be associated with biofilms, a key attribute linked to colonization (34). Both AdhE1 and LdhA may or may not be involved in virulence, and they may or may not be surface exposed, but what is important here is the demonstration that mucosal antibodies can block *C. difficile* colonization. It is also likely that there are other targets recognized by $TcdA_{26-39}$-specific antibodies, so the key points are that (i) cross-recognition of cell proteins by mucosal $TcdA_{26-39}$-specific antibodies can explain how the PP108 vaccine prevents both colonization and cytotoxicity and (ii) mucosal immunity is required for the prevention of *C. difficile* colonization. Since the $TcdA_{26-39}$ domain is part of toxin A, it is *prima facie* surprising that immunization with toxoids fails to generate cross-reactive antibodies. The explanation, though, may simply be that the tertiary structure of toxoid A fails to generate sufficient $TcdA_{26-39}$-specific antibodies in both quantity and avidity for cross-reaction to *C. difficile* vegetative cells or that it may be of the wrong isotype.

What is shown here is that mucosal antibodies targeting one or more antigens (or epitopes) block the interaction of the pathogen with the host and, as a result, prevent colonization. This finding suggests that to prevent CDI as well as relapse, an approach that addresses colonization must be considered (12). It is possible, then, that *C. difficile* colonization is particularly sensitive to interference, and this could be a factor in how other "nontoxin" approaches appear to work. For example, in this work, it is shown that the administration of nonrecombinant PY79 spores provided some level of protection, and for these animals, no colonization was present. For both rabbit anti-PY79 IgG and mouse anti-PY79 fecal IgA, some cross-recognition of *C. difficile* proteins was apparent, including the LdhA protein, which is supported by data from previous studies showing that oral dosing with *B. subtilis* probiotic spores reduces symptoms of CDI (35). However, there is no significant sequence homology between *B. subtilis* Ldh and that of *C. difficile*.

We wonder whether immune exclusion may, in part, explain the ability of nontoxigenic cells of *C. difficile* to suppress symptoms of CDI. Previous studies using pretreatment of animals with nontoxigenic strains of *C. difficile* demonstrated that the nontoxigenic strain can colonize the GI tract, and following challenge ~40 to 60 days later, animals were fully protected (21). It may therefore be of interest to investigate SIgA produced in animals dosed with atoxigenic *C. difficile* to determine whether it plays any role in preventing colonization. Finally, the ability to prevent adhesion using mucosal antibodies raises the possibility of developing specific immunity-based antiadhesion

therapies (36). In light of the studies described here, we believe that for complete protection against CDI, strategies that elicit mucosal immunity must be considered.

MATERIALS AND METHODS

Methods relating to the production of spores, antibodies, mutant constructions, and analysis of immune responses are described in the supplemental material.

Strains. PP108 is a recombinant strain of *B. subtilis* that expresses a carboxy-terminal domain of *C. difficile* toxin A (referred to here as $TcdA_{26-39}$ or A26-39 and carrying repeats spanning positions 26 to 39 [as defined in reference 37] and residues Ser_{2388} to Pro_{2706}) as a chimeric fusion to two individual *B. subtilis* spore coat proteins, CotB and CotC (22). PY79 is a nonrecombinant strain of *B. subtilis* and isogenic to PP108. *C. difficile* strain 630 (ribotype 012; toxin A positive and toxin B positive [38]) was used for challenge studies. A strain isogenic to 630 containing ClosTron insertional mutations in the *tcdA* (toxin A) and *tcdB* (toxin B) genes was obtained from N. Minton (9). ClosTron mutants isogenic to 630 and carrying insertions in the *sigK* gene (encoding sigma factor K of strain JP051) or the *cdeC* genes (strain SH2000) were made in this study (see methods in the supplemental material).

Immunizations. Golden Syrian hamsters (aged 5 to 6 weeks and weighing 120 to 130 g) were dosed ($n = 6$) with live or formaldehyde-inactivated PP108 spores and live PY79 spores (5×10^{10} spores/dose) in 4 dosing cycles (cycle 1, days 1, 2, and 3; cycle 2, days 14, 15, and 16; cycle 3, days 28, 29, and 30; cycle 4, days 42, 43, and 44). Dosing was done by utilizing combined s.o. administration, where the animals received 50 μl by sublingual administration and 150 μl via oral gavage. For sublingual administration, animals were anesthetized (isoflurane), and the test substance was allowed to adsorb for 30 min. Additional groups included those receiving the $rTcdA_{26-39}$ protein (10 μg/dose) by injection (i.m.) on days 1, 14, and 28. For immunization with toxoids, animals were given three i.m. injections (days 1, 14, and 28) of both toxoid A and toxoid B (Native Antigen Company) at 5 μg/dose, resuspended in an alum adjuvant in saline buffer. All animal work was performed under UK Home Office project license PPL 70/8276.

Challenge studies. Preliminary challenge experiments using nonimmunized hamsters were first conducted to confirm the infectious dose of 630 spores. The challenge commenced 14 days after the last immunization and when animals were transferred to individual IVCs (independently ventilated cages) and then treated with clindamycin (clindamycin-2-phosphate at 30 mg/kg body weight; Sigma). Twelve hours after antibiotic treatment, animals were dosed i.g. with 100 spores of strain 630. Animals were transferred to new cages every 2 days and monitored every 3 h until clear symptoms developed, after which infected animals were killed and considered unprotected. Symptoms were defined as "wet tail," where animals developed watery diarrhea, lethargy, and irritability and refused food.

Colonization. To determine colonization, feces was collected at 2 days postchallenge, homogenized in 70% ethanol, incubated overnight, serially diluted in sterile water, and plated onto ChromID plates (bioMérieux). Plates were incubated anaerobically (37°C) for 2 days before counting.

Immunological analysis. (i) Mice. Basic methodologies for immunological analysis of mice were described previously (22). Levels of serum anti-$TcdA_{26-39}$ IgG and fecal IgA from CD-1 mice were determined by an indirect ELISA. Greiner 96-well plates were coated with the purified $rTcdA_{26-39}$ (2 μg/ml) protein in phosphate-buffered saline (PBS) overnight at room temperature (RT). After this, the plates were blocked for 1 h at 37°C with 2% bovine serum albumin (BSA), and 2-fold serially diluted samples were added at a starting dilution of 1/50 in diluent buffer (0.01 M PBS [pH 7.4], 1% BSA, 0.05% Tween 20). Replicate samples were used together with a negative control (preimmune or naive serum). Plates were incubated for 2 h at 30°C before the addition of the appropriate horseradish peroxidase (HRP)-conjugated anti-mouse antibodies in diluent buffer. Plates were incubated for 1 h at RT and then developed by using the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate. Reactions were stopped by 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 as the maximum dilution of serum giving an absorbance reading of 0.1 over the ODs of naive samples.

(ii) Hamsters. ***(a) Serum IgG.*** Blood was withdrawn from the saphenous vein 2 days before challenge. Greiner Bio One 96-well high-binding plates were coated with proteins (50 μl/well). For anti-$TcdA_{26-39}$ responses, plates were coated with the $rTcdA_{26-39}$ protein (2 μg/ml) in PBS, while for antitoxin responses, plates were coated with toxins A and B (2 μg/ml; Sigma). Plates were left overnight at 4°C and then blocked for 1 h with 2% BSA diluted in PBS. Samples were loaded using 2-fold dilutions (diluent containing PBS, 0.05% Tween 20, and 1% BSA). The starting dilution for serum samples was 1/100. Samples were incubated for 2 h at RT before incubation with HRP-conjugated goat anti-hamster IgG antibodies (1/7,500) in diluent buffer (PBS, 1% BSA, 0.05% Tween 20). Plates were incubated for 1 h at RT and developed by using the TMB substrate (BioLegend). Reactions were stopped by using 2 M H_2SO_4, and the OD was read at 450 nm. Dilution curves were determined for each sample, and the endpoint titer for each sample was estimated according to the ODs of naive samples.

(b) Fecal IgA. Fecal samples were collected 2 days before challenge and broken up by using wooden sticks in extraction buffer at an approximate ratio of 1:5 (feces to buffer). The extraction buffer was made up in PBS containing 2% (vol/vol) fetal calf serum, penicillin-streptomycin (10 ml/liter) (catalog number P4333; Sigma), and Pierce protease inhibitor tablets (catalog number 88265; Thermo). Suspensions were shaken gently at 4°C for 4 h and then centrifuged (13,000 rpm for 15 min in a benchtop Microfuge), and the supernatant was retained. An indirect ELISA was performed as described above for serum but with the following modifications: (i) the starting dilution was 1/5 or 1/10, and incubation was done at 30°C for 2 h, and (ii) a conjugated mouse IgA-HRP antibody (Sigma) was used at a 1/1,000 dilution at RT for 1 h.

Identification of proteins cross-reacting with anti-TcdA$_{26-39}$. See the methods in the supplemental material for the construction of ClosTron mutants. rAdhE1, rLdhA, and rCdeC were purified by using Akta chromatography of His-tagged proteins. *E. coli* expression clones utilized pET28b vectors containing segments of *cdeC*, *adhE1*, and *ldhA* (see Table S1 in the supplemental material) as follows: Met1-Phe105 for *cdeC* (C-terminal His tag, 12.1 kDa), Lys2-Asn332 for *ldhA* (N-terminal His tag, 36.3 kDa), and Glu2-Gly420 for *adhE1* (N-terminal 45.2 kDa).

Statistics. The Mantel-Cox test was used for data in Fig. 1A and 3A, the Mann-Whitney test was used for data in Fig. 1B to D and 2, a paired *t* test was used for data in Fig. 4C and D, and an unpaired *t* test was used for data in Fig. 7.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.01060-16.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 3, PDF file, 0.1 MB.

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