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The *Clostridium difficile spo0A* Gene Is a Persistence and Transmission Factor

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***Clostridium difficile* is a major cause of chronic antibiotic-associated diarrhea and a significant health care-associated pathogen that forms highly resistant and infectious spores. Spo0A is a highly conserved transcriptional regulator that plays a key role in initiating sporulation in *Bacillus* and *Clostridium* species. Here, we use a murine model to study the role of the *C. difficile spo0A* gene during infection and transmission. We demonstrate that *C. difficile spo0A* mutant derivatives can cause intestinal disease but are unable to persist within and effectively transmit between mice. Thus, the *C. difficile* Spo0A protein plays a key role in persistent infection, including recurrence and host-to-host transmission in mice.**

Clostridium difficile is a Gram-positive, sporogenic, anaerobic bacterium that is the most common cause of antibiotic-associated diarrhea within health care systems of the developed world (24, 34). Perturbation of the hosts' indigenous microbiota by broad-spectrum antibiotic treatment, such as clindamycin, is a major risk factor for *C. difficile* colonization, disease, and transmission (4, 10). *C. difficile* colonization is associated with a spectrum of outcomes ranging from asymptomatic carriage, chronic diarrhea, and pseudomembranous colitis to multiple-organ dysfunction syndrome (6, 17). The first-line antibiotic treatment for *C. difficile* disease is vancomycin or metronidazole (39); however, recurrent disease (relapse with the same strain or reinfection with a different strain) occurs in ~15 to 35% of patients (1). Recurrence and transmission are relatively poorly understood aspects of *C. difficile* biology that likely underpin its persistence within health care systems.

During the past decade, distinct genetic clades of *C. difficile* have emerged that are responsible for epidemics within North America and Europe and continue to disseminate globally (18, 19). Among the most notable of these clades is the so-called "hypervirulent" variant of *C. difficile*, commonly genotyped as PCR ribotype 027 or North American pulsotype 1, which is associated with high rates of mortality and disease recurrence, as well as severe hospital outbreaks (7, 12, 36, 37). In addition, other genetic variants, such as *C. difficile* PCR ribotype 012, continue to be endemic in health care systems (2).

Sporulation, the production of metabolically dormant endospores from vegetative cells, is a property of several bacterial groups, including *Clostridium* and *Bacillus* (25, 38). In *Bacillus*, Spo0A is a key transcriptional regulator that is required in the early stages of sporulation (23). Orthologues of Spo0A are encoded by different members of the genera *Clostridium* and *Bacillus*, and genetic inactivation results in a sporulation-null phenotype in all species studied to date (9, 25, 28, 35). *C. difficile* produces highly infective endospores that are excreted by infected patients, allowing the oxygen-sensitive pathogen to retain viability outside the host (22). Spores of *C. difficile* can also resist commonly used disinfectant regimens and, as a result, are able to persist in the environment, generating a potential transmission reservoir that confounds standard infection control measures (5, 11, 20).

C. difficile is unusual among some spore-forming pathogens, including other clostridia, in being highly transmissible between humans. In contrast, there is little evidence of person-to-person transmission of *Clostridium botulinum* (32), *Clostridium tetani* (8), or *Bacillus anthracis* (30). Thus, transmissibility may be key to the continued survival and persistence of *C. difficile* in the human population, and it suggests that the pathogen is highly adapted to its niche. Here, we use a murine model of *C. difficile* infection to investigate the role of the *spo0A* gene in distinct PCR ribotypes 027 and 012 during infection and transmission.

MATERIALS AND METHODS

***C. difficile* strains and culture.** All *C. difficile* strains were grown for 24 to 48 h at 37°C under anaerobic conditions in a MACS MG-500 anaerobic workstation (Don Whitley Scientific, West Yorkshire, United Kingdom). *C. difficile* was routinely cultured in Wilson's broth with agitation (80 rpm) or on brain heart infusion (BHI) (Sigma, United Kingdom) agar plates with appropriate antibiotic selection unless otherwise stated. *Escherichia coli* strains were grown at 37°C using Luria-Bertani (LB) broth or agar with appropriate antibiotic selection. Table 1 describes the strains used in this study.

C. difficile was enumerated from fresh feces as previously described (21). Briefly, samples (100 mg feces/ml phosphate-buffered saline [PBS]) were serially diluted in PBS and plated on CCEY agar (Bioconnections, Whetherby, United Kingdom) supplemented with 0.1% taurocholate (Sigma, United Kingdom). This was done within 30 min of excretion. For

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source
Strains		
E. coli CA434	Conjugation donor	26
C. difficile		
Wild-type 630	Virulent and multidrug-resistant PCR ribotype 012, isolated from a patient with pseudomembranous colitis in Zurich, Switzerland (1985)	29
630	Erythromycin-sensitive derivative of wild-type *C. difficile* 630	16
R20291	Hypervirulent and epidemic PCR ribotype 027, isolated from a hospital outbreak in Stoke Mandeville, UK (2004–2005)	33
630Δ*spo0A*	*C. difficile* 630Δ*ermB spo0A*::*ermB*	This study
R20291Δ*spo0A*	*C. difficile* R20291 *spo0A*::*ermB*	This study
630Δ*spo0A*+p*spo0A*	Complemented 630Δ*spo0A* mutant	This study
R20291Δ*spo0A*+p*spo0A*	Complemented R20291Δ*spo0A* mutant	This study
Plasmids		
pMTL007	First-generation ClosTron plasmid with *catP* marker and intron containing *ermB* RAM; intron expression is induced using IPTG.	14
pMTL007C-E2	Second-generation ClosTron plasmid with *catP* marker and intron containing *ermB* RAM; contains a constitutive *fdx* promoter to direct intron expression	13
pRPF101	*E. coli-C. difficile* shuttle vector	This study
p*spo0A*	pRPF101 containing the 825-bp *spo0A* coding region (and upstream promoter)	This study

the enumeration of spores, samples were mixed with 100% ethanol (1:1 ratio) for 1 h at room temperature to kill vegetative cells, pelleted, washed in PBS, and cultured as described above.

Mutagenesis and genetic complementation. ClosTron technology was used to make targeted mutants in the *spo0A* gene of *C. difficile* strains R20291 (PCR ribotype 027) and 630 (PCR ribotype 012), as previously described (13, 14). Briefly, the group II Ll.LtrB intron was retargeted to *spo0A* by SOE PCR as previously described (13, 14). The retargeted intron, an antisense insertion between nucleotides 178 and 179, was then cloned into the HindIII and BsrGI sites of pMTL007 (*C. difficile* 630) or pMTL007C-E2 (*C. difficile* R20291) and transformed into the *E. coli* conjugation donor strain CA434. *C. difficile* strains 630 and R20291 were mated with the *E. coli* donor harboring the retargeted pMTL007 or pMTL007C-E2, respectively, as previously described (26). Transconjugants were selected for in the presence of thiamphenicol (15 μg/ml; Sigma-Aldrich, Dorset, United Kingdom). For pMTL007, mobilization of the intron to the *spo0A* gene of *C. difficile* 630 was induced using isopropyl-β-D-thiogalactopyranoside (IPTG) as previously described (14). pMTL007C-E2 is a version of pMTL007 in which the promoter of the group II intron is modified for a constitutive *fdx* promoter (13) and as such does not require IPTG induction to direct intron expression to the *spo0A* gene of *C. difficile* R20291. Potential transconjugants were then serially diluted onto plates containing lincomycin (15 μg/ml; *C. difficile* R20291; Sigma-Aldrich, Dorset, United Kingdom) or erythromycin (5 μg/ml; *C. difficile* 630; Sigma-Aldrich, Dorset, United Kingdom) to select for chromosomal insertion of the intron. Replica plating with lincomycin (15 μg/ml) or erythromycin (5 μg/ml) and thiamphenicol (15 μg/ml) was performed to select for the restored *ermB* retrotransposition-activated marker (RAM) that signals integration into the genome and loss of the *catP* gene encoding thiamphenicol resistance carried on the plasmid. Mutants were screened by PCR (see Table S1 in the supplemental material), sequencing, or Southern blot analysis to confirm the chromosomal integration of the intron within the desired genes and loss of plasmids pMTL007 and pMTL007C-E2.

To complement the *spo0A* mutants, the *C. difficile-E. coli* shuttle vector pRPF101 was constructed using the ColE1 replicon from pBlueScript II SK(+) (Agilent Technologies, Cheshire, United Kingdom), *catP* from pJIR418 (31), the *C. difficile* pCD6 replicon (26), and *oriT* from pJB665 (3). The wild-type *C. difficile* 630 *spo0A* gene and upstream promoter region were amplified using the oligonucleotide pair spo0A_A1F and spo0A_A2Rb (see Table S1 in the supplemental material), which were designed to enable cleavage with SalI, and cloned into pRPF101, yielding p*spo0A*. Since the amino acid sequences of Spo0A in both *C. difficile* R20291 and 630 are identical, only one plasmid was constructed for the complementation of both strains. p*spo0A* was initially transformed into *E. coli* CA434, followed by conjugation into the *spo0A* mutant derivatives as previously described (26), generating strains R20291Δ*spo0A*(p*spo0A*) and 630Δ*spo0A*(p*spo0A*), respectively. Thiamphenicol (15 μg/ml) was used to select for the p*spo0A*-containing cells, and cycloserine (250 μg/ml; Bioconnections, Whetherby, United Kingdom) and cefoxitin (8 μg/ml; Bioconnections, Whetherby, United Kingdom) were used to counterselect for *E. coli* CA434. Plasmid transfer was verified by p*spo0A* isolation and restriction analysis (data not shown).

Mouse infections. All experiments were carried out using 5- to 9-week-old specific-pathogen-free mice from colonies maintained at the Wellcome Trust Sanger Institute. Wild-type mice of C57BL/6 genetic background were pretreated with drinking water containing clindamycin (250 mg/liter; Apollo Scientific, Cheshire, United Kingdom) for 7 days. Clindamycin was then withdrawn for 24 h, and donors were infected via oral gavage or transmission, as indicated. When infected via oral gavage, mice received 10^7 vegetative cells and 10^5 spores (R20291 and 630 parental strains) or 10^7 vegetative cells (R20291Δ*spo0A* and 630Δ*spo0A* mutant derivatives). For relapsing infection experiments, mice received drinking water containing vancomycin hydrochloride (400 mg/liter; Sigma-Aldrich, Dorset, United Kingdom) for 7 to 10 days. The mice were housed in sterile cages with *ad libitum* access to food and water. All procedures and mouse handling were performed aseptically in a biosafety cabinet to contain spore-mediated transmission.

A clinical scoring system was employed to track the condition of the mice. Symptoms, including abnormal/hunched gait, piloerection, lethargy, and emaciation, were monitored. Moribund mice or mice displaying overt signs of disease were sacrificed. For pathological analysis, cecum tissue (0.5-cm tubular sections) was carefully excised, opened, and processed for hematoxylin and eosin staining, as previously described (21). All animal infections were performed in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986.

Competitive-index infections. Wild-type C57BL/6 mice ($n = 5$) were infected via oral gavage with 10^7 CFU in 0.2 ml PBS containing equal proportions of the *C. difficile* parental strain and the respective isogenic *spo0A* mutant. In order to determine the inoculum dose and ratio of

TABLE 2 Summary of transmission routes

Type of transmission (1 h)	Characteristics
Mingling	Donor and recipient mice were able to freely touch and interact with no restriction in movement; potential for coprophagy
Contact	Donor and recipient mice were separated by a single porous barrier and were able to touch each other; no potential for coprophagy
Airborne	Donor and recipient mice were separated by a double porous barrier and were unable to touch each other; no potential for coprophagy
Environmental	Recipient mice were housed in a contaminated donor cage, which had been left for 24 h under ambient oxygen conditions. Donor feces were removed to prevent transmission via coprophagy

parent to *spo0A* mutant, the inocula were grown on both CCEY plates (as a nonselective medium) and CCEY plates supplemented with 20 mg/ml lincomycin (which selects for the *ermB* gene inserted into the *spo0A* genes of both mutant derivatives). Fecal samples were then diluted as described above and plated on CCEY agar and CCEY agar supplemented with lincomycin, and the competitive index (CI) was determined by dividing the ratio of mutant to parent bacteria using the following formula: CI = (*spo0A* mutant/parental strain)$_{output}$/(*spo0A* mutant/parental strain)$_{input}$. The Mann-Whitney test was applied to the $\log_{10}$ values of the CI ratios to determine the statistical significance of the results.

Transmission experiments. Wild-type C57BL/6 donor mice ($n = 5$) were infected via oral gavage (10^7 CFU in 0.2 ml PBS). To measure transmission efficiency, naïve wild-type C57BL/6 recipient mice ($n = 5$) were pretreated with clindamycin as described above and used in one of four types of transmission assays as described in Table 2 and Fig. S1 in the supplemental material. Following transmission, recipient mice were individually housed, and the transmission efficiency was determined after 4 days by the isolation of the same strain of *C. difficile* in the feces that was used to infect the respective donor mice.

TcdA and TcdB quantification. *C. difficile* cultures were growth in Wilson's broth with shaking for 30 h and pelleted by centrifugation, and the supernatant was removed for TcdA and TcdB quantification. Total and spore counts were determined to ensure equal numbers of vegetative cells in the cultures. For TcdA quantification, microtiter plates were coated with capture antibody by adding 50 μl/well of a 2-μg/ml solution of anti-TcdA (tgcBiomics GmbH, Mainz, Germany) in PBS and incubating overnight at 4°C. The plates were then washed 3 times in 0.05% Tween20 in PBS (PBS-T) and blocked with 200 μl 1% bovine serum albumin (BSA) in PBS for 2 h at room temperature. Purified TcdA (tgcBiomics GmbH, Mainz, Germany) was diluted in 1% BSA-PBS (50 μl/well) and used to construct a standard curve. Culture filtrates (50 μl/well) were diluted as described above in order to generate readings within the linear range of the standard curve. Plates were then incubated at room temperature for 2 h, followed by washing in PBS-T as described above. The detection antibody (rabbit anti-*Clostridium difficile* TcdA; Antibodies-Online GmbH, Aachen, Germany) was diluted 1:5,000 in 1% BSA-PBS, added to the wells (50 μl/well), and incubated for 2 h at room temperature. After washing, polyclonal swine anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Dako, Cambridgeshire, United Kingdom) was diluted 1:1,000 in 1% BSA-PBS, added to the wells (50 μl/well), and incubated for 2 h at room temperature. Finally, the plates were washed, and 100 μl 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma-Aldrich, Dorset, United Kingdom) substrate was added for 30 min at room temperature in the dark; 50 μl 0.5 M H_2SO_4 was added to stop the reaction.

TcdB quantification was determined using a TcdB-specific enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (tgcBiomics GmbH, Mainz, Germany). Briefly, microtiter plates precoated with anti-TcdB capture antibody were coincubated with either *C. difficile* culture filtrates or purified TcdB (as described above) and anti-*C. difficile* TcdB-HRP and incubated for 1 h at 37°C. The plates were then washed, and 100 μl TMB substrate was added for 30 min at room temperature in the dark. The reaction was stopped with 50 μl 0.5 M H_2SO_4. The absorbances of all plates were measured at 450 nm on a FluoStar Omega (BMG Labtech, Bucks, United Kingdom). Data for TcdA and TcdB are from 3 independent experiments performed in triplicate.

Indirect immunofluorescence of *C. difficile* vegetative cells and spores. Cultures were washed in PBS and seeded onto microscope slides. Briefly, the slides were then fixed in acetone and blocked with 1% BSA in PBS. The wells were incubated with polyclonal rabbit sera (raised against pure wild-type *C. difficile* 630 spores) and polyclonal mouse sera (raised against 630Δ*spo0A* vegetative cells), washed in PBS, and reacted with Cy3-conjugated goat anti-rabbit IgG and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (each 1:1,000) in a humidified chamber in the dark. The slides were then mounted in ProLong Gold with DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen, Paisley, United Kingdom) and sealed, and immunofluorescence was assessed using an LSM510 Meta confocal microscope (Carl Zeiss Ltd., Herts, United Kingdom).

Antibodies were generated via subcutaneous delivery of *C. difficile* 630Δ*spo0A* into C57BL/6 mice (for anti-vegetative-cell sera; $n = 5$) or *C. difficile* 630 pure spores into rabbits (for anti-spore sera; $n = 2$). Briefly, the animals received 100 μl of an ~10^8-CFU/ml culture in each flank, followed by a series of boost immunizations and test bleeds. Sera were collected and stored at 4°C.

RESULTS

***C. difficile spo0A* is essential for spore formation.** We had previously sequenced and fully annotated the complete genomes of representative *C. difficile* ribotypes 027 (strain R20291) (33) and 012 (strain 630) (29). *C. difficile* R20291 and 630 both produce ~10^5 spores/ml after 48 h of growth in Wilson's broth, representing <1% of the total bacterial cells (Fig. 1A). To evaluate the impact of *spo0A* on growth and sporulation, defined insertional mutations were generated in the *spo0A* genes of either *C. difficile* R20291 or *C. difficile* 630, yielding mutant derivatives R20291Δ*spo0A* and 630Δ*spo0A*, respectively. The vegetative cells of both R20291Δ*spo0A* and 630Δ*spo0A* were morphologically indistinguishable from the respective parent strains when examined by light microscopy and displayed similar growth kinetics (data not shown). Both *C. difficile spo0A* mutant derivatives failed to produce any detectable spores or spore-like elements (15, 35), as determined by anaerobic culturing after ethanol shock (spores are resistant to ethanol) and indirect immunofluorescence using spore-specific antisera (Fig. 1).

To demonstrate that the asporogenous phenotype was directly attributable to the *spo0A* mutation, we complemented the mutated genes using the plasmid p*spo0A* harboring the wild-type *spo0A* gene under the control of its native promoter. We found that the complemented *spo0A* mutants produced spores at levels comparable to those of the parent ($P > 0.05$) and were morphologically indistinguishable (Fig. 1). Collectively, these observations indicate that the *spo0A* mutation is nonpolar and that expression of the *spo0A* gene is critical for vegetative cells to differentiate into spores in both *C. difficile* R20291 and 630.

***C. difficile spo0A* mutant derivatives cause acute disease in mice.** We previously described a *C. difficile* infection model in mice that mimics many aspects of *C. difficile* disease, persistence,

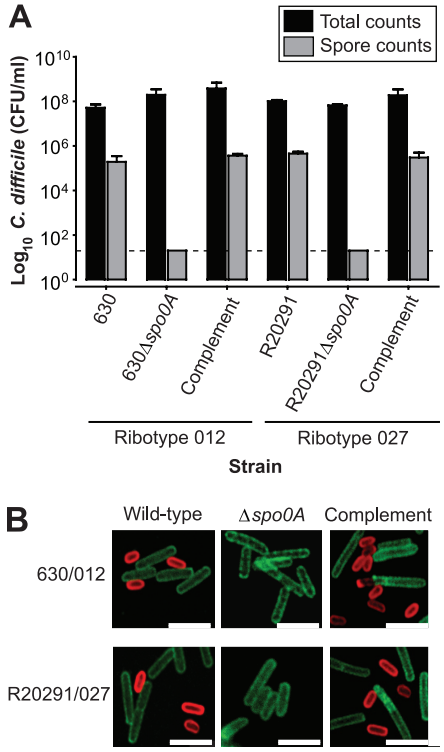

FIG 1 Genetic and phenotypic characterization of *C. difficile* 630 and R20291 *spo0A* mutants. (A) *C. difficile* cultures were grown in Wilson's broth for 48 h under anaerobic conditions, and then total cell and spore counts were determined. Genetic complementation of the *spo0A* mutation restored spore formation to levels statistically comparable to those of the parental strains ($P > 0.05$), according to the Student *t* test. The dashed horizontal line indicates the detection limit. The error bars indicate standard deviations. (B) Representative indirect-immunofluorescence images of *C. difficile* cultures stained with vegetative-cell-specific (green) and spore-specific (red) polyclonal antibodies and visualized with FITC-conjugated and Cy3-conjugated secondary antibodies, respectively. Scale bar = 5 μm.

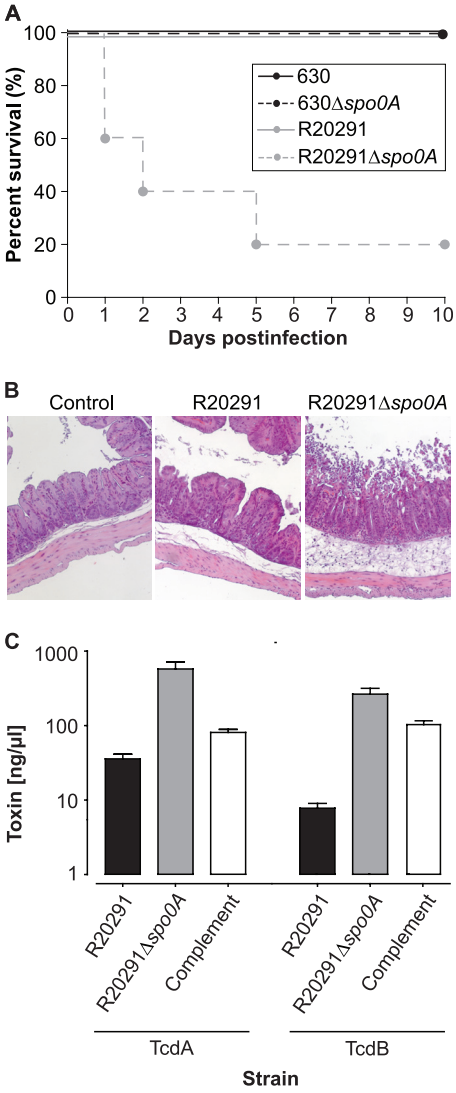

FIG 2 *C. difficile spo0A* mutants produce elevated levels of TcdA and TcdB and exhibit increased virulence in mice. (A) Kaplan-Meier survival curve of mice infected with *C. difficile* 630, R20291, 630Δ*spo0A*, or R20291Δ*spo0A*. (B) Representative images demonstrating epithelial cell damage in hematoxylin- and eosin-stained cecal sections of mice infected with *C. difficile* R20291 or R20291Δ*spo0A*. Magnification, ×20. (C) Sandwich ELISA indicating the relative levels of TcdA and TcdB produced by *C. difficile* R20291 or R20291Δ*spo0A* after 30 h of growth in Wilson's broth under anaerobic conditions. Data are from 3 independent experiments performed in triplicate. The error bars indicate standard deviations.

and transmission in humans (20–22). In order to investigate the role of the *spo0A* gene during the acute phase of *C. difficile* infection (days 2 to 4 postinfection), we pretreated mice with clindamycin and subsequently infected them with *C. difficile* R20291, *C. difficile* 630, or their equivalent *spo0A* mutant derivatives. Groups of mice infected orally with *C. difficile* 630 or 630Δ*spo0A* did not exhibit any overt signs of disease and had a 100% survival rate (Fig. 2A). In contrast, mice infected with *C. difficile* R20291 demonstrated notable signs of disease, such as hunched posture and ruffled fur; however, these clinical symptoms resolved by day 5 postinfection, and all the mice survived. Mice infected with *C. difficile* R20291Δ*spo0A* also displayed signs of overt disease, but this was more exaggerated than in R20291-infected mice, with the mice displaying hunched posture, piloerection, lethargy, dehydration, and emaciation. By day 5 postinfection, 80% of R20291Δ*spo0A*-infected mice were considered to be moribund according to a clinical scoring system and were consequently sacrificed. The remaining 20% resolved the disease and survived.

In order to identify any increased pathology associated with R20291Δ*spo0A* infection, we performed histologic analyses on the ceca of mice infected with *C. difficile* R20291 or R20291Δ*spo0A* at day 2 postinfection (Fig. 2B). At this time point, the mice shed equivalent levels (~10^8 CFU/gram fresh feces) of vegetative *C. difficile* R20291 or R20291Δ*spo0A*. Mice infected with *C. difficile* R20291 also shed spores (~10^6 CFU/gram fresh feces), in contrast to R20291Δ*spo0A*-infected mice, which did not shed spores. Uninfected clindamycin control mice did not exhibit any pathology. Conversely, at day 2 postinfection, mucosal damage was evident in R20291- and R20291Δ*spo0A*-infected mice, which included edema and immune cell infiltrate within the cecal mucosa. These observations, however, were much more notable in mice infected with R20291Δ*spo0A*, which exhibited more pronounced edema, epithelial surface damage, and acute infiltration. Putative pseudomembranes were also visible on the epithelial surface in R20291Δ*spo0A*-infected mice.

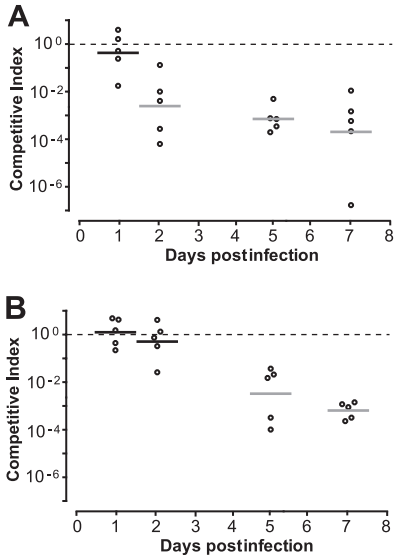

FIG 3 *C. difficile spo0A* mediates intestinal persistence in mice. Shown are the CI time courses of *C. difficile* R20291 and R20291Δ*spo0A* (A) and 630 and 630Δ*spo0A* (B). Individual CI values are illustrated as open circles, and the horizontal bars represent the geometric means. The second through fourth (A) and third and fourth (B) *spo0A* mutants are attenuated, as determined by the Mann-Whitney test.

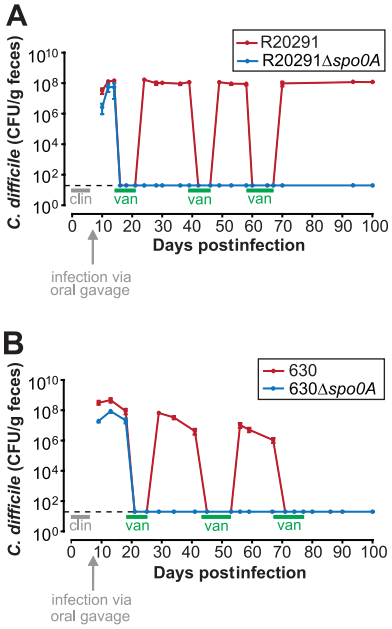

FIG 4 The *C. difficile spo0A* gene is required for relapsing disease in mice. (A and B) Representative fecal shedding profiles from mice ($n = 5$ per group) infected with *C. difficile* 630, R20291, 630Δ*spo0A*, or R20291Δ*spo0A*. The mice were pretreated with clindamycin (clin) (represented as a gray line) for 7 days prior to infection via oral gavage. Following infection, the mice received a series of 7- to 10-day courses of vancomycin (van) (represented as green lines) during which fecal shedding of *C. difficile* decreased to below the detection limit of the assay in all groups of mice. The dashed horizontal lines indicate the detection limit.

The increased virulence exhibited by *C. difficile* R20291Δ*spo0A* was not anticipated. We hypothesized that these observations might be related to increased toxin production. We therefore compared the levels of the major toxins TcdA and TcdB from broth grown cultures of wild-type *C. difficile* R20291 and R20291Δ*spo0A* using highly sensitive anti-TcdA- and anti-TcdB-specific ELISAs. Low-levels of TcdA and TcdB were detected during mid-exponential-phase growth in both R20291 and R20291Δ*spo0A* (data not shown). However, during stationary growth (30 h), significantly higher levels both of TcdA ($P = 0.0006$) and TcdB ($P = 0.0005$) were detected in *C. difficile* R20291Δ*spo0A* than in R20291 cultures (Fig. 2C). Complementation of the *spo0A* gene in R20291Δ*spo0A* reduced the levels of toxins produced, although both TcdA and TcdB were still slightly elevated compared to that produced by wild-type *C. difficile* R20291 ($P = <0.05$).

Therefore, we demonstrated that R20291 and R20291Δ*spo0A* are virulent in mice and that exaggerated virulence by R20291Δ*spo0A* during the acute phase of infection is associated with increased TcdA and TcdB production.

The *C. difficile spo0A* gene is required for persistent, relapsing infection in mice. We previously demonstrated that *C. difficile* isolated from infected hospital patients can asymptomatically persist in mice for several months (21). Therefore, we next assessed the contribution of the *C. difficile spo0A* gene to colonization and persistent infection. To assess this phenotype, CI experiments were performed in which groups of mice were infected with a mixture of equal numbers of parental strain *C. difficile* and the respective Δ*spo0A* mutant, enabling direct fitness comparisons *in vivo*. In the R20291 background, the Δ*spo0A* mutant was recovered at a significantly reduced level compared to wild-type R20291 (Fig. 3A). We observed that genetic disruption of *spo0A* resulted in reduced fitness by day 2 postinfection and continued to day 15 postinfection. Although *C. difficile* 630 does not cause a long-term persistent infection in our murine model, we found that the *C. difficile* 630Δ*spo0A* mutant was reproducibly cleared ~6 days earlier than its parental strain (data not shown). Additionally, the difference in relative fitness for the 630Δ*spo0A* mutant was statistically significant by day 5 postinfection (Fig. 3B). These results indicate that the *spo0A* gene of *C. difficile* contributes to intestinal colonization and persistent infection in mice.

Recurrent infection after cessation of antibiotic therapy is a hallmark feature of *C. difficile* persistence. Given that *C. difficile spo0A* may affect persistence, we were interested in whether Spo0A also influences relapsing infection. To test this, mice infected with R20291, R20291Δ*spo0A*, *C. difficile* 630, or 630Δ*spo0A* were treated with a 7-day course of oral vancomycin. During this period, the cages were regularly changed to reduce the potential for environmental transmission. We found that vancomycin therapy rapidly decreased the levels of fecal shedding of *C. difficile* to below the detection limit in all infected groups of mice (Fig. 4A and B), as expected, since these strains are sensitive to vancomycin. However, 3 days after vancomycin withdrawal, the mice infected with parental *C. difficile* R20291 or *C. difficile* 630 exhibited recurrent infection with the same isolate (i.e., relapse). With *C. difficile* 630, this relapsing phenotype was observed after the first two courses of vancomycin; however, after a third treatment, the relapse did not occur (Fig. 4B). In contrast, *C. difficile* R20291-infected mice consistently relapsed on cessation of treatment for up to 100 days (Fig. 4A). Significantly, mice infected with either *C. difficile* R20291Δ*spo0A* or 630Δ*spo0A* cleared the infection during the first

course of vancomycin and did not demonstrate relapse at any point during the 100-day monitoring period (Fig. 4A and B).

We hypothesized that recurrence in R20291- and 630-infected mice could be the result of either persistence of the bacterium within the host during vancomycin therapy or recolonization from environmental spores. To address this, mice infected with *C. difficile* R20291 or *C. difficile* 630 were treated with a 5-day course of vancomycin, after which the entire intestinal tract (small and large intestine and cecum) was removed and cultured for *C. difficile*. We consistently failed to detect any *C. difficile* in the intestinal tracts of mice receiving vancomycin therapy (data not shown). However, we could readily culture *C. difficile* from mouse chow, cage shavings, and bedding, as well as the fur, mouth, and feet of mice receiving vancomycin treatment, even though cages were changed during the vancomycin treatment. Spores were detected in all locations, indicating that Spo0A acts as a persistence factor, enabling *C. difficile* to remain in the environment generating an infection reservoir on cessation of antibiotic therapy.

***C. difficile spo0A* is required for efficient host-to-host transmission.** We previously used the murine infection model to demonstrate host-to-host transmission and the highly transmissible nature of various *C. difficile* ribotypes (20–22). Here, we used this model to investigate the role of the *C. difficile spo0A* gene in host transmission via distinct transmission routes (Table 2; see Fig. S1 in the supplemental material). During transmission experiments, the donor mice shed equivalent levels (~10^8 CFU/gram fresh feces) of vegetative *C. difficile* R20291, 630, or their respective *spo0A* mutant derivatives (Fig. 5A). Mice infected with *C. difficile* R20291 or *C. difficile* 630 also shed spores (~10^6 CFU/gram fresh feces), in contrast to mice infected with the *spo0A* mutants, where spores were undetectable (Fig. 5A).

Mingling donor mice that were shedding high levels of *C. difficile* R20291 or 630 with naïve recipient mice for 1 h (in cages without bedding) resulted in 100% transmission (Fig. 5B) ($n = 10$ for each strain). In contrast, mingling donors excreting *C. difficile* R20291Δ*spo0A* or 630Δ*spo0A* with naïve recipient mice resulted in only 40% or 20% transmission efficiency, respectively. In these experiments, coprophagy was regularly observed, and this behavior likely promotes *C. difficile* transmission. To further define the transmission route, we placed a porous wall between donor and recipient mice so that the mice could come into contact but coprophagy was blocked. During these experiments *C. difficile* R20291 and 630 were still transmitted at 100% efficiency, whereas the Δ*spo0A* derivatives failed to transmit at a detectable level (Fig. 5B, contact transmission). Next, we tested transmission between donor and recipient mice that were separated by a double porous wall that prevented coprophagy and direct contact but would still allow airborne transmission. In these experiments, *C. difficile* R20291 and 630 were transmitted at 60% efficiency, whereas the respective Δ*spo0A* derivatives failed to transmit (Fig. 5B, airborne transmission). Thus, Spo0A plays a key role in the transmission of *C. difficile* between hosts sharing a contained environment but with no direct contact.

Environmental *C. difficile* spores are proposed to be a significant transmission reservoir (20, 27). To mimic this situation, we contaminated cages (without bedding) by placing donor mice shedding *C. difficile* in cages for 1 h and then removed the feces and allowed the cages to stand overnight (~16 h) in a sterile environment so that only the aerobic-resistant spores survived. Exposure of naïve recipient mice to the environment contaminated

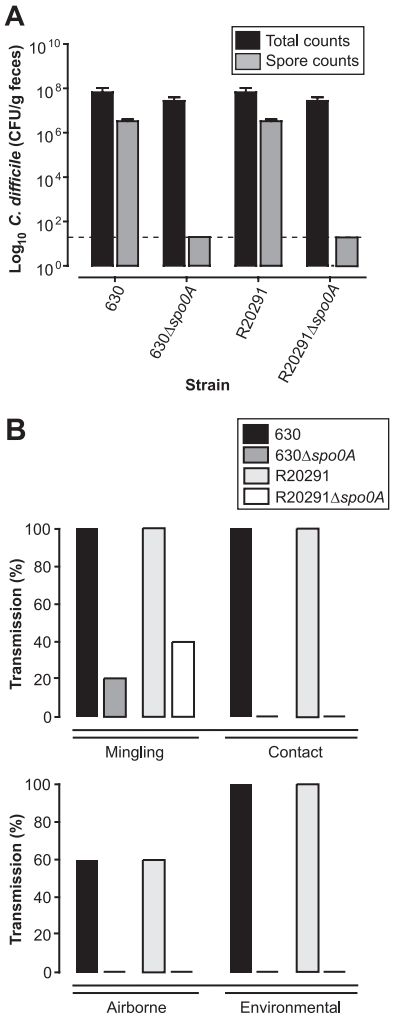

FIG 5 Host-to-host transmission of *C. difficile* is mediated by *spo0A*. (A) Average fecal shedding of *C. difficile* by mice ($n = 5$ per group) at 5 days postinfection. The dashed horizontal line indicates the detection limit. The error bars indicate standard deviations. (B) Transmission efficiencies of *C. difficile* 630, R20291, 630Δ*spo0A*, and R20291Δ*spo0A*, demonstrating the percentages of naïve recipient mice that acquired infection following exposure to infected donor mice via mingling, contact, airborne, or environmental transmission. The efficiency of transmission was determined as described in Materials and Methods.

with *C. difficile* R20291 or 630 spores resulted in 100% transmission to naïve recipient mice. In contrast, none of the naïve recipient mice exposed to *C. difficile* R20291Δ*spo0A*- or 630Δ*spo0A*-contaminated cages became colonized (Fig. 5B).

DISCUSSION

The availability of a murine infection model opens the way to study the genetic basis of *C. difficile* disease, relapse, persistence, and transmission. Here, we demonstrated for the first time that the *spo0A* gene of clinically relevant genetic variants of *C. difficile* is essential for persistent infection and efficient host-to-host transmission. Importantly, we also showed that a functional *spo0A* gene is required for relapsing infection after vancomycin therapy, and we further illustrated that the local environment (i.e., cage, food, mouse fur, etc.) may serve as a reservoir of *C. difficile*. Despite

multiple attempts, we could not culture *C. difficile* from the intestinal tracts of challenged mice during vancomycin treatment, indicating that maintenance of *C. difficile* within the intestinal tract was not a likely cause of relapse, although we cannot rule out the possibility that *C. difficile* persisted at very low levels or in a non-culturable form. We could, however, readily culture *C. difficile* from inside the cage during treatment. Given the highly transmissible nature of environmental spores, it is possible that relapsing infection was in fact reinfection with the same strain that contaminated the local environment before antibiotic treatment and persisted until after antibiotic therapy ended. The inability of *C. difficile spo0A* mutants to cause relapsing infection and form spores is consistent with this model. Indeed, antibiotic treatment of mice perturbs the intestinal microbiota and makes them extremely susceptible to *C. difficile* infection via environmental spores (21).

As expected, genetic inactivation of *spo0A* resulted in an asporogenous phenotype in *C. difficile* R20291 (ribotype 027) and 630 (ribotype 012) (35). In addition to not being able to form spores, we demonstrated that *C. difficile spo0A* mutants produced elevated levels of the toxins TcdA and TcdB *in vitro* compared to the respective wild-type parental strains. Therefore, Spo0A may negatively regulate toxin production, which was associated with increased virulence for mice for the *C. difficile* 027 variant. This finding was in contrast to the observations of Underwood et al. (35), which indicated that a *C. difficile* 630 *spo0A* mutant produced <10% of the parental levels of TcdA (35). This discrepancy may reflect the different methods used to quantify toxin levels. In the well-characterized model organism *Bacillus subtilis*, Spo0A regulates processes other than spore formation, such as efflux pumps and metabolism (23). Consequently, it is possible that, in addition to spore formation, there are other functions controlled by Spo0A that may play a role in persistence and transmission. Future experiments that define the *C. difficile* Spo0A regulon at the transcriptional and proteomic levels should identify persistence and transmission factors controlled by Spo0A in *C. difficile*.

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