3 The *Clostridium difficile spo0A* gene is a persistence and transmission factor

3.1 Publications arising from this chapter

The key findings from this chapter have resulted in the following publication (see Appendix 1):

Deakin LJ, Clare S, Fagan RP, Dawson LF, Pickard DJ, West MR, Wren BW, Fairweather NF, Dougan G, Lawley TD. (2012). The *Clostridium difficile spo0A* gene is a persistence and transmission factor. *Infect Immun* **80**(8): 2704-2711.

3.2 Introduction

3.2.1 Human-virulent *C. difficile*

During the past decade, distinct genetic clades of *C. difficile* have emerged that are responsible for epidemics within North America and mainland Europe and continue to disseminate globally (see Chapter 1.1.2.2). Amongst the most notable of these clades is the so called "hypervirulent" variant of *C. difficile*, commonly genotyped as PCR ribotype 027, which is associated with high rates of mortality and disease recurrence as well as severe hospital outbreaks (7-9, 206). In addition, other genetic variants such as *C. difficile* PCR ribotype 012 continue to be endemic in many healthcare systems (207).

3.2.2 *C. difficile* transmission

C. difficile produces highly infective endospores that are excreted by infected patients, allowing the oxygen sensitive pathogen to retain viability outside of the host (150). 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 (61, 151, 152).

The ability of a bacterium to transmit within an ecological niche is essential for its continued survival within the population. Spores are often cited as the primary transmission factor for *C. difficile*, and it has indeed been demonstrated that spores of the pathogen are highly infective and resistant to commonly used disinfectant regimens (61). The incidence of *C. difficile* increases within the hospital environment, whereby shedding of *C. difficile* by infected patients facilitates the spread of infection. Spores have been isolated from environmental surfaces as well as fomites including medical equipment and staff clothing (59, 208).

3.2.3 Antibiotic treatment of *C. difficile* infection: disease recurrence

First line antibiotic treatments for *C. difficile* disease are vancomycin or metronidazole (117), however recurrent disease (relapse with the same strain or reinfection with a different strain) occurs in ~15 to 35% of patients (60). In this sense, recurrence and transmission are relatively

poorly understood aspects of *C. difficile* biology that likely underpin its persistence within healthcare systems.

In the backdrop of endemic and epidemic CDAI, there is clearly a public health imperative to understand the transmission dynamics of the pathogen and a need to reduce the incidence of recurrent episodes that have now become a hallmark feature of *C. difficile* infection.

3.2.4 Animal models of *C. difficile* infection

A number of animal species have been used to model *C. difficile* infection, including piglets (209), guinea pigs (210), rats (111), rabbits (211), hamsters (143, 212, 213) and mice (98, 214). The golden Syrian hamster model of infection is one of the most widely used and has been employed for several decades to study CDAI. However, these animals are exquisitely susceptible to infection and can rapidly develop fulminant disease with severe enterocolitis, which is often lethal (143). Although great efforts have been taken to refine this model (213), it can be argued that it does not capture the spectrum of *C. difficile* disease observed in humans (215). In addition, due to the severe nature of the disease in hamsters, it is inherently more challenging to study some aspects of *C. difficile* disease, such as long-term persistence and recurrence in this model.

3.4.2.1 Murine model of *C. difficile* disease

In contrast to hamsters, mice are a potentially more accessible model through which to study infectious diseases. Molecular tools and reagents are more readily available for mice and there are thousands of genetically modified lines available for investigations (http://www.sanger.ac.uk/mouseportal/). Against this backdrop, the Wellcome Trust Sanger Institute has developed a murine model of *C. difficile* disease that has proved to be a valuable tool in studying aspects of *C. difficile* disease such as pathogenesis, transmission and virulence.

3.3 Aims of the work described in this chapter

In this chapter, I 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.

3.4 Results

3.4.1 *C. difficile spo0A* is essential for spore formation

The WTSI and collaborators have previously sequenced and fully annotated the complete genomes of individual isolates of *C. difficile* ribotypes 027 (strain R20291) (11) and 012 (strain 630) (10). *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 (Figure 3.1A). To

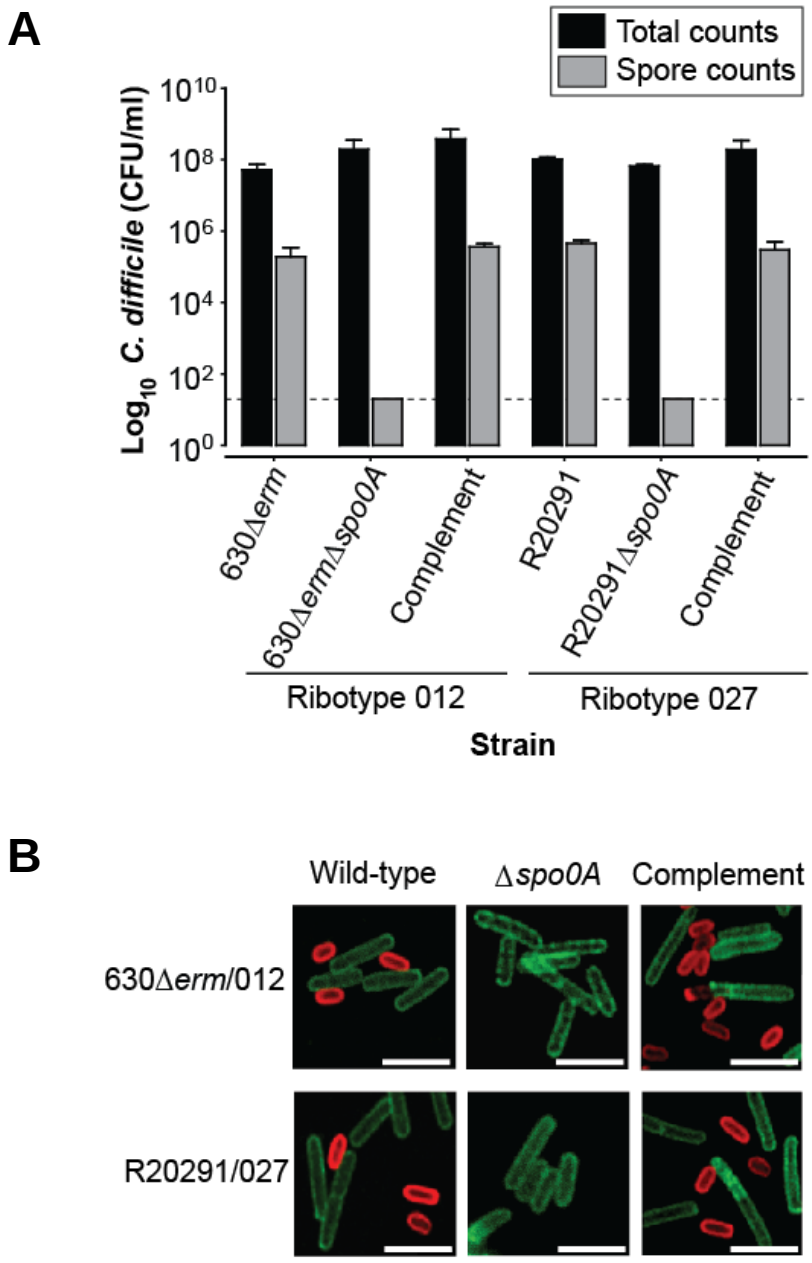

Figure 3.1. Genetic and phenotypic characterisation of *C. difficile* 630Δ*erm* and R20291 *spo0A* mutants. A) *C. difficile* cultures were grown in Wilson's broth for 48 h under anaerobic conditions and then total cell and spore counts were determined. Genetic complementation of the *spo0A* mutation restored spore formation to levels statistically comparable to the parental strains (P = > 0.05), according to the Student's t test. The dashed horizontal line indicates the detection limit. **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.

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* 630Δ*erm*, yielding mutant derivatives R20291Δ*spo0A* and 630Δ*erm*Δ*spo0A*, respectively, as described in Materials and methods chapter 2.2.5.9.

The vegetative cells of both R20291Δ*spo0A* and 630Δ*erm*Δ*spo0A* were morphologically indistinguishable from the respective parent strains when examined by light microscopy, and displayed similar general growth kinetics. However, both *C. difficile spo0A* mutant derivatives failed to produce any detectable spores or spore-like elements (177, 216), as determined by anaerobic culturing after ethanol shock (spores are resistant to ethanol) and indirect immunofluorescence using spore-specific anti-sera (Figure 3.1A and B).

To demonstrate that the asporogenous phenotype was directly attributable to the *spo0A* mutation, we complemented the mutated genes using the plasmid p*spo0A* harbouring the wild-type *C. difficile spo0A* gene under the control of the native promoter. We found that the complemented *spo0A* mutants produced spores at levels comparable to the parent ($P = > 0.05$) and that these spores were also morphologically indistinguishable (Figure 3.1A and B). Collectively, these observations indicate that the *spo0A* mutation is non-polar for spore formation and that expression of the *spo0A* gene is critical for vegetative cells to differentiate into spores in both *C. difficile* R20291 and 630Δ*erm*.

3.4.2 Antibiotic-induced murine model of *C. difficile* disease

CDAIs are precipitated primarily by the administration of antibiotics for therapy. As such, we pre-treated mice with clindamycin (a lincosomide antibiotic known to incite *C. difficile* disease in humans) to disrupt the intestinal microbiota. Groups of mice were subsequently independently orally infected with 10^7 CFU of either *C. difficile* strains R20291 or 630Δ*erm*, and colonisation was monitored by assessing *C. difficile* viable counts recovered from faeces (Figure 3.2).

Mice orally infected with *C. difficile* R20291 shed approximately 10^8 CFU/g fresh faeces and this level remained consistently high for the entire monitoring period (Figure 3.2). Thus, *C. difficile* R20291 infection can result in a long-term, persistent colonisation in this model. This is termed the "supershedder" state, and has been reported in *C. difficile* ribotype 017 (strain M68) (98) .

In contrast, *C. difficile* 630Δ*erm* did not establish a long-term persistent infection in this model. Following the initial infection, faecal shedding of *C. difficile* 630Δ*erm* remained high and comparable with R20291-infected mice for approximately 14 days. However, by day 15 post-infection the levels of *C. difficile* in the faeces had begun to decline, and by day 30 post-infection all mice had resolved the infection (Figure 3.2). Upon clearance, *C. difficile* 630Δ*erm* was undetectable in the faeces for the duration of the monitoring period.

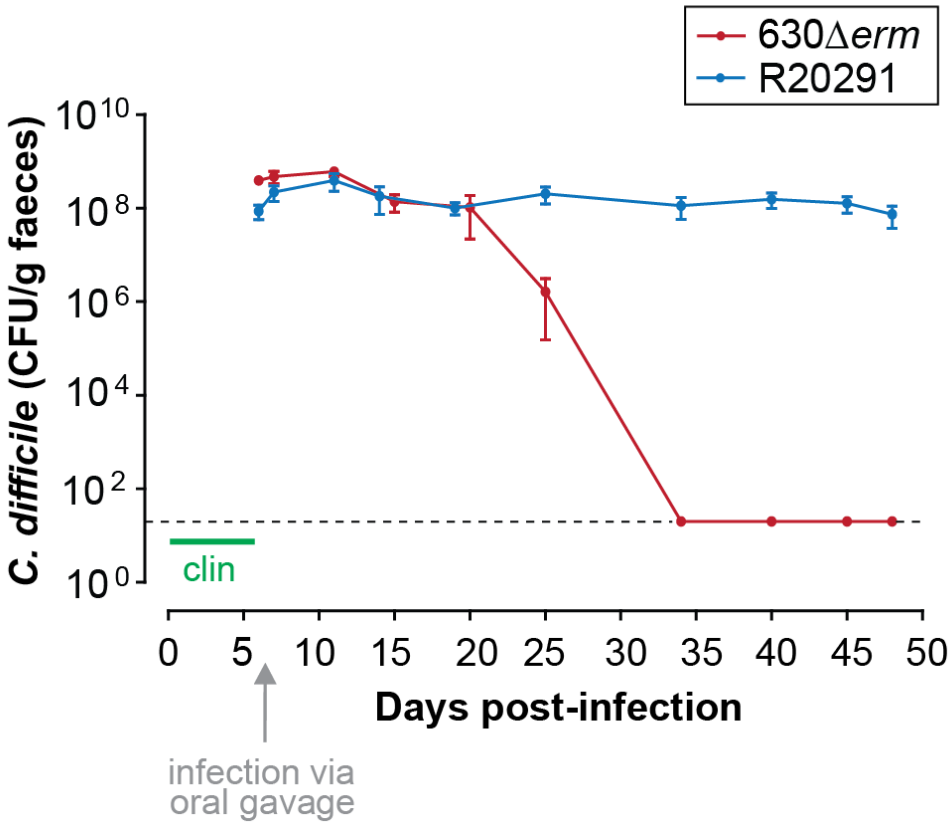

Figure 3.2. *C. difficile* R20291 can cause a long-term, persistent infection in mice. Representative faecal shedding profiles from mice (*n* = 5 per group) infected with *C. difficile* 630Δ*erm* or R20291. Mice were pre-treated with clindamycin (represented as a green line) for 7 d prior to infection via oral gavage. Following infection, colonisation was monitored by assessing viable counts recovered from faeces. The dashed horizontal line indicates the detection limit.

3.4.3 Role of *spo0A* in *C. difficile* pathogenesis

Spo0A is known to regulate the expression of many genes, including known virulence factors in other spore-forming pathogens such as *B. thuringiensis* (217) and *B. anthracis* (218).

However, the role of Spo0A in *C. difficile* disease, persistence and transmission had not yet been explored. A greater understanding of these aspects of *C. difficile* infection may help guide infection control measures and such information could have practical implications related to the management of hospital patients.

3.4.3.1 *C. difficile spo0A* mutant derivatives cause acute disease in mice

We previously described a *C. difficile* infection model in mice that mimics several aspects of *C. difficile* disease, persistence and transmission in humans (61, 98, 150). In order to investigate the role of the *spo0A* gene during the acute phase of *C. difficile* infection (day 2-4 post infection), we pre-treated mice with clindamycin and subsequently infected them with *C. difficile* R20291, *C. difficile* 630Δ*erm* or their equivalent *spo0A* mutant derivatives. Groups of mice infected orally with *C. difficile* 630Δ*erm* or 630Δ*erm*Δ*spo0A* did not exhibit any overt signs of disease and had a 100% survival rate (Figure 3.3).

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 post-infection and all mice survived. Mice infected with *C. difficile* R20291Δ*spo0A* also displayed signs of overt disease, but this was more exaggerated compared to R20291-infected mice with mice displaying hunched posturing, piloerection, lethargy, dehydration and emaciation. Within 5 days post-infection, 80% of R20291Δ*spo0A*-infected mice were considered to be

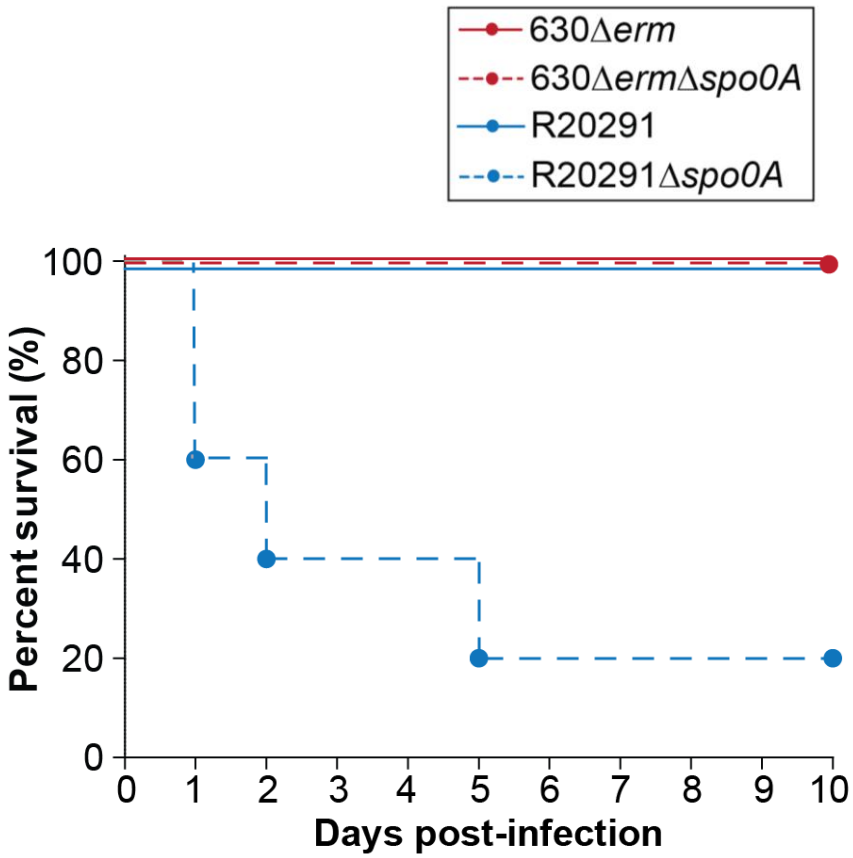

Figure 3.3. *C. difficile* R20291 *spo0A* mutants demonstrate increased virulence in mice. Kaplan-Meier survival curve of mice infected with *C. difficile* 630Δ*erm*, R20291, 630Δ*erm*Δ*spo0A* or R20291Δ*spo0A*. By day 5 post-infection, 80% of mice orally infected with *C. difficile* R20291Δ*spo0A* demonstrated severe signs of disease, including a hunched posture and lethargy and succumbed to infection.

moribund according to a clinical scoring system and were consequently sacrificed (Figure 3.3). The remaining 20% resolved disease and survived.

3.4.3.2 *C. difficile* Spo0A mediates pathological responses in mice

In order to identify any increased pathology associated with *C. difficile spo0A* mutant infections, we performed histologic analyses on the caeca of mice infected with *C. difficile* R20291, 630Δ*erm* or their respective *spo0A* mutant derivatives at day 2-4 post-infection. At these time-points, mice shed equivalent levels (~10^8 CFU/gram fresh faeces) of vegetative *C. difficile* R20291, R20291Δ*spo0A*, 630Δ*erm* or 630Δ*erm*Δ*spo0A*. Mice infected with *C. difficile* R20291 or 630Δ*erm* also shed spores (~10^6 CFU/gram fresh faeces) in contrast to R20291Δ*spo0A*- and 630Δ*erm*Δ*spo0A*-infected mice which did not, at least at a detectable level.

3.4.3.2.1 Pathological response to C. difficile R20291 infection

Uninfected, clindamycin control mice did not exhibit any pathology. Conversely, at day 2 post-infection mucosal damage was evident in R20291 and R20291Δ*spo0A*-infected mice, which included oedema and immune cell infiltrate within the caecal mucosa (Figure 3.4). These observations however were much more notable in mice infected with R20291Δ*spo0A*, which exhibited more pronounced oedema, epithelial surface damage and acute infiltration (Figure 3.4). Putative pseudomembranes were also visible on the epithelial surface of R20291Δ*spo0A*-infected mice.

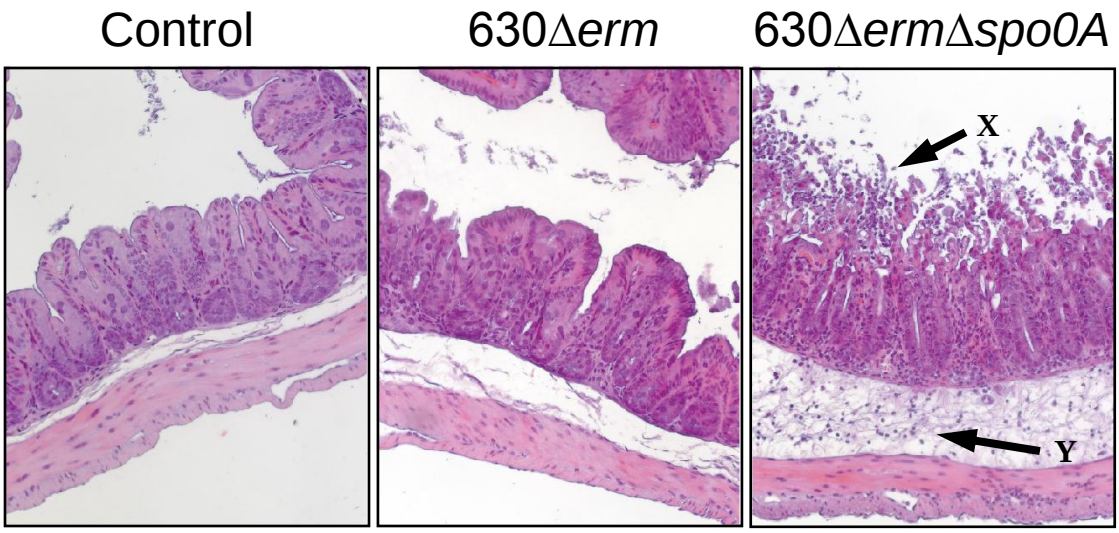

Figure 3.4. ***C. difficile*** **R20291** ***spo0A*** **mutants cause increased mucosal damage in mice.** Representative images demonstrating epithelial cell damage (arrow X) and acute infiltration arrow Y) in hematoxylin and eosin-stained caecum sections of mice infected with *C. difficile* R20291 or R20291Δ*spo0A* at day 2 post-infection. Magnification, x20.

Given the increased pathology associated with R20291Δ*spo0A*-infection, we decided to exploit electron and immunoelectron microscopy to both verify the observed histopathology and to provide a more detailed analysis of the infection. Using Transmission Electron Microscopy (TEM), we found that, again, control mice did not demonstrate any obvious pathology or signs of infection (Figure 3.5; top panel). R20291-infected mice, however exhibited some damage of the mucosal lining accompanied with immune cell infiltrate (Figure 3.5; top panel). Numerous *C. difficile* cells were evident, however these were largely contained to the lumen and no obvious invasion was noted.

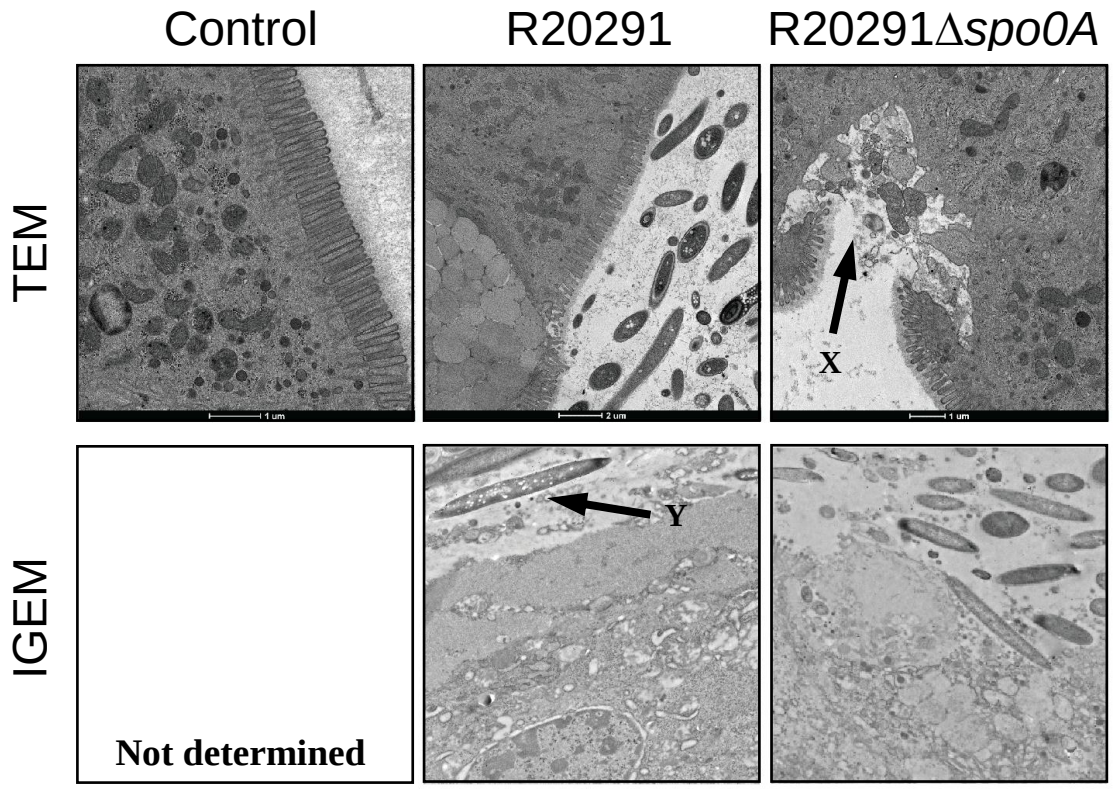

Figure 3.5. *C. difficile* R20291 Spo0A mediates pathological responses in mice. Top panel (TEM): transmission electron microscopy images, and bottom panel (IGEM): immune-gold electron microscopy images of mice infected with *C. difficile* R20291 or R20291Δ*spo0A* at day 2 post-infection. Post-infection damage was most notable in R20291Δ*spo0A*-infected mice, which was associated with microvillus effacement, endothelial cell necrosis and superficial invasion (arrow X). A greater ratio of *C. difficile* to non-*C. difficile* was also evident in the lumen of mice infected with *C. difficile* R20291Δ*spo0A*. Arrow Y indicates an immune-gold-labelled *C. difficile* R20291 vegetative cell.

The post-infection damage was greatest in R20291Δ*spo0A*-infected mice, which demonstrated acute mucosal damage with microvillus effacement and denudation (Figure 3.5; top panel). Endothelial cell necrosis and acute infiltration was also prominent. As with R20291-infected mice, numerous *C. difficile* cells were also visible, however this also appeared to be associated

with superficial invasion in mice infected with R20291Δ*spo0A* (Figure 3.5; top panel).

Using Immuno-Gold Electron Microscopy (IGEM), we were also able to estimate the number of *C. difficile* present, and thus also the ratios of *C. difficile* to non-*C. difficile* cells in local sections of caecum tissue (Figure 3.5; bottom panel). We found that the total numbers of *C. difficile* per unit area were approximately the same for R20291- and R20291Δ*spo0A*-infected mice. However, we noted that the ratios of *C. difficile* to non-*C. difficile* in the lumen were different, with a greater ratio of *C. difficile* present in mice infected with R20291Δ*spo0A*.

3.4.3.2.2 Pathological response to C. difficile 630Δerm infection

In contrast to *C. difficile* R20291, *C. difficile* 630Δ*erm* infection incited a much more modest pathological response. As before, uninfected, clindamycin control mice did not exhibit any pathology. At day 4 post-infection, some mucosal damage was apparent in mice infected with *C. difficile* 630Δ*erm*, such as oedema and some infiltration (Figure 3.6). These observations were more apparent in 630Δ*erm*Δ*spo0A*-infected mice, which demonstrated greater oedema and more pronounced immune cell infiltrate within the caecal mucosa compared to 630Δ*erm*-infected mice (Figure 3.6). Nonetheless, this was to a much more modest degree than was noted for ribotype 027 (R20291)-infected mice. Thus, in addition to not causing a long-term persistent infection, *C. difficile* 630Δ*erm* is also less pathogenic in our murine model of infection.

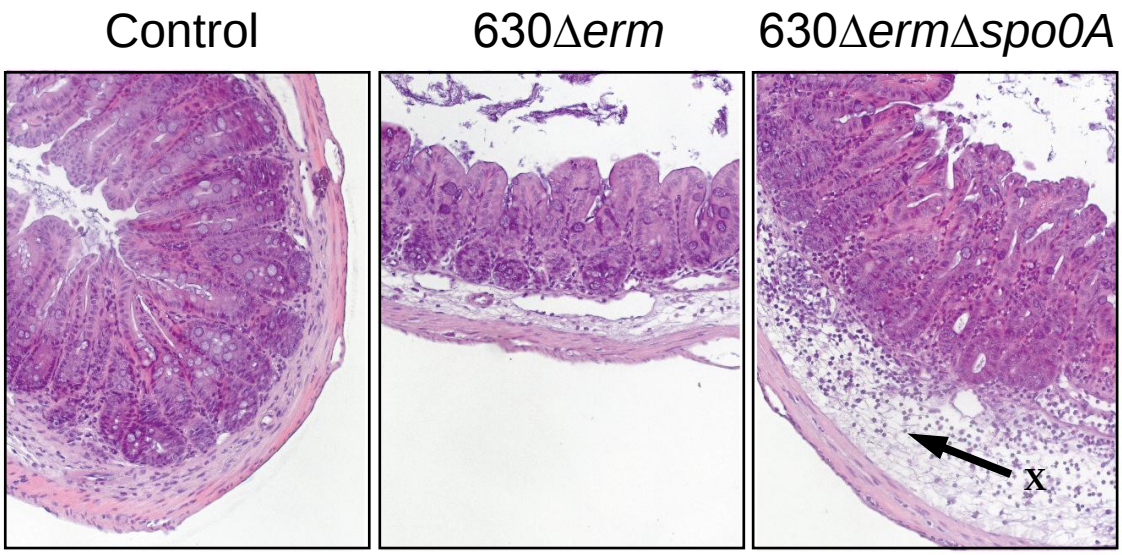

Figure 3.6. ***C. difficile* 630Δ*erm*Δ*spo0A* mutants cause increased mucosal damage in mice.** Representative images demonstrating epithelial cell damage in hematoxylin and eosin-stained caecum sections of mice infected with *C. difficile* 630Δ*erm* or 630Δ*erm*Δ*spo0A* at day 4 post-infection. Magnification, x20. Arrow X represents submucosal oedema and immune cell infiltrate, including the recruitment of polymorphonuclear neutrophils (PMNs).

3.4.3.3 Association between Spo0A and toxin synthesis

The association between spore formation and toxin production has been described previously, although the correlation is contentious. Reports describing positive regulatory associations between the two phenomena are opposed by those describing negative or minimal regulatory links. For example, in a recent publication, Underwood *et al*. (2009) outlined that *spo0A* deficient *C. difficile* produce less TcdA (177). On the other hand, a more recent paper by Saujet *et al*. (2011) demonstrated that SigH (an alternative sigma factor implicated in

sporulation initiation) markedly reduced *spo0A* transcription and concomitantly increased TcdA synthesis (182).

3.4.3.3.1 C. difficile R20291produces more TcdA and TcdB than 630Δerm strains

Using highly sensitive anti-TcdA and anti-TcdB specific ELISA assays, we demonstrate that *C. difficile* 630Δ*erm* produces less toxin than *C. difficile* R20291 under the growth conditions investigated, as shown in Figure 3.7A and B. This characteristic may theoretically be a contributory factor to R20291 "hypervirulence" and could explain, at least in part, why *C. difficile* 630Δ*erm* is less virulent in our model of infection. The difference in toxin production levels between 027 (R20291) and 012 (630Δ*erm*) ribotypes is consistent with previous observations (219). Additionally, purified TcdB from R20291 has previously been shown to have greater cytotoxic potency than 630 (11).

3.4.3.3.2 spo0A is a negative regulator of toxin production in C. difficile R20291

The increased virulence exhibited by *C. difficile* R20291Δ*spo0A* in our mouse model was not anticipated. We hypothesised that these observations may 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*. Low-levels of TcdA and TcdB were detected during mid-exponential growth in cultures of both R20291 and

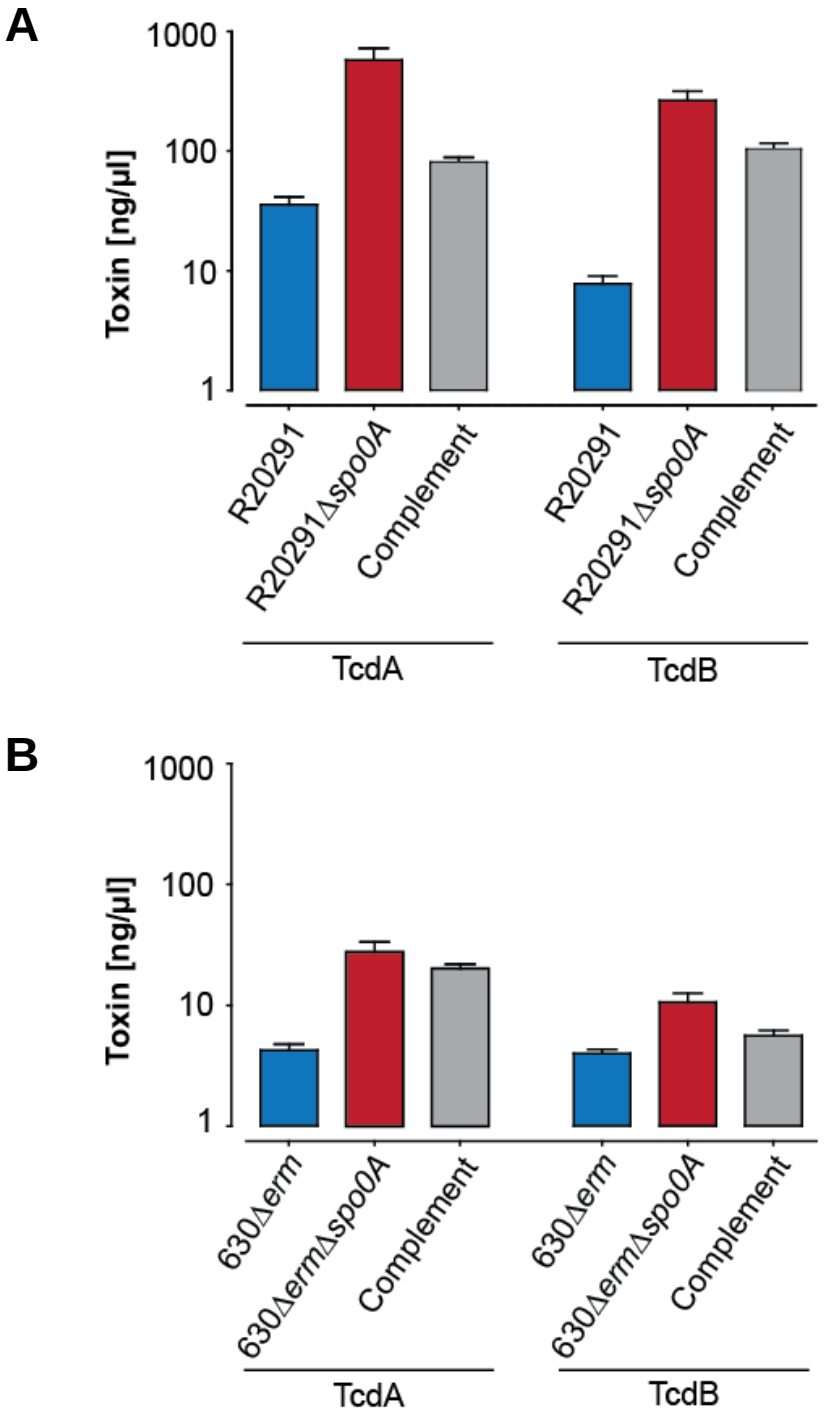

Figure 3.7. *C. difficile spo0A* mutants produce elevated levels of TcdA and TcdB. Sandwich ELISA indicating the relative levels of TcdA and TcdB produced by **A)** *C. difficile* R20291 derivatives and **B)** *C. difficile* 630Δ*erm* derivatives after 30 h of growth in Wilson's broth under anaerobic conditions. Data are from 3 independent experiments performed in triplicate. The error bars indicate standard deviations.

R20291Δ*spo0A* (data not shown). However, during stationary growth (30 h), significantly higher levels of both TcdA (P = 0.0006) and TcdB (P = 0.0005) were detected in *C. difficile* R20291Δ*spo0A* compared to R20291 cultures (Figure 3.7A). 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 demonstrate that R20291 and R20291Δ*spo0A* are virulent in mice and that exaggerated virulence by R20291Δ*spo0A* during the acute phase of infection is at least associated with increased TcdA and TcdB production. The increased toxin production by *spo0A* mutant derivatives may also be linked to the reduced bacterial diversity observed via IGEM in R20291Δ*spo0A*-infected mice, though this hypothesis clearly needs to be explored further.

3.4.3.3.3 Role of C. difficile 630Δerm Spo0A in toxin production

Low-levels of TcdA and TcdB were detected during mid-exponential growth by both 630Δ*erm* and 630Δ*erm* Δ*spo0A* (data not shown). During stationary growth (30 h), TcdA and TcdB were detected in all *C. difficile* 630Δ*erm* derivatives (Figure 3.7B). The levels of TcdA and TcdB were significantly higher in cultures of the *spo0A* mutant compared to those of the *C. difficile* parental strain (both P = < 0.05). The levels of TcdA and TcdB were both reduced upon genetic complementation with the *spo0A* gene, although this was not to the levels of

C. difficile 630Δ*erm* (P = < 0.05; Figure 3.7B).

We thus demonstrate that although *C. difficile* 630Δ*erm* produces less TcdA and TcdB than R20291 strains, the *spo0A* gene may nonetheless negatively regulate toxin production in this ribotype.

3.4.3.3.4 Attempt to quantify toxin from faecal samples

We have quantified both TcdA and TcdB levels *in vitro* as this is the most likely explanation for the exaggerated virulence phenotype in R20291Δ*spo0A*-infected mice. Additionally, we made multiple attempts at measuring the levels of TcdA and TcdB directly from the faeces of *C. difficile* infected mice, but have had no success.

It is important to note that precise and specific quantification of *C. difficile* toxin levels from faeces has never been reported, likely due to technical issues with faecal matter interfering with antibody-toxin interactions. Although these data would clearly be interesting, we do not believe that they would alter our conclusions about the role of *C. difficile spo0A* in virulence.

3.4.4 Role of *spo0A* in persistence and recurrence

Recurrent infection after cessation of antibiotic therapy is now a salient feature of *C. difficile*

disease and recurring episodes of diarrheoa are rapidly becoming the norm rather than the exception in elderly patients. As discussed in Chapter 1, recurrent disease can be the result of either (i) relapse with same strain that caused the initial infection, or (ii) re-infection with a different strain of the pathogen (122, 123). In this sense, recurrent disease is often considered to be a form of persistent infection, representing a constant background burden for the host, rather than a fortuitous sequelae of infection.

3.4.4.1 The *C. difficile spo0A* gene is required for persistent infection

We previously demonstrated that *C. difficile* isolated from infected hospital patients can asymptomatically persist in mice for several months (98). Therefore, we next assessed the contribution of the *C. difficile spo0A* gene to colonisation and persistent infection. To assess this phenotype, competitive index (CI) experiments were performed, whereby 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 (Figure 3.8A). We observed that genetic disruption of *spo0A* resulted in reduced fitness by day 2 post-infection and continued to day 15 post-infection (Figure 3.9A). Additionally, the difference in relative fitness for the 630Δ*erm*Δ*spo0A* mutant was statistically significant by day 5 post-infection (Figure 3.8B). Although *C. difficile* 630Δ*erm* does not cause a long-term persistent infection in our murine model, we found that

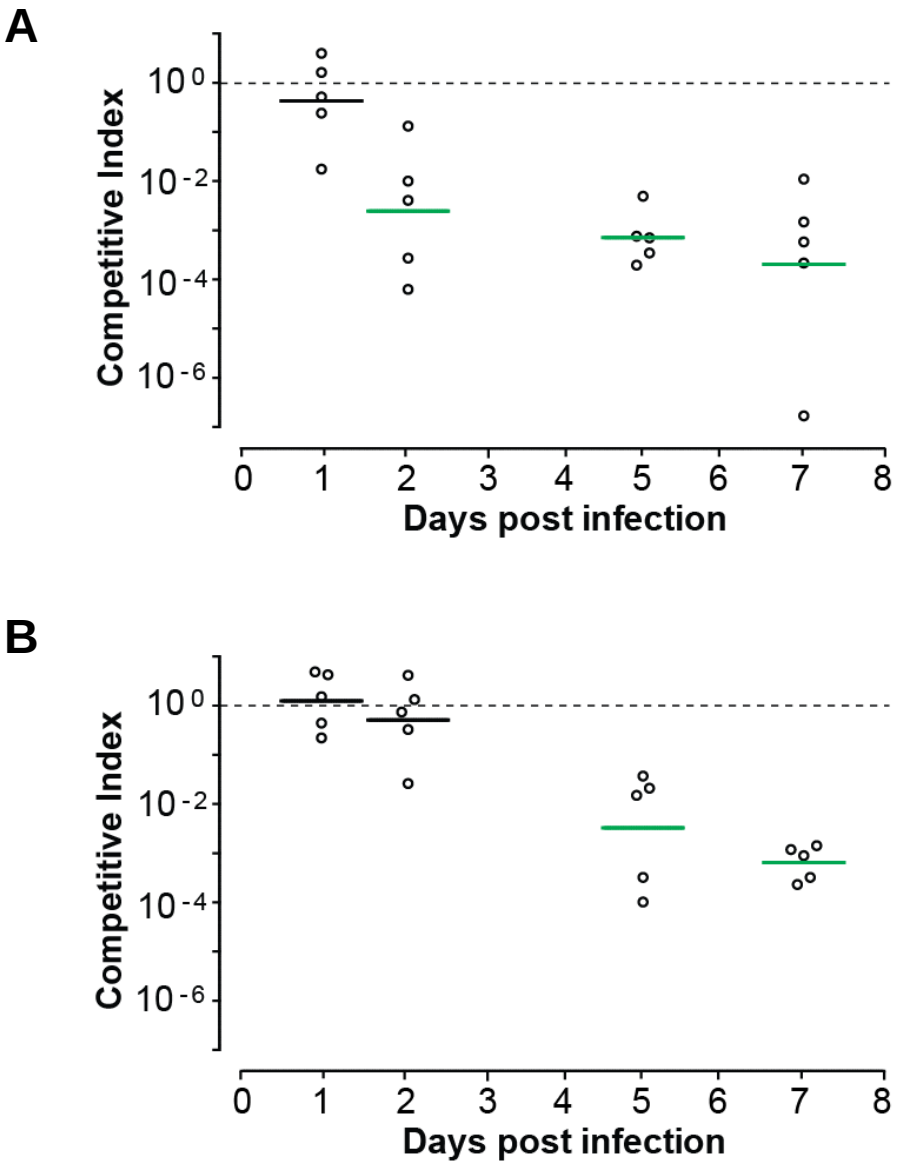

Figure 3.8. *C. difficile* Spo0A mediates intestinal persistence in mice. Competitive index (CI) time-course of **A)** *C. difficile* R20291 and R20291Δ*spo0A*, and **B)** 630Δ*erm* and 630Δ*erm*Δ*spo0A*. Individual CI values are illustrated as open circles, and horizontal bars represent the geometric means. Geometric means in green indicate that the *spo0A* mutant is attenuated, as determined by the Mann-Whitney test.

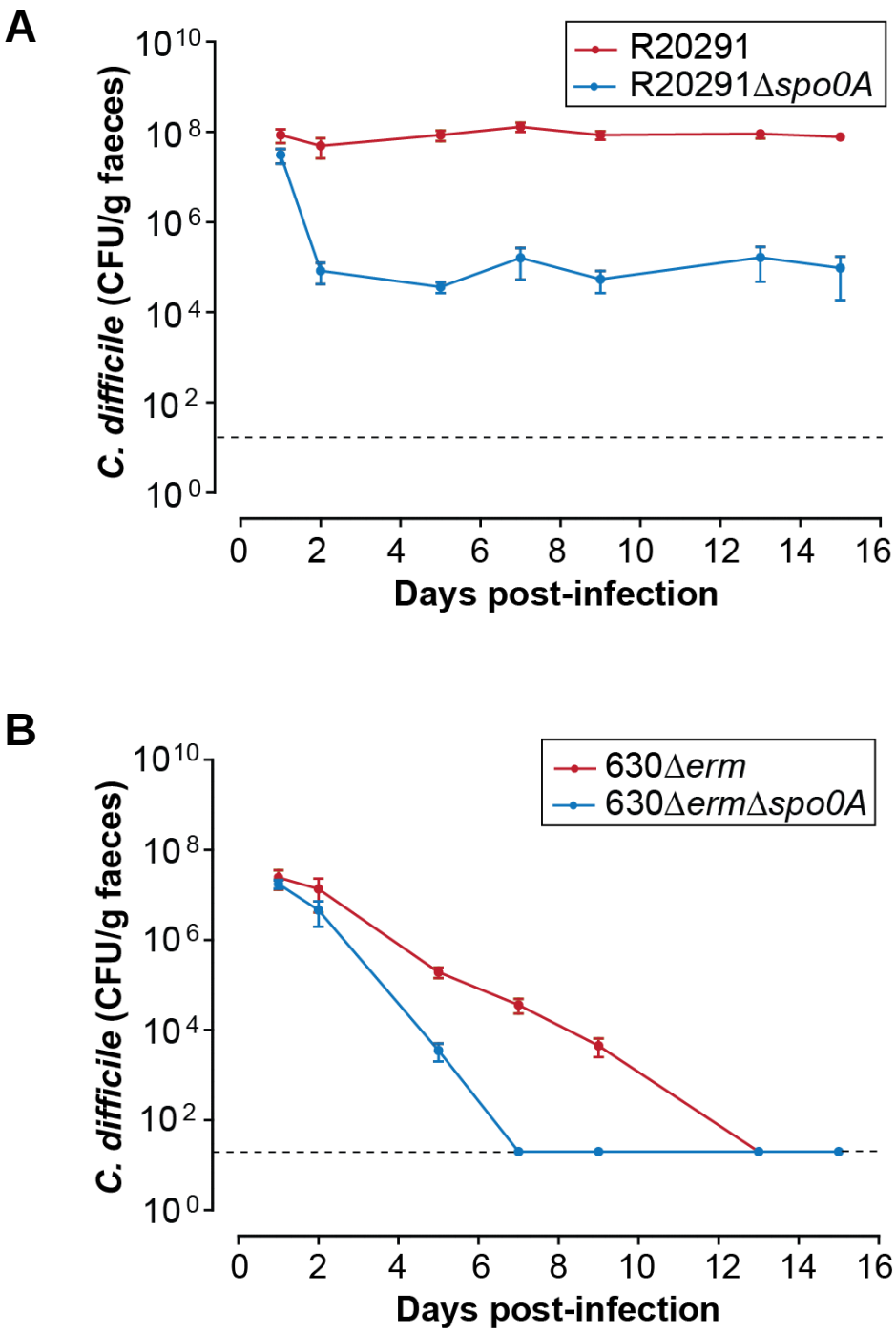

Figure 3.9. *C. difficile spo0A* gene is required for persistent colonisation in mice. Representative faecal shedding profiles from mice (*n* = 5 per group) infected with **A)** *C. difficile* R20291 or R20291Δ*spo0A*, or **B)** *C. difficile* 630Δ*erm* or 630Δ*erm*Δ*spo0A*. In both *C. difficile* ribotypes, genetic disruption of *spo0A* resulted in a reduced fitness *in vivo*.

the *C. difficile* 630Δ*erm*Δ*spo0A* mutant was reproducibly cleared ~6 days earlier than the parental strain (Figure 3.9B). These results indicate that the *spo0A* gene contributes to the intestinal colonisation and persistent infection of *C. difficile* in mice.

3.4.4.2 *C. difficile spo0A* mediates disease recurrence

Recurrent infection is defined by the complete subsidence of symptomatic disease followed by its subsequent reappearance. Given that *C. difficile spo0A* may affect persistence, we were interested in whether Spo0A also influences recurring infection. To test this, mice infected with R20291, R20291Δ*spo0A*, *C. difficile* 630Δ*erm* or 630Δ*erm*Δ*spo0A* were treated with a 7 day course of oral vancomycin. This is a standard therapy for patients experiencing severe systemic *C. difficile* disease. 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 faecal shedding of *C. difficile* to below the detection limit in all infected groups of mice (Figure 3.10A and B). This was 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* 630Δ*erm* exhibited recurrent infection with the same isolate.

With *C. difficile* 630Δ*erm*, this recurrence phenotype was observed after the first two courses of vancomycin, however after a third treatment the recurrent symptoms did not occur (Figure

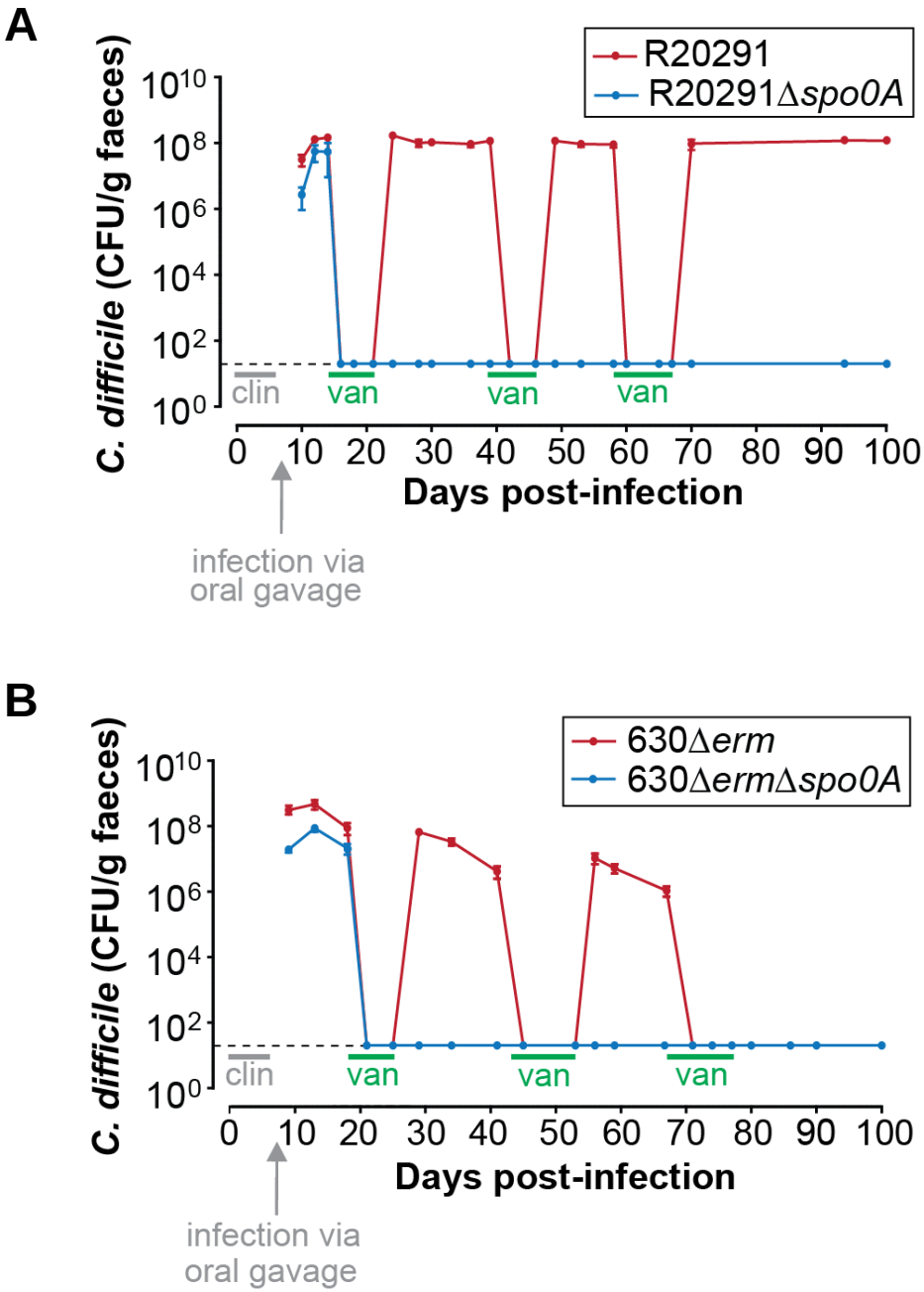

Figure 3.10. *C. difficile spo0A* gene is required for relapsing disease in mice. A and **B)** Representative faecal shedding profiles from mice (n = 5 per group) infected with *C. difficile* R20291, R20291Δ*spo0A*, 630Δ*erm* or 630Δ*erm*Δ*spo0A*. Mice were pre-treated with clindamycin (represented as a grey line) for 7 d prior to infection via oral gavage. Following infection, mice received a series of 7-10 d courses of vancomycin (represented as green lines) during which faecal shedding of *C. difficile* decreased to below the detection limit of the assay in all groups of mice. The dashed horizontal line indicates the detection limit.

3.10B). In contrast, *C. difficile* R20291-infected mice consistently exhibited disease recurrence on cessation of treatment for up to 100 days (Figure 3.10A). Significantly, mice infected with either *C. difficile* R20291Δ*spo0A* or 630Δ*erm*Δ*spo0A* cleared the infection during the first course of vancomycin and did not demonstrate recurrence at any point during the 100 day monitoring period (Figure 3.10A and B).

3.4.4.3 Disease recurrence is likely the result of relapse

Previous publications have indicated that approximately 50% of *C. difficile* recurrences are the result of re-exposure to the pathogen from an exogenous source such as other patients, healthcare workers, or from the environment, indicating that this is a key risk factor for disease recurrence (23). The remaining 50% of recurrences are estimated to be the result of a reactivation of the same strain that caused the initial infection, which had endogenously persisted within the host.

Thus, we hypothesised that recurrence in R20291 and 630Δ*erm*-infected mice could be the result of either persistence of the bacterium within the host during vancomycin therapy, or re-colonisation from environmental spores. To address this, mice infected with *C. difficile* R20291 or *C. difficile* 630Δ*erm* 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. 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 where 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.

In a study of hospital patients, Walters *et al*. (1983) observed that disease recurrence was associated with incomplete clearance of *C. difficile* from the bowel of infected patients receiving vancomycin treatment, though the authors acknowledged that a contaminated environment was a potential reason for relapse (58). Our findings, however are in direct contrast with Onderdonk *et al*. (1980) who reported that the levels of spores actually increased in the ceacal contents of *C. difficile*-infected mice during vancomycin treatment (220). Nonetheless, our data suggest that it is relapse (i.e., recurrence with the same strain) from an exogenous reservoir that was the source of infection in our model of infection.

3.4.5 Role of *spo0A* in *C. difficile* transmission

The central importance of *C. difficile* transmission is recognised (61, 98, 150). However, the molecular factors that influence transmission are unknown. A greater understanding of the

transmission dynamics of *C. difficile* is potentially of great importance to infection control and clinical practices.

3.4.5.1 *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 (61, 98, 150). Here, we use this model to investigate the role of the *C. difficile spo0A* gene in host transmission via distinct transmission routes, mimicking the situation commonly observed in hospital patients (see Materials and methods Table 2.3 and Figure 2.1). During transmission experiments, the donor mice shed equivalent levels (~10^8 CFU/gram fresh faeces) of vegetative *C. difficile* R20291, 630Δ*erm* or their respective *spo0A* mutant derivatives (Figure 3.11A). Mice infected with *C. difficile* R20291 or *C. difficile* 630Δ*erm* also shed spores (~10^6 CFU/gram fresh faeces) in contrast to mice infected with the *spo0A* mutants where spores were undetectable (Figure 3.11A).

Mingling donor mice that were shedding high-levels of *C. difficile* R20291 or 630Δ*erm* with naïve recipient mice for 1 h (in cages without bedding) resulted in 100% transmission (Figure 3.11B; n = 10 for each strain). In contrast, mingling donors excreting *C. difficile* R20291Δ*spo0A* or 630Δ*erm*Δ*spo0A* with naïve recipient mice resulted in only 40% or 20% transmission efficiency, respectively. In these experiments coprophagy was regularly observed and this behaviour likely promotes *C. difficile* transmission. To further define the

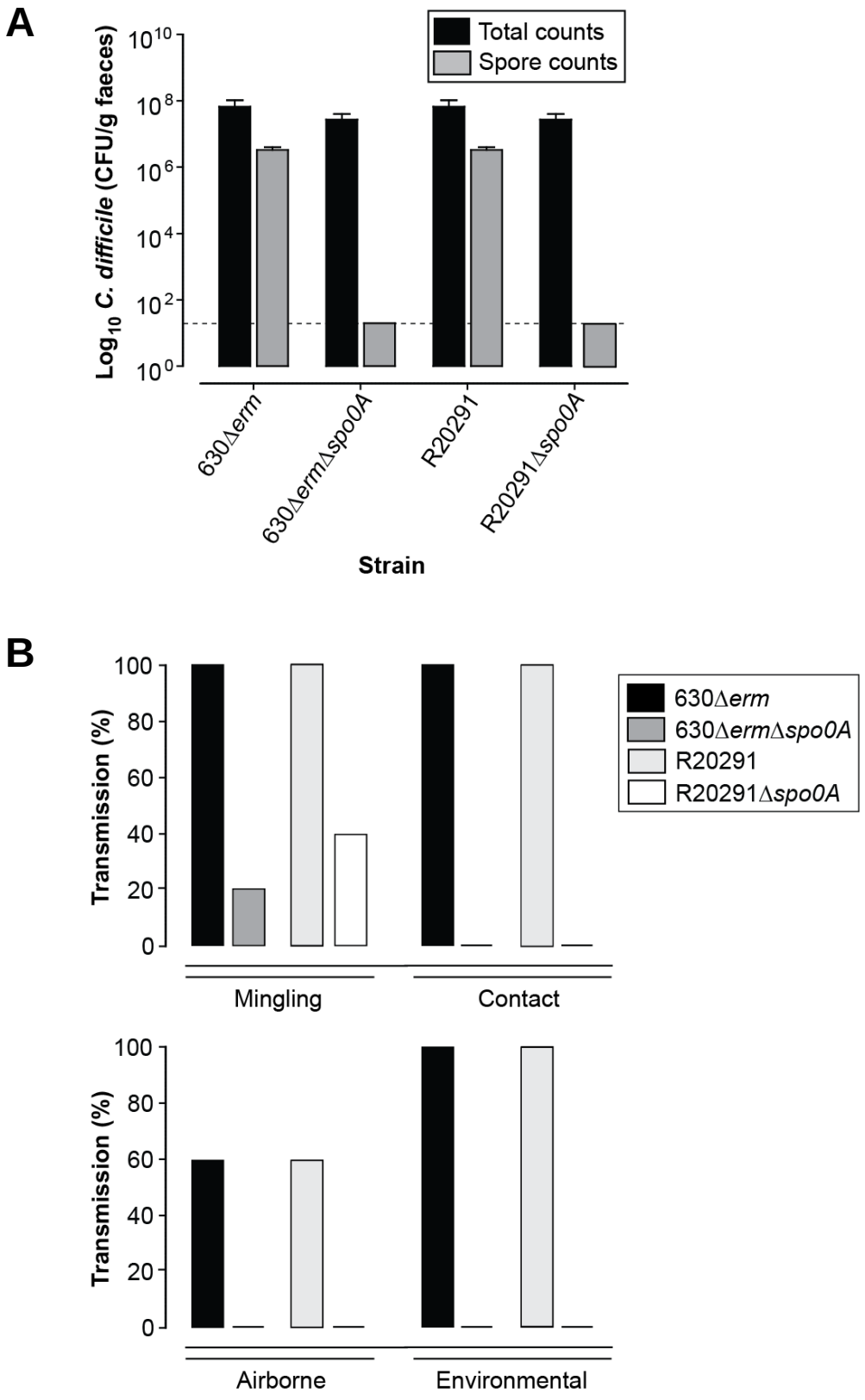

Figure 3.11. Host-to-host transmission of *C. difficile* is mediated by Spo0A. A) Average

faecal shedding of *C. difficile* by mice (n = 5 per group) at 5 d post-infection. The dashed horizontal line indicates the detection limit. **B)** Transmission efficiency of *C. difficile* 630Δ*erm*, R20291, 630Δ*erm*Δ*spo0A* or R20291Δ*spo0A*, demonstrating the percentage of naïve recipient mice that acquired infection following exposure to infected donor mice via mingling, contact, airborne or environmental transmission. Efficiency of transmission was determined as described in the Materials and Methods.

transmission route, we placed a porous wall between donor and recipient mice so that mice could come into contact but coprophagy was blocked. During these experiments *C. difficile* R20291 and 630Δ*erm* were still transmitted at 100% efficiency, whereas the *spo0A* mutant derivatives failed to transmit at a detectable level (Figure 3.11B; contact transmission).

Next, we tested transmission between donor and recipient mice that were separated by a double porous wall that prevents coprophagy and direct contact, but would still allow airborne transmission. In these experiments, the *C. difficile* R20291 and 630Δ*erm* were transmitted at 60% efficiency whereas the respective Δ*spo0A* derivatives failed to transmit (Figure 3.11B; 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 (61, 221). To mimic this situation, we contaminated cages (without bedding) by placing donor mice shedding *C. difficile* in cages for 1 h, and then removed faeces and allowed the cages to stand overnight (~16 h) in a sterile environment such that only the aerobic resistant spores survived. Exposure of naïve recipient mice to the environment contaminated with *C. difficile*

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*erm*Δ*spo0A*-contaminated cages became colonised (Figure 3.11B; environmental transmission).

3.5 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 demonstrate 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 show that a functional *spo0A* gene is required for relapsing infection after vancomycin therapy, and we further illustrate 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 tract of challenged mice during vancomycin treatment, indicating that maintenance of *C. difficile* within the intestinal tract was not a likely cause of disease recurrence, although we cannot rule out 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 re-infection *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 (98).

As expected, genetic inactivation of *spo0A* resulted in an asporogenous phenotype in *C. difficile* R20291 (ribotype 027) and 630Δ*erm* (ribotype 012) (177). In addition to not being able to form spores, we demonstrate *C. difficile spo0A* mutants produced elevated levels of the toxins TcdA and TcdB *in vitro* compared to the respective parental strains. Therefore, Spo0A may negatively regulate toxin production, which was associated with increased virulence for mice infected with the *C. difficile* 027 variant. This finding was in contrast to the observations of Underwood *et al*. (2009), which indicated that a *C. difficile* 630Δ*erm spo0A* mutant produced < 10% of the parental levels of TcdA (177). This discrepancy may reflect the different methods used to quantify toxin levels. However, it is also noteworthy that there are several further flaws that could explain these differences, the most obvious being the lack of genetic complementation to restore the sporulation and toxin expression phenotypes. Thus, it is not explicit what genetic lesions are contributing to these phenotypes.

The data presented in this chapter strongly suggests that Spo0A is required for persistence, disease recurrence and transmission of *C. difficile* in our clindamycin-induced mouse model of infection. However, it is important to note that *C. difficile* R20291Δ*spo0A* and 630Δ*erm*Δ*spo0A* derivatives are inherently resistant to clindamycin (owing to the ClosTron-mediated insertion of the *ermB* RAM). *C. difficile* R20291 and 630Δ*erm* parental strains do

not carry a functional copy of this gene and are consequently susceptible to clindamycin (see chapters 1.6.1.1 and 2.2.5.9). Although clindamycin has a short elimination half-life of approximately 2 - 4 hours (222), we were nonetheless unable to truly compare the colonisation, persistence and transmission kinetics of such strains in a like-for-like manner. Additionally, given the sensitivity *C. difficile* vegetative cells to oxygen, enumeration of the bacterium from the faeces inherently favours the recovery of the spore. In this sense, it may have been prudent to quantify the number of vegetative cells and spores directly from the site of infection. Clearly, however this would become the end-point of the experiment.

Furthermore, in the well-characterised model organism *B. subtilis*, Spo0A regulates processes other than spore formation, such as efflux pumps and metabolism (184). 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 level should identify persistence and transmission factors controlled by Spo0A in *C. difficile*. Additionally, this approach will enable us to identify any pleiotropic effects of the *spo0A* mutation besides sporulation, and link this back to its role in disease, relapse, persistence and transmission.