Use of *Bacillus subtilis* PXN21 spores for suppression of *Clostridium difficile* infection symptoms in a murine model

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**Abstract**

*Clostridium difficile* is the primary cause of nosocomial diarrhoea in healthcare centres of the developed world. Only a few antibiotics are available for treatment, and relapses are common in patients undergoing antibiotic therapy. New approaches are required to reduce reliance on antibiotics, the use of which represents a primary risk factor for development of *C. difficile* infections. Supplementation of the gut flora with probiotics represents a key area for producing more successful treatment options for *C. difficile* infection (CDI). In this study, spores of *B. subtilis* have been evaluated as a potential probiotic treatment against CDI. Using a murine model of infection, we demonstrate that oral administration of *B. subtilis* spores can attenuate the symptoms of infection. We further show that (1) suppression of symptoms was better if spores were administered post infection, and (2) germination of the spore to a vegetative cell may be an integral part of how CDI is suppressed. The results of this study highlight the potential of this bacterium as a probiotic treatment for CDI.

**Introduction**

*Clostridium difficile* is the primary aetiological agent of nosocomial diarrhoea in developed countries. Disease occurs when the normal colonic flora is disrupted, typically associated with antibiotic treatment. Dysbiosis of the microbiota of the gastro-intestinal (GI) tract allows ingested spores of *C. difficile* to germinate and proliferate. Clinical symptoms are mediated by production of two major exotoxins, toxin A and toxin B. Infection results in a variety of manifestations ranging from a nonsymptomatic carrier state to diarrhoea and inflammation of the gut. Severe infections can lead to development of pseudomembranous colitis and toxic megacolon which is potentially fatal (Rupnik *et al.*, 2009). Hospitalised elderly individuals receiving antibiotic treatment are the primary group at risk for developing this infection, although incidence in other groups such as pregnant woman (Rouphael *et al.*, 2008) and younger individuals is increasing (Wilcox *et al.*, 2008). Production of highly resistant spores during the infection cycle presents a major challenge to the control of infection, as spores play a significant role in the spread and persistence of disease (Dawson *et al.*, 2012; Deakin *et al.*, 2012).

The implementation of mandatory reporting across the UK in 2007 has resulted in a decrease in the number of cases as awareness of *C. difficile* has increased (HPA, 2009). However, the emergence of ‘hypervirulent’ strains and high relapse rates of infection have contributed to the persistence of this infection. A limited number of antibiotics are available for use in the treatment of *C. difficile* infection (CDI), and around 20% of treated cases will experience a recurrence of infection following treatment, representing a key challenge for treatment of CDI. Use of antibiotics is also a principle risk factor for development of infection. Due to these limitations in the use of antibiotics, alternative treatment strategies are being investigated. Most promising is faecal transplantation therapy consisting of an extract prepared from homogenised stool sample which is administered to the patient via a nasogastric tube (MacConnachie *et al.*, 2009). A second approach is that of the use of probiotics and/or prebiotics which have shown some effect in reducing symptoms of CDI. As an adjunct to antibiotic therapy, species of *Lactobacillus*, *Bifidobacteria* and *Saccharomyces boulardii* appear to reduce the incidence of CDI in human studies although none has shown full protection (Pochapin, 2000; Surawicz *et al.*, 2000; Wullt *et al.*, 2003). No
consensus has been reached as to how these bacterial supplements exert their effect, but one likely mechanism is by influencing the gut microbiota such that growth of *C. difficile* is inhibited. A number of human trials are in progress.

In this work, we used spores of *Bacillus subtilis*, a bacterial species already widely associated with probiotic use, to suppress symptoms of *C. difficile* CDI. Our objective was to assess the capacity of this bacterium, as a probiotic, to protect against disease.

**Methods**

**Strains**

(1) *B. subtilis*: PXN21 is one of fourteen strains in the commercial product Bio-Kult (Probiotics International Ltd, Lopen Head, UK) and was obtained from NCIMB where it is registered as NCIMB strain 30223. PY79 is a laboratory strain and derived from the type strain 168 (Youngman *et al.*, 1984). SC2376 gerD-cwlD is congenic to strain PY79 and carries a severe germination defect (Mauriello *et al.*, 2007).

(2) *Clostridium difficile*: R20291 is an epidemic strain of ribotype 027 and was obtained from T. Lawley (Wellcome Trust Sanger Institute, UK). *Clostridium difficile* VPI 10463 (provided by B. Wren, London School of Hygiene and Tropical Medicine, UK) was originally isolated in the USA in 1980 and has since been used as a reference strain.

**General methods**

Spores of *B. subtilis* strains were prepared in liquid medium by the exhaustion method as described previously (Harwood & Cutting, 1990). Heat-killed spores were prepared by autoclaving suspensions of spores at 120 °C for 20 min. Spores of *C. difficile* were prepared by growth on SMC agar plates using an anaerobic incubator (Don Whitley, UK) as described previously (Permpoonpattana *et al.*, 2011).

**Animal models of infection**

Female C57BL/6 mice (Charles River, UK) aged 6–8 weeks were used in all experiments, with animals housed in IVC units (Tecniplast, Italy). Sterile bedding was used in cages, and animals had *ad libitum* access to sterile water and food.

Two animal models were used to evaluate CDI: (1) a nonfatal colonisation model modified from that of Lawley (Lawley *et al.*, 2009) and (2) the fatal, severe, disease model of Chen *et al.* (Chen *et al.*, 2008).

(1) Nonfatal colonisation model: a single oro-gastric (o.g.) dose of clindamycin (30 mg kg⁻¹) was used 1 day prior to o.g. delivery of 1 × 10⁶ R20291 spores. Faecal samples were collected daily. Faecal samples were homogenised and then incubated with 70% ethanol for 20 min at RT. Samples were pelleted and ethanol removed before suspension in sterile water for serial dilution on brain heart infusion agar (BHI) supplemented with 0.1% cysteine and 0.1% sodium taurocholate for enumeration of ethanol-resistant spore counts.

(2) Fatal model: An antibiotic cocktail containing kanamycin (40 mg kg⁻¹), gentamicin (3.5 mg kg⁻¹), colistin (4.2 mg kg⁻¹), metronidazole (21.5 mg kg⁻¹) and vancomycin (4.5 mg kg⁻¹) was delivered via the o.g. route in a final volume of 200 µL per dose. Mice received three doses of the cocktail on consecutive days (D1, D2 and D3). On D5, mice were given a single o.g. dose of clindamycin (30 mg kg⁻¹). Animals were then infected with 1 × 10⁹ of VPI 10463 spores on D6. Animals were monitored for appearance of symptoms, with faecal samples and weights recorded daily. Animals were culled at the clinical end point of disease, which was considered when animals lost 20% of original body weight.

**Probiotic treatment**

Doses of 1 × 10⁹ spores in 200 µL of sterile H₂O were delivered by o.g. gavage. Animals received treatment daily: preinfection doses commenced on day 7 (where day 0 was the point of infection with *C. difficile*) with a total of seven doses received, and postinfection doses started on day 1, and administered until day 7 of study.

**Haematoxylin and eosin (H & E) staining**

C57Bl/6 mice (Charles River) were culled 3 days post infection with *C. difficile*. Colon and caecum were removed from animals that had received probiotic treatment either pre- or post infection, using nontreated and noninfected animals as controls. Tissue contents were carefully washed out and tissues fixed using 4% paraformaldehyde. Tissues were then sectioned, fixed on slides and stained with H&E (TUPI Manufacturing, Woodbridge, UK). Appearance of tissue was assessed microscopically once stained and images were taken using associated camera and software (GT Vision, Haverhill, UK).

**In vitro innate immunity tests**

RAW264.7 macrophage cells were seeded to six-well plates (3 × 10⁵) in antibiotic-free growth medium (DMEM with 10% foetal bovine serum) and incubated for 2 days to develop confluency. Cells were washed two
times with fresh growth medium then infected with media containing bacteria (spores or vegetative cells of \textit{B. subtilis} PXN21 at a CFU of $1 \times 10^7 \text{mL}^{-1}$).

For the Toll-like receptor 2 (TLR2) expression assay, a protocol previously used to study immunostimulatory properties of \textit{B. subtilis} (Huang et al., 2008a, b) was adapted for the assay. Cells were incubated for 4 h before washing two further times with sterile PBS. Macrophages were then lysed \textit{in situ} and total RNAs extracted using an RNeasy Kit (Qiagen, the Netherlands) as per manufacturer’s instructions. cDNA was produced using a Precision qScript Reverse Transcriptase Kit (PrimerDesign, Southampton, UK). qPCR used primers targeted at the TLR2 gene (forward: AAGAGGAAGCCCAAGAAAGC reverse: CAATGGGAATCCTGCTCACT), with β-actin (forward: AGAGGCAAATCGTGCCTGAC reverse: CAACTACGGCTTCGCTTTGC) as a reference gene. A three-step PCR cycle was run 50 times with the following conditions: 95 °C 15 s, 55 °C 30 s and 72 °C 10 s. Data were analysed using ROTORGENE 3000 software (Qiagen, the Netherlands).

For detection of cytokines, macrophages were incubated with antibiotic-free growth medium (DMEM with 10% foetal bovine serum) containing spores or vegetative cells of \textit{B. subtilis} PXN21 at a CFU of $1 \times 10^7 \text{mL}^{-1}$ bacteria for 24 h. Lipopolysaccharide, (Sigma, MO) was used as a positive control for activation of macrophages \textit{in vitro}. Supernatants were then removed, and detection of cytokines in the medium was carried out using commercial ELISA Kits (eBioscience, Hatfield, UK). Plates were read at 450 nm using a Spectramax microplate reader (Molecular Devices, CA).

**Results**

**Colonisation resistance**

Nonfatal colonisation of \textit{C. difficile} was induced in a murine model of infection by antibiotic treatment followed by infection with \textit{C. difficile} R20291 spores (Fig. 1). Levels of \textit{C. difficile} spores present in faecal samples demonstrated that oral delivery of \textit{B. subtilis} PXN21 spores both prior to and post infection had no significant effect on reducing \textit{C. difficile} colonisation. In all groups, peak levels of spores were detected on day two post infection, and although \textit{C. difficile} levels were highest in mice receiving no probiotic treatment, this was not significant ($P = 0.1$).

**Attenuation of symptoms in a model of fatal disease**

A study model utilising \textit{C. difficile} strain VPI 10463 to induce a fatal infection in mice was used to assess the ability of \textit{B. subtilis} PXN21 to influence the clinical outcome of infection. This model of infection utilised presothing with a cocktail of different antibiotics including vancomycin that was designed to mimic the clinical situation of antibiotic therapy in humans (Chen et al., 2008). PXN21 spores were administered to mice before and after infection with VPI 10463, and both pre- and post infection with \textit{C. difficile} increased survival compared with nontreated animals (Fig. 2a). In pilot studies, we showed that administration of PXN21 spores did not reduce the efficacy of vancomycin in treatment of CDI in mice (data not shown). Delivery of PXN21 spores prior to infection resulted in a survival rate of 41.6% while 66.6% survival was achieved in animals treated post-CDI (Fig. 2a); this compared to a survival rate of 16.6% in nontreated groups. Interestingly, weight profiles of infected animals added further complexity to our results (Fig. 2b). Animals that survived infection having received probiotic treatment prior to infection displayed less weight loss than those infected with PXN21 spores post infection. Post infection-treated animals displayed a similar weight profile to the nontreated group, despite having the highest rate of survival.

**Live \textit{B. subtilis} spores are required for suppression of infection**

Administering PXN21 spores post infection was more effective in preventing CDI than predosing. As killed
spores have been shown to have adjuvant properties and can enhance the immunity of prototype vaccines (Barnes et al., 2007; Huang et al., 2010), we addressed whether spore viability might affect survival in mice administered PXN21 spores post infection. As shown in Fig. 3, a dosing of mice post infection with killed spores showed almost no improvement on survival. By contrast, dosing with live spores markedly improved survival. As shown earlier (Fig. 2a), treatment post infection with live PXN21 spores increases survival rate but did not induce an improvement in the weight profile of infected animals.

Histopathology

H&E-stained tissue sections were produced to assess pathology affecting gut tissues in groups receiving B. subtilis PXN21 spores. Images from tissue sections suggest that in animals given PXN21 spores, the gut tissues display reduced pathology, with less damage to cell structure than in untreated animals (Fig. 4). Tissues from healthy, noninfected mice exhibited well-defined tissue structure and cell integrity (Fig. 4a). Toxin-mediated damage is evident in the breakdown of tissue structure and disruption of epithelial cell membranes (Fig. 4d). The level of

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**Fig. 2.** Suppression of CDI using PXN21 spores. Groups (n ≥ 6) of mice received daily o.g. doses of 10⁶ Bacillus subtilis PXN21 spores either preinfection with Clostridium difficile VP1 10463 spores (10⁶) or postinfection. The preinfection groups received 7 doses of PXN21 spores and postinfection seven doses of PXN21 after infection. (a) Kaplan–Meier survival plot for pre- and postinfection treatment groups. (b) % weight change for groups receiving either daily doses of PXN21 spores preinfection (◊), post infection (□) or no treatment (△).

**Fig. 3.** Live PXN21 spores are required for suppression of CDI. Groups of mice were infected with Clostridium difficile VPI 10463 as described in Fig. 2 but in this case dosed post infection with live PXN21 spores (10⁶) or heat-killed PXN21 spores. (a) Kaplan–Meier survival plot for pre- and postinfection treatment groups. (b) % weight change for groups receiving either daily doses of PXN21 spores preinfection (◊), postinfection (□) or no treatment (△).
damage in terms of cell structure and tissue integrity was reduced in groups receiving PXN21 spores both before (Fig. 4b) and after infection (Fig. 4c). However, PXN21 treatment did not completely protect tissues, and some cellular damage was present in PXN21 treated animals, with minor disruption of cell membranes apparent.

**Mechanisms of probiotic action**

One mechanism for how PXN21 spores might suppress symptoms of CDI includes stimulation of the innate immune responses. To address this, first we examined stimulation of the Toll-like receptor 2 gene (TLR2) and, second, induction of two proinflammatory cytokines, IL-6 and TNF-α. Using an *in vitro* assay, expression of the TLR2 gene was measured using real-time PCR (RT-PCR) in RAW264.7 macrophages infected with PXN21 spores (live or killed) and PXN21 vegetative cells. This approach has been used previously to show potential TLR2 stimulants as TLR2 agonists can upregulate the TLR2 gene (Liu *et al.*, 2001). As shown in Fig. 5, vegetative cells and live spores of PXN21 efficiently stimulated expression of the TLR2 gene implying an interaction with the TLR2 receptor. Levels of expression were consistent with that of purified peptidoglycan which is a known TLR2 agonist (Liu *et al.*, 2001). By contrast heat-killed PXN21 spores showed a marked (four- to fivefold) reduction in TLR2 expression. Induction of the proinflammatory cytokines IL-6 and TNF-α was only apparent in macrophages infected with live PXN21 spores, and virtually no activity was present in cells infected with heat-killed spores (Fig. 6).

**Discussion**

This study shows that oral administration of *B. subtilis* PXN21 spores can reduce symptoms of CDI in a murine model of disease. PXN21 is a component of a commercial probiotic product and of course numerous products carry spores of *B. subtilis* as their active ingredient (Permpoopattana *et al.*, 2012). In addition, some traditional foods such as natto contain live spores of *B. subtilis* and have...
been recognised as carrying health benefits (Hosoi & Kiuchi, 2004). There is also good data showing that oral consumption of probiotic bacteria or prebiotics can have some effect in reducing the symptoms or occurrence of CDI. As an adjunct to antibiotic therapy, species of *Lactobacillus*, *Bifidobacteria* and *S. boulardii* appear to reduce the incidence of CDI in human studies although no study has yet shown full protection (Pochapin, 2000; Surawicz et al., 2000; Wullt et al., 2003). Moreover, no consensus has been reached as to how these bacterial supplements exert their effect. A number of mechanisms can be considered. First, by influencing the gut microbiota such that growth of *C. difficile* is inhibited possibly by out-competing for available nutrients or possibly by secretion of antimicrobials. This is the basis of treatments that focus on supplementation of the gut flora to prevent *C. difficile* from maintaining a niche within the GI tract. Faecal transplants, for example, while unpleasant, have been demonstrated to have a high success rate in resolving persistent infection (Gough et al., 2011) although carry risk of further infections unless samples are heavily screened prior to use. More recently, ‘synthetic stools’ have been developed (Lawley et al., 2012; Petrof et al., 2013), selecting particular microorganisms that are able to resolve infections through rebuilding the microbial communities in the gut without the unpleasant and uncertain aspect of faecal transplants. Supplementation of the gut flora with a complex or even minimalist mix of microorganisms mimics the protection against colonisation by *C. difficile* that the normal healthy microbial community of the gut provides. Single strains of bacteria are less likely to be able to successfully act as a substitute for the complex microbial communities of the gut. However, used alongside conventional antibiotics, particular strains could provide enough influence in the gut environment to reduce the symptoms of infection and aid restoration of the gut flora. A second mechanism would be by stimulation of a robust innate immune response in the gut-associated lymphoid tissue (GALT). The importance of the innate immune response in CDI has been demonstrated previously, with mice carrying deficiencies in immune signalling (Lawley et al., 2009) more vulnerable to infection. Direct stimulation of the innate immune receptor TLR5 has also been demonstrated to successfully protect against...
disease in infected mice (Jarchum et al., 2011). Finally, for toxin producing pathogens, it is possible that probiotic bacteria might subtract toxins produced in the gut lumen. This is particularly true for probiotic spores as these entities have been shown able to efficiently adsorb toxins onto the surface of the spore (Huang et al., 2010).

Our studies show here support the role of innate immunity as we have shown that PXN21 spores can upregulate expression of TLR2. Peptidoglycan is one of many ligands associated with TLR2 induction and was used as a control in our in vitro experiments. Spores carry peptidoglycan in their cortex, a layer lying beneath the spore coat; however, when autoclaved spores were used, induction of TLR2 was substantially reduced. Vegetative cells of PXN21 showed strong induction of TLR2, so we assign this to peptidoglycan exposed on the cell envelope. This suggests that spore germination and release of the growing vegetative cell might be critical to induction of TLR2. We base this assumption on the fact that when the spore is inactivated by autoclaving, it is not broken but remains intact, so it is unlikely that peptidoglycan within the spore cortex is released in significant quantities. In other studies, TLR2 was shown to be activated by live B. subtilis PY79 spores but not by heat-killed PY79 spores or spores unable to germinate (Huang et al., 2008a, b). Interestingly, in other work, we have shown that live spores of B. subtilis PY79 were shown to significantly delay symptoms of CDI in a hamster model of infection (Permpoonpattana et al., 2011). Although hamsters did eventually die from CDI, PY79 spores were clearly shown to delay fatalities and most likely this is due to germination and release of live B. subtilis vegetative cells. A clear result of TLR2 activation would be induction of pro-inflammatory cytokines, and we have confirmed here that (1) both IL-6 and TNF-α were induced and (2) induction did not occur with heat-killed PXN21 spores. Again, proof that this must arise from germination of live spores to the vegetative cell comes from indirect evidence using germinating and nongerminating spores of PY79.

It is not clear why dosing of spores beginning at the onset of CDI (post dosing) was superior to pre dosing. Innate immunity is normally short-lived and would be induced pre- and post dosing and should not really affect the outcome. One possibility, spores might have some level of adsorption or subtract toxins produced by C. difficile, and this process might be more effective if spores are dosed at the time infection begins. We have found that PXN21 spores can adsorb C. difficile toxin A in vitro but have not yet addressed whether this might occur in vivo. One problem with this explanation is practicalities of adsorption in the GI tract due to the interference of the gut microbiota. We are essentially sceptical but cannot completely exclude the possibility that with regular doses of spores, some level of adsorption could occur in the intestine.

Alternatively, prior dosing of probiotics could allow the probiotic strain to colonise the gut of the animal prior to infection allowing for some level of protection through supplementation of the gut flora. Bacillus subtilis can survive and reproduce in the murine gut (Hoa et al., 2001; Tam et al., 2006), and in the case of PXN21, this strain has been shown to persist in the GI tract of mice for 18 days following a single oral dose of spores (Permpoonpattana et al., 2012). Although often considered a soil-based organism, it is likely that B. subtilis has adapted for at least transient colonisation of the animal GI tract (Hong et al., 2009; Schyns et al., 2014). Reduced success of predosing could therefore be due to individual variation in animals in terms of successful colonisation.

Clearly, suppression was not complete and animals succumbed to CDI. We are not necessarily alarmed by this, and it is quite possible that the dose of PXN21 spores used was not at an optimal level. It is also possible that the dosing regimen might need adjustment, and we cannot rule out that excessive dosing might induce some level of tolerance. PXN21 spores as a treatment for CDI might have some value, and we state only that symptoms are significantly suppressed. If translated to humans, spores might have some utility possibly as an adjunct therapy with no apparent side effects.

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References


