Assessing the role of *p*-cresol tolerance in *Clostridium difficile*

Lisa F. Dawson, Richard A. Stabler and Brendan W. Wren

Correspondence
Brendan W. Wren
brendan.wren@lshtm.ac.uk

Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK

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Clostridium difficile is an important nosocomial pathogen, resulting in antibiotic-associated disease ranging from mild diarrhoea to the life-threatening pseudomembranous colitis. Upon antibiotic exposure, it is believed that the normal bowel microflora of patients is disrupted, allowing *C. difficile* to proliferate. Significantly, *C. difficile* is among only a few bacteria able to ferment tyrosine to *p*-cresol, a phenolic compound that is toxic to other microbes via its ability to interfere with metabolism. Therefore, the ability of different *C. difficile* strains to produce and tolerate *p*-cresol may play an important role in the development and severity of *C. difficile*-associated disease. In this study, it was demonstrated that two *C. difficile* hypervirulent 027 strains (Stoke Mandeville and BI-16) are more tolerant to *p*-cresol than other *C. difficile* strains including 630, CF4 and CD196. Surprising, it was shown that *Clostridium sordellii* also has a high tolerance to *p*-cresol, suggesting an overlap in the tolerance pathways in these clostridial species.

INTRODUCTION

Clostridium difficile is the most frequent cause of nosocomial diarrhoea worldwide (Bartlett, 1994; Kelly & LaMont, 1998). *C. difficile*-associated disease (CDAD) is a major health care problem that can lead to patient isolation, ward closures and, in extreme cases, hospital closure (Riley *et al.*, 1995). The population at risk is substantial, including patients on antimicrobial therapy, but also the immunocompromised and the elderly.

Disturbingly, the reported incidence of CDAD has increased significantly in the last decade, with highly virulent strains causing outbreaks of increased severity in North America and Europe (Loo *et al.*, 2005; McDonald *et al.*, 2005; Pépin *et al.*, 2005; Warny *et al.*, 2005). The origin of these strains (called BI/NAP1/027 hypervirulent strains) is uncertain, although it has been proposed that increased use of fluoroquinolone antibiotics may give the strains a selective advantage (Pépin *et al.*, 2005).

CDAD characteristically occurs after treatment with broad-spectrum antibiotics, which disrupt the normal gut microflora, allowing *C. difficile* to become established, although the mechanism(s) by which *C. difficile* establishes itself in the gut under these conditions is unclear. CDAD covers a wide range of diseases from asymptomatic or mild diarrhoea to moderately severe diarrhoea, and even pseudomembranous colitis, which can be fatal (Johnson & Gerding, 1998). Host factors and genetic differences in *C. difficile* strains account for some of the differences in

disease severity, but the status of the human gut microflora prior to and after infection is also likely to be important, particularly in the case of relapse.

C. difficile produces a number of factors that contribute to its virulence, including two related toxins, called toxin A and toxin B (Lyerly *et al.*, 1986). However, production of these toxins cannot completely explain *C. difficile* pathogenesis. In recent years, increasing numbers of pathogenic strains have been reported that have truncated versions of toxin A and/or B (Borriello *et al.*, 1992; Depitre *et al.*, 1993; Pituch *et al.*, 2003; Toyokawa *et al.*, 2003; van den Berg *et al.*, 2004). Our understanding of other factors involved in *C. difficile* pathogenesis and survival in the gut is poor.

It has been demonstrated that *C. difficile* produces a phenolic compound, *p*-cresol, via the degradation of tyrosine (Scheline, 1968), and can withstand up to 0.5% *p*-cresol, a concentration that inhibits the growth of other anaerobic bacteria (Hafiz & Oakley, 1976). To date, the mechanism and relevance of *p*-cresol production and tolerance have not been demonstrated. Our hypothesis is that it may provide the bacterium with a competitive advantage over other intestinal microflora and consequently contribute to the progression of CDAD. When the *C. difficile* strain 630 genome sequence was scrutinized (Sebaihia *et al.*, 2006), an intriguing genetic pathway was discovered that allows *C. difficile* to produce the bacteriostatic compound *p*-cresol (4-methylphenol) from tyrosine via the intermediate *p*-hydroxyphenylacetate (pHPA). The genes involved include those encoding components of pHPA decarboxylate (CD0153–CD0155, *hpdBCA*) (Sebaihia *et al.*, 2006), which catalyse the decarboxylation of pHPA to yield *p*-cresol (Selmer & Andrei, 2001).

Abbreviations: CDAD, *Clostridium difficile*-associated disease; pHPA, *p*-hydroxyphenylacetate.

Given the undoubted and unusual ability of *C. difficile* to produce and tolerate *p*-cresol, the aim of this study was to ascertain whether different strains of *C. difficile*, including the hypervirulent 027 strains, were able to tolerate different concentrations of *p*-cresol, and to compare the relative *p*-cresol tolerance with other clostridial species.

METHODS

Bacterial strains and culture conditions. The strains for this experiment were chosen from the hypervirulent clade and the toxin-variable clade (A^-B^+) as defined by Stabler *et al.* (2006) using Bayesian analysis of microarray data (Table 1). The strains covered a range of clostridial species, including *C. difficile* R20291 (Stoke Mandeville), a hypervirulent ribotype 027 strain, isolated from a recent outbreak in Stoke Mandeville (provided by Jon Brazier, Anaerobe Reference Laboratory, Cardiff, UK), which caused infection of over 300 people, along with an epidemic *C. difficile* 027 strain BI-16 (provided by Dale Gerding, Edward Hines Jr Veterans Affairs Hospital, Hines, IL, USA), which are both fluoroquinolone-resistant. *C. difficile* CD196 was the primary 027 ribotype (provided by Michel Popoff, Institut Pasteur, Paris, France), an historical strain, which is fluoroquinolone-sensitive. *C. difficile* CF4 (provided by Dale Gerding) was chosen because it falls in the A^-B^+ clade (Fig. 1), which lacks toxin A, thought to be an important virulence factor, whilst *C. difficile* strain 630 (Wüst & Hardegger, 1983) has been fully sequenced by the Wellcome Trust Sanger Institute (Sebahia *et al.*, 2006), thus providing useful additional information. *C. difficile* 630 was isolated in Switzerland from a patient with severe pseudomembranous colitis (provided by Dr Peter Mullany, Eastman Dental Institute, London, UK). *Clostridium perfringens* NCTC 8237 and *Clostridium sordellii* NCTC 6929 were provided by Peter Donachie (London School of Hygiene and Tropical Medicine).

C. difficile was routinely cultured on brain heart infusion (BHI) agar or BHI broth, containing *C. difficile* supplement (Oxoid) and 0.05 % cysteine. *C. perfringens* and *C. sordellii* were cultured on BHI agar or BHI broth with 0.05 % cysteine. All cultures were undertaken in an anaerobic cabinet at 37 °C in a reducing anaerobic atmosphere (10 % CO₂, 10 % H₂, 80 % N₂). To obtain cells during exponential phase (OD₆₀₀ 0.3–0.4), three single colonies were inoculated into pre-equilibrated medium in an anaerobic chamber, with shaking at 50 r.p.m. on an orbital shaker. When OD₆₀₀ 0.3–0.4 was reached, liquid cultures were inoculated 1:10 into BHI broth pre-equilibrated to the desired temperature and anaerobic atmosphere, containing 0.025, 0.05, 0.1 or 0.2 % (v/v) *p*-cresol (Sigma) alongside an untreated control. The cultures were incubated in an anaerobic chamber with shaking at 50 r.p.m. on an orbital shaker. Every hour for 4–7 h, 0.5–1 ml

culture was removed from the anaerobe chamber in sealed cuvettes and the OD₆₀₀ was measured.

Viability assays. Viability assays were performed using the growth conditions outlined above. After 4 h incubation in the *p*-cresol (0–0.2 %), serial dilutions were performed in pre-equilibrated PBS and plated in triplicate onto BHI plates, which were incubated for 24 h under the anaerobic conditions described above. Colony counts were determined for all of the test conditions and calculated as c.f.u. (ml culture)⁻¹. The *p*-cresol stress c.f.u. data were normalized to the untreated control and expressed as a percentage of the untreated control. Data were presented in graph form using GraphPad Prism version 4.02, and analysed using a two-tailed Student's *t*-test with a cut-off value of *P* < 0.01 in Microsoft Excel to determine whether any of the strains showed a significant difference in level of tolerance to *p*-cresol compared with *C. difficile* 630.

RESULTS AND DISCUSSION

Growth curves with varying concentrations of *p*-cresol (0–0.2 %) were produced for *C. difficile* strains R20291, BI-16, CD196, 630 and CF4 (Fig. 1). It appeared that the *C. difficile* 027 strains R20291 and BI-16 were significantly more tolerant to *p*-cresol than the historical *C. difficile* 027 strain CD196, and were more tolerant than *C. difficile* strains 630 and CF4 (Fig. 1a–e). This indicates that the newly emerging hypervirulent strains of *C. difficile* may tolerate a higher level of *p*-cresol, which, in turn, may be linked to the level of *p*-cresol production by these strains.

Growth curves were also undertaken for *C. perfringens* (NCTC 8237) and *C. sordellii* (NCTC 6929) over a similar time course with the same concentrations of *p*-cresol (0–0.2 %). Both strains appeared to tolerate similarly high concentrations of *p*-cresol, in line with the hypervirulent *C. difficile* 027 clinical isolates R20291 and BI-16.

Viability assays were then performed with a selection of the strains to determine the number of c.f.u. after exposure to various concentrations of *p*-cresol (0–0.2 %) (data not shown, except for 0.1 %; Fig. 2). The viability assays clearly showed that there was a significant increase in viable c.f.u. for *C. difficile* R20291 compared with *C. difficile* 630 (Fig. 2), with a value of *P* = 4.29 × 10⁻⁹, when treated with 0.1 % *p*-cresol, thus demonstrating that *C. difficile* R20291 tolerates a higher level of *p*-cresol than *C. difficile* 630, as

Table 1. List of strains

Strain	Information	Ribotype
<i>C. difficile</i> 630	Patient isolate from Switzerland, which caused pseudomembranous colitis	012
<i>C. difficile</i> R20291 (Stoke Mandeville)	Severe outbreak strain from Stoke Mandeville Hospital, fluoroquinolone-resistant	027
<i>C. difficile</i> BI-16	Epidemic fluoroquinolone-resistant strain	027
<i>C. difficile</i> CD196	Original 027 ribotype, fluoroquinolone sensitive	027
<i>C. difficile</i> CF4	A^-B^+ strain, lacking toxin A, asymptomatic	017
<i>C. sordellii</i> NCTC 6929	Causes myonecrosis and gangrene	–
<i>C. perfringens</i> NCTC 8237	Causes necrotic enteritis, food poisoning and gas gangrene	–

Fig. 1. Growth curves for *p*-cresol tolerance: (a) *C. difficile* 630, (b) *C. difficile* CD196, (c) *C. difficile* R20291, (d) *C. difficile* BI-16, (e) *C. difficile* CF4, (f) *C. perfringens*, (g) *C. sordellii*. The trends were reproducible for each strain. ■, Untreated bacteria; ▲, 0.025 % *p*-cresol; ▼, 0.05 % *p*-cresol; ◆, 0.1 % *p*-cresol; ●, 0.2 % *p*-cresol.

also indicated by the growth curves (Fig. 1). Also, in line with the growth-curve data, *C. difficile* CD196 and CF4 were not significantly different from *C. difficile* 630 in terms of *p*-cresol tolerance at a 99 % confidence interval ($P=0.062$) (Fig. 2). The hypervirulent *C. difficile* strains R20291 and BI-16 were more tolerant to *p*-cresol than the

more historical *C. difficile* strains 630 and CD196. This evolutionary difference between the historic and epidemic strains may be a contributory virulence factor of the *C. difficile* 027 strains, which have spread rapidly throughout North America and Europe, causing widespread disease.

Fig. 2. Survival of various clostridial species in 0.1 % *p*-cresol. The *C. difficile* strains chosen were: R20291, 630, CD196 and CF4, together with *C. perfringens* NCTC 8237 and *C. sordellii* NCTC 6929. *, Significant difference ($P < 0.01$; Student's *t*-test) compared with strain 630.

It was noted that, after 24 h on BHI agar plates, there was some growth for all of the strains treated with 0.2 % *p*-cresol. This was limited to relatively few colonies on the neat and lower-dilution plates (less than 0.009 % of untreated) (data not shown), which was most likely a result of germination of spores present in the cultures. It was clear from the growth curves that none of the strains tested was able to grow in 0.2 % *p*-cresol.

Viability assays for the other clostridial species indicated that *C. perfringens* had a similar level of tolerance for *p*-cresol to *C. difficile* 630 (Fig. 2), whereas, interestingly, *C. sordellii* was able to tolerate a similar level of *p*-cresol to the hypervirulent *C. difficile* R20291 strain (Fig. 2). This is of particular interest as it may indicate an overlap in tolerance pathways, or may suggest that the mechanism for *p*-cresol tolerance has been transferred horizontally or may have evolved via the acquisition of point mutations in similar pathways. However, Sivsammie & Sims (1990) revealed using GLC that neither *C. perfringens* nor *C. sordellii* produce *p*-cresol. To address this issue further, BLASTN analysis was performed with the current published genome sequences available for *C. perfringens* strains ATCC 13124 and SM101 (Myers *et al.*, 2006), with the *C. difficile* 630 genes *hpdA*, *hpbB* and *hpdC*, which encode the decarboxylase responsible for converting *p*HPA to *p*-cresol. No homologues were identified in *C. perfringens*. Currently, there is no genome sequence available for *C. sordellii*; however, there may be an overlapping pathway that has been adapted by *C. sordellii* to enable tolerance of *p*-cresol. It has been shown that several clostridial species produce *p*HPA from tyrosine, but do not decarboxylate it to *p*-cresol (Elsden *et al.*, 1976). Interestingly, in *Lactobacillus* strains, *p*-cresol, 5-hydroxyskatol and methylcatechol are all produced using the same method of decarboxylation (Yokoyama & Carlson, 1981), adding further support to the possibility of overlapping degradation pathways.

The current data from viability assays, along with the GLC data (Sivsammie & Sims, 1990), indicate that the mechanism of *p*-cresol tolerance may not be linked to the production of *p*-cresol, as *C. sordellii* tolerates *p*-cresol, yet does not produce it. This hypothesis is supported by preliminary data from a *hpdB* mutant constructed in an erythromycin-sensitive *C. difficile* 630 strain ($\Delta 630erm$), which indicate that, although the strain lacks the decarboxylase (via mutation) that enables the conversion of *p*HPA to *p*-cresol, it is able to tolerate the same level of *p*-cresol as the wild type (L. F. Dawson, unpublished data).

Future experiments will measure *p*-cresol tolerance from a larger selection of *C. difficile* strains, and will also measure the extent of *p*-cresol production from various strains using a combination of GLC, NMR and MS.

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