Bioavailability of the anti-clostridial bacteriocin thuricin CD in gastrointestinal tract

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Thuricin CD is a two component narrow spectrum bacteriocin comprising two peptides with targeted activity against Clostridium difficile. This study examined the bioavailability of thuricin with a view to developing it as an effective antimicrobial against intestinal infection. One of the peptides, Trn-β, was found to be degraded by the gastric enzymes pepsin and α-chymotrypsin both in vitro and in vivo, whereas Trn-α was resistant to digestion by these enzymes and hence was detected in the intestinal porcine digesta following oral ingestion by pigs. In order to determine if spores of the producing organism Bacillus thuringiensis DPC 6431 could be used to deliver the bacteriocin to the gut, spores were fed to 30 mice (approx. 10^8–2×10^9/c190/10^8 per animal) and their germination, growth and production of thuricin in the gastrointestinal tract (GIT) of the animals was monitored. Almost 99% of the spores delivered to the GIT were excreted in the first 24 h and neither Trn-α nor Trn-β was detected in the gut or faecal samples of the test mice, indicating that ingestion of B. thuringiensis spores may not be a suitable vehicle for the delivery of thuricin CD. When thuricin CD was delivered rectally to mice (n=40) and C. difficile shedding monitored at 1, 6, 12 and 24 h post-treatment, there was a >95% (>1.5 log units) reduction of C. difficile 027 in the colon contents of infected mice (n=10) 1 h post-treatment compared with the control group (n=10; P<0.001). Furthermore, 6 h post-treatment there was a further 1.5 log reduction in C. difficile numbers (n=10) relative to the control group (n=10; P<0.05). These results would suggest that rectal administration of thuricin may be a promising mode of delivery of thuricin CD to the colon.

INTRODUCTION

Clostridium difficile is the leading aetiological agent responsible for nosocomial diarrhoea in the developed world and the most common cause of pseudomembranous colitis. Moreover, it is also responsible for up to 25% of all cases of antibiotic associated diarrhoea, with the elderly being most at risk (for a review see Rupnik et al., 2009). Currently, antibiotic therapy for C. difficile associated diarrhoea is almost exclusively vancomycin and metronidazole, with a number of alternative antibiotics currently undergoing clinical trials (Shah et al., 2010; Fox, 2011). Fidaxomicin has recently been approved for clinical use and demonstrated superior clinical outcomes compared with vancomycin (Cornely, 2012). While the emergence of resistant strains is infrequent, recent studies have shown that there is a trend towards decreased susceptibility (Brazier, et al., 2008; Baines, et al., 2008; Huang et al., 2009; Gerding, 2010). Bacteriocins such as lacticin 3147 and nisin have previously been shown to inhibit C. difficile in vitro (Bartoloni et al., 2004; Rea et al., 2007). However, lacticin 3147 has been shown not to survive transit through the mammalian gut (Gardiner et al., 2007). More recently thuricin CD,

Abbreviations: GIT, gastrointestinal tract; RP, reversed-phase; RSM, reconstituted skimmed milk; RSMT, RSM containing thuricin; WDA, well diffusion assay.
produced by _Bacillus thuringiensis_ DPC 6431, has been found to have a narrow inhibition spectrum _in vitro_, with potent activity against a wide range of clinical isolates of _C. difficile_, including the virulent ribotype 027 (Rea et al., 2010). Thuricin CD is a two component bacteriocin (Trn-α and Trn-β) which belongs to a new group of bacteriocins called sactibiotics (Rea et al., 2011a), which are post-translationally modified with unusual sulphur to α-carbon linkages. In addition _ex vivo_ studies have shown little collateral damage to human gut microbiota (Rea et al., 2011b). However, to exploit thuricin as an alternative or adjunct treatment for _C. difficile_ infection the biological activity needs to be assessed _in vivo_. In this study three approaches were undertaken to determine the fate of thuricin CD _in vivo_: (1) oral administration of thuricin in milk to pigs as a model of the human gut, (2) delivery of thuricin CD by the oral ingestion of spores of the producing organism _B. thuringiensis_ DPC 6431, and (3) rectal delivery of the unprotected peptides to the colon using the mouse as a model and _C. difficile_ as the target organism.

**METHODS**

**Bacterial strains used.** _C. difficile_ EM304 and _C. difficile_ ATCC 63593 were grown routinely from −80 °C stocks on Fastidious Anaerobic Agar (LAB M) containing 7.5% defibrinated horse blood at 37 °C under anaerobic conditions. _C. difficile_ ATCC 63593 was used as a target organism in well diffusion studies and _C. difficile_ EM304 was used in murine studies as a target organism in the rectal delivery study of thuricin CD. _C. difficile_ strains were grown in reinforced Clostridium medium (RCM; Reinforced Clostridium Medium Merck KGaA, Darmstadt, Germany) overnight at 37 °C under anaerobic conditions. _B. thuringiensis_ DPC 6341, used for thuricin CD production, and a rifampicin-resistant variant (DPC 6768) of _B. thuringiensis_ DPC 6341, used to produce a spore crop, were routinely subcultured on brain–heart infusion (BHI) agar (Brain Heart Infusion Merck KGaA, Darmstadt, Germany) and grown aerobically at 37 °C. All strains were stored long-term at −80 °C on Microbank beads (Pro-Lab).

**Animal experiments.** The pig experiment was approved by the Moorepark animal ethics committee and was conducted in accordance with the EU Council Directive 91/630/EEC, which lays down minimum standards for the protection of pigs, and EU Council directive 98/58/EC, which concerns the protection of animals kept for farming purposes. All procedures involving mice were approved by the UCC Animal Experimentation Ethics Committee (approval number 2011/017).

**Treatment of peptides with digestive enzymes.** The purified thuricin peptides Trn-α and Trn-β (35 μg ml⁻¹), separated by reversed phased high pressure liquid chromatography (RP-HPLC) as described (Rea et al., 2010), were treated with 77 μg pepsin ml⁻¹ (in 10 mM HCl) or 77 μg z-chymotrypsin ml⁻¹ as previously described (Gardiner et al., 2007). The presence of the intact peptides following exposure to pepsin and z-chymotrypsin was determined over time using MALDI-TOF MS. Lacticin 3147 Ltn-α (degraded by z-chymotrypsin) and the cell-free supernatant of _Lactobacillus gasseri_ DPC 6480 were used as positive controls for z-chymotrypsin and pepsin, respectively. Aliquots were taken at hourly intervals up to 2 h (pepsin) and up to 5 h (z-chymotrypsin) and MALDI-TOF MS was used to detect the intact peptides. Biological activity was assessed using the well diffusion assay (WDA) as described (Rea et al., 2010), by combining the Trn-α and Trn-β pre- and post-treatment using _C. difficile_ ATCC 63593 as the indicator organism.

**Production of thuricin CD for pig feeding trial.** Thuricin CD was produced as described by Rea et al. (2010) with some modifications as follows: partially purified thuricin CD was prepared by passing the cell-free supernatant through a column filled with XAD beads (Sigma) and the peptides were eluted using 70% 2-propanol pH 2.0. The 2-propanol was removed through rotary evaporation and the pH of the resulting fraction was adjusted to >6.0 with 1 M NaOH and the fraction mixed with 500 ml 20% reconstituted skimmed milk powder (RSMT). The mixture was then freeze-dried in a Virtis Advantage Wizard 2.00 freeze dryer. The activity (AU ml⁻¹) of the freeze-dried preparation was determined using WDA as described previously (Rea et al., 2010).

**Pig feeding trial.** Sixteen male pigs (large white × landrace) were selected 1 week after weaning (28 days), tagged and individually penned in thermostatically controlled rooms. To accustom the animals to drinking milk, the pigs were each fed 200 ml of 20% reconstituted skimmed milk (RSM), in addition to the basal diet, daily for 1 week prior to the commencement of the trial. Intake of milk was recorded daily.

At the end of this period eight pigs with the best history of consuming milk were selected for experiment. On the evening before slaughter the basal diet was removed, with only water being available to the pigs on an _ad libitum_ basis. Two hours before slaughter four pigs were fed with 200 ml 20% RSM (controls) and four pigs were fed 200 ml RSMT, which equated to the ingestion of 10⁸ AU thuricin CD. When the allocation of milk was consumed the pigs were each fed −140 g starter diet (Starrrite 88; Nutec Naas) to which ferric oxide had been added at a concentration of 1.5%. Ferric oxide was added to the feed as a marker to identify how far the feed had travelled through the pig gastrointestinal tract (GIT) prior to slaughter. Two hours after consumption of the RSM or RSMT the animals were slaughtered by captive bolt followed by immediate exsanguination. The stomach was clamped above and below and removed. The gastric contents were removed, the pH was immediately adjusted to 7 using 1 M NaOH, and samples were stored on ice. Samples were also taken from the duodenum, the ileum (15 cm above the ileocecal junction), the jejunum (105 cm from the ileocecal junction) and the caecum and immediately stored on ice. The passage of the feed through the GIT was recorded as indicated by the presence of ferric oxide (red colour) in GIT contents.

**Detection of Trn-α and Trn-β in the porcine digesta.** One hundred milligrams of digesta was mixed with 900 μl 0.1% trilfluoroacetic acid, vortexed well and allowed to stand at room temperature for 30 min. The sample was then centrifuged (5 min, 16 000g), the supernatant was passed through a Strata E C18 SPE column (Phenomenex), the column was washed with 1 ml 30% ethanol and the peptides were eluted with 70% 2-propanol pH 2.0. The peptides were then purified using RP-HPLC and the active fractions identified using MALDI-TOF MS as previously described (Rea et al., 2010). Biological activity was assessed using the WDA with _C. difficile_ ATCC 59635 as the target organism (Rea et al., 2007).

**Delivery of _B. thuringiensis_ spores and detection of spores and/or vegetative cells in the murine gut.** A spore suspension of _B. thuringiensis_ DPC 6341 in sterile distilled water was heat treated at 70 °C for 30 min to kill vegetative cells. BALB/c mice were used in this experiment. Five mice (n=5) were used as negative controls and 30 as test mice. The test mice were divided randomly into six groups (n=5) and 100 μl heat treated spore suspension (10⁸–2 × 10⁶ spores) was given by oral gavage. Each group of five animals was kept in a separate cage.

The control animals were sacrificed at time 0 (prior to feeding) to get a baseline count to ensure that there was no background count on the
BHI agar containing rifampicin, and test mice were sacrificed at time 0.5 h and 1, 3, 5, 7 and 10 days after ingestion. The abdomen was dissected and the entire gut (stomach plus small and large intestine) was removed and kept on ice. Faecal pellets were collected from the cage the day prior to that on which the animals were sacrificed. The bedding of the mice was changed daily to determine the number of spores excreted each day. Both the gut material and faecal droppings were kept on ice after collection and processed within 2 h of sampling (Fig. 1). The sampling plan for the enumeration of *B. thuringiensis* spores/vegetative cells in the GIT contents or faecal pellets is outlined in Fig. 1. BALB/c mice (n=30) were fed with *B. thuringiensis* spores (10^5–2×10^8 spores per mouse). Mice droppings were collected daily from each cage and bedding was changed until animals were sacrificed.

**Enumeration of spores and vegetative cells of *B. thuringiensis* in the GIT and faecal pellets of mice.** The gut samples were weighed, opened using sterile scissors and sliced into small pieces of 1–2 mm length. The gut tissue/contents and the faecal pellets were suspended separately in 0.5 ml phosphate buffer (50 mM, pH 7.0) and mixed for 60 s, and a portion of the sample was heat treated for 30 min at 70 °C to kill the vegetative cells of *B. thuringiensis*. The viable cells of both heat treated and untreated samples were enumerated by plating 10-fold serial dilutions on BHI agar containing rifampicin (50 μg ml^-1). Plates were incubated aerobically at 37 °C for 48 h.

**Rectal inoculation of mice with *C. difficile* suspension and administration of thuricin peptides**

**Preparation of *C. difficile* cultures for rectal administration.** *C. difficile* EM304 ribotype 027, which had previously been isolated from a clinical case of *C. difficile* infection (Rea et al., 2012), was used to inoculate 20 female C57BL/6 mice aged between 8 and 10 weeks. An overnight culture of *C. difficile* EM304 was centrifuged (2 min, 13 400 g) and the pellet washed twice in maximum recovery diluent (Oxoid). The percentage of vegetative cells in the culture preparation used to infect the mice was determined by treating the cell suspension with 50% ethanol for 60 min before plating the ethanol treated and control samples on cycloserine-cefoxitin egg yolk agar (CCEY agar; LAB M) containing lysozyme (5 mg l^-1). The plates were incubated anaerobically at 37 °C for 48 h. The culture was maintained under anaerobic conditions until use.

**Rectal administration of thuricin peptides.** Prior to the experiment, the mice were anaesthetized with an intraperitoneal injection of 65 mg ketamine kg^-1 and 13 mg xylazine kg^-1. One hundred microlitres of the cell suspension containing 10^5 c.f.u. *C. difficile* EM304 was introduced 2 cm deep rectally into each mouse using a flexible catheter. Fifteen minutes later 50 μl purified thuricin CD (2.5 mg made up in 10% ethanol and water) was introduced rectally into each of 10 test mice and 100 μl distilled H2O containing 10% ethanol was introduced into each of 10 control mice. One hour, 6 h, 12 h and 24 h following the administration of the antimicrobial the test and control mice were sacrificed and the entire GIT from below the caecum to the rectum was removed, weighed and placed in the anaerobic work station (Don Whitley). The colon was opened under anaerobic conditions using sterile scissors and a 1:10 dilution made in Maximum Recovery Diluent (Oxoid) which had been previously boiled and cooled anaerobically. The samples were well macerated in the diluent before being diluted further. The samples were then serially diluted, in duplicate, and 100 μl of each dilution plated on freshly prepared CCEY agar containing lysozyme (Lab M). Plates were incubated anaerobically for 48 h and typical colonies counted. Results were expressed as c.f.u. (g mouse colon)^-1.

**Statistical analysis.** Statistical analyses were carried out by ANOVA one-way analysis using Minitab version 15.

**RESULTS**

In previous studies we had demonstrated that thuricin CD was effective in killing *C. difficile* in a model of the distal gut and faecal pellets in mice. In the current study, we aimed to determine if thuricin CD was effective in killing spores of *C. difficile* in the GIT in mice. The fate of thuricin CD in the GIT was measured at different time points after rectal administration. The results showed that thuricin CD was effective in killing spores of *C. difficile* in the GIT in mice.
human colon (Rea et al., 2011b). However, to be effective in vivo, thuricin would need to be delivered to the colon in a bioavailable form. Consequently, this study first investigated the fate of orally ingested thuricin in pigs and then evaluated its efficacy when introduced rectally in a mouse model.

**Oral administration of unprotected thuricin CD to pigs**

The presence of Trn-α and Trn-β in the digesta of the four pigs that consumed the RSMT was investigated using a combination of solid phase extraction, RP-HPLC and MALDI-TOF MS. The thuricin peptides in milk were readily detected by both RP-HPLC (Fig. 2a) and MALDI-TOF MS (Fig. 2b) and the biological activity in milk was confirmed (Fig. 2c) in the spray dried powder. The transit of the feed was monitored by observing the presence of ferric oxide in the digesta. In all cases there was ample evidence showing that a portion of the feed had travelled at least as far as the ileum before slaughter. Trn-α was detected in the stomach of one test pig; in the stomach and jejunum of a second test pig and in the ileum of a third test pig. In the fourth pig, which ingested only 60% of the RSMT, neither peptide was detected. Trn-β was not detected in the GIT content of any of the test pigs (Fig. 2d, e). The presence of active Trn-α in the HPLC fraction (labelled fraction X in Fig. 2d) of the test pigs was confirmed by supplementation of the presumptive positive HPLC fraction with purified Trn-β at a concentration which on its own did not give a zone of inhibition in WDA. Supplementation of the presumptive Trn-α HPLC fraction (fraction X) with Trn-β restored biological activity when assayed by WDA using *C. difficile* as the target organism (Fig. 2f). The susceptibility of Trn-β but not Trn-α to the digestive enzymes pepsin and α-chymotrypsin was confirmed in vitro when the purified peptides were exposed to these enzymes. Using MALDI-TOF MS Trn-α was detected 2 h and 5 h after treatment with pepsin and α-chymotrypsin respectively while Trn-β was not detected.

**Fig. 2.** RP-HPLC chromatogram (a), MALDI-TOF MS spectrum (b) and WDA (c) of freeze-dried milk, showing the presence of the two peptides Trn-α and Trn-β in spray dried milk before ingestion. RP-HPLC chromatogram indicating the presence of putative Trn-α (fraction X) (d), MALDI-TOF MS showing correct molecular mass for Trn-α (e), and confirmation of the presence of biologically active Trn-α (fraction X) (f) in the digesta of pigs 2 h after oral consumption of thuricin by supplementation with purified Trn-β, which restored biological activity of thuricin.
after 1 h treatment with either enzyme. The loss of biological activity of Trn-β after treatment with the digestive enzymes was demonstrated by the absence of zones of inhibition surrounding the wells containing the enzyme treated peptides in plates seeded with C. difficile.

### Oral administration of *B. thuringiensis* DPC 6431 to mice

Spores of Bacillus species are used commercially as probiotics and competitive exclusion agents for animals and humans to protect against infectious agents. For this reason, we set out to establish whether *B. thuringiensis* DPC 6431 spores could germinate and produce thuricin CD in the GIT. The experimental design enabled the estimation of the total number of spores and/or vegetative cells shed by each group during the 10 day experiment period (Fig. 1). The mice shed spores rapidly and *B. thuringiensis* DPC 6431 was detected in the faecal pellets within 30 min after ingestion (Fig. 3). The maximum shedding of *B. thuringiensis* spores occurred in the first 24 h, when nearly 99% of spores were recovered from both the gut samples and faecal pellets (Fig. 3). Shedding of the spores decreased steadily between days 2 and 5 and no spores or vegetative cells were detected after day 6. In all animals tested, over 99% of *B. thuringiensis* was shed as spores (Fig. 3), indicating that little or no germination of spores occurred during transit of the animal’s GIT. Fig. 3 (inset graph) shows a very sharp rise of *B. thuringiensis* spores shed in the faeces in the first 24 h and a gradual reduction of spores shed up to day 5; no spores or vegetative cells were detected after day 6. Over a 10 day period, the numbers of vegetative cells in the mouse GIT did not rise above 100, indicating little or no germination of the *B. thuringiensis* spores. There was no evidence of presence of Trn-α or Trn-β in the faecal pellets or GIT contents when a combination of C18 solid phase extraction and MALDI-TOF MS was used for detection.

### Rectal delivery of thuricin CD

To overcome the problems of enzymic degradation of Trn-β in the upper GIT, a thuricin CD suspension was prepared and delivered to the mouse colon via the rectum. *C. difficile* EM304 (ribotype 027) isolated from a patient with active *C. difficile* infection (Rea et al., 2012) was used as the test organism. Using a flexible catheter, 100 μl (containing 10^5–2×10^7 c.f.u.) of an overnight culture of *C. difficile* was introduced rectally about 2 cm deep into the mouse colon. This cell suspension contained <1% spores as determined by differential counting of the cell suspension before and after ethanol treatment. The mean *C. difficile* count recovered from the control mice (n=10) 1 h after infection was 1.4×10^5 (g mouse colon)^−1, whereas in the test mice (n=10) there was a 1.5 log unit reduction (P<0.001) in *C. difficile* count 60 min following the administration of the bacteriocin (Fig. 4). Six hours post-treatment *C. difficile* was undetected in 6/9 test mice and in 3/10 control mice [1.5 log unit reduction; P<0.05]. However, no *C. difficile* was recovered 12 and 24 h post-treatment in the test (n=10) or control (n=10) animals.

### DISCUSSION

Oral administration of antimicrobial compounds is one of the more common routes for delivery of drugs to sites within the GIT. However, oral delivery of therapeutic agents whose target is the colon poses a formidable challenge in that the drugs need to be stable in the acid conditions of the
stomach and not be broken down or absorbed in the upper GIT. With respect to bacteriocins, this poses an additional challenge in that many peptides are readily degraded by digestive enzymes in the stomach and upper digestive tract and therefore may fail to reach their target site. Some bacteriocins have been successfully delivered orally to the GIT in vivo. For example, feeding a salivaricin producing strain of *Lactobacillus salivarius* UCC 118 protected mice against *Listeria monocytogenes* infection whereas feeding of a non-bacteriocin variant of the same strain had no effect (Corr et al., 2007). In another example, bacteriocin-producing cultures have also been shown in livestock to alter rumen flora and decrease shedding of bacterial pathogens (for a review see Diez-Gonzalez, 2007). The lantibiotics nisin and lactacin 3147 have both been shown to have anti-
*Clostridium difficile* activity (Bartoloni et al., 2004; Rea et al., 2007). However, in vivo studies showed that both these bacteriocins are degraded by digestive enzymes in the upper mammalian GIT. This poses significant challenges for oral administration when the target is a pathogen affecting the colon (Bernbom et al., 2006; Gardiner et al., 2007). The results from this study demonstrate that Trn-β is particularly susceptible to degradation by digestive enzymes in the upper GIT. The 3D structure of thuricin CD, which has been determined (Sit et al., 2011), may explain the differences in susceptibility of these α and β peptides to enzymatic degradation. Both enzymes cleave preferentially either before or after aromatic residues, which in Trn-β are near the terminus of the peptide and so would be particularly susceptible to enzymatic cleavage, whereas in Trn-α the aromatic residue is within the cyclic ring, which makes it inaccessible to enzyme cleavage. However, irrespective of the difference in their susceptibility to enzymes, we have shown in previous studies that both peptides are required for optimal activity (Rea et al., 2010).

Historically *Bacillus* spores have been used as probiotics in both humans and animals (Cutting, 2011). Previous studies have shown that some *Bacillus* species could germinate and grow in the GIT of humans and mice (Hoa et al., 2001; Tam et al., 2006; Hong et al., 2009a, b). Spores have the advantage over vegetative cells of being resistant to the low pH of the stomach, ensuring the delivery of high numbers to the GIT. The producing organism of thuricin BD, *B. thuringiensis* DPC 6431, was isolated from the human GIT under anaerobic conditions and produced the bacteriocin anaerobically. However, as only 1 c.f.u. was detected in the faecal sample of one volunteer, there is no evidence to suggest that this organism had colonized the gut of that individual but rather the organism was just in transit in the gut. In the study reported here there was no evidence of spore germination, and no thuricin CD was detected in the GIT of the test mice in vivo.

An alternative method of drug delivery to the colon is via the rectum through enema preparations or suppositories. In this way, the peptide degrading enzymes of the upper digestive tract are bypassed, thus increasing the possibility of peptides such as thuricin CD reaching their target site without being degraded by digestive enzymes. In this study, we have demonstrated clearly in vivo that the bacteriocin thuricin CD administered via the rectum was effective in reducing the pathogenic *Clostridium difficile* strains by over 1.5 log units 1 h post-treatment and a further significant reduction was observed 6 h post-treatment, indicating that biological activity was retained for at least 6 h post-treatment. However, the limitation of this type of model is that, due to the rapid transit of material through the murine GIT, as seen from our study with oral feeding of spores, *Clostridium difficile* was cleared naturally in both control and test animals 12 h post-treatment.

However, our results suggest that the concept of rectal administration of bacteriocins such as thuricin may be an avenue worthy of further investigation for the successful administration of peptide antimicrobials because they retain their activity post-inoculation when the digestive enzymes in the upper GIT are bypassed.

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