A novel antimicrobial peptide significantly enhances acid-induced killing of Shiga toxin-producing Escherichia coli O157 and non-O157 serotypes

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Shiga toxin-producing Escherichia coli (STEC) colonizes the human intestine, causing haemorrhagic colitis and haemolytic uraemic syndrome (HUS). Treatment options are limited to intravenous fluids in part because sublethal doses of some antibiotics have been shown to stimulate increased toxin release and enhance the risk of progression to HUS. Preventative antimicrobial agents, especially those that build on the natural antimicrobial action of the host defence, may provide a better option. In order to survive the acid stress of gastric passage, STEC is equipped with numerous acid resistance and DNA repair mechanisms. Inhibition of acid-induced DNA repair may offer a strategy to target survival of ingested STEC. We report here that brief pretreatment with a novel antimicrobial peptide, which was previously shown to inhibit bacterial DNA repair, significantly and profoundly reduces survival of acid-stressed O157 : H7 and non-O157 : H7 STEC seropathotypes that are highly associated with HUS. Reduction in survival rates of STEC range from 3 to 5 log. We also show that peptide/acid treatment results in little or no increase in toxin production, thereby reducing the risk of progression to HUS. This study identifies the peptide wrwyr as a potential new candidate for a preventative antimicrobial for STEC infection.

INTRODUCTION

Shiga toxin-producing Escherichia coli (STEC) is a serious food- and water-borne pathogen that causes a variety of illnesses ranging from mild diarrhoea to severe, bloody diarrhoea (haemorrhagic colitis), and life-threatening systemic sequelae including haemolytic uraemic syndrome (HUS) (Karmali, 2004; Nataro & Kaper, 1998). The development of systemic complications is attributed to the production of prophage-encoded Shiga toxins (Karmali, 2004; Scheiring et al., 2008). Although the serotype O157 : H7 is most highly associated with outbreaks and severe disease (including HUS) in North America, the non-O157 serotypes also constitute a serious public health threat and are reported to be associated with as many as 20 % of North American HUS cases (Banatvala et al., 2001; Karmali et al., 2003). The non-O157 serotypes have also been frequently associated with HUS, particularly in Latin America, Australia and Europe, and at least 200 strains of these serotypes have now been associated with human disease (Karmali et al., 2003). Recent evidence suggests that many of the non-O157 : H7 strains have acquired additional virulence factors that correlate with outbreak and HUS potential in humans (Coombes et al., 2008). The World Health Organization has identified the virulent non-O157 STEC as a public health priority (Coombes et al., 2008). These results provide persuasive evidence of the need for prevention strategies that are equally effective against both O157 and non-O157 STEC strains.

Currently, there is no specific therapeutic treatment for STEC infection other than intravenous fluids (Ake et al., 2005; Palermo et al., 2009; Tarr et al., 2005; Bavaro, 2009). Doctors strongly advise against treatment with antibiotics, nonsteroidal anti-inflammatory agents or anti-motility agents, in part because studies have shown that sublethal

Received 22 December 2010
Revised 17 March 2011
Accepted 24 March 2011

Abbreviations: HJ, Holliday junction; HUS, haemolytic uraemic syndrome; STx, Shiga-like toxin; STEC, Shiga toxin-producing Escherichia coli.
antibiotic doses may stimulate STEC toxin production and promote the development of HUS in STEC-infected individuals (Scheiring et al., 2008). Consequently, research aimed at preventing the establishment of the primary infection may offer the best strategy for preventing STEC-associated illness.

STEC, like all enteric pathogens, must survive exposure to the extreme acidity associated with gastric passage. STEC is well equipped with a variety of acid resistance mechanisms as well as repair mechanisms to combat acid-induced DNA damage (Foster, 2004; Lin et al., 1996). Exposure to low pH results in extensive depurination of DNA which, if not repaired, can have lethal consequences for the pathogen (Kunkel, 1984). Holliday junctions (HJs) and stalled replication forks are DNA structures that are central to DNA damage repair. Failure to resolve these intermediates can seriously compromise bacterial viability (Cox et al., 2000; Kuzminov, 1999; Michel et al., 2007). Therefore, inhibition of acid-induced DNA repair should enhance acid killing of STEC and may provide a novel antimicrobial strategy for preventing STEC infection.

Segall and colleagues have identified several peptides that prevent resolution of HJ intermediates by trapping the protein-bound HJ intermediate (Boldt et al., 2004; Cassell et al., 2005). Among the best characterized of these is the D-amino acid hexapeptide wrwycr. This peptide binds HJs and other branched DNA intermediates, the latter with lesser affinity and stability, and by doing so, is able to inhibit a number of HJ processing enzymes in a structure-selective manner (Kepple et al., 2005, 2008). During site-specific recombination mediated by bacteriophage lambda integrase, an HJ intermediate is generated after the first round of DNA cleavage, strand exchange and ligation, and is resolved in a second round of the same catalytic steps (Azaro & Landy, 2002). Peptide wrwycr prevents the resolution of the HJ intermediates to either products or substrates (Boldt et al., 2004).

Peptide wrwycr, when oxidized, functions as a disulfide-linked homodimer and is relatively hydrophobic (Boldt et al., 2004), thereby enhancing its passage through both the lipid bilayer membrane as well as the peptidoglycan layer in order to access HJ. It is composed of D-amino acids, which renders it resistant to in vivo peptidase degradation. The peptide WRWYCR, with the same sequence but composed of L-amino acids, interacts with HJs with a Ka of 14 nM, inhibits several HJ resolution enzymes with an IC50 of 5–64 nM and is also bactericidal against non-pathogenic E. coli, although it is less antimicrobial than wrwycr (Gunderson & Segall, 2006; Kepple et al., 2005, 2008). The peptide wrwycr has also been shown to inhibit Salmonella enterica growing inside murine macrophages (Su et al., 2010) and to have antimicrobial activity against non-pathogenic E. coli (Gunderson & Segall, 2006). Importantly, wrwycr was found to have no toxic effect on J774A.1 macrophage-like cells and murine peritoneal macrophages in the study by Su et al. (2010).

In the current study, we hypothesized that if the lethal effects of DNA lesions due to low pH in the stomach could be enhanced by pretreatment with the peptide wrwycr, we could target killing of newly ingested STEC without damaging the beneficial commensal flora of the gut. This strategy could be used to develop an antimicrobial which could be applied to potentially contaminated foods prior to ingestion so as to enhance the killing effect of gastric acid stress. The goals of this study included: firstly, to determine if pretreating STEC with peptide wrwycr influences bacterial survival after acute acid stress; secondly, to examine the impact of treatment conditions including temperature, wrwycr concentration and acid exposure time on STEC survival; and finally, to evaluate the impact of wrwycr/acid treatment on Shiga toxin production. The study includes STEC strains representative of each of the O157:H7 and non-O157:H7 seropathotypes highly associated with human disease (Karmali et al., 2003). The findings provide the first evidence that wrwycr in combination with exposure to acid, similar to that typically encountered during ingestion, significantly and dramatically reduces survival of STEC strains of seropathotypes most highly associated with HUS without a concomitant increase in Shiga toxin production. Accordingly, wrwycr could serve as an antimicrobial agent for prevention of STEC-induced food-borne illness.

**METHODS**

**Bacterial strains and growth conditions.** Seven STEC strains (Table 1), kindly provided by Dr Karmali, Public Health Agency of Canada, were used in this study. Karmali et al. (2003) classified STEC based on the relative frequency with which each of the serotypes is associated with serious and epidemic human disease. A summary of the strains used in this study, their STEC seropathotypes and their association with outbreaks of human disease and development of severe disease (HUS) is provided in Table 1. Bacteria were maintained as glycerol stocks at −80 °C. Prior to use, bacteria were streaked for single colonies on LB agar. Single colonies were used to inoculate LB broth for overnight culture at 37 °C with shaking. Fresh cultures from glycerol stocks were prepared for each individual experiment in order to maintain the original clinical characteristics of the stock. Bacterial viability was assessed by serial dilution and plating on LB agar.

**Synthesis of wrwycr.** The peptide wrwycr was synthesized with a C-terminal amide group, purified to >95% purity at Sigma-Genosys or Biosynthesis and dissolved in 50% DMSO as described previously (Gunderson & Segall, 2006). A wrwycr stock solution (10 mM) was maintained in 50 or 100% DMSO. Final DMSO concentrations were either 0.5 or 1.0% and DMSO at an appropriate concentration was added in the absence of wrwycr to control for DMSO effects.

**Survival assays.** Overnight LB broth cultures were diluted 1:6 in LB broth and grown to mid-exponential phase (OD600 0.4–0.6). For acid stress survival studies, bacteria were pelleted, resuspended in LB either pH 7.0 or pH 2.5 (adjusted with HCl). The first sample taken immediately after bacteria were resuspended was designated time 0; sampling and plating were done four times for a total of 3 h incubation. Bacterial viability was assessed by serial dilution and plating on LB agar.

For the peptide/acid treatment survival studies, bacterial pellets as above were resuspended in either 0.5 x PBS at pH 7.0, or 0.5 x PBS
and wrwrwycr (concentrations ranging from 25 to 75 μM) at pH 7.0. The first sample, immediately following suspension in PBS, was serially diluted, plated and was designated ‘unstressed’. Bacteria were incubated with wrwrwycr at room temperature, 30 °C or 37 °C for 5 min, after which the suspensions were acidified to pH 2.5 by the addition of 0.5 × PBS pH 1.2. The second sample immediately following wrwrwycr treatment was serially diluted, plated and designated time 0. Subsequent samples were obtained for 15, 30, 60 and 120 min at pH 2.5 and room temperature, 30 °C or 37 °C. Viability was assessed by serial dilution and plating on LB agar. Earlier experiments had identified 25 μM wrwrwycr as the lowest effective dose for enhanced acid-induced killing of non-pathogenic E. coli and we tested concentrations ranging from 25 to 75 μM wrwrwycr in our assays with STEC.

**Shiga toxin production.** The effect of peptide treatment on Shiga-like toxin (STx) production was measured using a Vero cell cytotoxicity assay as described previously (House et al., 2009). Purified stock solutions of STx1 and STx2 (100 μg ml⁻¹) were kindly provided by Dr Lingwood, Hospital for Sick Children, Toronto, ON, Canada. Vero cells (ATCC) were cultured at 37 °C, 5% CO₂ in complete medium (minimal essential medium supplemented with 5% fetal bovine serum, 100 IU penicillin ml⁻¹, 100 μg streptomycin ml⁻¹). For the cytotoxicity assay, cells were seeded at 20,000 cells in 200 μl per well in 96-well plates and cultured overnight to form a monolayer. Serial dilutions of purified STx1, STx2 or STx extracts from STEC strains (described below) were prepared in triplicate in complete medium and 50 μl was added per well. The plates were incubated for 72 h after which viable, adherent cells were fixed with well of 10 % acetic acid in water. Crystal violet staining was quantified by Gentry & Dalrymple (1980). Briefly, overnight cultures of representative strains of these seropathotypes. which to compare the impact of peptide on acid sensitivity clearly establish that the STEC seropathotypes, like non-pathogenic E. coli, are sensitive to acid to varying degrees and more importantly, they provide a baseline against which to compare the impact of peptide on acid sensitivity of these seropathotypes.

**Statistical analysis.** Results are expressed as means ± SD. Friedman two-way analysis of variance and Kruskal–Wallis one-way analysis of variance were used for statistical analysis of intergroup comparisons. Pairwise t-tests were performed for paired data. P < 0.05 (confidence interval of 95%) was considered significant.

## RESULTS

### Survival of STEC seropathotypes after acid stress

All the O157 and non-O157 STEC strains used in this study showed a significant decrease in viability after 3 h in pH 2.5 LB broth (Fig. 1). For the two O157:H7 strains tested, there was a 3 log decrease in survival after 3 h exposure to pH 2.5 (strain 86-24, Fig. 1a; strain D103F5, not shown). Similarly, all of the non-O157 strains tested showed a significant loss of viability after acid stress with varying degrees of sensitivity. Strains from seropathotype B showed survival losses at pH 2.5 ranging from a 2 log decrease over 3 h (CL106, Fig. 1b) to a 3–5 log decrease after 1 h (CL1, data not shown). Seropathotype C strain N99-4390 was particularly sensitive to acid, with no detectable survival after 2 h at pH 2.5 (Fig. 1c). The non-pathogenic strain MG1655 showed a 3 log loss after 3 h exposure to acid stress with a plot similar to Fig. 1(a) (data not shown). The non-human, non-pathogenic strain EC2-211 showed a 2 log loss over 3 h (Fig. 1d), in a manner similar to pathogenic strain CL106 (Fig. 1b). These results clearly establish that the STEC seropathotypes, like non-pathogenic E. coli, are sensitive to acid to varying degrees and more importantly, they provide a baseline against which to compare the impact of peptide on acid sensitivity of these seropathotypes.

### Peptide treatment significantly augments acid-induced killing of STEC O157:H7

In our initial experiments with wrwrwycr/acid treatment, we examined the effect of various incubation temperatures, times and peptide concentrations using the O157:H7 strains, 86-24 (Fig. 2) and D103F5 (not shown). When STEC O157:H7 was incubated with 25 μM wrwrwycr at room temperature for 5 min followed by room temperature incubation at pH 2.5, bacterial survival was significantly reduced.
Antimicrobial peptide enhances acid killing of STEC

Fig. 1. Survival after acid stress. Survival of strains 86-24 (a), CL106 (b), N99-4390 (c) and EC2-211 (d) after incubation in LB at 37 °C at either pH 7.0 (black bars) or pH 2.5 (grey bars). Each experiment includes two biological replicates and three technical replicates. Asterisks indicate a statistically significant difference relative to the corresponding control, confidence interval 95 %. Error bars indicate SD.

Fig. 2. Peptide wrwycr/acid treatment. The impact of temperature, time and peptide concentration on survival of STEC strain 86-24 after 5 min incubation with PBS (black bars), 25 μM wrwycr (grey bars), 50 μM wrwycr (dark-grey bars, c only) or 75 μM wrwycr (white bars, c only) followed by timed acid stress exposure. Both wrwycr and acid stress incubations were conducted at room temperature (a), 30 °C (b) or 37 °C (c). Asterisks indicate a peptide treatment with a statistically significant difference relative to untreated control, confidence interval 95 %. U, Unstressed. Error bars indicate SD.
decreased over the total 2 h period and, at each time interval, there was a 2–3 log decrease in viability of the wrwycr/acid-treated samples relative to non-wrwycr/acid-treated samples. Similar results were observed when 25 μM wrwycr and acid treatments were both conducted at either 30 °C (Fig. 2b) or 37 °C (Fig. 2c). Fig. 2(c) also demonstrates a significant dose-dependent increase in peptide/acid killing of STEC O157 : H7 using wrwycr concentrations ranging from 0 to 75 μM. Survival was increasingly compromised with increasing concentrations of peptide, with no survival detected with 50 μM wrwycr coupled with 120 min of acid stress or 75 μM wrwycr coupled with just 60 min of acid stress.

Finally, since the peptide treatment could serve as a pre-ingestion intervention strategy (i.e. the pathogen is exposed to the peptide prior to ingestion), we also wanted to test the effect of wrwycr incubation at room temperature followed by a 37 °C acid stress, the latter part of which would be typically encountered during gastric passage. When a 5 min room temperature incubation with 25 μM wrwycr was followed by a 37 °C incubation at pH 2.5, survival of the wrwycr/acid-treated samples relative to the non-wrwycr/acid-treated samples was dramatically decreased (Fig. 3a). Since these conditions provided the most pronounced antimicrobial effect at the lowest concentration of wywycr tested, they were selected for the subsequent survival experiments with the different seropathotypes.

**Peptide treatment significantly enhances acid-induced killing of non-O157 : H7 STEC**

Using these conditions, we determined that wrwycr pretreatment significantly reduces the survival of acid-stressed samples relative to untreated acid-stressed samples of STEC seropathotypes A, B and C, all of which are highly associated with outbreaks and severe human disease (Fig. 3). Both strains from seropathotype A (D103F5 and 86-24) were significantly more sensitive to the acid after exposure to wrwycr, with no detectable survival after 1 h of wrwycr/acid treatment (86-24, Fig. 3a; D103F5, not shown). Strains from seropathotypes B and C were even more sensitive to wrwycr/acid treatment with CL1 (seropathotype B), CL106 (seropathotype B) and N99-4390 (seropathotype C) showing no detectable survival after 15, 30 and 30 min, respectively, of wrwycr/acid treatment (CL106, Fig. 3b; CL1, not shown; N99-4390, Fig. 3c). The non-pathogenic strain EC2-211 of seropathotype E (of bovine origin and

![Fig. 3. Comparison of survival of O157 and non-O157 strains after peptide/acid treatment. Survival after incubation with wrwycr for 5 min at room temperature followed by timed 37 °C acid stress exposure. Survival of control (incubation in PBS followed by pH 2.5 treatment (black bars)) or sample (incubation in 25 μM wrwycr in PBS followed by pH 2.5 treatment (grey bars)) of strain 86-24 (seropathotype A) (a), strain CL106 (seropathotype B) (b), strain N99-4390 (seropathotype C) (c), strain EC2-211 (seropathotype E) (d) or MG1655 (e). Each experiment includes two biological replicates and three technical replicates. Asterisks indicate a statistically significant difference relative to the corresponding control, confidence interval 95 %. U, Unstressed. Error bars indicate SD.](image)
not associated with human disease) showed a significant 5 log decrease in survival after 1 h wrwycr/acid treatment with modest but significant survival decreases relative to the acid/treatment alone (Fig. 3d). Finally, wrwycr/acid treatment of the non-pathogenic E. coli strain MG1655 significantly reduced survival relative to the untreated, acid-stressed sample, with a 3 log reduction in viability within 30 min and no viable bacteria detected at 1 h (Fig. 3e). This behaviour was similar to that of strains from seropathotype A.

We note that the detection limit of this assay is 50–100 c.f.u. ml\(^{-1}\) which could still represent an infectious dose of STEC. However, the results clearly indicate dramatic and significant survival loss for all of the STEC pathogenic strains, decreasing from 10\(^8\) c.f.u. ml\(^{-1}\) to below the detection limit of 50–100 c.f.u. ml\(^{-1}\) after a 5 min wrwycr treatment followed by 15–60 min at pH 2.5 (Fig. 3a–c, grey bars). In contrast, in the absence of wrwycr, we were still able to detect 10\(^3\)–10\(^4\) c.f.u. ml\(^{-1}\) of each of these strains after 30–120 min of similar acid exposure (Fig. 3a–c, black bars).

**Peptide/acid treatment has minimal impact on toxin production**

Finally, we sought to determine whether wrwycr/acid treatment alters the production of active STx. An established quantitative Vero cell assay was used. The results showed little or no significant difference in STx production after wrwycr treatment of strains representing seropathotypes A, B and C. The standard curves for STx1 and STx2 (Fig. 4d) were used to generate CD\(_{50}\) values: 1.5 × 10\(^{-7}\) μg ml\(^{-1}\) for STx1 versus 3 × 10\(^{-7}\) μg ml\(^{-1}\) for STx2. Fig. 4 shows the cytotoxicity curves for STx periplasmic extracts from strains representing each of the seropathotypes at pH 2.5 with and without wrwycr. Using the CD\(_{50}\) values from the standard curves, we found a minor 2.3-fold increase in the CD\(_{50}\) value for the periplasmic extract from strain 86-24 (seropathotype A) after wrwycr/acid treatment relative to acid treatment alone. Strain CL106 (seropathotype B) and N99-4390 (seropathotype C) showed no change in periplasmic toxin production after peptide treatment (Fig. 4b, c). Secreted STx extracts were similarly tested (results not shown) and we found no change in the CD\(_{50}\) value for strain 86-24 (seropathotype A), a 1.5-fold decrease in the CD\(_{50}\) value for strain CL106 (seropathotype B) and a fivefold increase in the CD\(_{50}\) value for secreted STx for strain N99-4390 (seropathotype C). These changes are relatively small when compared with studies that report increases in STx production of 13–20-fold with subinhibitory concentrations of the antibiotic fosfomycin (Wolf et al., 1997; Ito et al., 1997), and up to 169–436 % increases in the presence of the antibiotic ciprofloxacin (Walterspiel et al., 1992).

**DISCUSSION**

STEC infection is a serious public health concern due to its ability to cause haemorrhagic colitis as well as HUS, the primary cause of paediatric renal failure. Since the strains that are highly associated with human diarrhoeal disease outbreaks and progression to HUS typically belong to seropathotypes A, B and to a lesser extent C (Karmali et al., 2003), strategies to prevent infection with these three

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**Fig. 4.** Effect of wrwycr/acid stress treatment on periplasmic STx production as measured by the Vero cell cytotoxicity assay for STEC strains 86-24 (a), CL106 (b) and N99-4390 (c). Following the indicated treatment, periplasmic extracts were prepared from each strain and diluted serially on Vero cells. After 72 h, adherent, viable cells were stained with crystal violet and absorbance was read at 570 nm. Results are expressed as the mean absorbance ± SD versus the dilution of extract and are representative of three independent experiments. ▲, Control (no STx); ●, pH 2.5; ■, pH 2.5 + 25 μM wrwycr. Standard curves for STx1 (▲) and STx2 (●) are shown in (d). Error bars indicate SD.
In contrast, when acid stress was preceded by a 5 min incubation at pH 2.5.

Strains from all three seropathotypes associated with severe disease showed significant decreases in survival of strains from seropathotypes A, B or C after 15–60 min of wrwycr treatment. The finding that wrwycr in combination with acid treatment significantly and profoundly reduces survival of STEC seropathotypes associated with severe human disease provides an exciting new opportunity for envisioning a pre-ingestion antimicrobial treatment for the prevention of STEC infection.

ACKNOWLEDGEMENTS

This research was supported by a Natural Sciences and Engineering Research Council (NSERC) of Canada Discovery Grant to D.B.F. A.M.S. was supported by NAID grant ROI-058253A1. J.V.K. was supported by an NSERC postdoctoral fellowship and S.L.T. was supported by a postdoctoral fellowship awarded by Ryerson University. We gratefully acknowledge A. Laursen at Ryerson University for his advice and assistance with the statistical analysis.

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Antimicrobial peptide enhances acid killing of STEC


Edited by: V. Sperandio

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