Role of uropathogenic Escherichia coli OmpT in the resistance against human cathelicidin LL-37

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Keywords
outer membrane protease; antimicrobial peptide; cathelicidin; uropathogenic Escherichia coli; urinary tract infection.

Abstract
Uropathogenic Escherichia coli (UPEC) strains are among the most prevalent causative agents of urinary tract infections. To establish infection, UPEC must overcome the bactericidal action of host antimicrobial peptides. Previously, the enterohaemorrhagic E. coli outer membrane protease, OmpT, was shown to degrade and inactivate the human antimicrobial peptide LL-37. This study aims to investigate the involvement of UPEC OmpT in LL-37 degradation. An ompT deletion mutant was generated in the prototypical UPEC strain CFT073. Western blot analysis showed that the OmpT protein level is moderate in CFT073. In agreement, OmpT was shown to partially cleave LL-37. However, no difference in the minimum inhibitory concentration of LL-37 was observed between CFT073 and the ompT mutant. Plasmid complementation of ompT, which led to increased OmpT levels, resulted in complete cleavage of LL-37 and a fourfold increase in the minimum inhibitory concentration. The analysis of other UPEC isolates showed similar OmpT activity levels as CFT073. Although UPEC OmpT can cleave LL-37, we conclude that the low level of OmpT limits its contribution to LL-37 resistance. Collectively, these data suggest that UPEC OmpT is likely accompanied by other LL-37 resistance mechanisms.

Introduction
Escherichia coli is a commensal of the intestinal tract in healthy humans. However, certain pathotypes of E. coli have acquired specific virulence traits that enable them to cause a wide range of intestinal and extra-intestinal diseases (Kaper et al., 2004; Croxen & Finlay, 2010). Uropathogenic E. coli (UPEC) strains account for about 80% of urinary tract infections (UTIs) (Sivick & Mobley, 2010; Hannan et al., 2012). UPEC originate from the intestinal tract, enter the urethra and ascend to the bladder to cause cystitis. When left untreated, UPEC can further colonize the kidneys to cause pyelonephritis and eventually urosepsis.

Antimicrobial peptides (AMPs) are critical components of the innate immune system. They are small (usually 20–50 amino acids) cationic and amphipathic molecules expressed by epithelial cells and neutrophils (Zasloff, 2002; Hancock & Sahl, 2006). AMPs are synthesized as inactive precursors that are processed into biologically active peptides by host proteases. AMPs protect mucosal surfaces by both bactericidal and immunomodulatory activities. In humans, there are two major groups of AMPs, the cathelicidin LL-37 and the defensins. In the urinary tract, AMPs are secreted by uroepithelial cells to maintain sterility (Zasloff, 2007). In healthy individuals, LL-37, ribonuclease 7 (RNase 7), α-defensin 5 (HD-5) and β-defensin 1 (hBD-1) are constitutively expressed by the uroepithelium (Valore et al., 1998; Chromek et al., 2006; Spencer et al., 2011, 2012). During infection, uroepithelial cells secrete larger quantities of LL-37 and HD-5 (Chromek et al., 2006; Spencer et al., 2012). In addition, secretion of β-defensin 2 is induced (Lehmann et al., 2002). Other cell types such as infiltrating neutrophils also contribute to increased release of α-defensins and LL-37 (Chromek et al., 2006). Furthermore, LL-37 expression can be increased by a variety of stimuli including vitamin D (Hertting et al., 2010). Chromek
et al. (2006) showed that human (LL-37) and murine (mCRAMP) cathelicidins play a key role during bladder infection and provided evidence that cathelicidin expression and secretion are increased during UPEC urinary tract colonization.

Bacterial pathogens have evolved several strategies to resist killing by AMPs. For example, they produce capsule polysaccharides that shield their cell surface, they covalently modify their lipopolysaccharide (LPS) to reduce binding of AMPs or they down-regulate AMP expression by host cells (Gruenheid & Le Moual, 2012). The mechanisms used by UPEC to resist AMPs are poorly described. Some pathogens of the Enterobacteriaceae family possess outer membrane (OM) proteases of the omptin family, exemplified by E. coli OmpT (Hritonenko & Stathopoulos, 2007). Omptins share high amino acid sequence identity (45–80%), and the active site faces the extracellular environment. Omptins have been shown to impact bacterial virulence by cleaving host proteins such as plasminogen and AMPs (Haiko et al., 2009). The vast majority of UPEC clinical isolates possess the ompT gene, but its role in pathogenesis remains unclear (Foxman et al., 1995; Kanamaru et al., 2003).

A previous study has shown that CroP, the OmpT homologue in the murine enteric pathogen Citrobacter rodentium, degrades mCRAMP (Le Sage et al., 2009). Another study revealed that OmpT of enterohaemorrhagic E. coli (EHEC) cleaves LL-37 at dibasic sequences, and the resulting proteolytic fragments are devoid of bactericidal activity (Thomassin et al., 2012a). Strikingly, enteropathogenic E. coli (EPEC) expressed ompT at much lower levels than EHEC, resulting in markedly slower cleavage of LL-37 and reduced OmpT-mediated resistance to this AMP (Thomassin et al., 2012a). Thus, the contribution of OmpT to AMP resistance in E. coli appears to be pathotype specific. This study aims to assess the importance of UPEC OmpT in LL-37 resistance.

### Material and methods

#### Strains, growth media and reagents

Strains used in this study are listed in Table 1. Bacteria were grown overnight at 37 °C with aeration in Luria–Bertani (LB) broth or in N-minimal medium adjusted to pH 7.5 and supplemented with 0.2% glucose and 1 mM MgCl2, as previously described (Thomassin et al., 2012a). Human urine was pooled from three healthy adults and filter-sterilized prior to use. Media were supplemented with chloramphenicol (30 µg mL⁻¹), kanamycin (50 µg mL⁻¹) or DL-diaminopimelic acid (50 µg mL⁻¹) where applicable. Restriction enzymes and iProof DNA polymerase were from New England Biolabs and Bio-Rad, respectively. LL-37 was synthesized with a purity of > 85% (BioChemia), and polymyxin B (PMB) was purchased from Sigma.

#### Construction of ompT deletion mutant in UPEC CFT073

DNA purification, cloning and transformation were performed according to standard procedures (Sambrook et al., 1989). The CFT073 ompT deletion mutant was generated by sacB gene-based allelic exchange (Donnenberg & Kaper, 1991). Genomic DNA was used as a template to amplify the region upstream of ompT, using primers UP1 (CTAGTCTAGATCTCCAGAAGCCAGATTAT) and UP2 (CCCCCTCGAGCATAAAAGGTCTCCATCTCG). The region downstream of ompT was amplified using primers

### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant characteristics</th>
<th>Source</th>
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<tbody>
<tr>
<td>UPEC CFT073</td>
<td>Wild-type</td>
<td>Mobley et al. (1990)</td>
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<tr>
<td>UPEC ΔompT</td>
<td>CFT073 ΔompT</td>
<td>This study</td>
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<tr>
<td>UPEC ΔompT(pUPompT)</td>
<td>CFT073 ΔompT expressing ompT from pUPompT</td>
<td>This study</td>
</tr>
<tr>
<td>UPEC 536</td>
<td>Wild-type UPEC O6:K15:H31</td>
<td>Brzuszkiewicz et al. (2006)</td>
</tr>
<tr>
<td>UPEC J96</td>
<td>Wild-type UPEC O4:K6</td>
<td>Hull et al. (1981)</td>
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<tr>
<td>UPEC UTI89</td>
<td>Wild-type UPEC O18:K1:H7</td>
<td>Mulvey et al. (2001)</td>
</tr>
<tr>
<td>UPEC ABU83972</td>
<td>Wild-type UPEC OR:K5:H-</td>
<td>Andersson et al. (1991)</td>
</tr>
<tr>
<td>EPEC E2348/69</td>
<td>Wild-type EPEC O127:H6</td>
<td>Levine et al. (1978)</td>
</tr>
<tr>
<td>EPEC ΔompT</td>
<td>E2348/69 ΔompT</td>
<td>Thomassin et al. (2012a)</td>
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<tr>
<td>EPEC ΔompT(pEPompT)</td>
<td>E2348/69 ΔompT expressing ompT from pEPompT</td>
<td>Thomassin et al. (2012a)</td>
</tr>
<tr>
<td>E. coli y7213</td>
<td>thr-1 leuB6 hua21 lacY1 glnV44 recA1 asdA4 thi-1 RP4-2-Tc::Mu [-pir] Kan’</td>
<td>Roland et al. (1999)</td>
</tr>
<tr>
<td>Plasmids</td>
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<td>pRE112</td>
<td>Sucrose-sensitive (sacB) suicide plasmid, Cm’</td>
<td>Edwards et al. (1998)</td>
</tr>
<tr>
<td>pUPompT</td>
<td>CFT073 ompT deletion construct in pRE112</td>
<td>This study</td>
</tr>
<tr>
<td>pWSK129</td>
<td>Low-copy number plasmid, Kan’</td>
<td>Wang &amp; Kushner (1991)</td>
</tr>
<tr>
<td>pUPompT</td>
<td>CFT073 ompT cloned into pWSK129</td>
<td>This study</td>
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UP3 (CCGCTCGAGTAAACGTTAATAGATTTTCTC) and UP4 (CACCAGGCCTCCCTTGATTTGAAAATGGC-GCAT). The resultant PCR products were treated with XhoI (italicized sequences) and ligated together. This ligation product was used as the template for a PCR using primers UP1 and UP4. The resultant PCR product was gel-purified, treated with XbaI and SacI (italicized sequences) and ligated into the corresponding restriction sites of pRE112, generating plasmid pΔUmpT. The pΔUmpT construct was verified by sequencing and conjugated into wild-type UPEC CFT073 using E. coli χ7213 as the donor strain. Integration of the plasmid into the chromosome was selected for by plating bacteria on LB agar supplemented with chloramphenicol. Chloramphenicol-resistant transformants of CFT073 were then cultured on peptone agar containing 5% sucrose to isolate colonies that were sucrose resistant. Sucrose resistant colonies were also tested for chloramphenicol sensitivity to ensure loss of the suicide plasmid. Deletion of ompT was verified by PCR using primers UP1 and UP4 and by sequencing the PCR product at the McGill University and Genome Québec Innovation Centre. The ompT complementation plasmid was constructed by PCR – amplifying the ompT promoter and open-reading frame from genomic DNA using primers UP5 (CTAGTCCTAGTTAAATGCTTAC) and UP6 (CTAGGAGCTGACCGAGGTAAATGCCGCTTAC). The resultant PCR product was cloned into the XbaI and SacI restriction sites of plasmid pWSK129, generating plasmid pUPompT.

Western blotting

Bacterial cells grown in N-minimal medium to an optical density (OD600) of 0.5 were pelleted by centrifugation, resuspended in Laemmli sample buffer and boiled for 5 min. Whole cell lysate samples were resolved by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore). Membranes were blocked with 5% (w/v) skim milk powder in Tris-buffered saline. OmpT was detected using a rabbit polyclonal antibody raised against the C. rodentium OmpT homologue, CroP, and an anti-rabbit IgG coupled with horseradish peroxidase (HRP). Membranes were developed using chemiluminescent HRP substrate (Millipore).

Fluorescence resonance energy transfer assays

The synthetic fluorescence resonance energy transfer (FRET) substrate containing the underlined dibasic site [2Azb-SLGRKIQ-K(Dnp)-NH2] was purchased from AnaSpec. Cells were pelleted and resuspended to an OD600 of 0.5 in phosphate-buffered saline (PBS, pH 7.4). The FRET substrate (final concentration of 3 μM) was transferred into a quartz cuvette equipped with a stir bar, and 1.5 mL of cells was added after 30 s. Fluorescence emission (λ = 430 nm) was monitored for 1 h at 22 °C with an excitation wavelength set at 325 nm using a Varian Eclipse spectrofluorometer. Data were normalized using PBS instead of cells.

Proteolytic cleavage of LL-37

Culture aliquots (∼107 CFU) were incubated with 10 μg of LL-37 in a total volume of 25 μL of N-minimal medium for 60 or 90 min. Bacterial cells were pelleted by centrifugation, and supernatants were combined with Tris–Tricine sample buffer (Bio-Rad). Aliquots (5 μL) were boiled for 5 min and resolved by Tris–Tricine SDS-PAGE (10–20% acrylamide; Bio-Rad). After fixation for 30 min in 5% (v/v) glutaraldehyde, gels were stained with Coomassie blue G-250.

Minimum inhibitory concentrations

Minimum inhibitory concentrations (MICs) were determined in 96-well microtitre plates using the broth microdilution method (Wiegand et al., 2008). Bacteria (5 × 105 CFU) were added to each well. Twofold serial dilutions of the tested AMP were added to the row of wells, and the plates were incubated at 37 °C for 24 h. The MIC was the lowest concentration of AMP that did not permit any visible growth as determined by the absence of turbidity. MIC determination was repeated three times.

SYTOX green assays

SYTOX green assays were performed in 96-well black microtitre plates, according to manufacturer’s instructions (Molecular Probes). Briefly, bacterial cells were grown to an OD600 of 0.5 in N-minimal medium, and 4 × 107 CFU were added to each well. Mixtures of SYTOX green nucleic acid stain (final concentration 5 μM) with LL-37 (40 μg mL−1) or N-minimal medium were added to the appropriate wells. Plates were incubated for 1 h in the dark at 37 °C, and fluorescence was measured (excitation λ, 504 nm; emission λ, 523 nm). Data shown were normalized against cells treated with SYTOX green alone.

Results

Presence and activity of OmpT in UPEC CFT073

To test whether UPEC OmpT plays a role in degrading human LL-37 during infection, an ompT deletion mutant strain, as well as a plasmid-complemented strain, was generated in the UPEC CFT073 strain. Both the mutated and complemented strains grew as the parent strain (data not shown). The OmpT protein levels in these CFT073 strains
were compared to the previously described EPEC E2348/69 strains by Western blot analysis using an antiserum raised against CroP, the C. rodentium ompT that is 74% identical to OmpT at the amino acid level (Thomassin et al., 2012a).

As shown in Fig. 1a, a faint band corresponding to OmpT that migrated slightly lower than the expected molecular weight (32 kDa) was detected in wild-type CFT073. This band was absent from the CFT073 ΔompT mutants and, as expected, below detection limit in wild-type E2348/69 (Thomassin et al., 2012a). Upon complementation with the ompT gene under control of their respective native promoters expressed from low-copy number plasmids, intense OmpT bands were observed (Fig. 1a). The slight difference in SDS-PAGE migration of CFT073

Fig. 1. Presence and activity of OmpT in CFT073 and E2348/69. (a) Western blot of whole cell lysates using a polyclonal CroP antibody. Data are representative of three independent experiments. (b) Cleavage of the FRET substrate by CFT073 (black) and E2348/69 (grey) strains grown in N-minimal medium (solid lines) or urine (dashed line). Fluorescence was measured every 30 s for 1 h. Data are representative of three independent experiments. (c) Bacterial strains were incubated with the SYTOX green nucleic acid stain and LL-37. Emitted fluorescence was measured after 1 h. Relative fluorescence (%) indicates 100*(emitted fluorescence strain/ emitted fluorescence wild-type). Data are representative of three independent experiments performed in triplicate. Error bars represent the standard error of triplicate samples. Statistical significance was assessed using a one-way analysis of variance and Tukey's post hoc comparison test (**p < 0.001).
OmpT may be caused by a difference of three amino acids with E2348/69 (Rath et al., 2009). These Western blot data indicate that the level of OmpT is slightly higher in CFT073 compared to E2348/69. To test the proteolytic activity of these strains, cleavage of the synthetic FRET substrate containing the dibasic sequence RK was monitored over time (Fig. 1b). In agreement with our Western blot analysis, the measured fluorescence was higher in wild-type CFT073 than wild-type E2348/69. The CFT073 and E2348/69 ΔompT strains had consistently lower fluorescence than the wild-type strains, indicating that OmpT is proteolytically active (Fig. 1b). In contrast, the complemented strains showed much higher levels of fluorescence. Similar OmpT activity levels were observed for CFT073 wild type when grown in either N-minimal or human urine (Fig. 1b). The OmpT protein levels in Fig. 1a agree well with the OmpT proteolytic activity levels shown in Fig. 1b. Together, these data indicate that the OmpT protein level in CFT073 is somewhat similar to the OmpT level observed previously for E2348/69 (Thomassin et al., 2012a).

Membrane integrity assays

To further explore the role of CFT073 and E2348/69 OmpT in the protection against LL-37-mediated lysis, membrane integrity was assessed using the SYTOX green stain that fluoresces upon DNA binding following membrane disruption (Roth et al., 1997). A similar increase in fluorescence was observed for both the wild-type and ΔompT CFT073 and E2348/69 strains, indicating no significant difference in bacterial lysis (Fig. 1c). In contrast, a much lower level of fluorescence was obtained for the complemented strains. Taken together, these data indicate that OmpT protects the bacterial membrane from LL-37 only when present at high levels.

Activity of OmpT in other UPEC isolates

To determine whether other UPEC isolates have similar OmpT activity as CFT073, cleavage of the FRET substrate was monitored for the UPEC isolates 536, J96, UTI89 and ABU83972 (Fig. 2). Although there are slight differences in emitted fluorescence between isolates, all these UPEC isolates exhibit roughly similar proteolytic activities. These data suggest that there are no major differences in OmpT activity among these UPEC isolates.

UPEC OmpT cleaves human LL-37

To determine whether UPEC OmpT plays a role in LL-37 resistance, MIC values of LL-37 were determined for these
strains. As shown in Table 2, the MIC of LL-37 for CFT073 wild type was 16 μg mL⁻¹. Similarly to what was observed for E2348/69 (Thomassin et al., 2012a), the deletion of CFT073 ompT did not affect the MIC. In contrast, a fourfold increase in the MIC of LL-37 was observed for the CFT073-complemented strain (Table 2). The MICs of LL-37 for isolates 536, J96 and UTI89 were similar to the MIC obtained for CFT073. Only the isolate ABU83972 was slightly more susceptible. The cyclic lipopeptide PMB, which was previously shown to not be a substrate for ompTins, was used as a control (Le Sage et al., 2009; Thomassin et al., 2012a). As expected, the MIC values of PMB were overall similar (0.5–1 μg mL⁻¹) for all isolates (Table 2).

Taken together, these data indicate that OmpT-mediated LL-37 resistance requires high OmpT protein and activity levels at the OM. They also suggest that UPEC OmpT may play a marginal role in LL-37 resistance, as previously proposed for EPEC OmpT (Thomassin et al., 2012a).

**Genomic context of ompT**

The genomic contexts of UPEC, EPEC and EHEC ompT were compared to explain the discrepancies observed among E. coli pathotypes (Fig. 4). The UPEC and EPEC ompT genes were found around 0.6 Mb within the same genomic context. In contrast, EHEC EDL933 ompT gene was found around 1.7 Mb in a different context (Fig. 4) (Thomassin et al., 2012b). Alignment of UPEC isolates and EPEC E2348/69 ompT promoter regions showed 97–99% identity at the nucleotide level (data not shown), suggesting that the UPEC and EPEC ompT genes are regulated similarly. In contrast, the EHEC ompT promoter region is only ~75% identical to UPEC isolates, likely explaining the observed differences. Given the heterogeneity among UPEC isolates, we cannot rule out that in some cases, the ompT gene may be found in different genomic contexts, resulting in different OmpT protein levels and, in turn, different contribution to LL-37 resistance.

**Discussion**

To establish and maintain infection, UPEC must overcome LL-37 secreted by uroepithelial cells and neutrophils. One means by which bacteria can resist LL-37 is through proteolytic degradation. We previously reported that EHEC and EPEC OmpT degrade LL-37 to different extents due to differential ompT expression (Thomassin et al., 2012a). In this study, the contribution of UPEC OmpT to LL-37 degradation was investigated. We found that UPEC OmpT protein and activity levels were overall similar to those observed for EPEC OmpT, suggesting that UPEC OmpT plays a marginal role in LL-37 resistance under these experimental conditions.

The possibility remains that expression of UPEC ompT is upregulated during infection by stimuli that were not present under our experimental conditions. However, two lines of evidence suggest that this possibility is unlikely. First, CFT073 grown in human urine or N-minimal medium had similar OmpT activity levels (Fig. 1b). Second, from an in vivo transcriptome study, the ompT expression levels decreased by 0.5- to twofold for UPEC clinical isolates collected from infected urine when compared to ex vivo growth in urine (Hagan et al., 2010). These data provide a valuable indication that ompT expression may not be dramatically changed in vivo. However, given that most UPEC isolates have the ompT gene, it remains to be determined whether ompT expression changes upon association with uroepithelial cells or invasion and subsequent formation of intracellular bacterial communities (Foxman et al., 1995; Kanamaru et al., 2003).

<table>
<thead>
<tr>
<th>Strain</th>
<th>LL-37</th>
<th>PMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFT073</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>CFT073 ΔompT</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>CFT073 ΔompT(pUPompT)</td>
<td>64</td>
<td>1</td>
</tr>
<tr>
<td>536</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>J96</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>UTI89</td>
<td>16</td>
<td>0.5</td>
</tr>
<tr>
<td>ABU83972</td>
<td>8</td>
<td>0.5</td>
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</tbody>
</table>

Fig. 4. Genomic context of the ompT gene in *Escherichia coli* pathotypes. Schematic representations of the genomic regions containing the ompT gene in UPEC strains (a), EPEC (b) and EHEC (c). Genes, represented by arrows, and intergenic regions were drawn to scale. Gene names are according to GenBank entries of the published sequences.
AMPs play a major role in maintaining sterility in the urinary tract (Zasloff, 2007). Various AMPs, including LL-37, were reported to have increased expression and secretion during infection (Chromek et al., 2006; Spencer et al., 2012). In healthy children, the median LL-37 concentration in urine was 0.3 ng mL\(^{-1}\), whereas in children with UTIs, it increased to 2.4 ng mL\(^{-1}\) (Chromek et al., 2006). These reported concentrations are at least three orders of magnitude lower than the MIC value of LL-37 for UPEC strains (Table 2). This could suggest that OmpT-mediated degradation of LL-37 is not the only resistance mechanism for this AMP in UPEC. However, RNase 7, which is considered as an AMP, is present at high concentrations during UTIs (7.2 to 11.2 \(\mu\)g mL\(^{-1}\)) and has multiple dibasic motifs in its sequence, making it a putative OmpT substrate (Spencer et al., 2011, 2013). On the other hand, hBD-1 and HD-5, which are present in infected urine at a somewhat similar concentration range as LL-37, are unlikely to be OmpT substrates because they lack dibasic motifs in their amino acid sequences.

Other AMP resistance mechanisms are likely implemented by UPEC to overwhelm the action of LL-37, hBD-1 and HD-5 within the urinary tract. For example, UPEC curli fimbriae have been reported to bind LL-37 directly, shielding the bacterial membrane (Kai-Larsen et al., 2010). Many other mechanisms, including the production of capsular polysaccharides, exopolysaccharides, efflux pumps and lipopolysaccharide modifications, may play a larger role than OmpT in UPEC resistance to urinary tract AMPs.

**Acknowledgements**

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**References**


