The Outer-Membrane Protease Family of Omptins in Uropathogenic *Escherichia coli*

Isabelle Desloges

Department of Microbiology and Immunology,
McGill University, Montreal
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Abstract

Uropathogenic *Escherichia coli* (UPEC) causes approximately 85% of community acquired urinary tract infections (UTIs). In order to cause infection in the urinary tract, UPEC must overcome innate immune defenses such as antimicrobial peptides (AMPs). Antimicrobial peptides have both antibacterial and immunomodulatory properties that keep the bladder epithelium free of bacterial colonization. OmpT, an outer-membrane protease that plays a role in AMP resistance, has previously been identified as a UPEC virulence factor. We assessed OmpT activity in a collection of 58 UPEC clinical isolates of increasing disease severities. The activity was significantly higher in symptomatic groups compared to fecal isolates. Since the *ompT* gene is present in 85-97% of UPEC clinical isolates, we screened for the presence of *ompT* and other possible members of the OmpT subfamily such as *ompP* and *arlC*. This screen revealed the presence of *arlC* in eight symptomatic isolates while *ompP* was not found in any isolates. Heterogeneity of OmpT activity was due to differential gene expression of both *ompT* and *arlC* in clinical isolates causing cystitis. Furthermore, OmpT and ArlC showed different substrate specificities towards known AMPs of the urinary tract. Finally, *arlC*, and potentially *ompP*, should also be included as UPEC virulence genes and be screened for in newly isolated bacterial strains.

Résumé

Les *Escherichia coli* uropathogènes causent approximativement 85% des infections urinaires acquises dans la communauté. Pour causer des infections urinaires, les *E. coli* uropathogènes doivent surmonter les défenses innées du système immunitaire telles que les peptides antimicrobiens. Les peptides antimicrobiens ont à la fois des propriétés antimicrobiennes et des propriétés immunomodulatoires. OmpT, une protéase de la membrane externe qui joue un rôle dans la défense contre les peptides antimicrobiens, a précédemment été identifiée comme un facteur de virulence chez les *E. coli* uropathogènes. Nous avons évalué l'activité de la protéase OmpT dans une collection de 58 isolats cliniques causant des infections de sévérité croissante. L'activité était signicativement plus élevée dans les groupes symptomatiques comparativement aux isolats provenant des excréments. Étant donné que le gène *ompT* est présent dans 85-97% des *E. coli* uropathogènes, nous avons investigué pour la présence de ce gène ainsi que pour la présence des autres membres de la famille OmpT tels qu' *ompP* et *arlC*. Ce dépistage a révélé la présence de *arlC* dans huit isolats symptomatiques alors que *ompP* n'as pu être repéré dans aucun isolat de notre collection. L' hétérogénétité de l'activité de OmpT est due à la différente expression des gènes *ompT* et *arlC* dans les isolats cliniques causant des cystites. En outre, OmpT et ArlC ont démontrés différentes spécificités de substrat envers des peptides antimicrobiens connus des voies urinaires. Finalement, *arlC*, et potentiellement *ompP*, devraient être inclus dans la caractérisation des *E. coli* uropathogènes en tant que gènes de virulence.

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Contributions of Authors

Chapter 1 (Literature Review)

This chapter was written by ID with the help of HLM for edits.

Chapter 2 (Manuscript)

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This chapter was written by ID and HLM. SG and JLT both contributed to edits of the manuscript. JT did the southern blot (Figure S3). JML did the qPCR for *ompT* and *arlC* of the cystitis isolates (Figure 3A). JLT designed primers for *ompT* screen of the isolates and for the *arlC* plasmid construct (Table S1). AP and AM provided the clinical isolates strain collection. KD helped for the sequencing of the three cystitis strains (cystitis 1, cystitis 6 and cystitis 11). ID performed all other experiments (Fig.1, Fig.2, Fig.3B, Fig.4, Fig.S1, Fig.S2, Table 1, Table 2, Table S1, and Table S2).

Chapter 3 (General Discussion and Future Directions)

This chapter was written by ID with the help of HLM for edits.

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List of Abbreviations

AA- Amino acid

AMP- Antimicrobial Peptides

APEC- Avian Pathogenic *Escherichia coli*

AU- Arbitrary Unit

Bp- Base Pairs

CFU- Colony Forming Unit

DC- Dendritic Cell

EDTA- Ethylenediaminetetraacetic acid

EHEC-Enterohemmoragic *Escherichia coli*

ELISA-Enzyme-Linked Immunoabsorbent Assay

EPEC- Enteropathogenic *Escherichia coli*

ExPEC- Extra-intestinal Pathogenic *Escherichia coli*

FRET- Fluorescence Resonance Energy Transfer Assay

GI- Gastro-Intestinal

hCAMP18- Human Cationic Antimicrobial Peptide 18

hBD-2- Human β-Defensin 2

HD-5- Human α-Defensin 5

IBC- Intracellular Bacterial Community

IgA- Immunoglobulin A

IL-Interleukin

InPEC- Intestinal Pathogenic Escherichia coli

KDa- Kilo Dalton

LB- Luria-Bertani Broth

LPS- Lipopolysaccharide

NTEC- Necrotoxigenic Escherichia coli

OM- Outer-membrane

OMP- Outer-membrane Protease

PAI- Pathogenicity Island

PBS-Phosphate Buffer Saline

QIR- Quiescent Intracellular Reservoir

qPCR- Quantitative Polymerase Chain Reaction

SDS- Sodium Dodecyl Sulfate

SDS-PAGE- Sodium Dodecyl Sulfate Polyacrylamide Gel

T1P- Type 1 Pili

THP- Thamm-Horsfall Protein

USA- United States of America

UPEC- Uropathogenic *Escherichia coli*

Chapter 1-Literature Review

1 Enterobacteriaceae

Enterobacteriaceae is the family of prokaryotes, which is part of the phylum *proteobacteria*. It includes a large portion of Gram-negative bacteria of genera such as *Salmonella*, *Escherichia* and *Klebsiella* [1]. The evolutionary classification shows that *Enterobacteriaceae* is part of the class of *γ-proteobacteria* and the order of *Enterobacteriales* (Figure 1) [1]. The *Enterobacteriaceae* family includes both pathogenic and non-pathogenic bacteria. Some can colonize specific human body sites and cause disease, while others are found in the environment in soil or water [1, 2]. *Enterobacteriaceae* is responsible for half of the nosocomial infections in the United States of America (USA) alone [3]. They are characterized by a rod shape, the inability to sporulate, a facultative oxygen utilization and the ability to ferment various sugars [2]. The most studied member of the *Enterobacteriaceae* family is *Escherichia coli.*

1.1 Escherichia coli

E. coli strains can either be non-pathogenic (commensals) or pathogenic (pathogens) [4]. Commensal *E. coli* reside in the gastro-intestinal (GI) tract of humans and are part of their microbiota [5]. They are the most abundant facultative anaerobe present in the human gut even if they are highly outnumbered compared to other bacterial species [5]. Pathogenic *E. coli* evolved from non-pathogenic strains through acquisition of virulence genes by mutations and horizontal gene transfer that enhanced their genomic diversity [4, 6]. These extra virulence factors, compared to their commensals counterparts, allowed them to cause disease [6]. *E. coli* strains are classified according to their serogroup, which is determined by their lipopolysaccharide (LPS) O-antigen, their capsule K-antigen and their flagella H-antigen [1].

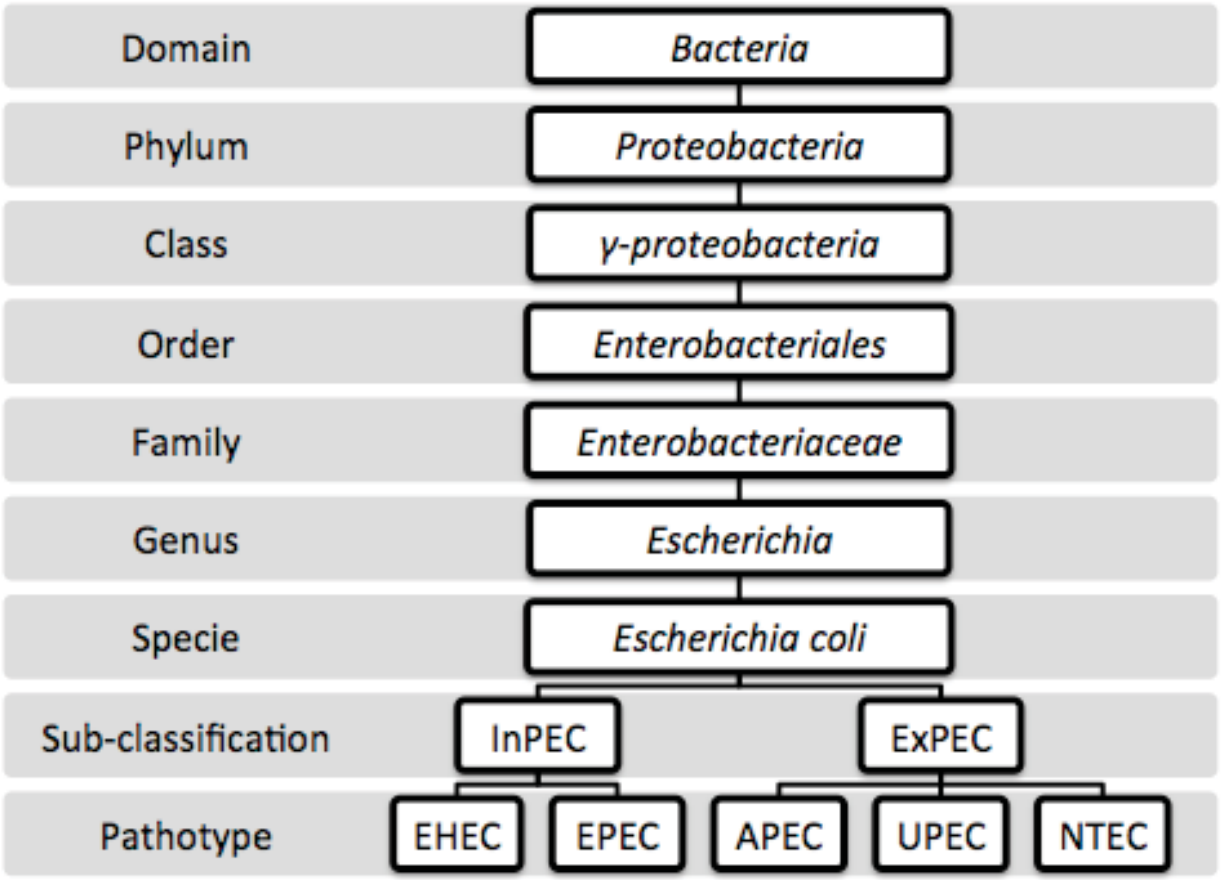

Figure 1. Classification of *Escherichia coli* pathotypes. Enterohemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC) are part of intestinal pathogenic *E. coli* (InPEC). Avian pathogenic *E. coli* (APEC), uropathogenic *E. coli* (UPEC) and necrotoxigenic *E. coli* (NTEC) are part of extra-intestinal pathogenic *E. coli* (ExPEC) [1].

Pathogenic *E. coli* are divided in two categories, indicative of the body site they can colonize: intestinal pathogenic *E. coli* (InPEC) and extra-intestinal pathogenic *E. coli* (ExPEC) [7]. InPEC infects the GI tract while ExPEC infects other body sites than the intestine such as the urinary tract, the meninges, and the bloodstream [7].

1.1.1 *E. coli* pathotypes

Most studied InPEC include enterohemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC), two pathotypes that cause diarrheal diseases in humans [7]. However, other pathotypes are part of InPEC such as Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), Adherent Invasive *E. coli* (AIEC) and Diffusely adherent *E. coli* (DAEC) [8].

ExPEC pathogens cause a variety of disease pathologies including urinary tract infection and meningitis [7]. ExPEC infects both humans and animals [7]. The main pathotypes infecting humans are Uropathogenic *E. coli* (UPEC), Meningitis-associated *E. coli* (MNEC) and Necrotoxigenic *E. coli* (NTEC). ExPEC comprises many others pathotypes that infect a wide variety of animals such as avian pathogenic *E. coli* (APEC), which infects chickens [7]. However, the majority of ExPEC is part of the UPEC pathotype that causes urinary tract infections (UTIs) [9-11].

Specific virulence actors are necessary for each of these pathotypes to cause disease in their particular niche. For example, only EHEC, EPEC and EIEC encode a Type III secretion system. This allows them to inject effectors proteins into host cells [12]. Both EPEC and EHEC are

known as attaching and effacing pathogens that form pedestal like structure when bound to intestinal epithelial cells [13]. EIEC is in fact very similar to *Shigella* and share most of its virulence factors including a second type of Type III secretion system encoded on large plasmids [14]. ETEC is known to encode a wide variety of enterotoxins. Theses toxins are both heat-labile and heat-stable toxins that allow ETEC to colonize the small intestine mucosa [15]. EAEC encoded both cytotoxins and enterotoxins and forms large biofilms on the intestinal epithelia [16]. DAEC and AIEC are known for their specific adherence to intestinal epithelial cells [17]. However, AIEC are able to invade the cells upon attachment while DAEC only adheres to the cell surface using the F1845 adhesin [17].

1.1.1.1 Extra-Intestinal Pathogenic *Escherichia coli*

ExPEC can be mistaken with commensal organisms during their passage through the GI tract since it does not cause intestinal disease [7, 18]. However, once they reach a specific body site, they can colonize it, and cause disease [7, 18]. Diverse sites of infection lead ExPEC pathogens to develop a wide array of virulence factors specific for the body niche that they infect [19]. For example, the K1 capsule is necessary for NTEC to cause disease in the brain while NTEC isolates that lack K1 capsule resemble, in gene content, fecal isolates [20, 21].

An array of virulence factors that has previously been identified as important in ExPEC pathogenesis is shown in Table 1 [18, 22]. However, there are several factors to consider when talking about the importance of a virulence factor, not only do bacteria need to encode a virulence factor but they also need to express it [19]. Some of these virulence factors such as *fimH* are necessary in order to cause infection of the urinary tract.

2 Urinary Tract Infections

UTIs are one of the most common community acquired infections, which result in enormous health consequences and cost burden all around the world [23, 24]. UTIs affect mostly women and in 25% of cases, a recurrent infection will occur within 6 months after the initial infection [23, 24]. UTIs are diagnosed by the presence of at least 10^3 colony-forming units (CFU) per mL in midstream urine of infected individuals [25]. UTIs refer to infection of different parts of the urinary tract such as the bladder, the kidneys and more severely, the bloodstream [26]. UTIs are divided in complicated and uncomplicated infections [23]. Complicated infections result from a compromised patient such as an individual suffering from urinary blockage, kidney stones or immune-deficiency, while uncomplicated infections affect normally healthy individuals [27]. UTIs can be caused by a wide variety of pathogens [25].

2.1 UTI-Causing Pathogens

Both bacteria and fungi can cause UTIs [28]. However, in the majority of cases, UTIs are the result of bacterial infection [28, 29]. Complicated UTIs are caused, in order of incidence, by UPEC, *Enterococcus* species, *Klebsiella pneumoniae*, *Candida* species, *Staphylococcus aureus*, *Proteus mirabilis* and *Pseudomonas aeroginosa* [28]. Uncomplicated UTIs are caused, in order of incidence, by UPEC, *K. pneumoniae*, *Staphylococcus saprophyticus*, *Enterococcus fecaelis*, Group B *Streptococcus*, *P. mirabilis, P. aeroginosa*, *S. aureus* and *Candida* species [28, 30]. In uncomplicated UTIs, UPEC is the causative agent of approximately 85% of infections [26, 29].

2.1.1 UPEC

Pathogenic *E. coli* pathotypes (from both InPEC and ExPEC) can be classified in four phylogenetic groups: A, B1, B2 and D, which is representative of their genetic origin [31]. These different groups are heterogenous in their virulence gene content [32]. Most UTIs are caused by group B2 and to a lesser extent, group D [33]. In ExPEC, groups A and B1 have been associated with commensal strains that have smaller genome lengths, less virulence genes, and are generally associated with a lower number of UTIs [33-35]. Compared to the other phylogenetic groups, phylogenetic group B1 is able to persist in the environment [35]. In a mouse model of UTI, phylogenetic group B2 is associated with high infectivity and lethality while group A and B1 have much lower lethality rates [36]. However, virulence genes can be horizontally transferred to strains from group A and B1 and make them more prone at causing disease in the urinary tract [32, 37-39]. In contrast, InPEC pathogens are associated with groups A, B1 and D [35].

UPEC possesses an array of virulence factors to subvert the hostile environment of the urinary tract [9, 22, 40]. For this purpose, UPEC strains are very heterogenous in their genetic content [31, 40, 41]. For example, transcriptome analysis of UPEC prototypical strain CFT073 revealed the increased expression of five iron acquisition systems, capsule genes, LPS synthesis and antibiotic resistance genes in a mouse model of cystitis [41].

2.1.1.1 CFT073

CFT073 UPEC prototypical strain from phylogenetic group B2 was isolated from a woman suffering from severe pyelonephritis in Maryland, USA [42]. This strain displayed enhanced hemolytic activity compared to other UPEC strains from the same bacterial collection [42]. It

was sequenced in 2002 and its virulence genes, that are absent from commensal strain K-12, are grouped into five pathogenicity islands (PAIs) [43, 44]. Surprisingly, this bacterial strain does not possess any extra-chromosomal DNA element [43].

2.1.2 UPEC Route of Infection

UPEC causing UTIs usually come from feces. After passing through the GI tract, it ascends the urethra to reach the bladder [25, 45]. From the bladder, where UPEC can infect both asymptomatically (asymptomatic bacteriuria (ABU)) and symptomatically (cystitis), it can reach the kidneys (pyelonephritis) and can cause permanent scaring [25, 45]. From the kidneys, UPEC can access the bloodstream through an unknown mechanism and cause urosepsis [25, 28, 45].

The human urinary tract is normally a sterile environment [46, 47]. However, new evidence suggests that UPEC can colonize the urinary tract, mostly the bladder, without causing any disease (ABU) [48, 49]. The new terms urinary microbiome or virobiome reflect the microorganism community that can live in the urinary tract [50]. The urinary microbiome is composed of a diverse array of both Gram-positive and Gram-negative bacteria [51]. Due to the impossibility to grow most of these bacterial strains *in vitro*, 16S rRNA sequencing was used to identify all microorganisms present in the urinary tract of healthy individuals [48, 49, 51-54]. However, the role of these microorganisms in the urinary tract is still unclear. These bacteria and viruses are believed to prevent colonization by pathogenic bacteria and, by doing so, prevent infection [55-57]. However, carriage of an ABU strain for extended periods of time can be detrimental for the health of the individual [58]. Interestingly, bacteria causing ABU are present

in the bladder in higher numbers than pathogens causing cystitis [55]. ABU is diagnosed by the presence of at least 10^5 CFU/mL of urine while cystitis only counts 10^3 CFU/mL [55].

UPEC route of infection starts when UPEC ascends the urethra and comes into contact with the bladder epithelium [45]. The first important step for colonization is bacterial binding to the bladder epithelium. Adherence is mediated by Type 1 pili (T1P) binding to mannosylated receptors present on bladder epithelial cells [59]. Binding to the uroepithelium leads to UPEC subsequent internalization by umbrella cells (the outer most layer of differentiated bladder cells) in a vesicle dependent fashion [60, 61]. Once inside bladder cells, a vast majority of the bacteria will be exocytosed [61]. A minority of bacteria will evade this mechanism and enter the cytosol to form intracellular bacterial communities (IBCs) that consist of a biofilm of 10^4-10^5 bacteria (Figure 2) [62]. When intracellular bacteria stop replicating, they enter another stage of their infection cycle in which they form a quiescent intracellular reservoir (QIR) [63]. These QIRs prevent immune detection of bacterial cells and allow their release in the bladder lumen to cause recurrent UTIs (Figure 2) [63, 64].

Different virulence factors are important for UPEC pathogenesis depending on which part of the urinary tract it is infecting [41, 65-67]. The different urinary defenses that UPEC faces in different parts of the urinary tract can affect bacterial survival and fitness during infection [30, 68].

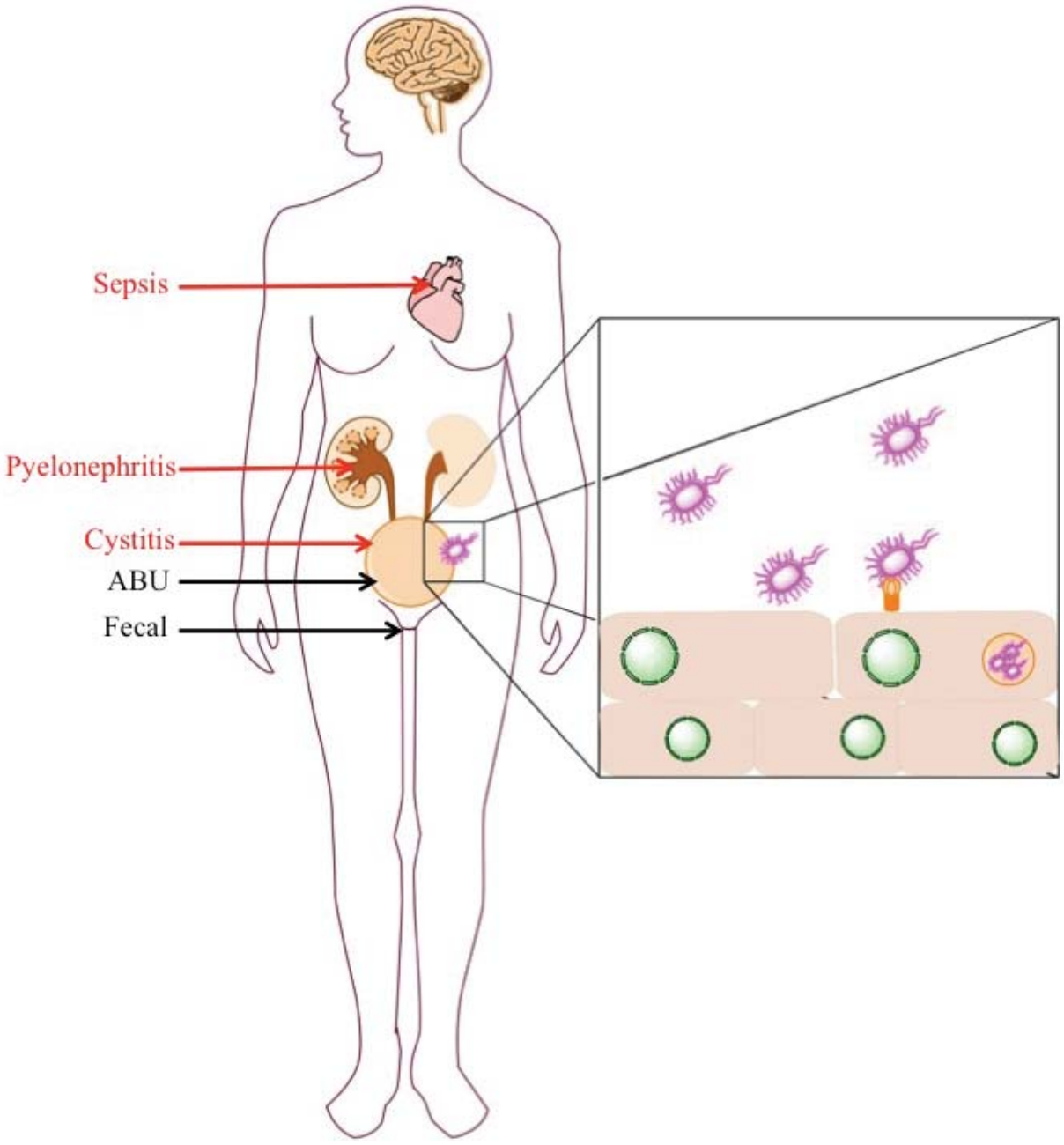

Figure 2. UPEC can infect multiple body sites and cause recurrent infections. UPEC originates from feces. It ascends the urethra to colonize the bladder. Asymptomatic bacteriuria (ABU) is characterized by the asymptomatic colonization of the bladder, while a symptomatic bladder infection is called cystitis. Ascension of UPEC into the kidneys leads to pyelonephritis [38]. From the kidneys, UPEC can reach the bloodstream and cause urosepsis. UPEC (pink) binds to bladder epithelium through the Type 1 pili (orange) and forms intracellular bacterial communities (IBCs) [52, 55]. The persistence of these IBCs leads to recurrence of infection. Black depicts non-disease pathologies while red indicates disease states [55].

2.2 Bladder Defenses Against Pathogens

UPEC causing UTIs needs to overcome natural defenses of the urinary tract in order to cause infection [69]. This normally sterile environment is hostile to pathogen colonization due to several defense mechanisms developed by the bladder [70, 71]. The bladder defenses can be divided in two categories: physical and chemical defenses.

2.2.1 Physical Defenses

Bladder physical defenses include mechanical killing or physical removal of bacterial pathogens to prevent infection [72]. Those physical defenses include cell exfoliation, urine flow, low pH and high osmolality of urine [73, 74].

The bladder epithelium is covered with a thin mucus layer, different from the one found in the intestinal tract, that prevents bacteria from directly binding to uroepithelial cells [75]. However, once UPEC is bound to bladder cells, adherent UPEC has to overcome the shear force of urine in order to establish infection in the bladder [76]. Loosely adherent bacteria can detach and be ejected by the flow of urine coming from the kidneys [77]. Urination creates a force that exfoliates the first layer of umbrella cells and subsequently removes bound bacteria [77]. However, urine flow has been shown to reinforce the binding of T1P to bladder epithelium of previously attached UPEC cells [78].

Another physical defense of the bladder is low urine pH, which leads to a stressful environment for bacterial pathogens such as UPEC, that need to survive this acidic environment in order to cause infection [79]. The pH can vary from 4.5-8 but the mean pH of urine in healthy individuals

is 5.6 for males and 5.7 for females [80]. Bacterial growth is inhibited at a pH lower than 6.5 [70]. High osmolality usually cause bacteria to lyse due to the osmotic pressure [81]. The mean osmolality of urine in healthy individuals is 750 mmol/kg in males and 640 mmol/kg in females [80]. In order to survive in the bladder lumen UPEC needs to overcome the osmotic pressure of urine [73].

2.2.2 Chemical Defenses

Chemical defenses of the urinary tract include the secretion of a multitude of proteins such as Tamm-Horsfall Protein (THP), secretory immunoglobulin A (IgA) and antimicrobial peptides (AMPs), that play an important role in the defense of the urinary tract against uropathogens [47, 82-84]. Chemical bladder defenses also include the immune system activation, both innate and adaptive, that help prevent bacterial bladder colonization [69, 71].

THP is the most abundant urinary protein produced by the kidneys [85, 86]. THP plays a very important role to prevent bacterial infection of the bladder [82]. It binds to FimH, the tip protein of the T1P, and subsequently prevents bacterial attachment to bladder epithelial cells [82, 87]. THPs have immunomodulatory properties in the urinary tract such as dendritic cells (DCs) activation [88].

Immune response to pathogens in the urinary bladder is important for pathogen clearance [89]. Antibodies and AMPs play a role in the bladder immune response. For example, secreted IgAs prevent pathogen binding to bladder epithelia [83, 90]. FimH is able to repress NF-κB activation, which induces cell apoptosis [78, 91]. However, TLR-4 activation by UPEC LPS leads to the

activation of NF-κB, which induces secretion of pro-inflammatory cytokines such as interleukin (IL)-6 and IL-8 [92]. IL-8 attracts neutrophils to the site of infection and allows the release of specific AMPs such as LL-37 [93, 94]. AMPs play an important role in the defense of the urinary tract against pathogens [69].

2.2.2.1.1 Antimicrobial Peptides

AMPs are part of the body's innate immune response [95, 96]. They are small proteins variable in length (12-50 amino acid (AA)) and structure [95, 96]. They contribute to keeping the epithelial lining of the bladder and other body epithelia free of bacterial colonization [47, 95]. They have both antimicrobial and immunomodulatory properties [95]. They have a bactericidal action against Gram-positive bacteria, Gram-negative bacteria and fungi [95, 96]. AMPs are positively charged peptides that bind preferentially to negatively charged bacterial membranes, but not to neutral host cell membranes [97, 98]. AMPs are then thought to insert in the bacterial membrane, which results in cytoplasmic content leakage in the periplasm and subsequent bacterial lysis [97, 99, 100].

AMPs have been shown to play an important role in the defense of the urinary tract against pathogens [46, 47, 101, 102]. Several categories of antimicrobial peptides are secreted in the bladder including defensins, cathelicidins and ribonucleases (Figure 3) [102-105].

2.2.2.1.1.1 Defensins

Defensins are the most widespread AMPs as they can be found in plants, insects, and mammals [106]. They are small peptides (3-5 kilo Daltons (kDa)) and possess, in their structure, three or

four disulphide bridges [107, 108].They are classified in three categories (α, β and θ-defensins) depending on their tertiary structure (Figure 3) [108]. Defensins expressed in the bladder include human ß defensin 1 (hBD-1), which is constitutively expressed, human ß defensin 2 (hBD-2) and human α defensin 5 (HD-5), which are both induced upon infection [104, 105].

2.2.2.1.1.2 Cathelicidins (LL-37)

The human cathelicidin LL-37 is a 4.5 kDa positively charged α-helical amphipathic peptide (Figure 3) [109, 110]. It is synthesized as a precursor protein called human cationic antimicrobial peptide-18 (hCAP18) that is processed into LL-37 after the removal of the cathelin domain [111]. LL-37 is in a monomer form at acidic pH but oligomerizes at physiological pH (pH=7.5) [112]. The significance of this oligomerization for the mechanism of action of LL-37 is still unknown [112].

LL-37 is expressed in a variety of tissues and cell types in the human body such as neutrophils, bone marrow myeloid cells, skin, lungs, urinary tract, and gut epithelia [47, 113-117]. In the urinary tract, LL-37 production is induced upon infection and can be measured in the urine of patients suffering from UTIs [101]. The role of cathelicidins in mouse infection models of UTIs remains unclear [101, 118]. One study showed that mice deficient in mouse cathelicidin CRAMP production have high bacterial burden and are more susceptible to infection [101]. While another study involving a mouse model of cystitis showed that mice deficient in the murine cathelicidin

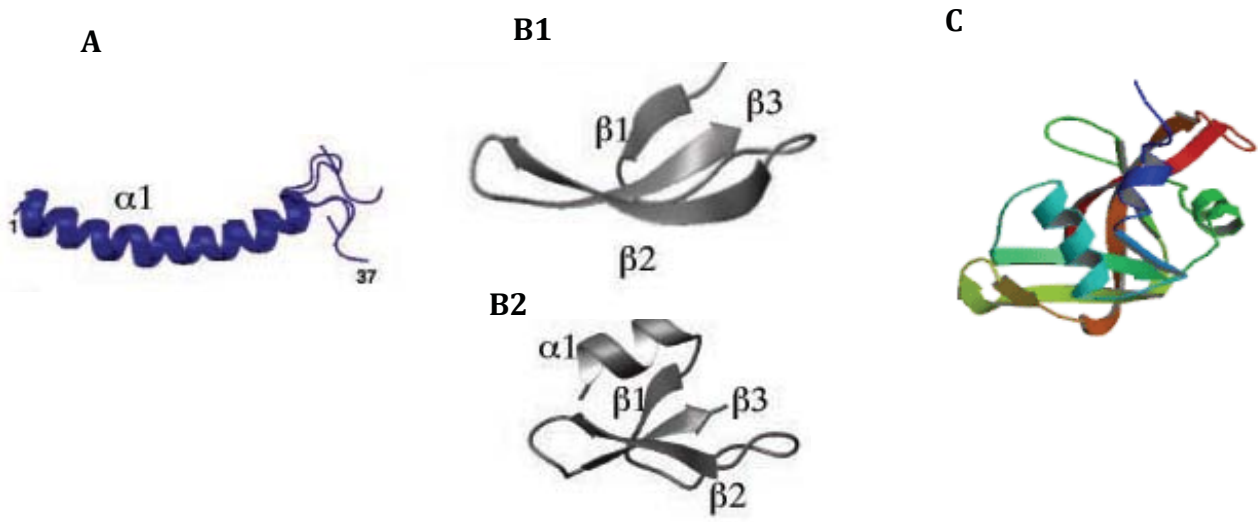

Figure 3. Antimicrobial peptides present in the urinary bladder. (A) LL-37 is a α-helical amphipathic peptide. (B1) Human β-defensin 1 consists of three β-strands. (B2) Human α-defensin 5 has one α-helix and three β-strands. (C) RNase 7 is composed of three α-helices and seven β-strands [100, 101, 102, 103, 112].

CRAMP were more resistant to infection by the prototypical strain UTI89 due to an increase in inflammatory response [118].

2.2.2.1.1.3 Ribonucleases (RNases)

A few RNases, part of the ribonuclease A family in humans, have antimicrobial properties that are independent of their RNase activity [119]. Although the mechanism by which these RNases carry their antimicrobial function is unknown, it is known to be independent of their RNase activity [120]. The RNases that have antibacterial properties are RNase 2, RNase 3, RNase 5, RNase 6, RNase 7 and RNase 8 [103, 121-124]. Recently, RNase 7 has been shown to play an important role in the urinary tract [102, 125].

RNase 7 has potent antimicrobial properties against Gram-negative bacteria, Gram-positive bacteria and fungi such as *Candida albicans* [126, 127]. It is secreted by keratinocytes in different epithelia of the body such as the skin and the bladder [126]. In the urinary tract, RNase 7 is found in the kidneys and the bladder with higher expression in the bladder epithelium [102]. It is present in detectable amount by enzyme-linked immunosorbent assay (ELISA) in healthy human urine and its production is up regulated upon infection [102, 125]. RNase antibacterial activity requires the presence of charged basic amino acids (AAs) on its surface [120]. For RNase7, only one out of three basic AAs surface clusters is necessary for antibacterial activity [120].

2.2.2.1.1.4 Antimicrobial Peptide as Therapeutics

The antibacterial activity of AMPs, combined with the paucity of new antibiotics in the pharmaceutical industry pipeline, would make them good candidates for treatment of multi-drug resistant bacteria [128]. AMPs act as natural intrinsic antibacterial agents and have low potency to induce resistance, which gives them a great advantage for their use as therapeutics [129, 130]. They have already been used for the topical treatment of various skin infections [131-133]. It is however difficult to treat intestinal diseases or UTIs with these peptides due to their poor stability and absorption at these body sites [134]. Some have approached the problem in a different manner and tried to increase the natural production of AMPs by tissues [135-137]. LL-37 production, for example, is enhanced by vitamin D and butyrate [138, 139]. These peptides are through to induce low levels of resistance due to the very broad nature of their target (bacterial membrane) compared to antibiotics that usually have very specific targets, that can be mutated, within the bacterium [129]. The rise in resistance to AMPs among bacterial communities, including commensals, is low due to the fact that most AMPs are only induced upon infection and almost completely absent from healthy individuals [129].

3 UPEC Genetic Heterogeneity

UPEC is a very heterogenous pathotype in terms of the virulence genes it encodes (Table 1). Those virulence genes are present in pathogenicity islands (PAIs), or within mobile genetic elements such as prophages or plasmids.

Table 1. Virulence factors important for ExPEC pathogenesis

Gene	Role	Reference
Adhesins		
fimH	D-mannose–specific adhesin, type 1 fimbriae	[22]
papAH	Major structural subunit of P fimbriae	[22]
papC	Pilus assembly, central region of pap operon	[22]
papEFG	Minor tip pilins, connect PapG to PapA	[22]
sfaS	Pilus tip adhesion, S fimbriae	[22]
focG	F1C fimbrial adhesin	[9]
iha	Adhesion siderophone	[9]
tsh	Temperature sensitive hemmaglutinin	[9]
hra	Heat-resistant agglutinin	[9]
afa	Dr-brinding adhesin	[9]
Iron related		
fyuA	*Yersinia* siderophore receptor	[22]
iutA	Ferric aerobactin receptor gene involved in iron transport	[22]
iroN	Salmochelin	[9]
ireA	Siderophore receptor	[9]
Protectins		
kpsMTII	Group II capsular polysaccharide synthesis	[22]
kpsMTIII	Group III capsular polysaccharide synthesis	[22]
K1	K1 capsule	[9]
K2	K2 capsule	[9]
K5	K5 capsule	[9]
Toxins		
cnf-1	Cytotoxic necrotizing factor 1	[22]
hlyA/D	α-Hemolysin	[22]
sat	Secreted autotransporter toxin	[9]
pic	Serine protease	[9]
vat	Vacuolating toxin	[9]
astA	Entero aggregative *E.coli* toxin	[9]
Miscellaneous		
ompT	Outer-membrane protease	[140]
usp	Uropathogenic-specific protein	[9]
traT	Serum resistance associated factor	[9]
Iss	Increased serum survival	[9]
H7 fliC	Flagellin	[9]
malX	Pathogenicity island marker	[44]

3.1 Pathogenicity Islands

Pathogenic *E. coli* virulence factors are organized in gene clusters called PAIs that give bacteria a fitness advantage and allow colonization of specific body sites [4]. PAIs are absent from commensal *E. coli* strains and are most often more than 10,000 base pairs (bp) in size [141]. These areas rich in virulence genes are usually different in Guanine-Cytosine content and AA codon usage than the rest of the bacterial genome [44, 141]. The genetic organization of PAIs usually starts with a tRNA gene and ends by direct repeat elements [141]. PAIs often contain mobile genetic elements that are involved in DNA mobility such as transposons, phages, or integrases [141].

3.2 Mobile Genetic Elements

Mobile genetic elements are DNA fragments that can move from one place to another within the bacterial genome or to another bacterial cell [142]. This DNA mobility can be seen in three forms: conjugation, transformation, and transduction [142]. Conjugation is the transfer of a plasmid from one bacterium to another using a specialized apparatus called conjugation pilus [142, 143]. Usually, the proteins necessary for this conjugation pilus are encoded on the plasmid that is being transferred [142, 144]. Transformation involves the transfer of genes between closely related bacteria through the use of proteins that are encoded on the bacterial chromosome [142, 145, 146]. Transduction is the transfer of DNA through viruses called bacteriophages [147]. These viruses can insert their DNA into the bacterial genome and stay inactive permanently in the bacteria (prophages) or have the ability to make viral capsid and move from one bacterium to the other [147]. Occasionally, when bacteriophages exit the bacterial cell, they can leave with a portion of the bacterial genome with them [142].

3.3 Plasmids

Some plasmids are self-transmissible and can be conjugated from one bacterium to the next, while other plasmids do not have this property or lost it over time [148]. Their lengths can vary from a few thousand bp to over 300,000 bp [149]. There are different types of plasmids and a bacterium can have more than one plasmid as long as they have compatible origins of replication [149, 150]. This is necessary in order for the bacterium to replicate these plasmids successfully [150]. These different origins can be used to classify plasmids and where they come from [150]. However, initial plasmid classification was done according to specific genes present on the plasmids such as colicins (ColV, ColB, ColM, ColBM) [151]. Colicins are antibacterial agents produced by pathogenic bacteria to kill their susceptible counterparts [152]. Although chromosomal and plasmid DNA evolved independently in bacterial strains, certain plasmid types are associated with specific bacterial pathotypes [153]. For example, plasmids associated with ExPEC pathotype are the ColV and Vir plasmids [153, 154].

Plasmids are known to encode a variety of virulence genes that give a growth advantage during infection [155]. They are notorious for the large number of antibiotic resistance genes they can carry such as the *bla* gene, which encodes for a β-lactamase [156]. Antibiotic resistance genes are most often encoded on extra-chromosomal DNA [157]. The rate of resistance associated with extra-chromosomal DNA (plasmids) is much higher than resistance acquired through gene acquisition or gene mutation [157, 158]. However, gene mutation for antibiotic resistance is ten times more frequent in pathogenic bacteria compared to commensals [159]. The genes associated with β-lactam and aminoglycoside resistance have been the most common among bacterial

pathogens [156, 158]. These antibiotic resistance genes, along with other virulence factors, are important for UPEC pathogenesis.

4 UPEC Virulence Factors

The UPEC pathotype is heterogenous in genomic content and possesses a multitude of virulence factors such as adhesins, toxins, capsules, iron uptake systems, and hemolysins, which allows them to colonize the urinary tract (Table 1) [160, 161]. These virulence factors are either inserted in the genome (chromosome), by insertion elements and transposons, or encoded on plasmids [162].

4.1 Adhesins

UPEC strains encode a vast variety of different adhesins that are regulated depending on the body site the bacterium is colonizing [163, 164]. These adhesins include T1P, P pili, S pili and Dr adhesins (Table 1) [9]. T1P is very important for bladder colonization [164]. It is encoded by the *fim* operon, which is present in most *E. coli* strains and even in some other *Enterobacteriaceae* genera [165, 166]. FimH, the tip protein of the T1P, is responsible for binding manosylated residues on glycoproteins of host epithelial cells [167]. Its main binding target on bladder epithelial cells is uroplakin Ia although it can also bind to other cell surface proteins such as integrins [168, 169]. It also acts as an invasin to allow UPEC invasion of bladder cells [59]. Its expression is highly upregulated *in vivo* in a mouse model of cystitis [41].

P fimbriae are involved in colonization of the kidneys [170]. Expression of P fimbriae is turned on when uropathogens leave the bladder through the ureters to reach the kidneys [163]. P fimbria

bind to di-galactosides present on kidney epithelium and are highly expressed in strains causing pyelonephritis [163].

4.2 Toxins

UPEC can produce several toxins including haemolysin, cytotoxic necrotizing factor and autotransporter toxins [22]. UPEC is known to possess *hylA*, a gene that encodes for an α-haemolysin [9]. UPEC strains possessing this toxin are associated with clinically severe UTIs [171]. Since the urinary tract is an environment that is very poor in nutrients, secretion of HylA is thought to allow UPEC to lyse bladder epithelial cells in order to release nutrients in the bladder lumen [172]. Some toxins such as HylA and cytotoxic necrotizing factor 1 (CNF-1) are known to be secreted by outer-membrane vesicles (OMVs) to reach host cells [173]. CNF-1 acts in the host cell cytosol by activating Rho family GTPase, which affects many cell components and signalling pathways [174]. CNF-1 is also known to increase bladder cell exfoliation [175]. Autotransporter toxins, also known as Type V secretion toxins, include the secreted autotransporter toxin (Sat) and the vacuolating autotransporter toxin (Vat) and are often encoded by UPEC [176].

4.3 Iron acquisition

UPEC possesses multiple iron scavenging systems [177]. Iron is very important for bacterial growth but is usually present at very low concentration at infection sites such as the urinary tract [178]. One effective way of scavenging iron is the use of siderophores [179]. Those molecules secreted by the bacteria in its environment have a very high affinity for ferric iron (Fe^{3+}), which allows bacterial pathogens to bring the iron back into the cell [179]. Commensal bacterium K-12 has one siderophore system called enterobactin. UPEC, which encodes enterobactin, also

possesses salmochelin, yersiniabactin and aerobactin [180]. Deletion mutants for one of these siderophores were outcompeted in a UTI mouse model by their wild-type counter parts [180]. However, UPEC does not solely use siderophores to scavenge iron [180]. It also uses iron receptors present at the outer-membrane that bind iron and carries it back inside the bacterial cell. Prototypical UPEC strain CFT073 encodes 14 outer-membrane iron receptors [181].

4.4 Antimicrobial Peptide Resistance

Both pathogenic and commensal bacteria have developed mechanisms to resist AMPs [182-184]. Some of the resistance mechanisms used by bacteria involve the modification of the bacterial surface charge [182, 185]. They include lipopolysaccharide (LPS) modification or production of a neutral group 4 capsule to prevent binding of the AMPs to the bacterial membrane [182, 186]. Bacteria may also produce curli fimbriae that can bind AMPs and prevent them from reaching the bacterial membrane [187, 188]. Bacteria can also use ABC transporters to pump the AMPs out of the bacterial cell [184]. Other mechanisms include production of surface proteases that cleave and inactivate AMPs, such as the omptin family of proteases [189-191]. Defensins are resistant to proteolysis, whereas cathelicidins are highly susceptible to bacterial proteases [183].

5 Proteases

Proteases are enzymes that cleave peptide bonds [192]. There are seven types of proteases: threonine proteases, aspartic acid proteases, metalloproteases, cysteine proteases, serine proteases, glutamic acid proteases, and a last group that includes all other proteases [192]. Proteases are usually found within mobile genetic elements such as transposons, plasmids or prophages [192]. They are not essential for bacterial replication and survival, but can be

important for bacterial virulence [192]. Proteases are at the forefront of host pathogen interactions; both secreted and membrane bound proteases can either degrade or process host proteins [191, 193-196]. The omptin family of proteases can degrade those host proteins such as AMPs and Fas ligand [191, 197].

5.1 Omptins

Omptins are outer-membrane proteases found in a number of pathogens from the *Enterobacteriaceae* family such as *Yersinia pestis* (Pla), *Salmonella enteria* (PgtE), *Shigella flexneri* (IcsP), *Citrobacter rodentium* (CroP) and *E. coli* (OmpT, OmpP and ArlC) (Figure 4) [189, 198-203]. They are transmembrane β-barrels composed of ten antiparallel ß-strands linked by four periplasmic loops and five extracellular loops that surround the active site (Figure 5) [204, 205]. Omptins are associated with LPS and require this interaction for activity [206-208]. In the extracellular active site, there are two pairs of amino acids that are important for activity; Asp^{206}-His^{208} and Asp^{84}-Asp^{86} [204, 209]. These residues hold a water molecule in the active site, which induces cleavage between AA residues of the substrate [204, 210]. The sequence of the five periplasmic loops of omptins influences which substrates they are able to cleave [211]. Omptins usually cleave between basic AA residues [191, 212-214]. However, omptin specificity depends on the AAs adjacent to the basic residues in the peptide sequence [214]. Different omptins have different physiological substrates [191, 203]. For example, Pla cleaves plasminogen while OmpT cleaves smaller peptides such as AMPs [191, 203]. Based on these their different AA identity and their ability to activate plasminogen, omptins were divided in two sub-categories: OmpT-like and Pla-like subfamilies (Figure 5) [215].

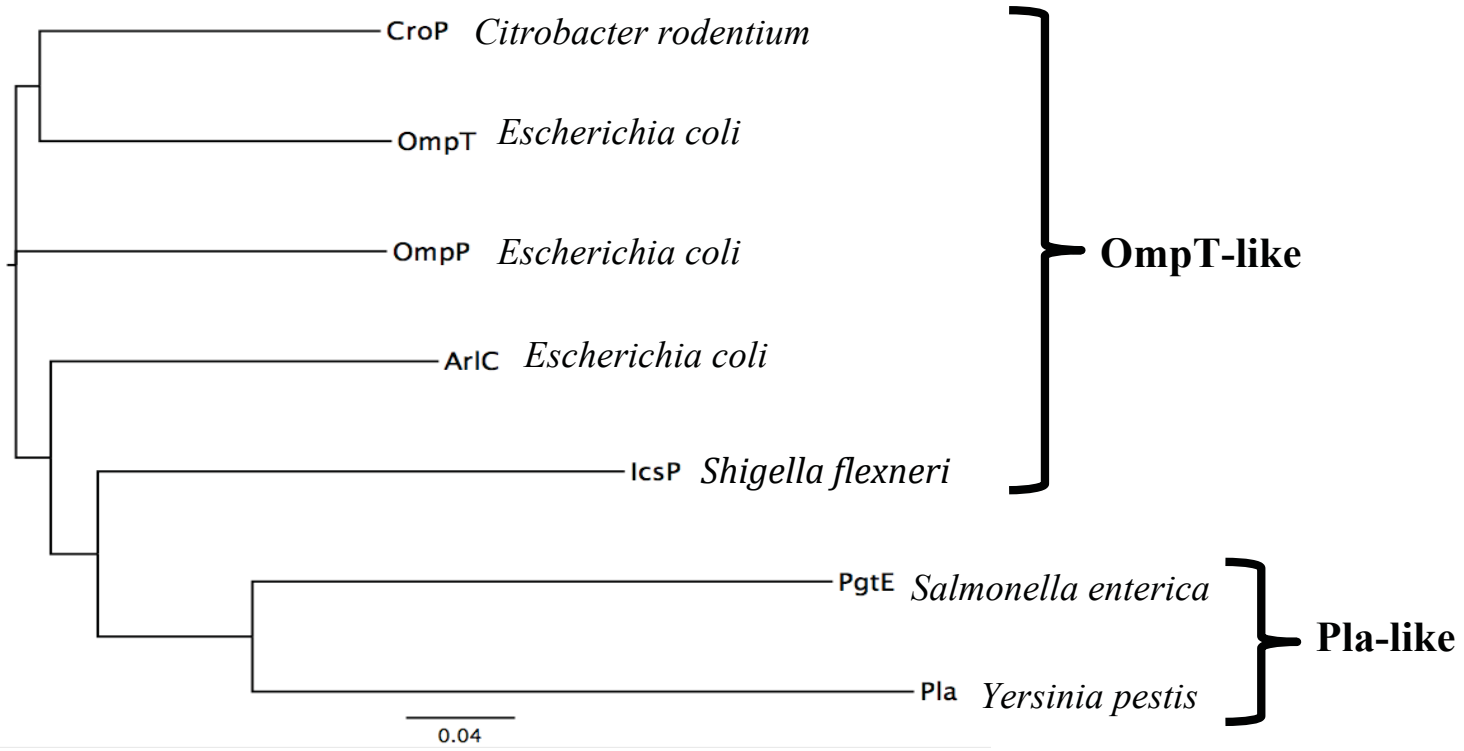

Figure 4. Phylogenetic tree of the omptin family of proteases. OmpT, OmpP and ArlC, the three omptins of *E. coli*, are approximately 74% identical at the amino acid level. Due to their substrate specificity and their amino acid identity, they were divided in two categories: OmpT-like and Pla-like [234].

Table 2. Presence of omptins in prototypical *Escherichia coli* strains

Strain	Chromosomal *ompT*	Plasmid *ompT*	Plasmid *arlC*	Plasmid *ompP*
Commensals				
BL21 Laboratory strain	-	-	-	-
K-12 Commensal	+	-	-	+
InPEC				
EPEC Intestinal disease	+	-	-	-
EHEC Intestinal disease	+	-	-	-
NRG857c Colitis	+	P	+	-
ExPEC				
CFT073 Pyelonephritis	+	-	-	-
IHE3034 Meningitis	++	-	-	-
ST131 Sepsis	+	P	+	-
1303 Bovine Mastitis	+	-	-	+
APEC O1 Avian colibacillosis	+	P	+	-

Table legend:

+ : Presence of the gene

++: Presence of the gene twice

- : Absence of the gene

P : Pseudogene

5.1.1 Omptins in *E. coli*

E. coli may encode up to three omptins: *ompT*, *ompP,* and *arlC* [140, 201, 202]. These three omptins are approximately 74% identical at the AA level [201]. Several combinations of omptins can be found in different *E. coli* strains (Table 2). Some strains such as NRG857c encode both *ompT* and *arlC* while other strains such as IHE3034 can encode the *ompT* gene twice [201, 216, 217]. However, no *E. coli* strain has been reported to encode all three omptins.

5.1.1.1 OmpT

OmpT is the chromosomally-encoded omptin present in a large proportion of *E. coli* strains [140]. The *ompT* gene is part of a prophage [140, 209]. OmpT has been reported to cleave an array of proteins such as protamine, T7 RNA polymerase, ferric enterobactin receptor, fusion proteins, colicins and the human antimicrobial peptide LL-37 [190, 191, 218-221]. OmpT has also been shown to activate plasminogen into plasmin *in vitro*, although to a much smaller extent than Pla [205, 222]. In addition, OmpT can undergo auto-proteolysis between Lys^{217}-Arg^{218} [223]. OmpT has a strong preference for arginine in the first position of its active site and lysine, glycine or valine in the second one [224]. Most often, alanine and valine flank those residues [224].

OmpT has been identified as an important virulence factor in UPEC with an increased incidence in strain causing cystitis, pyelonephritis and urosepsis compared to asymptomatic strains [140]. OmpT activity of various UPEC prototypical strain have been tested and displayed low activity levels [225]. However, its activity in UPEC clinical isolated has not yet been tested. OmpT is mostly associated with the phylogenetic group B2 [226]. It is also found at a higher proportion in disease causing UPEC isolates (more than 85% of isolates) compared to fecal isolates (60% of

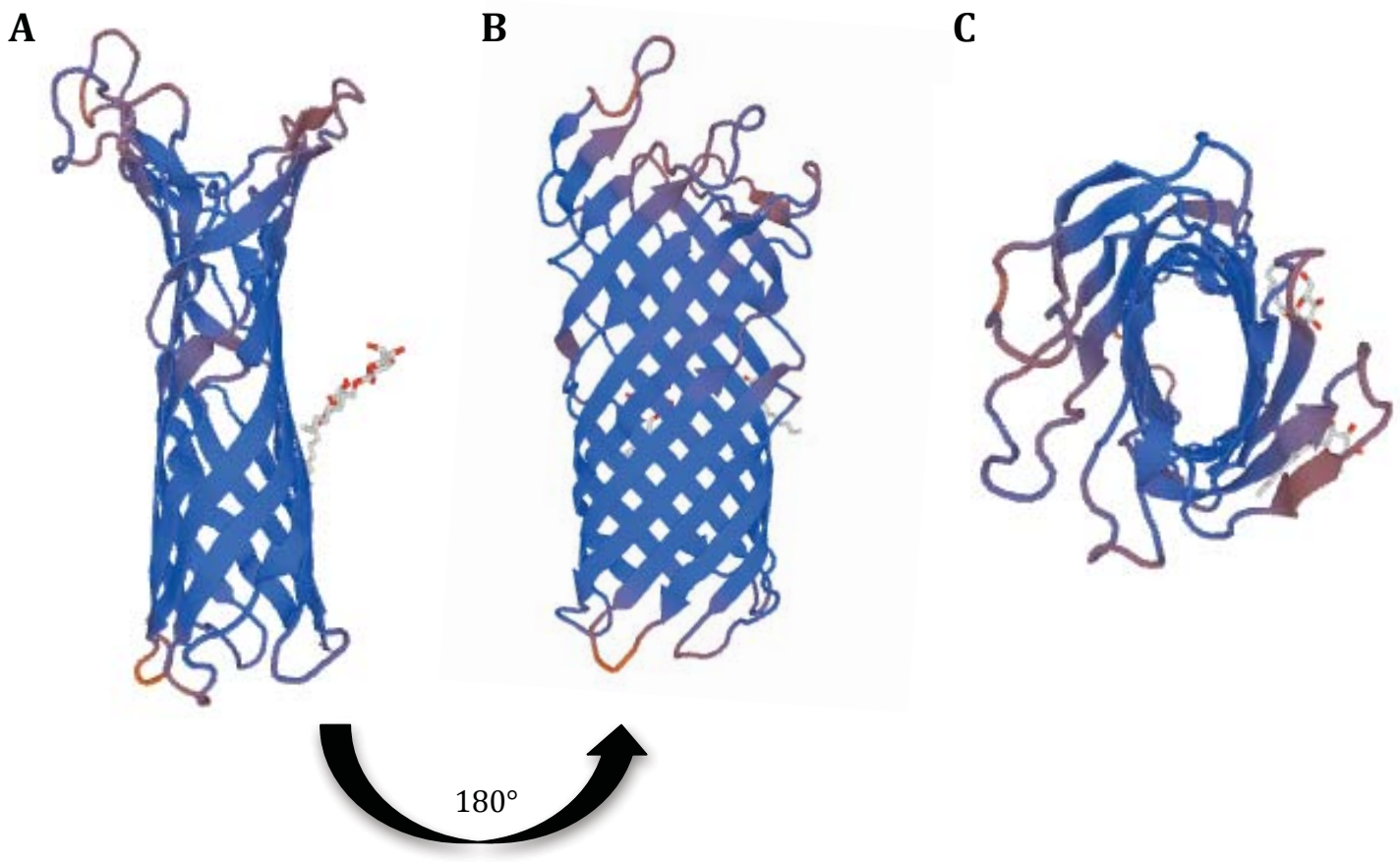

Figure 5. Model structure of OmpT. Representation of OmpT (blue) and its loops (orange). (A) Side view with LPS binding in grey. (B) View from the other side (180°) of OmpT, hiding the LPS binding site. (C) Top view of the OmpT active site [205].

isolates) [226]. A previous study has shown that UPEC OmpT from prototypical strain CFT073 is able to cleave LL-37, although to a limited extent [225]. Furthermore, OmpT seems to play an important role in resistance to cationic peptides isolated from urine [190].

5.1.1.2 OmpP

OmpP was initially identified on the F plasmid of commensal *E. coli* K-12 [202]. It has also recently been sequenced in two *E. coli* strains that cause mastitis in cattle [227]. It is also encoded on a Vir plasmid, which is associated with septicemic *E. coli* strains in both cattle and humans, but an early stop codon makes the protein non-functional [228, 229]. The physiological substrate of OmpP has not yet been discovered. However, OmpP substrate specificity has been elucidated [212]. OmpP, aside from cleaving between basic residues (lysine and arginine), prefers threonine, arginine or alanine as the amino acids flanking these basic residues [212]. OmpP can also accommodate the amino acid serine in its active site and cleave between serine and arginine about 120 fold better than OmpT [212].

5.1.1.3 ArlC

ArlC has been described in an adherent-invasive *E. coli* (AIEC) strain (NRG 857c), isolated from an individual suffering of colitis, on a large virulence plasmid as part of a PAI called PI-6 that is proposed to play a role in resistance against AMPs [230]. This PAI comprises a *mig-14* ortholog, a sugar epimerase and an omptin (*arlC*) [230]. *In vitro*, ArlC provides protection against AMPs such as CP10A, CP28 and LL-37 but not to ethylenediaminetetraacetic acid (EDTA) and sodium dodecyl sulfate (SDS) in a minimum inhibitory concentration assay [201]. The *arlC* deletion strain did not show any fitness defect *in vivo* in a chronic intestinal colonization model [201].

ArlC can also be found in a number of sequenced strains on databases [230-233]. Most of these are ExPEC strains found in poultry (APEC) [232, 233]. Interestingly, *arlC* is always found on plasmids as part of the same AMP resistance PAI as initially described. ArlC's physiological role has yet to be elucidated.

Rationale and Objectives

UPEC is associated with 85% of community acquired UTIs, which cost billions of dollars in health care services worldwide [23, 29]. Since AMPs act as natural antibiotic of our body they may be used as an alternative therapeutic approach against antibiotic resistant UPEC strains [129, 130, 135, 136].

However, some bacterial pathogens have evolved mechanisms to resist specific AMPs [183]. We have shown that OmpT from the EPEC and EHEC pathotypes cleave and inactivate LL-37 [191]. The levels of OmpT production in EPEC and EHEC are reflective of the levels of LL-37 in their respective infectious niches [183]. The urinary tract encompasses a large variety of AMPs including LL-37, which is secreted upon UPEC infection [47, 101]. OmpT activity of UPEC prototypical strain CFT073 revealed that poor *ompT* expression probably limits the role of OmpT in LL-37 resistance of CFT073 [225].

OmpT is an important virulence factor in UPEC and is present in a large proportion of disease causing UPEC clinical isolates [33, 140]. However, activity of OmpT in UPEC clinical isolates has not yet been studied. The objective of this work is to assess OmpT activity in a collection of UPEC clinical isolates from different disease severities (from fecal to urosepsis) and screen for the presence of other omptins in UPEC. We hypothesize that OmpT activity in UPEC clinical isolates will vary according to the disease severity they cause.

Chapter 2- Identification of an OmpT-like Protease with Different Substrate Specificity from OmpT in Uropathogenic *Escherichia coli* Clinical Isolates

Isabelle Desloges[1], James A. Taylor[1], Jean-Mathieu Leclerc[1], Jenny-Lee Thomassin[1], Andrea Portt[2], Ken Dewar[3, 4], Gregory T Marczynski[1], John D Spencer[5], Amee Manges[2], Samantha Gruenheid[1,4] and Hervé Le Moual[1,4,6§]

[1] Microbiology and Immunology Department, McGill University, Montreal, QC, H3A 2B4, Canada

[2] School of Population and Public Health, University of British Columbia, Vancouver, BC, V6T 1Z9, Canada

[3] McGill University and Genome Quebec Innovation Center, McGill University, Montreal, QC, H3A 0G1, Canada

[4] Microbiome and Disease Tolerance Centre, McGill University, Montreal, QC, H3A 2B4, Canada

[5] Division of Nephrology, Nationwide Children's Hospital, Columbus, OH, 43205, USA

[6] Faculty of Dentistry, McGill University, Montreal, QC, H3A 1G1, Canada

[§]Corresponding author

Corresponding author information: herve.le-moual@mcgill.ca (514-398-6235)

Running Head: Heterogeneity of OmpT activity in UPEC

Abstract

Background. Antimicrobial peptides (AMPs), including LL-37 and RNase7, are components of the host innate immune response of the urinary tract. Bacterial pathogens, including uropathogenic *Escherichia coli* (UPEC), must resist AMP-activity to cause urinary tract infections. Omptin proteases, such as *E. coli* OmpT, can cleave and inactivate LL-37. The presence of the *ompT* gene in UPEC strains is recognized as an important virulence determinant, although its role in pathogenesis remains unclear.

Methods. Multiplex PCR was used to detect known UPEC virulence factors including *ompT* and *ompT*-like proteases, *arlC* and *ompP*, in 58 clinical isolates. Protease activity was monitored by FRET assay. In addition, *ompT* and *arlC* expression levels were measured by qPCR. The contribution of OmpT and ArlC to AMP-resistance was monitored by AMP-cleavage assay.

Results. Our data show that *ompT* and *arlC* are more prevalent in UPEC clinical isolates causing symptomatic infection. Omptin activity was heterogenous among the different isolates, due to the presence of *arlC*. In addition, AMP-cleavage assays showed that OmpT preferentially cleaves LL-37, whereas ArlC cleaves RNase7.

Conclusions. The prevalence of OmpT-like proteases in UPEC clinical isolates causing symptomatic infection and ability to cleave AMPs suggest that both OmpT and ArlC should be considered UPEC virulence factors.

Key words. UPEC, outer-membrane proteases, omptins, urinary-tract infections, cystitis, antimicrobial peptides, cathelicidin, OmpT

Background

Urinary tract infections (UTIs) are among the most common bacterial infections and are associated with huge health care costs. Uropathogenic *Escherichia coli* (UPEC) is the causative agent of nearly 85% of community-acquired UTIs [234, 235]. UPEC strains belong to a subset of extraintestinal pathogenic *E. coli* (ExPEC) that cause infections outside of the gastrointestinal tract [7]. UPEC, which colonizes the human gut without consequence, can colonize the periurethral area and infect the urinary tract in an ascending manner, resulting in diseases ranging from asymptomatic bacteriuria (ABU) to cystitis, pyelonephritis and eventually urosepsis [236]. UPEC isolates commonly belong to the *E. coli* phylogenetic groups B2 and D [34]. UPEC strains are characterized by high genomic diversity and have acquired a vast array of genes encoding virulence factors, which are part of either genomic pathogenicity islands or mobile elements such as plasmids and prophages. A number of prototypical UPEC strains, including the urosepsis strain CFT073, have been sequenced and extensively studied [237].

To colonize the uroepithelium, UPEC has to overcome the innate defenses of the urinary tract, including antimicrobial peptides (AMPs) [47, 238]. Both uroepithelial and immune cells produce various AMPs including cathelicidin LL-37, a-defensin 5, b-defensin 1 and ribonuclease 7 (RNase 7) [102, 104, 239]. Previous studies have shown that human LL-37 production is increased upon UPEC infection and can be measured in the urine of patients suffering from UTIs [101, 240]. However, the murine cathelicidin CRAMP did not appear to protect mice against UPEC in a cystitis infection model [118]. More recently, it was shown that large amounts of human RNase 7 (14.5 kDa) are secreted upon UPEC infection and that this AMP contributes to the defense of the urinary tract against bacteria [102, 125].

The *ompT* gene has been identified as an important virulence factor and is present in 85-97% of UPEC isolates [241]. In *E. coli*, OmpT is a chromosomally-encoded outer-membrane protease belonging to the omptin family. Omptins are found in a number of Gram-negative pathogens and contribute to virulence by cleaving host or bacterial proteins [242]. We previously showed that the OmpT proteases of enterohemorrhagic *Escherichia coli* (EHEC), enteropathogenic *Escherichia coli* (EPEC) and the UPEC strain CFT073 cleave and inactivate the human cathelicidin LL-37 [191, 225]. However, the low levels of OmpT produced by CFT073 probably limits its contribution to LL-37 resistance [225]. In addition to the chromosomally-encoded *ompT* gene, two plasmid-encoded *ompT*-like genes were previously identified in *E. coli* [201, 202]. These genes, called *ompP* and *arlC*, encode omptins that have approximately 74% amino acid identity to OmpT. The *ompP* gene was initially found on the F plasmid of *E. coli* K12 [243]. More recently, it has been identified within plasmids found in two pathogenic ExPEC strains causing bovine mastitis [227]. The *arlC* gene has only been characterized in the adherent-invasive *E. coli* (AIEC) strain NRG 857c [201]. The *arlC* gene is part of a pathogenicity island (PAI) that was reported to play a role in resistance against AMPs, including the human cathelicidin LL-37 [201].

In this study, we analyzed a collection of 12 fecal ExPEC clinical isolates and 46 UPEC from five groups of patients with infections of increasing clinical severity (ABU, cystitis, pyelonephritis and urosepsis). All isolates were tested for the presence of the *E. coli* omptin genes and for omptin protease activity. In all groups, heterogenous OmpT activity was observed. In some isolates, high OmpT activity could be correlated with the presence of the plasmid-

encoded *ompT*-like gene, *arlC*. In addition, we showed that OmpT and ArlC have different substrate specificities and preferentially cleave LL-37 and RNase 7, respectively.

Material and Methods

Bacterial Strains and Growth Conditions

58 ExPEC isolates originating from patients suffering of UTIs were randomly selected from the Manges collection [244]. Isolates were divided into 5 groups based on disease severity. The fecal isolates were recovered from feces (n=12), ABU isolates were from patients with asymptomatic bacteriuria (n=10), cystitis isolates were from patients with cystitis (n=12), pyelonephritis isolates were from patients with pyelonephritis (n=12) and sepsis isolates from patients with urosepsis (n=12). Bacteria were grown in Luria-Bertani broth (LB) or in N-minimal medium (50mM Bis-Tris, 5mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 0.5 mM KH_2PO_4, 0.1% casamino acids) adjusted to pH 7.5 and supplemented with 1.4% glucose and 1mM $MgCl_2$.

Multiplex PCR of UPEC Virulence Genes

Genomic DNA was isolated from all 58 *E. coli* isolates using the Puregene Yeast/Bact. kit (Qiagen). Phylogenetic groups were determined as described in [245], using the primer pairs listed in Table S1. To characterize the virulence genes of the isolates, primer sequences were obtained from previous studies [22] or designed *de novo* for this study (Table S1). Three multiplex PCR experiments were performed as follows: pool 1: *hylA* (1177 bp), *papAH* (720 bp), *fimH* (508 bp), *kspMTIII* (392 bp), and *papEF* (336 bp); pool 2; *papC* (200 bp), *sfaS* (240 bp), *cnf1* (498 bp), *fyuA* (880 bp), *iutA* (300 bp), *kpsMTII* (272 bp); pool 3: *arlC* (852 bp), *ompT* (670 bp) and *fimH* (508 bp) and *ompP* (648 bp). PCR reaction conditions were as follow: 95°C for 3 minutes, (94°C for 30 seconds, 63°C for 3 seconds, 68°C for 3 minutes) repeated 25 times and a final extension of 10 minutes at 72°C [22].

Construction of Plasmids Expressing *ompT* and *arlC*

The *ompT* and *arlC* genes were amplified from genomic DNA isolated from the cystitis-6 isolate using the primer pairs ompT_3F/ompT_3R and arlC_1F/arlC_1R, respectively (Table S1). PCR fragments were cleaved with XbaI and SacI restriction enzymes (New England Biolabs) and cloned into plasmid pWSK129 cleaved with the same enzymes, generating plasmids pC6*ompT* and pC6*arlC* (Table S2).

Fluorescence Resonance Energy Transfer (FRET) Activity Assay

The FRET substrate containing the dibasic motif (RK) in its center (2Abz-SLGRKIQI-K(Dnp)-NH2) was purchased from Anachem. Bacteria were grown in N-minimal medium and normalized to an OD_{595nm} of 0.5. Bacterial cells were pelleted and resuspended in phosphate-buffered saline (PBS). Bacteria (75 µL) were mixed in a 96-well plate with 75 µL of the FRET substrate (final concentration 3 µM). Fluorescence was monitored for 1 h at 25°C using a Biotek FLx 800 plate reader. The excitation and emission wavelengths were 325 and 430 nm, respectively. Data were normalized by subtracting the background fluorescence of the FRET substrate in PBS.

Quantitative RT-PCR

Quantitative RT-PCR (qPCR) was performed as previously described [191]. Briefly, bacterial strains were grown to an OD_{595nm} of 0.5 in N-minimal medium. Total RNA was isolated using TRIzol reagents (Invitrogen) and treated with TURBO DNase I (Ambion) to remove any residual DNA. The absence of DNA was confirmed by qPCR using the primer pair rpoD_F/rpoD_R (Table S1). RNA (100 ng) was reverse transcribed using Superscript II (Invitrogen) with 0.5 µg

of random hexamer primers (Sigma Aldrich). A reaction mixture without Superscript II was also included and was used as negative control. qPCR reactions were performed in a Rotor-Gene 3000 thermal cycler (Corbett Research) using the Maxima SYBR Green qPCR kit (Thermo Scientific), according to the manufacturer's instructions. Primers used are listed in Table 1. The relative expression levels were calculated by normalizing the threshold cycle (C_T) of *ompT* and *arlC* transcripts to the C_T of *rpoD* using the $2^{-\Delta CT}$ method [246].

Western Blotting

Bacteria were grown in N-minimal medium and normalized to an OD_{595nm} of 0.5. Bacterial cells grown in N-minimal medium were pelleted and resuspended in 2 × electrophoresis sample buffer. Samples were resolved on a 10% SDS-PAGE gel and transferred to a PVDF membrane. Membranes were blocked for 1 hour in Tris-buffered saline (TBS) supplemented with 5% skim milk and incubated overnight with the polyclonal anti-CroP antibody in TBS supplemented with 5% BSA. A goat anti-rabbit secondary antibody conjugated with HRP was applied to the membrane for 1hour and chemiluminescent HRP substrate was added for visualization.

Southern Blotting

Genomic DNA from the cystitis isolates and from strains CFT073 and CFT073 Δ*ompT*, was isolated as described above and cleaved with EcoRV. Southern blotting and hybridization were performed as previously described [247] using Hybond-XL membranes (GE Healthcare). The probes for *ompT* and *arlC* were PCR-generated using Bestaq DNA Polymerase (Diamed) and the primer pairs ompT_5F/ompT_5R and arlC_F/arlC_2R, respectively (Table S1). The probe was radiolabelled with dATP[a-32P] (Perkin Elmer) using the RadPrime kit (Invitrogen).

AMP Cleavage Assays

Bacteria were grown in N-minimal medium and normalized to an OD_{595nm} of 0.5. Bacterial cells were resuspended in PBS and aliquots corresponding to 10^7 × CFU were incubated for 1h at a 1:4 (v/v) ratio with 2 μg/mL LL-37 (BioChemia) or 1 μg/mL RNase 7 purified as described previously [125]. LL-37 samples were resolved on 10-20% Tris-Tricine gels (BioRad) and RNase 7 samples were resolved on 20% SDS-PAGE gels. Gels were fixed using 20% (v/v) glutaraldehyde for 30 min, rinsed with water and stained for 1h in G-250 Coomassie blue stain. Gels were destained in 20% (v/v) acetic acid.

Statistical Analysis

Statistical analysis was done using GraphPad Prism version 5.0. Fisher's Exact test was performed to compare incidence of virulence genes within severity groups of UPEC clinical isolates with a p value <0.05 being significantly different. FRET activity was assessed using a TWO Way ANOVA with Newman-keuls Multiple comparison test with a p value <0.05 representing * and a p value <0.01 representing **, both were regarded as being statistically significant.

Results

Phylogenetic and virulence profile of UPEC isolates

UPEC isolates are predominantly from phylogenetic groups B2 and D, while phylogenetic groups A and B1 are mainly associated with Intestinal Pathogenic *E. coli* (InPEC) strains [34]. Therefore, we determined the phylogenetic grouping of our 58 clinical isolates categorized into the fecal (n=12), ABU (n=10), cystitis (n=12), pyelonephritis (n=12) and sepsis (n=12) groups. Most isolates from the ABU, cystitis and pyelonephritis groups associated with UTIs belong to the phylogenetic group B2 and, to a lesser extent, D (Table 1). In contrast, isolates from the sepsis group were predominantly from group D (Table 1). Finally, isolates from the fecal group had the most variable phylogenetic grouping (Table 1). Overall, this distribution reflects the general trend that UPEC strains mainly belong to phylogenetic groups B2 and D.

The 58 isolates were further characterized by testing for the presence of 12 recognized UPEC virulence genes using multiplex PCR (Figure S1). Our data showed variations consistent with previous studies reporting that UPEC is a heterogenous species [248-251]. The *iutA* gene, involved in iron uptake, showed significantly lower and higher incidences in the fecal and sepsis groups, respectively (Table 2). All adhesion and fimbrial genes (*papAH*, *papC*, *papEF*, and *fimH*) were increasingly present with the higher disease severities. The distribution of virulence genes between asymptomatic (i.e. fecal and ABU) and symptomatic (i.e cystitis, pyelonephritis and sepsis) groups was similar, with the exception of the *fuyA* and *ompT* genes for which the incidence was higher in the symptomatic groups (Table 2). In agreement with previous studies, we found that *ompT* is present in 89% of the UPEC isolates causing symptomatic infection (Table 2) [252].

Table 1. Phylogenetic distribution of the UPEC clinical isolates.

	Phylogenetic groups				
	A	B1	B2	D	Total (n=58)
Fecal (n=12)	4	1	3	4	12
ABU (n=10)	2	1	5	2	10
Cystitis (n=12)	2	1	6	3	12
Pyelonephritis (n=12)	1	2	5	4	12
Urosepsis (n=12)	0	2	0	10	12
Total (n=58)	9	7	19	23	58

Table 2. Prevalence of major virulence factors in the UPEC clinical isolates.

Gene	Fecal (n=12)	ABU (n=10)	Cystitis (n=12)	Pyelonephritis (n=12)	Sepsis (n=12)	*p**
iutA	1	8	8	6	12	***0.0270***
fimH	12	8	12	11	12	*0.1397*
papAH	3	6	6	4	10	*0.4173*
papC	3	6	6	6	10	*0.1780*
papEF	4	7	6	6	10	*0.4279*
sfaS	1	1	2	2	0	*1.0000*
fyuA	9	7	12	11	11	***0.0437***
kspMTII	7	7	7	7	8	*1.0000*
kspMTIII	0	0	1	1	0	*0.5209*
cnf1	4	4	4	4	0	*0.3641*
hylA	4	3	4	1	1	*0.2078*
ompT	7	7	12	10	10	***0.0418***

* *p* value calculated using Fisher's exact test. Significance shown in bold when $p < 0.05$.

Variability of omptin proteolytic activity among UPEC isolates

We previously showed that omptin proteases cleave the FRET substrate (2Abz-SLGRKIQI-K(Dnp)-NH2) [183, 201, 225, 253]. This assay was used to measure the omptin-mediated proteolytic activity of the 58 UPEC isolates and compare these activities to that of the reference UPEC strain CFT073. As shown in Fig. 1, the omptin activity of the isolates was highly heterogenous, both within and between groups. The omptin activity of the isolates of the fecal group was significantly lower than that of the 3 symptomatic groups (cystitis, pyelonephritis and sepsis) (Fig.1). The mean activity of the isolates from the fecal group (0.75 +/- 0.14) was lower than that of strain CFT073. In contrast, the mean activity of the isolates from the symptomatic groups was higher than that of CFT073. Importantly, extensive variability in omptin activity was observed within groups (Fig. 1). Isolates for which the *ompT* gene was not detected by PCR showed basal activity levels (triangles in Fig. 1). Isolates harboring the *ompT* gene showed a wide range of omptin activity. The cystitis group exhibited the most heterogenous OmpT activity. Some cystitis isolates had 3-fold higher omptin activity than CFT073. These results reveal high variability of omptin activity among UPEC isolates.

OmpT-like proteases in UPEC

In addition to the chromosomally-encoded *ompT* gene, plasmid-borne *ompT*-like genes *ompP* and *arlC* are present in several *E. coli* strains [201, 202]. These OmpT-like proteins are approximately 74% identical to OmpT in amino acid sequence and ArlC is known to cleave the FRET substrate [201]. To determine whether the presence of *ompT*-like genes in some isolates may account for the heterogeneity of OmpT activity observed in Fig. 1, a multiplex-PCR screen was performed for *ompT, ompP* and *arlC*. This PCR screen showed that the *ompP* gene was

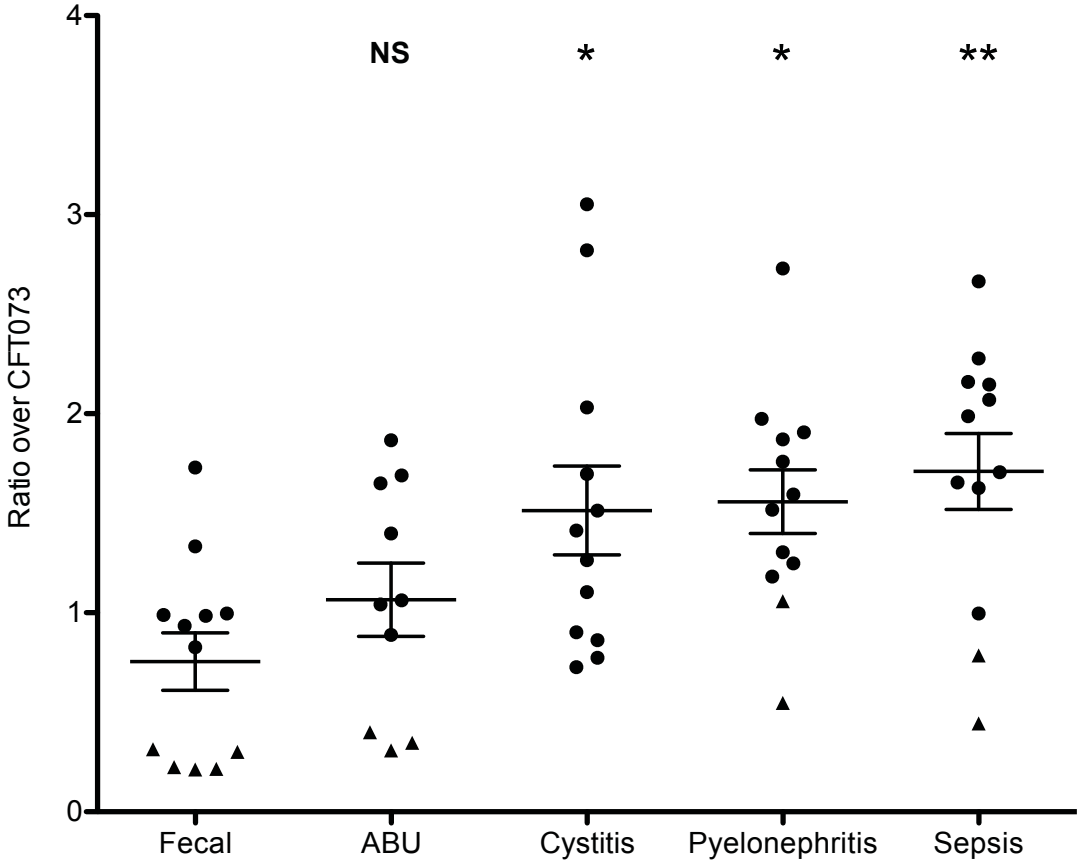

Figure 1. OmpT activity in the UPEC clinical isolates. FRET assay performed on 58 clinical isolates of different disease severity compared as a fold change of the area under the curve to the reference strain CFT073 after 60 minutes of incubation with synthetic substrate. Black circles indicate isolates with *ompT* and black triangles indicates absence of *ompT*. Statistical analysis was performed by One-way ANOVA test followed by Newman-keuls Multiple comparison test for all groups against the activity of the fecal group.

absent from all the isolates (data not shown). In contrast, the *arlC* gene was present in 8 of the 58 isolates (Fig. 2). Strikingly, the *arlC* gene was absent from asymptomatic isolates and was only present in isolates from the symptomatic groups ($P = 0.0192$). Most isolates harboring the *arlC* gene also contained *ompT* and generally had high proteolytic activity. The only exception was isolate 6 of the pyelonephritis group that lacked *ompT* but encoded *arlC* (Fig. 2); this isolate exhibited moderate proteolytic activity (Fig. 1). Southern blot analysis confirmed the presence of *arlC* in isolates 1, 6 and 11 of the cystitis group (Fig. S3). In addition, it showed that two *ompT* are encoded in isolates 7 and 8 of the cystitis group (Fig. S3). Together, these data show that isolates with the highest omptin activity harbor both the *ompT* and *arlC* genes.

Variability of *ompT* and *arlC* expression among UPEC cystitis isolates

To further understand the heterogenous proteolytic activity of UPEC isolates, we focused on the cystitis group in which all the isolates harbor the *ompT* gene and have the widest range of omptin activity. We measured the expression levels of *ompT* and *arlC* by qPCR using primer pairs specific for each gene. Expression of the *ompT* gene was heterogenous in the cystitis isolates (Fig. 3A). With the exception of cystitis 11, all the isolates had higher *ompT* expression levels than CFT073. As expected from the multiplex-PCR screen and southern blot, only the isolates cystitis 1, 6 and 11 isolates express *arlC*. Cystitis isolates 1 and 6, which showed the highest *ompT* and *arlC* expression levels (Fig. 3A), also had the highest omptin activity (Fig. 3B). Although both *ompT* and *arlC* are present in cystitis 11, the low expression levels of these genes are consistent with the low proteolytic activity obtained for this isolate (Fig. 3). These data indicate that variability of the omptin activity of the isolates is caused by both the presence of the *ompT* and *arlC* genes, in addition to variable expression levels of these genes.

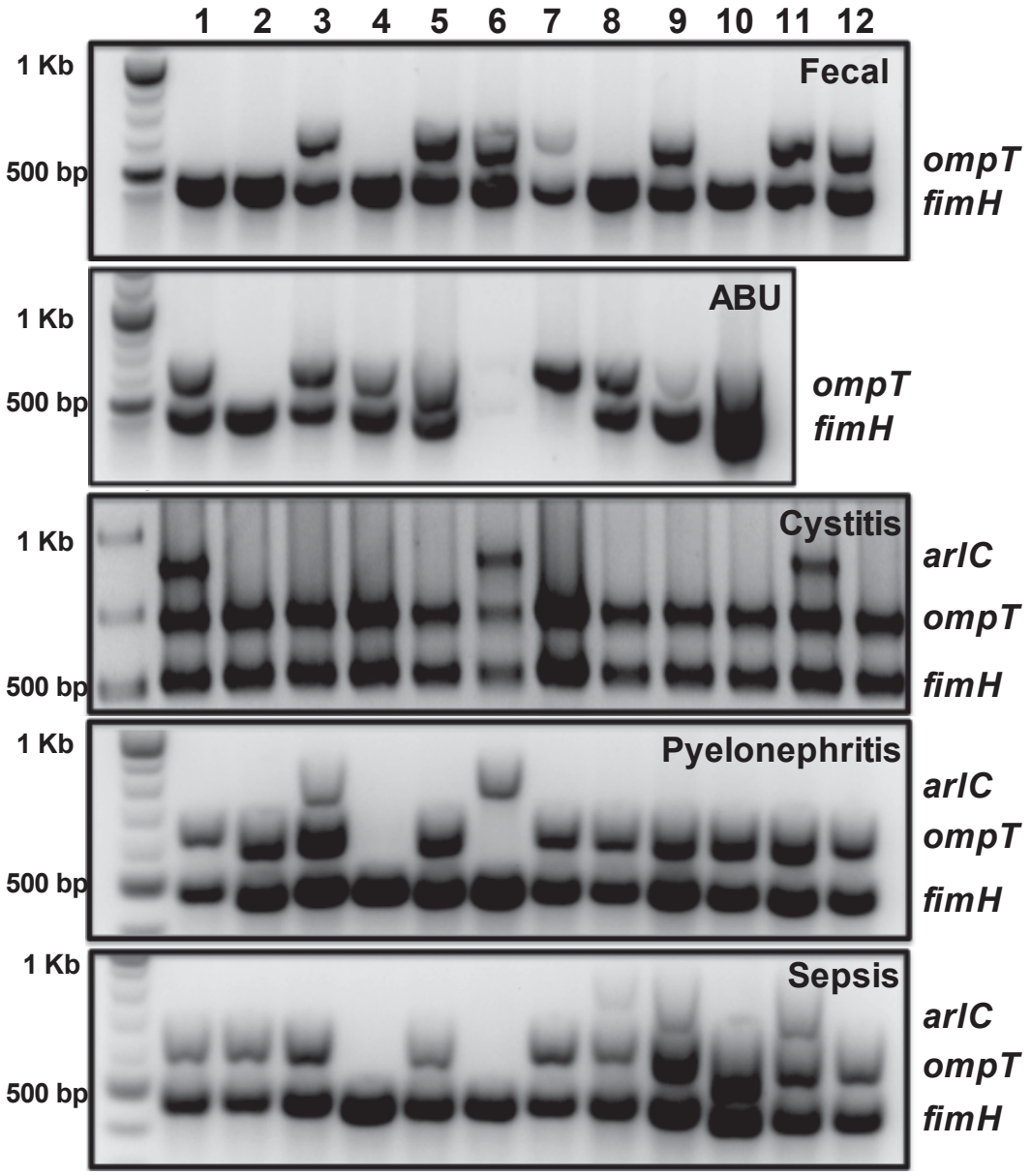

Figure 2. Multiplex PCR of the *ompT* and *ompT*-like genes in the UPEC clinical isolates. Amplification by multiplex PCR of *ompT* (670 bp), *arlC* (852 bp) and *fimH* (508 bp) in 58 UPEC clinical isolates of different severities. Amplification of *fimH* was used as a positive control.

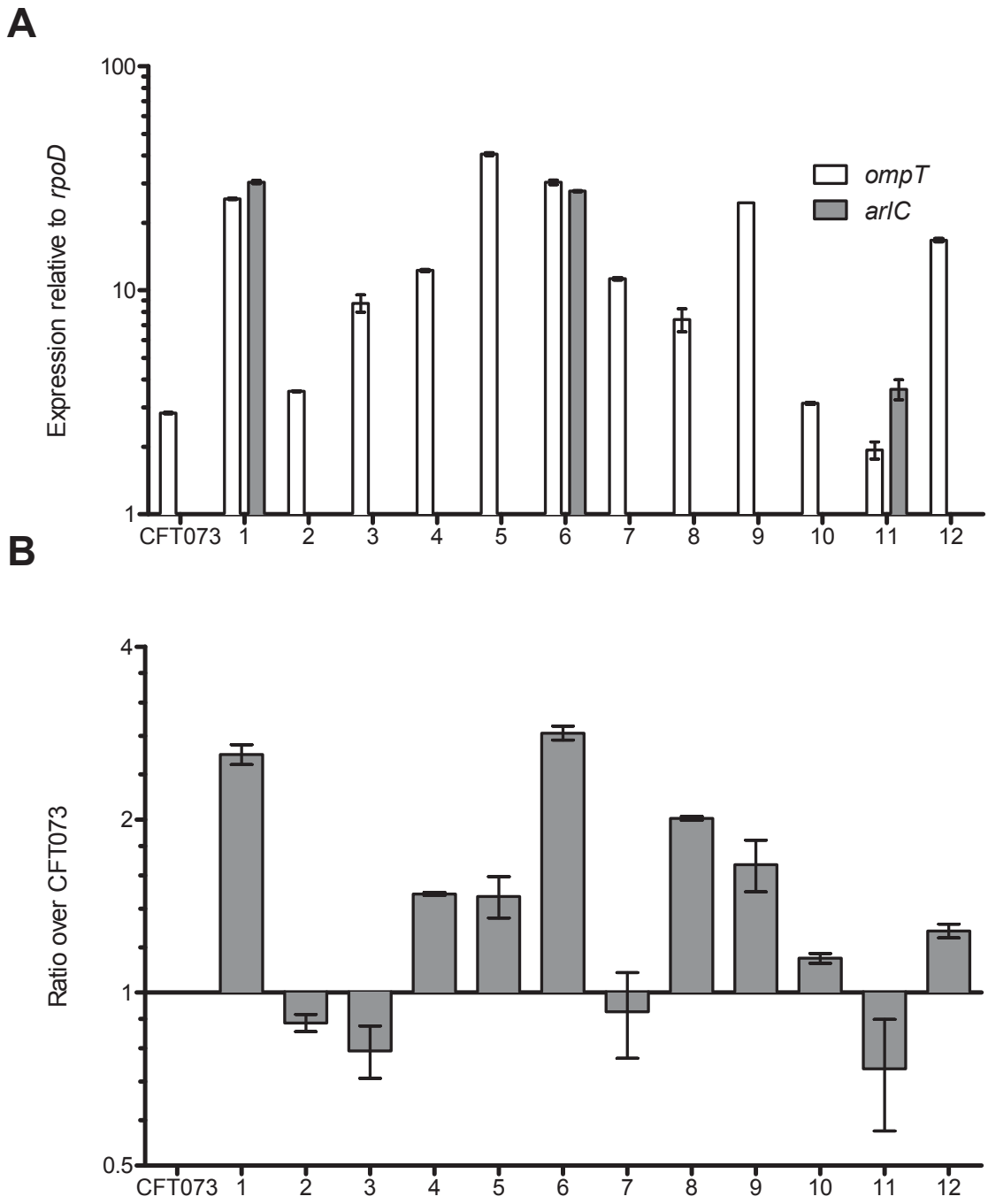

Figure 3. Expression of omptin genes and proteolytic activity of omptins in cystitis-causing isolates. (A) Quantitative RT-PCR of *ompT* and *arlC* in 12 cystitis clinical isolates as a ratio over the expression of *rpoD*. (B) FRET activity of 12 cystitis clinical isolates expressed as a fold change of the area under the curve to the reference strain CFT073 after 60 minutes of incubation with synthetic FRET substrate. Statistical analysis made by One way ANOVA test followed by Newman-keuls Multiple comparison test.

arlC is present on plasmids

To determine the genomic context of the *ompT* and *arlC* genes, isolates 1, 6 and 11 of the cystitis group were sequenced on a PacBio platform. For these three isolates, *ompT* was present on the bacterial chromosome and *arlC* was part of large plasmids (79-200 kbp; data not shown). The amino acid sequences of ArlC were 100% identical to ArlC in NRG857c [201]. Although the three plasmids were different, *arlC* is part of the same pathogenicity island previously reported to play a role in AMP resistance (data not shown).

OmpT and ArlC have different substrate specificities

OmpT was shown to cleave and inactivate the human AMP LL-37 *in vitro* [191]. In contrast, the ArlC physiological substrates remain unclear, although ArlC has been proposed to play a role in AMP resitance [201]. To assess whether OmpT and ArlC have similar substrate specificities, both genes were cloned into plasmid pWSK129 and expressed in *E. coli* BL21, which lacks all *E. coli* omptin genes (Table S2). Both *ompT* and *arlC* were expressed at similar levels in BL21, as determined by qPCR (Fig. 4A). Western blot analysis showed similar protein levels of both OmpT and ArlC in BL21 (Fig 4B). In addition, both OmpT and ArlC showed high proteolytic activity against the FRET substrate (Fig. 4C). Next, the ability of both proteases to cleave the AMPs LL-37 and RNase 7 was assessed. Both OmpT and ArlC cleaved LL-37, although ArlC cleaved LL-37 to a lesser extent than OmpT (Fig. 4D). In contrast, OmpT didn't cleave RNase 7, whereas ArlC partially cleaved it (Fig. 4D). These results show that OmpT and ArlC are active against different substrates.

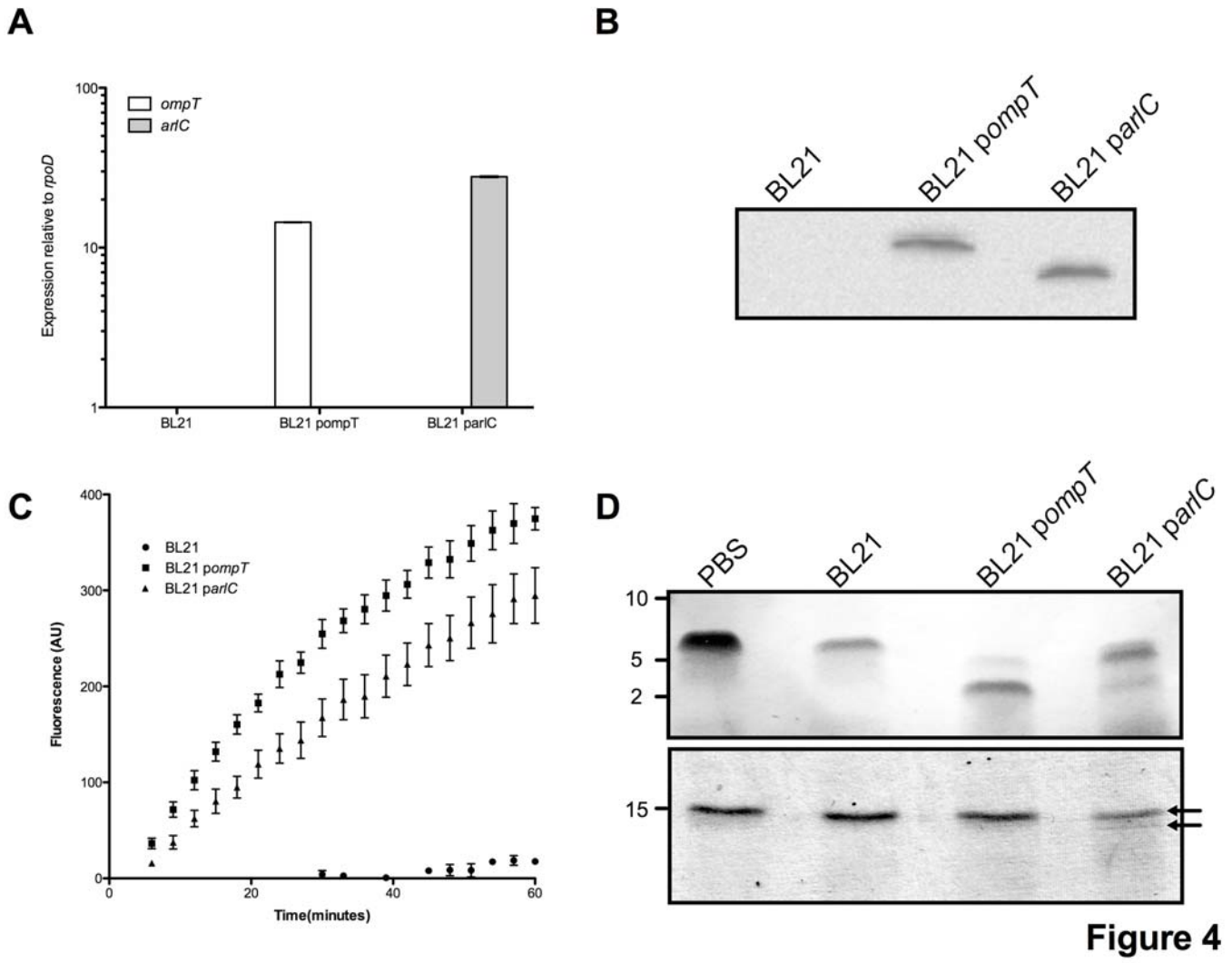

Figure 4. OmpT and ArlC substrate specificities. (A) Quantitative RT-PCR of *ompT* and *arlC* of BL21 pC6*ompT* and BL21 pC6*arlC* as a ratio over the expression of *rpoD*. (B) Western blot analysis of BL21 pC6*ompT* and BL21 pC6*arlC* using anti-CroP antibody [253]. (C) FRET activity of BL21 pC6*ompT* and BL21 pC6*arlC* for 60 minutes. (D) Cleavage of LL-37 and RNase 7 by BL21 pC6*ompT* and BL21 pC6*arlC* after 60 minutes.

Discussion

The chromosomally-encoded *ompT* gene is associated with UPEC virulence [241]. This study confirms that the *ompT* gene is more prevalent in symptomatic than in asymptomatic isolates (Table 2). Furthermore, the omptin activity is significantly higher in isolates causing symptomatic infection compared to isolates of the fecal group (Fig. 1). However, the exact role of OmpT during infection remains unclear. Previous studies have suggested that OmpT from the UPEC strain CFT073 is involved in adhesion, invasion and/or inactivation of AMPs [225, 254]. This study shows extensive heterogeneity of omptin activity among UPEC clinical isolates, which is due, in part, to variations in *ompT* expression and to the presence of the plasmid-encoded *ompT*-like gene *arlC*. In addition, we found that OmpT and ArlC, which are ~ 74% identical at the amino acid level, preferentially cleave LL-37 and RNase 7, respectively. Therefore, the presence of two different outer-membrane proteases with different substrate specificities may provide an additional fitness advantage to UPEC strains.

One of the main finding of this manuscript is the heterogeneity of omptin activity among isolates (Fig. 1). Our study provides two explanations that are not mutually exclusive. (1) Our qPCR analyses showed variable expression levels of the *ompT* and *arlC* genes among isolates (Fig. 3A). For example, a 20-fold difference in *ompT* expression was observed between isolates 5 and 11 of the cystitis group (Fig. 3A). This finding is not unprecedented, since it was previously shown that *ompT* expression was 32-fold higher in EHEC than in EPEC [191]. This differential expression level was attributed to differences in the promoter sequences of EHEC and EPEC [191]. The *ompT* promoter sequences of isolates 1, 6 and 11 were compared to the *ompT* promoter sequences of EHEC and EPEC. High sequence similarities were observed between

isolate 6 and EHEC and between isolate 11 and EPEC (data not shown), which further suggests that variations in the promoter sequence may be responsible for the differential *ompT* expression and, in turn, activity.

In addition, it is possible that some transcriptional factors regulating *ompT* expression are absent or differentially expressed in some isolates. This could explain the differences between activity and expression levels in isolates cystitis 2, 3 and 11 in which expression levels are higher than CFT073 but have lower FRET activity. (2) The variability of omptin activity is also due to the presence of the plasmid-encoded *ompT*-like *arlC* gene in some isolates. The plasmid-encoded *arlC* gene, which was first described in the AIEC strain NRG857c [201], can also be found on plasmids harbored by various human ExPEC strains causing meningitis and sepsis as well as avian *E. coli* strains (APEC). Strikingly, the other plasmid encoded *ompT*-like gene, *ompP*, was not found among the 58 isolates analyzed. The fact that *ompP* has been recently identified on plasmids harbored by the ExPEC strains 1303 and ECC-1470 causing bovine mastitis [227], may suggest that *ompP* could be found in UPEC. This possibility is supported by the fact that farm animals are food reservoirs for *E. coli* causing UTIs [255].

So far, it is unclear whether OmpT and OmpT-like proteases cleave the same substrates. This study shows that OmpT and ArlC have different specificities towards the AMPs LL-37 and RNase 7, although both OmpT and ArlC cleave the FRET substrate with similar efficiency (Fig. 4C). We showed that OmpT cleaves LL-37 extensively, but not RNase 7. In contrast, ArlC cleaves both LL-37 and RNase 7 to limited extents. This clearly indicates that OmpT and ArlC should have different substrates *in vivo*.

In conclusion, this study revealed the presence of the plasmid borne *ompT*-like *arlC* gene in some UPEC isolates. Virulence profiling of UPEC strains most often includes the *ompT* gene [22]; this study reinforces the importance to screen for multiple omptin genes. The systematic screening of both *ompT*, *arlC* and possibly *ompP* should be considered for future characterization of UPEC isolates.

Acknowledgements

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We thank Dr. S. Sagan for the gift of labeling reagents for Southern hybridization.

Supplementary Data

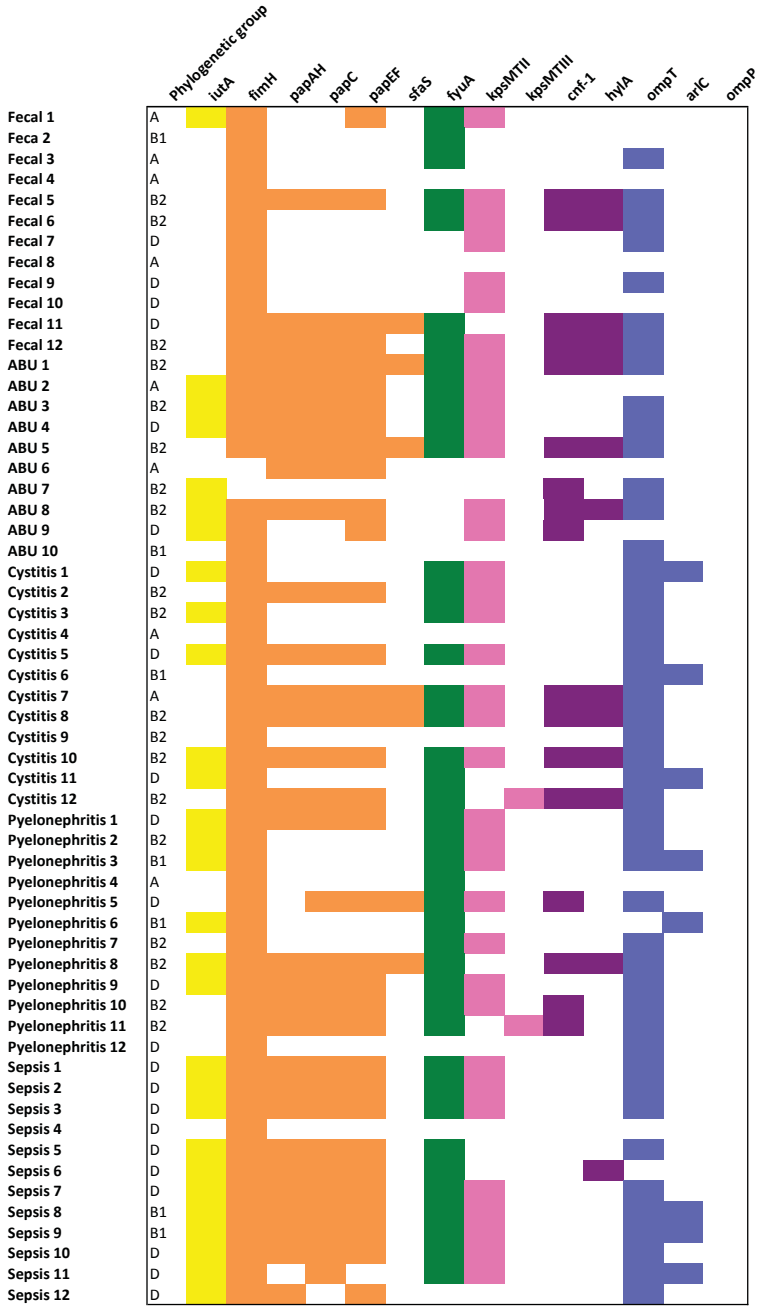

Figure S1. UPEC virulence genes and phylogenetic groups incidence among 58 clinical isolates causing different disease severity. Colors corresponding to; yellow (PTJ-100 related genes), orange (adhesins), green (iron-related), pink (protectins), purple (toxins) and blue (miscellaneous).

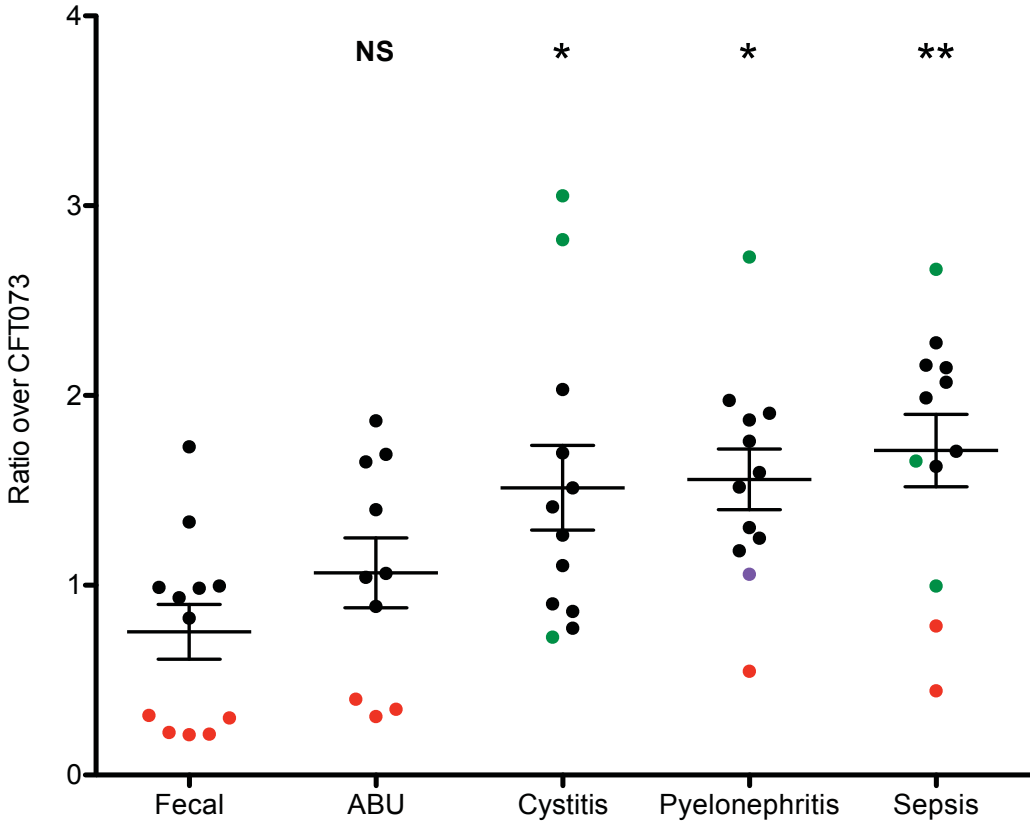

Figure S2 - FRET activity of UPEC clinical isolates. FRET assay performed on 58 clinical isolates of different disease severity compared as a fold change of the area under the curve to the reference strain CFT073 after 60 minutes of incubation with synthetic substrate. Red indicates isolates with no omptin, purple indicates isolates with ArlC, black indicates isolates with OmpT and green indicates isolates with both OmpT and ArlC Statistical analysis made by One way ANOVA test followed by Newman-keuls Multiple comparison test.

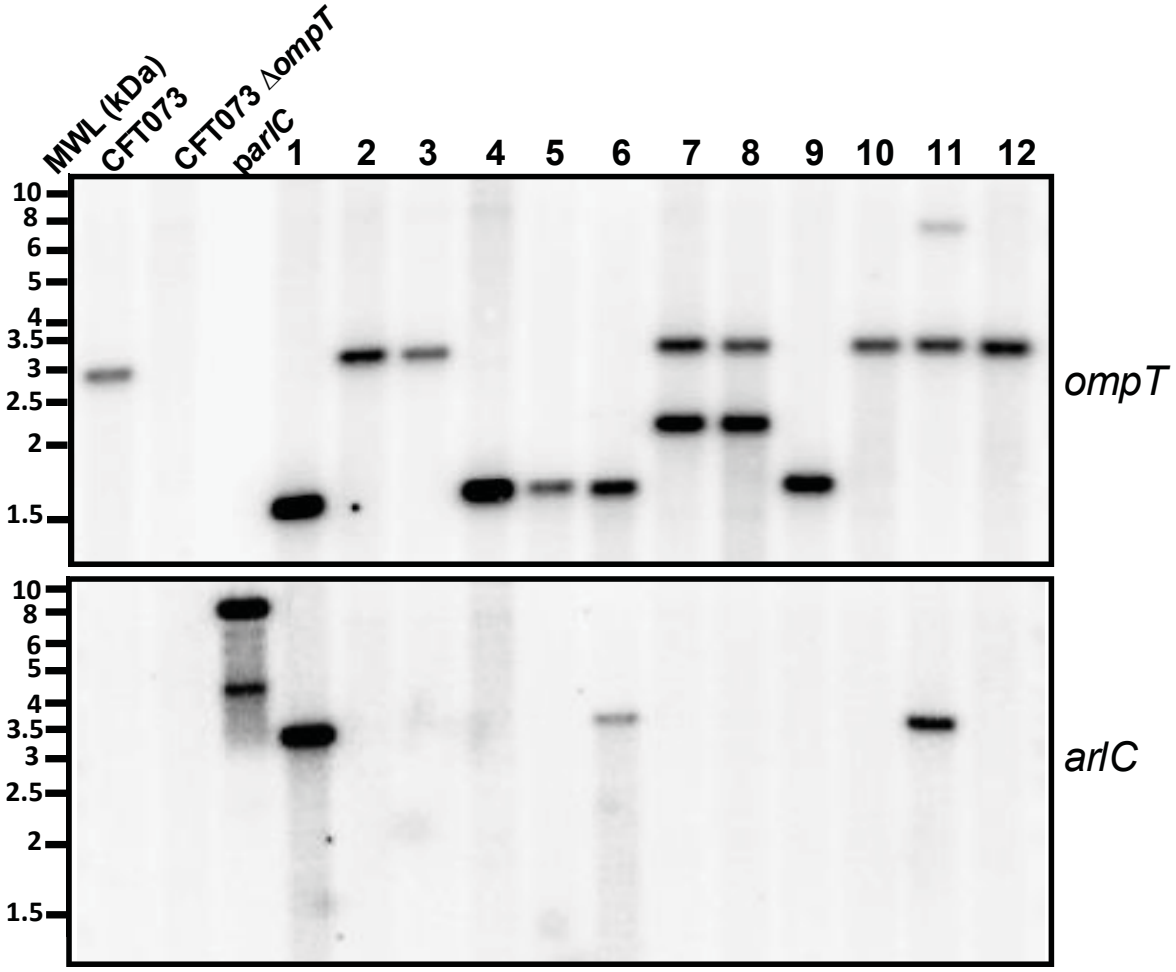

Figure S3 – Southern blot of *ompT* and arlC in 12 cystitis clinical isolates. Southern blot of *ompT* and *arlC* from DNA isolated from 12 cystitis causing clinical isolates digested with EcoRV.

Table S1. Strains and plasmids used in this study

Strains	Description	Source
UPEC CFT073	Wild type UPEC O6:K2:H1	[42]
UPEC Δ*ompT*	CFT073Δ*ompT*	[225]
BL21(DE3)	Wild type BL21 F^- *dcm ompT* *hsdS*$_B$ (r_B^- m_B^-) *gal*	Norvagen
BL21 p*ompT*	BL21 expressing *ompT* from p*ompT*	This study
BL21 p*arlC*	BL21 expressing *arlC* from p*arlC*	This study
Plasmids		
pWSK129	Low copy plasmid (Kan^R)	[256]
p*ompT*	Cystitis 6 *ompT* cloned into pWSK129	This study
p*arlC*	Cystitis 6 *arlC* cloned into pWSK129	This study

Table S2. Primers used in this study

Gene	Role	Primers	Size (bp)	Reference
pTJ100 related genes				
iutA	Ferric aerobactin receptor gene involved in iron transport	F: GGCTGGACATCATGGGAACTGG R: CGTCGGGAACGGGTAGAATCG	302	Johson & Stell (2000)
Adhesins				
fimH	D-mannose–specific adhesin, type 1 fimbriae	F: TGCAGAACGGATAAGCCGTGG R: GCAGTCACCTGCCCTCCGGTA	508	Johson & Stell (2000)
papAH	Major structural subunit of P fimbriae	F: ATGGCAGTGGTGTCTTTTGGTG R: CGTCCCACCATACGTGCTCTTC	717	Johson & Stell (2000)
papC	Pilus assembly, central region of pap operon	F:GTGGCAGTATGAGTAATGACCGTTA R: ATATCCTTTCTGCAGGGATGCAATA	205	Johson & Stell (2000)
papEF	Minor tip pilins, connect PapG to PapA	F:GCAACAGCAACGCTGGTTGCATCAT R:AGAGAGAGCCACTCTTATACGGACA	326	Johson & Stell (2000)
sfaS	Pilus tip adhesion, S fimbriae	F: GTGGATACGACGATTAACTGTG R: CCGCCAGCATTCCCTGTATTC	244	Johson & Stell (2000)
Iron related				
fyuA	*Yersinia* siderophore receptor	F: TGATTAACCCCGCGACGGGAA R: CGCAGTAGGCACGATGTTGTA	787	Johson & Stell (2000)
Protectins				
kpsMTII	Group II capsular polysaccharide synthesis	F: GCGCATTTGCTGATACTGTTG R: CATCCAGACGATAAGCATGAGCA	272	Johson & Stell (2000)
kpsMTIII	Group III capsular polysaccharide synthesis	F: TCCTCTTGCTACTATTCCCCCT R: AGGCGTATCCATCCCTCCTAAC	392	Johson & Stell (2000)
Toxins				
cnf-1	Cytotoxic necrotizing factor 1	F: AAGATGGAGTTTCCTATGCAGGAG R: CATTCAGAGTCCTGCCCTCATTATT	498	Johson & Stell (2000)
hlyA	α-Hemolysin	F: AACAAGGATAAGCACTGTTCTGGCT R: ACCATATAAGCGGTCATTCCCGTCA	1177	Johson & Stell (2000)
Miscellaneous				
ompT	Aspartate protease	1F: TTTGATGCCCCAGATATCTATCGG 1R: GGCTTTCCTGATATCCGGCCATG 2F:TTATAGCTTTACAGCCAGAGGTGG 2R: TTCTTTTTCCTGGGTCATAG	479	This study
arlC	Omptin	F: GATTCTTGCTACTGCACTCTCAGCTCC R: CTGGAGTACAGAGAAGTATCACC	852	This study
ompP	Omptin	F: TGCTTCTGATTTCTTCGGCC R: GTAGTTTGTCTTACATAATGCTC	648	This study
Phylogenetic typing				
chuA	Heme transport gene	F: GACGAACCAACGGTCAGGAT R: TGCCGCCAGTACCAAAGACA	279	Clermont et al. (2000)
yjaA	Gene of unknown function from *E.coli* K-12 genome	F: TGAAGTGTCAGGAGACGCTG R: ATGGAGAATGCGTTCCTCAAC	211	Clermont et al. (2000)
TSPE4.C2	Anonymous DNA fragment	F: GAGTAATGTCGGGGCATTCA R: CGCGCCAACAAAGTATTACG	152	Clermont et al. (2000)
Complementation				
ompT	Omptin from Cystitis 6 clinical isolate	3F: CCACGACTTAGAAGTTCCTAGAACG 3R:GCGAGCTCAAATCTGGTTAACTTCGTTAA	1500	This study
arlC	Omptin from Cystitis 6 clinical isolate	1F: CTAGGAGCTCCCCGGCATAAAGTGTCC 1R: CATGTCTAGAATCGTTGAGCACATATAC	1270	This study
qPCR primers				
ompT	Aspartate protease	4F: CAGCGGCTGGGTGGAAGCAT 4R: ACCCGATTCCATGCGCCTTCA	168	Thomassin et al. (2012)
arlC	Omptin	2F: AGGATCACCTATCGTAGCGATGT 2R: CGGTTCCATGTTCCTTCGACATAA	113	This study
rpoD	RNA polymerase	F: GCTGGAAGAAGTGGGTAAAC R: TAATCGTCCAGGAAGCTACG	126	This study
Southern blot primers				
ompT	Aspartate protease	5F:ATGCGGGCGAAACTTCTGGGAATAG 5R: TCCCAATTAATTGCACCTTTAATAATT	250	This study

Chapter 3- General Discussion and Future Directions

As UPEC is a ubiquitous pathogen, it is important to understand its pathogenicity and how it interacts with host factors [29, 161]. Host factors such as AMPs are important in the defense of the human urinary tract against pathogen colonization [101, 104, 105]. OmpT, which play a role in AMP resistance in *E. coli*, is a recognized virulence factor in UPEC [22, 140]. However, OmpT activity in five UPEC prototypical strains, including CFT073, have been assessed and all strains displayed limited proteolytic activity [225].

This work demonstrates that OmpT activity in 12 fecal ExPEC and 46 UPEC clinical isolates of various disease severities (from ABU to sepsis) was highly variable and heterogenous between isolates. Activity was significantly higher in disease causing groups when compared to fecal isolates. This reinforces the idea that OmpT may be important for UPEC virulence. This might be explained by differences in promoters, which lead to variable *ompT* expression among the clinical isolates. This was previously demonstrated in EPEC and EHEC, where their different promoters lead to different expression of the *ompT* gene [191]. Quantitative PCR of *ompT* in the isolates causing cystitis revealed, once again, a very heterogenous expression of *ompT*. Promoter alignment from sequenced isolates (Cystitis 1, Cystitis 6 and Cystitis 11) revealed differences in promoter that could explain the variation in *ompT* expression between strains. Some have promoters similar to EPEC, which has low *ompT* expression, while others have a promoter similar to EHEC, which has a high *ompT* expression [191].

However, this heterogeneity may also be due to the presence of other members of the omptin family such as OmpP and ArlC, the two plasmid-encoded omptins of *E. coli*. Screening for

previously identified omptins in *E. coli* revealed that *ompT* is widely present in UPEC clinical isolates with a higher incidence in isolates causing disease. Furthermore, only a small proportion of disease causing isolates harbored the *arlC* gene, while none of the non-disease causing isolates encoded it. None of the isolates harboured *ompP*. This incidence might indicate the importance of *ompT* and *arlC* in UPEC virulence. Southern blot analysis of the clinical isolates causing cystitis revealed that two isolates (Cystitis 7 and Cystitis 8) harboured two copies of the *ompT* gene. Sequencing of both *ompT* genes would reveal if they are of full length and functional. Some sequenced isolated have been shown to encode two copies of the *ompT* gene such as ExPEC strain IHE3034 [217]. The presence of two gene copies could once again emphasize the importance of OmpT as a virulence factor in UPEC pathogenesis.

Using a BL21 strain expressing *ompT* and *arlC* from a plasmid with their natural promoters demonstrates that OmpT and ArlC have different substrate specificities. Both omptins have similar FRET activity but OmpT and ArlC were able to degrade LL-37 and RNase 7 to different extents. Elucidating the difference in ability of OmpT and ArlC to cleave different substrates is crucial to understand their mechanism of action and to potentially discover the physiological substrate of ArlC.

In conclusion, this work demonstrates the importance of OmpT as an active factor in symptomatic UPEC clinical isolates. Furthermore, we showed the importance to screen for other omptins that can be found in *E. coli,* such as ArlC and that OmpT and ArlC have different substrate specificities.

Investigating the role of ArlC in pathogenesis is important to understand why only disease causing strains, within our collection, encode *arlC*. One way to investigate and find a potential physiological substrate for ArlC is by using a peptide array containing human peptides sequences to see which one ArlC can cleave. Theses peptide arrays could be used to compare the three omptins present in *E. coli* (OmpT, OmpP and ArlC) and their potential to cleave different substrates. Since theses peptides libraries contain a large variety of peptides, the chance of ArlC's substrate being found is quite high. Recently, this approach has been used to reveal a new Pla substrate; Fas ligand [197]. This experiment would be useful in order to find ArlC's physiological substrate. However, in order to perform this experiment, OmpT, OmpP and ArlC need to be purified. Using bacteria, for such a study, might give false-positive results due to potential interference by other bacterial proteases. Our laboratory has previously purified the omptin of *C. rodentium* CroP and previous reports of OmpT purification (after modification of the auto-degradation site) have been published [204, 253].

Initial sequencing of three cystitis causing strains (Cystitis 1, Cystitis 6 and Cystitis 11) containing both *ompT* and *arlC* confirmed the presence of *ompT* on their chromosomes and *arlC* on large plasmids. It also confirmed the presence of *arlC* in the antimicrobial peptide resistance locus in which it was previously described. However, often more information can be drawn from these sequences. For example, the PAIs in which *arlC* is encoded contains a putative colicin V (*ColV*) gene between *arlB* and *arlC* (Figure 6). Cystitis 11 even has *ColV* flanking *arlC* on both sides. Since OmpT has previously been shown to cleave some colicins (colicin A and colicin E2), it would be of interest to investigate if ArlC is able to cleave colicin V using a cleavage assay [221, 257]. Colicins are usually encoded next to their immunity protein, which prevent the

secreting bacteria to get killed by its own colicins. Immunity proteins have also been shown to limit colicin E2 cleavage by OmpT [257]. However, in this PAI, no immunity protein can be found encoded next to *ColV*. This could indicate the potential role of ArlC in colicin cleavage. ArlC could prevent killing of the bacterium itself and potentially protect it against other secreted colicins present in its environment.

Recently, OmpT has been shown to play a role in adhesion and invasion into bladder epithelia in a mouse model of cystitis [254]. It would be of interest to explore the role of ArlC in adhesion and invasion in bladder cell lines as well as in a cystitis mouse model. The best experimental approach would be to create gene knockouts of *ompT* and *arlC* in one of the sequenced clinical isolates (Cystitis 6 for example) and assess the individual roles of *ompT* and *arlC* in infection in a pathogenic bacterial background. Another experimental approach would be to use the well-studied UPEC prototypical strain CFT073 as a background to overexpress *ompT* and *arlC* from a cloned expression vector. However, the exact impart that ArlC will have in a mouse infection is unknown. It is important to note that mouse AMPs are different than human AMPs and that establishing the role and impact of omptin proteases in a mouse model does not always translate into humans. ArlC did not show an important role in virulence in a mouse model of colitis, but could play an important role in UTIs since the presence of ArlC in our clinical isolate collection is far greater than any other report of the presence of ArlC in InPEC strains.

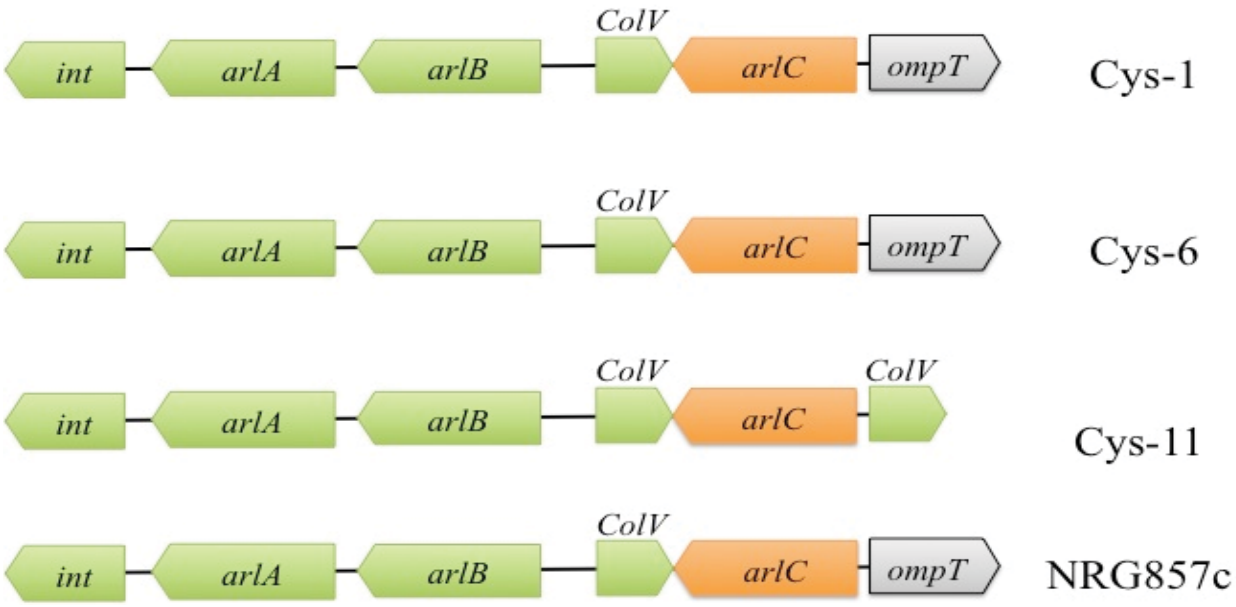

Figure 6. ArlC genomic context of three cystitis-causing strains and colitis strain NRG857c. The *arlC* gene (orange) of three cystitis-causing strains that have both *ompT* and *arlC* within thie genomes. Pseudogenes are represented in grey. The *inT* gene is a prophage integrase gene. The *arlA* gene encodes a mig-14 resistance gene, *arlB* encodes a sugar epimerase and *arlC* encodes an OmpT homologue. *ColV* encodes a putative colicin [183].

Little is known about UPEC resistance to AMPs and recent findings indicate high amounts of previously known AMPs present in the urinary tract. UPEC has been known to resist to LL-37 using OmpT to a small extent as well as curli fimbriae [225, 258]. The heterogenous activity of OmpT within clinical isolates suggests that they might use other mechanism of resistance against AMPs present in the urinary tract. In future work, the other mechanisms of AMP resistance in clinical isolates that have low OmpT activity, similarly to prototypical strain CFT073 or that do not encode *ompT,* would be of interest [225]. This can be achieved by looking for the presence of different LPS, capsules, or curli fimbriae in those isolates all of which are known AMP resistance mechanism in Gram-negative bacteria. More specifically, UPEC might use a group 4 capsule to resist HD-5 that is present in the urinary tract since that capsule is know to be important for HD-5 resistance by EPEC in the small intestine [182].

The future development of therapeutics against UPEC pathogens is necessary due to their increasing antibiotic resistance. There are three main ways to develop therapeutics using AMPs; treat infections by directly using AMPs, enhance the natural body production of AMPs, or block bacterial resistance to AMPs. AMPs can directly treat an infection when applied to the side of infection in a skin wound for example [131]. This might provided more of a challenge in order to treat urinary tract infections due to the difficult access to the infection site. Increased natural body production of AMPs can be achieved with the ingestion of specific nutrients by the human body. For example, LL-37 production can be enhanced with vitamin D and/or butyrate [138, 139]. Blocking bacterial resistance to AMPs can be reached, in the case of OmpT, by the use of proteases inhibitors [253]. These omptin inhibitors would increase bacterial killing by naturally occurring AMPs of the body. Such an inhibitor was discovered for the murine omptin CroP and

also shows an inhibitory effect towards OmpT and Pla [253]. This inhibitor, called Aprotinin, could be used in combination with AMP treatments or antibiotics to increase the chances of curing bacterial infections [253]. Although there is no magic solution to bacterial resistance to antibiotics, turning back to intrinsic body resistance might be an effective way of combatting antibiotic resistant bacteria.

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