Identification and characterization of OmpT-like proteases in uropathogenic *Escherichia coli* clinical isolates

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Abstract

Bacterial colonization of the urogenital tract is limited by innate defenses, including the production of antimicrobial peptides (AMPs). Uropathogenic *Escherichia coli* (UPEC) resist AMP-killing to cause a range of urinary tract infections (UTIs) including asymptomatic bacteriuria, cystitis, pyelonephritis, and sepsis. UPEC strains have high genomic diversity and encode numerous virulence factors that differentiate them from non-UTI causing strains, including *ompT*. As OmpT homologues cleave and inactivate AMPs, we hypothesized that high OmpT protease activity-levels contribute to UPEC colonization during symptomatic UTIs. Therefore, we measured OmpT activity in 58 UPEC clinical isolates. While heterogeneous OmpT activities were observed, OmpT activity was significantly greater in UPEC strains isolated from patients with symptomatic infections. Unexpectedly, UPEC strains exhibiting the greatest protease activities harboured an additional *ompT*-like gene called *arlC* (*ompTp*). The presence of two OmpT-like proteases in some UPEC isolates led us to compare the substrate specificities of OmpT-like proteases found in *E. coli*. While all three cleaved AMPs, cleavage efficiency varied on the basis of AMP size and secondary structure. Our findings suggest the presence ArlC and OmpT in the same UPEC isolate may confer a fitness advantage by expanding the range of target substrates.

1 Introduction

Urinary tract infections (UTIs) are among the most common cause of bacterial infections requiring antibiotic treatment (Flores-Mireles, Walker, Caparon, & Hultgren, 2015; Foxman, 2014; Hooton & Stamm, 1997). The majority of community acquired UTIs (70-95%) and recurrent UTIs are caused by uropathogenic *Escherichia coli* (UPEC) (Flores-Mireles et al., 2015; Nielubowicz & Mobley, 2010). The human gut acts as a reservoir for UPEC strains where they form part of the fecal flora (Kaper, Nataro, & Mobley, 2004; Moreno et al., 2006). Following colonization of the periurethral area, UPEC infect the urinary tract in an ascending manner, resulting in diseases ranging from asymptomatic bacteriuria (ABU), cystitis, pyelonephritis and sepsis (Hooton, 2012). UPEC strains have high genomic diversity and encode numerous virulence factors that differentiate them from non-UTI causing strains (Johnson, 1991; Lloyd, Rasko, & Mobley, 2007; Najafi, Hasanpour, Askary, Aziemzadeh, & Hashemi, 2018; Norinder, Koves, Yadav, Brauner, & Svanborg, 2012). These virulence factors contribute to disease progression allowing UPEC to colonize the uroepithelium, produce toxins, scavenge metabolites, and evade the host immune system (Schwab, Jobin, & Kurts, 2017; Terlizzi, Gribaudo, & Maffei, 2017).

Bacterial colonization is limited in the upper urogenital tract by several mechanisms including urine flow, chemical properties of urine, epithelial cell shedding, influx of immune cells including neutrophils upon bacterial stimulation, and secretion of soluble proteins and peptides by epithelial cells (Spencer, Schwaderer, Becknell, Watson, & Hains, 2014; Weichhart, Haidinger, Horl, & Saemann, 2008). Secreted proteins and antimicrobial peptides (AMPs) form part of the innate immune defenses of the urogenital tract and act through immunomodulation, indirect anti-colonization activity or direct bacterial killing (Kai-Larsen et al., 2010; Zasloff, 2007). AMPs are small (12-50 amino acids), cationic, amphipathic

peptides that exert bactericidal action by interacting with anionic bacterial membranes to form pores resulting in bacterial lysis (Jenssen, Hamill, & Hancock, 2006). Two types of AMPs are detected in the urogenital tract: defensins that form small disulfide bond stabilized ß-sheets and the α-helical cathelicidin LL-37 (Chromek et al., 2006; Lehmann et al., 2002; Valore et al., 1998). In addition, the urogenital tract produces large structured antimicrobial proteins called ribonucleases (RNase) (Spencer et al., 2011; Spencer et al., 2013). Human α-defensin 5 (HD5), human ß-defensins (hBD) 1 and 2, LL-37 and RNase 7 are thought to prevent bacterial colonization as they are constitutively expressed in the urinary tract (Kjolvmark, Akesson, & Pahlman, 2017; Spencer et al., 2012). During UTIs HD5, hBD2, LL-37 and RNase 7 production increases, suggesting an active role in bacterial clearance (Chromek & Brauner, 2008; Chromek et al., 2006; Nielsen et al., 2014; Spencer et al., 2012; Spencer et al., 2013). Remarkably, increased cathelicidin expression and LL-37 secretion is triggered a few minutes after bacteria encounter uroepithelial cells. This suggested role for AMPs in UTI immune defense is consistent with reports that UPEC strains are generally more resistant to AMPs than commensal *E. coli* strains that do not colonize the urogenital tract (Chromek et al., 2006).

Gram-negative bacteria use several mechanisms to resist killing by AMPs, including capsules, efflux pumps, LPS modifications, and proteases (Gruenheid & Le Moual, 2012). Omptin proteases are found in the Gram-negative outer bacterial membrane and have a conserved active site with features of both aspartate and serine proteases (Kramer et al., 2001; Vandeputte-Rutten et al., 2001). With their active sites facing the extracellular environment, omptins contribute to virulence by cleaving a variety of proteins and peptides (Haiko, Suomalainen, Ojala, Lahteenmaki, & Korhonen, 2009). Both substrate specificity and amino acid identity are used to classify omptins into Pla-like and OmpT-like subfamilies. Pla readily

cleaves the proenzyme plasminogen into active plasmin to promote bacterial dissemination during both bubonic and pneumonic plague (Lathem, Price, Miller, & Goldman, 2007; Sodeinde et al., 1992; Zimbler, Schroeder, Eddy, & Lathem, 2015). OmpT rapidly cleaves and inactivates AMPs, including LL-37, protamine, and a synthetic peptide optimized to have maximum antibacterial activity called C18G (Brannon, Thomassin, Desloges, Gruenheid, & Le Moual, 2013; Stumpe, Schmid, Stephens, Georgiou, & Bakker, 1998; Thomassin, Brannon, Gibbs, Gruenheid, & Le Moual, 2012). OmpT-mediated AMP inactivation is thought to support host colonization by some pathogenic *E. coli* strains (Thomassin, Brannon, Gibbs, Gruenheid, & Le Moual, 2012). In addition to OmpT, two OmpT-like proteases have been described in *E. coli* strains (Kaufmann, Stierhof, & Henning, 1994; McPhee et al., 2014; Zhuge et al., 2018), these genes, called *ompP* and *arlC* (*ompTp*) encode proteins that have approximately 74% amino acid identity to OmpT. While the physiological substrates of OmpP and ArlC are unknown, OmpP has been shown to cleave the AMP protamine and ArlC is associated with AMP resistance (Hwang et al., 2007; McPhee et al., 2014).

The *ompT* gene is present in the genome of 85-97% of UPEC clinical isolates and is used in epidemiological studies to identify virulent UPEC strains, yet its function across clinical isolates remains unclear (Foxman, Zhang, Palin, Tallman, & Marrs, 1995). As OmpT and OmpT-like omptins play roles in resistance to host-produced AMPs, we hypothesized that high OmpT protease activity-levels contribute to UPEC colonization during symptomatic UTIs. To test this hypothesis, we detected *ompT* and measured OmpT activity in a collection of 58 UPEC clinical isolates from groups of patients with infections of differing clinical severity (fecal, ABU, UTI [cystitis and pyelonephritis] and sepsis). Heterogeneous OmpT activity was observed and in some isolates high protease activity was correlated with the presence of an additional *ompT*-like gene called *arlC* (*ompTp*). The presence of two OmpT-

like proteases in some UPEC isolates led us to compare the substrate specificity of the three *E. coli* omptins (OmpT, OmpP and ArlC). We found that OmpT, OmpP and ArlC all cleave AMPs, although cleavage efficiency of different AMP-types varied. Our results suggest that the presence of multiple omptins allows UPEC to cleave at least two major subsets of AMPs encountered during infection.

2 Material and Methods

2.1 Bacterial Strains and Growth Conditions

58 ExPEC isolates originating from patients diagnosed with extraintestinal infections or from the urine or stool of healthy individuals were obtained from the Manges collection. Included isolates were randomly selected from the *E. coli* category to ensure they were representative. Isolates were divided into 4 groups based on disease type. Fecal isolates (n = 12) were recovered from the feces of healthy subjects in Québec Canada (2009-2010), ABU isolates (n = 10) were from patients with asymptomatic bacteriuria in California USA (2005-2006) (Manges, Johnson, & Riley, 2004), UTI isolates (n = 24) were recovered from patients with cystitis in Québec Canada (2005-2007) (Manges, Tabor, Tellis, Vincent, & Tellier, 2008) and cystitis or pyelonephritis in California USA (1999-2000) (Manges, Dietrich, & Riley, 2004), and sepsis isolates (n = 12) were from patients with sepsis in California USA (2001-2003) (Manges, Perdreau-Remington, Solberg, & Riley, 2006). Bacterial strains used in this study are listed in Table 1. Bacteria were routinely cultured in lysogeny broth (LB; 10% (w/v) tryptone, 5% (w/v) yeast extract, 10% (w/v) NaCl) or in N-minimal medium (50 mM Bis-Tris, 5 mM 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, supplemented with 1.4% glucose and 1 mM $MgCl_2$ (UPEC isolates) or with 0.5% glucose and 1 mM $MgCl_2$ (all other strains). Bacteria were cultured at 37°C with aeration (220 rpm).

2.2 Multiplex PCR of UPEC Virulence Genes

Total DNA (genomic and large-plasmid DNA) was isolated using the Puregene Yeast/Bact. kit (Qiagen). Phylogenetic groups were determined as described in (Clermont, Bonacorsi, & Bingen, 2000), using primer pairs listed in Table 2. To detect virulence genes present in the isolates, primer sequences were obtained from previous studies (Johnson & Stell, 2000) or

designed *de novo* for this study (Table 2). 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); *ompP* (648 bp).

2.3 Fluorescence Resonance Energy Transfer (FRET) Activity Assay

The FRET substrate containing a dibasic motif (RK) in its center (2Abz-SLGRKIQI-K(Dnp)-NH2) was purchased from Anachem. Bacteria were grown in N-minimal medium to mid-exponential phase and normalized to an OD_{595nm} of 0.5. Bacterial cells were pelleted and resuspended in phosphate-buffered saline (PBS). Bacteria (~ 2.25 x 10^7 CFU in 75 µL) were mixed in a 96-well plate with 75 µL of the FRET substrate (final concentration 3 µM). Fluorescence (λ Ex 325 nm, λ Em 430 nm) was monitored for 1 h at 25°C using a Biotek FLx 800 plate reader. Data were normalized by subtracting the background fluorescence of the FRET substrate in PBS.

2.4 Plasmid construction

The *ompT* and *arlC* genes were PCR-amplified from DNA isolated from the UPEC UTI clinical isolate 6, also called cystitis 6, using their respective primer pairs ompT_cf/ompT_cr and arlC_cf/arlC_cr (Table 2). PCR fragments were treated with XbaI and SacI and ligated into plasmid pWSK129 treated with the same enzymes, generating plasmids pWSK*ompT* and pWSK*arlC* (Table 1). The *ompP* gene was PCR-amplified from XL1-Blue DNA using primer pair ompP_cf/ompP_cr. PCR-products were treated with XbaI and PstI and ligated into pWSK129 treated with the same enzymes to generate plasmid pWSK*ompP*. The *pla* gene under control of the *croP* promoter was subcloned from pYC*pla* (Brannon, Burk, et al., 2015)

using XbaI and SacI and ligated into pWSK129 previously treated with the same enzymes, generating pWSK*pla*.

2.5 Southern Blotting

Total DNA was isolated and treated with EcoRV. Southern blotting and hybridization were performed as previously described (Taylor, Ouimet, Wargachuk, & Marczynski, 2011) using Hybond-XL membranes. Probes for *ompT* and *arlC* were PCR-generated using primer pairs ompT_sf/ompT_sr and arlC_sf/arlC_sr, respectively (Table 2). Probes were radiolabelled with dATP [α-32P] using the RadPrime kit (Invitrogen). The pWSK*arlC* plasmid was used as the positive control for the *arlC* probe.

2.6 Quantitative RT-PCR

Quantitative RT-PCR (qPCR) was performed as previously described (Thomassin, Brannon, Gibbs, et al., 2012). 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 residual DNA. The absence of DNA was confirmed by qPCR using the primer pair rpoD_qf/rpoD_qr. RNA (100 ng) was reverse transcribed using Superscript II (Invitrogen) with 0.5 μg of random hexamer primers. A reaction mixture without Superscript II was also included and was used as the 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 2. 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 (Livak & Schmittgen, 2001).

200 **2.7 Whole genome sequencing**

201 Sequencing was performed on a PacBio platform (Pacific Biosciences). Genomic DNA
202 samples were purified using the Gentra® Puregene® kit (Qiagen) and sheared to 20 kb using
203 g-tubes (Covaris). Libraries were prepared using the template preparation kit from Pacific
204 Biosciences. A single SMRT cell was sequenced to generate data sets including unique sub-
205 reads with a minimum length of 3 kb. Genome assemblies of sequence reads were generated
206 using a combination of HGAP/Celera/Quiver following Pacific Biosciences
207 recommendations. The complete chromosome and plasmid sequences were submitted to
208 GenBank.

209

210 **2.8 Preparation of whole-cell lysates and outer-membrane fractions**

211 Bacteria were grown in N-minimal medium until mid-exponential phase and normalized to an
212 OD_{595nm} of 0.5. For whole-cell lysate samples, bacterial cells were pelleted and resuspended
213 in 1/10 volume of 2X ESB (Thomas et al., 2005). Outer-membrane fractions were isolated as
214 follows: bacterial cultures were centrifuged at 2500 rpm for 10 min and pellets were
215 resuspended in 1.5 mL low-salt buffer (100 mM NaPi buffer [pH 7], 5 mM EDTA and 10%
216 glycerol). Samples were supplemented with 10 uL PMSF and sonicated. Samples were then
217 centrifuged at 5500 rpm for 10 min. Supernatants were collected and centrifuged at 65000
218 rpm for 30 min at 4°C. Pellets were resuspended in 2 mL sarcosyl buffer (10 mM Tris [pH
219 7.5], 5 mM $MgCl_2$ and 2% sarcosyl) and incubated for 30 minutes at 10°C. Samples were
220 then centrifuged for 60 min at 45000 rpm and the pellet containing outer membranes was
221 resuspended in buffer (20 mM Tris-HCl pH 7.5 and 10% glycerol). Outer membrane samples
222 were combined 1:1 with 2X ESB and boiled for 10 minutes prior to loading samples on an
223 SDS-PAGE gel.

224

225 **2.9 Western blotting**

226 Whole-cell lysate and outer-membrane fractions were resolved on a 10% SDS-PAGE gel and
227 transferred to a polyvinylidene fluoride membrane. Membranes were blocked for 1 h in Tris-
228 buffered saline (TBS) supplemented with 5% skim milk and incubated overnight with the
229 polyclonal anti-CroP antibody (Thomassin, Brannon, Gibbs, et al., 2012). Membranes were
230 washed extensively with TBS and incubated for 1 h with a goat anti-rabbit secondary
231 antibody conjugated with HRP. Membranes were washed and developed using
232 chemiluminescent HRP substrate.

233

234 **2.10 Plasminogen activation assay**

235 Bacteria were grown in N-minimal medium to mid-exponential phase and normalized to an
236 OD_{595nm} of 0.5. Bacterial cells were pelleted and resuspended in ½ volume of phosphate-
237 buffered saline (PBS; final 6 $x10^8$ CFU/mL). In a 96 well plate, 178 µL of bacteria and 20 µL
238 of 45 mM VLKpNA (Sigma Aldrich) were combined. Baseline assays were performed at
239 OD_{405nm}. After 5 min, 4 µg of plasminogen substrate was added and absorbance (405nm) was
240 measured every 10 min for 400 minutes at 37 °C with agitation before every reading.

241

242 **2.11 Proteolytic cleavage of AMPs**

243 Bacteria were grown in N-minimal medium to mid-exponential phase, washed and
244 normalized to an OD_{595nm} of 0.5 in PBS. Aliquots of bacteria (10^7 CFU) were combined 1:4
245 (v/v) with 2 µg/µL LL-37, mCRAMP, C18G or Magainin II (BioChemia) or 1 µg/µL RNase
246 7 and incubated at room temperature for various time points. Bacteria were separated from
247 peptide cleavage products by centrifugation and supernatants were combined 1:1 with 2X
248 ESB then boiled and frozen at -20°C. Peptide cleavage products were resolved on 10-20%
249 Tris-Tricine gels (BioRad) and RNase 7 samples were resolved on 20% SDS-PAGE gels.

Peptides were fixed in the gel by incubation in 20% (v/v) glutaraldehyde for 30 min; gels were rinsed with water and peptides stained for 1h with Coomassie blue G-250 stain. Gels were destained in 20% (v/v) acetic acid.

2.12 Circular dichroism spectroscopy

Experiments were performed on a Jasco J-810 spectropolarimeter (Easton, MD). AMPs (200 µg/ml in PBS) were placed in a quartz cuvette with a path length of 0.1 cm and spectra were recorded from 260 to 195 nm. Samples were scanned three times at 20°C using a bandwidth of 1 nm, a time response of 2 sec and a scan rate of 100 nm/min. Spectra were corrected by subtracting the background spectrum of PBS and values were converted from ellipticity to mean residue ellipticity (MRE; degree $\times$ cm^2 $\times$ $dmol^{-1}$).

2.13 Statistical Analyses

Data were analyzed using GraphPad Prism software. Normality was verified using D'Agostino-Pearson normality test. Fisher's Exact test was performed to compare incidence of virulence genes within severity groups of UPEC clinical isolates. FRET activity was assessed using a two way ANOVA with Tukey's post test. P value $\leq .05$ being significantly different.

3 RESULTS

3.1 Phylogenetic and virulence profile of UPEC isolates

UPEC isolates from patients with different disease severities were obtained from the Manges collection (Manges, Dietrich, et al., 2004; Manges et al., 2001; Manges, Johnson, et al., 2004; Manges et al., 2006; Manges et al., 2008). Although UPEC strains are heterogeneous, clinical isolates from UTIs predominantly belong to *E. coli* phylogenetic groups B2 and D (Johnson, Delavari, Kuskowski, & Stell, 2001). To confirm that our isolates are generally representative of UPEC clinical strains we determined the phylogenetic grouping of our 58 clinical isolates categorized into the fecal (n=12), ABU (n=10), UTI (cystitis and pyelonephritis; n=24), and sepsis (n=12) groups. Most isolates from the ABU and UTI groups associated with UTIs belong to the phylogenetic group B2 and, to a lesser extent, D (Table 3). In contrast, isolates from the sepsis group were predominantly from group D (Table 3). Finally, isolates from the fecal group had the most variable phylogenetic grouping with 5/12 isolates belonging to phyogenetic groups A and B1 (Table 3). Overall, this distribution is in agreement with previous reports showing that UPEC strains mainly belong to *E. coli* phylogenetic groups B2 and D (Johnson et al., 2001).

The 58 isolates were further characterized using multiplex PCR to detect 12 recognized UPEC virulence genes (Table. 4). Our data showed variations consistent with previous studies reporting that UPEC is a heterogeneous pathotype (Marschall et al., 2012; Maynard et al., 2004; Norinder et al., 2012; Poey, Albini, Saona, & Lavina, 2012). The *fimH* gene, involved in UPEC adherence, was present in all but 2 ABU isolates (Table 4). There was a difference in the distribution of virulence genes *fyuA* and *ompT* for which the incidence was significantly higher in symptomatic (i.e UTI and sepsis) groups than asymptomatic (i.e. fecal and ABU) groups (Table 4). No other genes showed a significant difference in incidence between

293 asymptomatic and symptomatic groups. In agreement with previous studies, we found that
294 *ompT* is present in 89% of the UPEC isolates associated with symptomatic infections (Table
295 4).
296
297 **3.2 Variability of omptin proteolytic activities among UPEC isolates**
298 OmpT preferentially cleaves substrates between two consecutive basic residues (Dekker, Cox,
299 Kramer, & Egmond, 2001; McCarter et al., 2004). Therefore, to assess OmpT proteolytic
300 activity we measured cleavage of a FRET substrate (2Abz-SLGRKIQI-K(Dnp)-NH_2) that
301 contains a dibasic motif in its center (Brannon, Burk, et al., 2015; Brannon et al., 2013;
302 McPhee et al., 2014; Thomassin, Brannon, Gibbs, et al., 2012). Cleavage of the substrate by
303 the 58 UPEC isolates was monitored by measuring fluorescence emission over time and
304 compared with substrate cleavage by the previously characterized reference UPEC strain
305 CFT073 (Brannon et al., 2013). As shown in Fig. 1A, omptin activity of the isolates was
306 heterogeneous between groups. Isolates for which the *ompT* gene was not detected by PCR
307 showed basal activity levels (red triangles in Fig. 1A), whereas isolates harbouring the *ompT*
308 gene showed a wide range of omptin activity. The omptin activity of the isolates of the fecal
309 group was significantly lower than that of the 2 symptomatic groups (UTI and sepsis) (Fig.
310 1A). The mean activity of the isolates from the fecal group (0.75 ± 0.5) was lower than that of
311 strain CFT073. In contrast, the activity means of the symptomatic groups (1.54 ± 0.66 and
312 1.71 ± 0.66) were higher than that of CFT073. Extensive variability in omptin activity was
313 also observed within groups (Fig. 1A). The UTI group exibited the most heterogeneous
314 omptin activity and some isolates from the UTI group had 3-fold higher omptin activity than
315 CFT073. Together, these results indicate that omptin activity is variable among fecal and
316 UPEC clinical isolates.
317

3.3 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 (Kaufmann et al., 1994; McPhee et al., 2014; Zhuge et al., 2018). These OmpT-like proteins are approximately 74% identical to OmpT. To determine whether the presence of *ompT*-like genes in some isolates may account for the heterogeneity of OmpT activity observed in Fig. 1A, multiplex-PCR screens were performed to detect *ompT, ompP* and *arlC*. The *ompP* gene was not detected in any of the isolates (data not shown). In contrast, the *arlC* gene was present in 8 of the 58 isolates (Fig. 1B). Strikingly, *arlC* was only present in symptomatic isolates, which was statistcially significant according to a Fisher's exact test (P = .0445). Most isolates harbouring the *arlC* gene also contained *ompT* and generally had higher proteolytic activity (green circles, Fig. 1A) than CFT073. This is consistent with the report that ArlC cleaves the FRET substrate (McPhee et al., 2014). Isolate 18 from the UTI group did not have *ompT* but harboured *arlC* (Fig. 1B); this isolate exibited moderate proteolytic activity (purple triangle in Fig. 1A). Together these data show that among commensal and clinical isolates, higher omptin activity is associated with symptomatic disease and isolates with the greatest omptin activity harbour both the *ompT* and *arlC* genes.

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

To further understand omptin activity among UPEC isolates, we selected 12 isolates from the UTI group (Table 1) for further analysis because they have the most heterogeneous omptin activity. The presence of *ompT* genes in these isolates was confirmed by Southern blot analysis (Fig. 2A). This analysis also indicated that two *ompT* genes may be present in isolates 7, 8 and 11. Consistent with the multiplex PCR results, *arlC* was detected in UTI isolates 1, 6 and 11 (Fig. 2A). Next, qPCR was used to measure the expression levels of *ompT* and *arlC*. In agreement with our activity assay, *ompT*-levels were heterogeneous among these

343 UTI isolates (Fig. 2B and 2C). Only three isolates (2, 10 and 11) had similar expression levels
344 as the reference strain CFT073, whereas all other isolates had higher *ompT* expression levels
345 than the reference strain. As expected from the multiplex-PCR screen and Southern blot, *arlC*
346 expression was only detected in UTI 1, 6 and 11 isolates. UTI isolates 1 and 6, which showed
347 the highest *ompT* and *arlC* expression levels also had the highest omptin activity-levels (Fig.
348 2C). Although both *ompT* and *arlC* are present in UTI isolate 11, they have low expression
349 levels, which is consistent with the low omptin activity observed (Fig. 2C). These data
350 indicate that heterogeneous omptin activity-levels are associated with both the presence and
351 the different expression levels of the *ompT* and *arlC* genes.
352
353 **3.5 *arlC* is present on plasmids**
354 To determine the genomic context of the *ompT* and *arlC* genes, isolates 1, 6 and 11 of the UTI
355 group were sequenced on a PacBio platform. These isolates were then renamed cystitis 1,
356 cystitis 6 and cystitis 11. Detailed descriptions of genomes and gene features are found in
357 Appendix (Appendix Figs. 1AB, 2AB, 3). In all three isolates, *ompT* was located within the
358 bacterial chromosome and *arlC* was part of large plasmids (79-200 kbp; Appendix Fig. 1A
359 and 2A). In addition, the *ompT* gene was invariably located downstream of *nfrA* and *ybcH*
360 (Fig 3A). Some differences were noted in the genomic context of *ompT* among the clinical
361 isolates. In cystitis 1 and 6 the *envY* gene, encoding a transcriptional regulator of porin
362 synthesis, is inserted between *ybcH* and *ompT* (182 bp downstream of *ybcH,* 512 bp upstream
363 of *ompT*). The *appY* gene, encoding a transciptional activator, is located 249 bp downstream
364 of the *ompT* gene in cystitis 1, whereas *ymcE*, encoding a putative cold shock gene, is located
365 186 bp downstream of *ompT* in cystitis 6. In cystitis 11 the *ompT* gene is located 657 bp
366 downstream of *ybcH* and 272 bp upstream of *ybcY*; this is the same genomic context as that in
367 UPEC strains CFT073, UTI89, 536, J96 ABU83972 and EPEC strain e2348/69, all of which

were reported to have low omptin activity (Fig. 3A, (Brannon et al., 2013; Thomassin, Brannon, Gibbs, et al., 2012; Thomassin, Brannon, Kaiser, Gruenheid, & Le Moual, 2012)). For all isolates, the predicted amino acid sequence of ArlC is 100% identical to ArlC identified in adherent-invasive *E. coli* (AIEC) strain NRG857c (McPhee et al., 2014). Although the three plasmids harbouring *arlC* were different (Appendix Fig. 2A and B), *arlC* was present in all cases as part of pathogenicity island PI-6 previously reported to play a role in AMP resistance (Fig. 3B, McPhee et al., 2014).

3.6 Comparative analysis of OmpT, OmpP and ArlC

With the unexpected detection of *arlC* among the UPEC clinical isolates, we hypothesized that the presence of a second or even a third omptin protease within a single species may provide an advantage by expanding the potential range of substrates cleaved. Therefore, we sought to compare the substrate specificities of these proteases. As OmpT undergoes auto-cleavage during purification (Kramer, Zandwijken, Egmond, & Dekker, 2000; Vandeputte-Rutten et al., 2001) and mutagenesis of residues to stabilize the protein results in a significant decrease in FRET substrate cleavage ((Kramer et al., 2000); unpublished data Thomassin JL and Brannon JR) it was not possible to purify these proteases and directly compare their activities. Instead, we produced OmpT, OmpP and ArlC in *E. coli* BL21, a laboratory strain that lacks omptin proteases. To test their production and correct localization in BL21, omptin proteins were detected by western blot analysis from both whole cells and outer-membrane preparations (Fig. 4A). To determine if the proteases were active in BL21, FRET substrate cleavage was monitored over time. As expected, BL21 with empty vector did not cleave the FRET substrate, whereas the three omptins readily cleaved the FRET substrate (Fig. 4B). This demonstrates that when produced in BL21, ArlC, OmpP and OmpT are found in the outer membrane and are proteolytically active.

Omptin proteases are generally subdivided into OmpT-like or Pla-like subfamilies. These subfamilies differ in their ability to cleave plasminogen to activate it into active plasmin, with Pla-like omptins producing active plasmin more readily than OmpT-like omptins (Haiko et al., 2009; Kukkonen et al., 2001). To verify that the three omptin proteases belong in the OmpT-like subfamily, we tested their ability to cleave plasminogen into plasmin. Consistent with their presence in the outer membrane, all three omptins cleaved plasminogen to a greater extent than BL21 alone (Fig. 4C). There was no difference in their ability to activate plasminogen. Compared with the positive control, Pla produced in BL21, the *E. coli* omptins converted significantly less plasminogen into plasmin. These data are consistent with previous publications (Brannon, Burk, et al., 2015; Kukkonen et al., 2001; McPhee et al., 2014) and suggest that all three omptins found in *E. coli* belong to the OmpT-like subfamily of omptin proteases.

Omptin proteases belonging to the OmpT-like subfamily have been associated with AMP cleavage (Le Sage et al., 2009; Stumpe et al., 1998; Thomassin, Brannon, Gibbs, et al., 2012). Previous work has shown that OmpT from EPEC, EHEC, and UPEC cleave the human cathelicidin LL-37. Although ArlC was shown to play a role in AMP-resistance (McPhee et al., 2014), and OmpT and OmpP are reported to exhibit similar substrate specificities (Hwang et al., 2007; McCarter et al., 2004), their ability to cleave different AMPs has not been directly compared. Therefore, we investigated the ability of the *E. coli* omptins to cleave the synthetic cationic peptide C18G and various cathelicidins Magainin II (*Xenopus laevis*), mCRAMP (*Mus musculus*) and LL-37 (*Homo sapiens*). As expected, AMPs incubated with BL21 did not show any degradation or cleavage products, indicating that BL21 does not contain intrinsic proteases that cleave these AMPs (Fig 5A). OmpT cleaved all peptides by the

418 first time point tested (2 min C18G; 15 min mCRAMP, Magainin II and LL-37; Fig. 5A).
419 Similarly to OmpT, OmpP readily cleaved C18G and Magainin II within 2 and 30 min,
420 respectively. In contrast, OmpP only cleaved small amounts of mCRAMP after 60 min and
421 did not appear to cleave LL-37 (Fig. 5A). ArlC cleaved mCRAMP, C18G and Magainin II by
422 the first time point tested (2 min C18G; 15 min mCRAMP and Magainin II), but only a small
423 amount of LL-37 cleavage was observed after 60 minutes. Substrate properties, such as size
424 and secondary structure are known to influence omptin activity (Brannon, Thomassin,
425 Gruenheid, & Le Moual, 2015; Hritonenko & Stathopoulos, 2007). Given that all three
426 proteases readily cleave the FRET substrate and C18G, the striking differences in ability to
427 cleave Magainin II, mCRAMP and LL-37 are likely due to intrinsic differences between
428 OmpT, OmpP and ArlC. Although all peptides tested contain sites with two consecutive basic
429 residues, they range in size from 18-37 amino acids (Fig. 5B). OmpP cleaved smaller peptides
430 such as C18G and Magainin II more readily than the larger mCRAMP and LL-37. As ArlC
431 cleaved mCRAMP more rapidly than OmpT, peptide length might not be the limiting factor
432 for this protease. Peptide secondary structure also influences omptin activity (Brannon,
433 Thomassin, et al., 2015), therefore, we used circular dichroism spectroscopy to determine the
434 secondary structure of these AMPs (Fig. 5C). Under our experimental conditions, only LL-37
435 is α-helical, while mCRAMP, C18G and Magainin II are unstructured (Fig. 5C). While
436 peptide structure did not affect OmpT activity, ArlC did not appear to cleave the only α-
437 helical AMP (Fig. 5BC). Together these findings suggest that OmpT, OmpP and ArlC have
438 differences in substrate cleavage specificities.
439
440 We previously reported that disulfide bonds present in defensins render them resistant to
441 OmpT-mediated proteolysis (Thomassin, Brannon, Kaiser, et al., 2012). Yet ArlC was shown
442 to contribute to bacterial survival in the presence of human defensins (McPhee et al., 2014),

443 suggesting that unlike OmpT, ArlC might cleave AMPs that are stabilized by disulfide
444 bridges. RNase 7 contains four disulfide bridges, three dibasic sites (Fig. 6A) and is abundant
445 in the urinary tract (Spencer et al., 2011; Spencer et al., 2013). The presence of dibasic sites
446 suggests that RNase 7 might be an omptin substrate; therefore, we sought to investigate if
447 there was a difference in omptin-mediated cleavage of this peptide. Under our experimental
448 conditions, OmpT and OmpP did not cleave RNase 7 (Fig. 6B). After 60 min incubation with
449 ArlC, an RNase 7 cleavage product appeared, with more cleavage product appearing after 90
450 min. While cleavage appears limited, ArlC was the only OmpT-like omptin able to cleave
451 RNase 7. Taken together, these data indicate that ArlC, OmpP, and OmpT have different
452 substrate specificities, suggesting that the presence of multiple omptin proteases in a single
453 bacterial strain may enhance AMP-resistance by increasing the range of substrates cleaved.

4 DISCUSSION

Detection of specific genes, including *ompT*, is often used to characterize virulent clinical UPEC isolates (Johnson et al., 2001; Najafi et al., 2018). Previous studies have suggested that OmpT from the UPEC strain CFT073 is involved in adhesion, invasion and/or inactivation of AMPs (Brannon et al., 2013; He et al., 2015). While the presence of *ompT* is associated with virulent strains, its precise contribution remains unclear, as UPEC clinical isolates have highly variable genetic sequences (Schreiber et al., 2017). In addition, we previously observed large differences in OmpT protein activity due to differential *ompT* expression (Thomassin, Brannon, Gibbs, et al., 2012; Thomassin, Brannon, Kaiser, et al., 2012) suggesting that the presence of the *ompT* gene may not entirely correlate with its activity levels in different UPEC clinical isolates. In this study we hypothesized that OmpT activity correlates with increased disease severity among UPEC clinical isolates. To test this hypothesis, we systematically measured omptin activity in 58 *E. coli* isolates representing colonization and a range of clinical outcomes. Increased omptin activity was correlated with clinical UPEC strains isolated from patients with symptomatic UTIs (UTI and sepsis groups).

Omptin activity was heterogeneous among the clinical isolates, and could be related with differential *ompT* expression and the presence of a second OmpT-like protease, *arlC*. For example, a 20-fold difference in *ompT* expression was observed between isolates 5 and 11 of the UTI group (Fig. 2B). This finding is not unprecedented, since it was previously shown that *ompT* expression was 32-fold higher in EHEC than in EPEC (Thomassin, Brannon, Gibbs, et al., 2012). Differential *ompT* expression-levels in EHEC and EPEC were attributed to differences in distal promoter sequences found more than 150 bp upstream of the *ompT* start codon (Thomassin, Brannon, Gibbs, et al., 2012). An EPEC-like *ompT* distal promoter sequence and genomic context was also correlated with low OmpT activity in UPEC

479 reference strains (Brannon et al., 2013). Therefore, it was not surprising that the EPEC-like
480 promoter in cystitis (UTI) isolate 11 resulted in low *ompT* expression and OmpT activity. The
481 insertion of *envY* in the intergenic space between *nfrA* and *ompT* correlated with the increased
482 *ompT* expression and OmpT activity levels observed in cystitis (UTI) isolates 1 and 6 (Figs.
483 2BC and 3A). These data further suggest that variations in distal promoter sequences are
484 responsible for differential *ompT* expression and, in turn, proteolytic activity observed. It is
485 also possible that in addition to differences in the promoter regions, transcription factors or
486 post-transcriptional factors regulating *ompT* expression are absent or differentially expressed
487 in some isolates. Another explanation for heterogeneous omptin activity observed in this
488 study can be attributed to the presence of a second plasmid-encoded omptin, *arlC,* in some
489 isolates. The *arlC* gene was first identified as part of a large virulence plasmid of the AIEC
490 strain NRG857c (McPhee et al., 2014). BLAST searches in the NCBI database revealed that
491 *arlC* can also be found on plasmids harboured by various human ExPEC strains isolated from
492 patients with meningitis and sepsis, as well as avian *E. coli* strains (Appendix Fig. 2B). While
493 we did not detect *ompP* in our study, *ompP* is present in some UPEC strains that were
494 collected and sequenced by the Broad Institute ("E.coli UTI Bacteremia initiative," 2019).
495 This opens the possibility that any combination of *ompT*-like omptin may be present in a
496 given UPEC strain.
497
498 Omptins belonging to the OmpT-like subfamily are known to have subtle differences in
499 substrate specificity (Brannon, Thomassin, et al., 2015; Hwang et al., 2007; McCarter et al.,
500 2004). Studies using peptide libraries to compare OmpP and OmpT activity showed both
501 omptins preferentially cleave substrates between two consecutive basic residues, but that
502 OmpP appears to have a slight preference for Lys in the P and P' sites (Hwang et al., 2007).
503 In addition to subtle differences in amino acid motif preference, peptide size and secondary

504 structure also impact substrate specificity (Brannon, Thomassin, et al., 2015; Haiko et al.,
505 2009; Hritonenko & Stathopoulos, 2007). For example, AMP α-helicity was shown to be a
506 determining factor for proteolytic activity of the OmpT-like omptin, CroP, from *Citrobacter*
507 *rodentium* (Brannon, Thomassin, et al., 2015). While ArlC, OmpP and OmpP all readily
508 cleave small unstructured substrates, such as the FRET substrate and C18G, differences in
509 cleavage efficiency were noted for larger or more structured AMPs. OmpP did not cleave
510 Magainin II as efficiently as C18G and did not cleave larger substrates such as mCRAMP,
511 LL-37 and RNase 7 (Figs. 4A, 5AB, 6B). These findings suggest that larger peptides might be
512 excluded from the OmpP active site. While OmpT and ArlC cleaved the FRET substrate,
513 C18G, Magainin II and mCRAMP relatively efficiently, there was a striking difference in LL-
514 37 and RNase 7 cleavage (Figs. 4A, 5A, 6B). Given the similarity in size of mCRAMP and
515 LL-37, and the ability of ArlC to cleave RNase 7, it is unlikely that the 3 amino acid size
516 difference accounts for the marked difference in cleavage efficiency. It is possible that ArlC
517 does not cleave α-helical AMPs, but instead cleaves unstructured and disulfide bond-
518 stabilized peptides. While this possibility requires further study, it is supported by the finding
519 that an *arlC* deletion strain is more susceptible to killing by human defensins (McPhee et al.,
520 2014). Altogether, these findings suggest the presence ArlC and OmpT in the same UPEC
521 isolate may confer a fitness advantage by expanding the spectrum of target substrates.
522

5 Conclusions

Here we show that increased omptin activity is associated with UPEC strains causing symptomatic UTIs. Extensive heterogeneity of omptin activity among UPEC clinical isolates was is due to variations in *ompT* expression and to the presence of a plasmid-encoded *ompT*-like gene *arlC*. Our findings support current profiling practices of UPEC strains that include the *ompT* gene (Johnson & Stell, 2000), but suggest that additional screening for *arlC* should be considered as both genes were exclusively harboured in UPEC strains associated with symptomatic infection. Altogether our findings suggest that the presence of two different omptins in a UPEC strain may provide an additional fitness advantage by expanding the range of AMPs cleaved during UTIs.

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548 **Conflict of Interest**

549 The authors have declared that no conflict of interest exists.

550

551 **Author contributions**

552 AP, ID, JAT, JLT, JML, and JRB performed experiments. AM, GTM, HLM, ID, JDS, JLT,
553 KD, and SG conceived and designed experiments. HLM, ID, JLT, and SG analyzed the data.
554 HLM and ID wrote early drafts of the manuscript. JLT wrote and reviewed later drafts with
555 support from all other authors.

556

557 **Dedication**

558 This publication is dedicated to Dr. Hervé LeMoual who passed away on March 3rd 2018; he
559 was a great mentor that always encouraged his trainees to follow their passions.

560

561 **Ethics statement**

562 None required.

563

564 **References**

565

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Table 1. Strains and plasmids used in this study

Strains	Description	Source
XL1-Blue	*endA1 gyrA96*(nal^R) *thi-1 recA1 relA1 lac glnV44* F'[::Tn10 *proAB*$^+$ *lacI*^{q} Δ(*lacZ*)M15] *hsdR17*($r_K^-m_K^+$)	Stratagene
GMS 002A	O11:NM; coded as Fecal 1	(Aslam et al., 2014)
GMS 003A	Coded as Fecal 2	Manges strain collection
GMS 005A	Coded as Fecal 3	Manges strain collection
GMS 006E	Coded as Fecal 4	Manges strain collection
GMS 008A	Coded as Fecal 5	Manges strain collection
GMS 009B	Coded as Fecal 6	(Aslam et al., 2014)
GMS 010A	Coded as Fecal 7	Manges strain collection
GMS 012A	Coded as Fecal 8	Manges strain collection
GMS 015A	Coded as Fecal 9	Manges strain collection
GMS 016D	Coded as Fecal 10	Manges strain collection
GMS 017A	Coded as Fecal 11	Manges strain collection
GMS 018A	Coded as Fecal 12	Manges strain collection
10001U001	Coded as asymptomatic bacteriuria 1	(Manges, Johnson, et al., 2004)
10003U002	Coded as asymptomatic bacteriuria 2	(Manges, Johnson, et al., 2004)
10004U001	Coded as asymptomatic bacteriuria 3	(Manges, Johnson, et al., 2004)
10013U005	Coded as asymptomatic bacteriuria 4	(Manges, Johnson, et al., 2004)
10014U005	Coded as asymptomatic bacteriuria 5	(Manges, Johnson, et al., 2004)
10017U005	Coded as asymptomatic bacteriuria 6	(Manges, Johnson, et al., 2004)
1001006	Coded as asymptomatic bacteriuria 7	(Manges, Johnson, et al., 2004)
10005004	Coded as asymptomatic bacteriuria 8	(Manges, Johnson, et al.,

		2004)
10006001	Coded as asymptomatic bacteriuria 9	(Manges, Johnson, et al., 2004)
10012007	Coded as asymptomatic bacteriuria 10	(Manges, Johnson, et al., 2004)
CLSC 36	O1:H42; isolated from a patient with cystitis; coded as UTI 1	(Manges et al., 2008)
MSHS 100	O2:H7; isolated from a patient with cystitis; coded as UTI 2	(Manges et al., 2008)
MSHS 1070	Isolated from a patient with cystitis; coded as UTI 3	(Manges et al., 2008)
MSHS 233	O9:H32; isolated from a patient with cystitis; coded as UTI 4	(Manges et al., 2008)
MSHS 434	O73:H18; isolated from a patient with cystitis; coded as UTI 5	(Manges et al., 2008)
MSHS 472	O82:NM; isolated from a patient with cystitis; coded as UTI 6	(Manges et al., 2008)
MSHS 635	Isolated from a patient with cystitis; coded as UTI 7	(Manges et al., 2008)
MSHS 637	Isolated from a patient with cystitis; coded as UTI 8	(Manges et al., 2008)
MSHS 689	Isolated from a patient with cystitis; coded as UTI 9	(Manges et al., 2008)
MSHS 415	O6:H1; isolated from a patient with cystitis; coded as UTI 10	(Manges et al., 2008)
MSHS 133	O24:NM; isolated from a patient with cystitis; coded as UTI 11	(Manges et al., 2008)
MSHS 769	O4:H5; isolated from a patient with cystitis; coded as UTI 12	(Manges et al., 2008)
UTI PI 486	O11:Neg; isolated from a patient with pyelonephritis; coded as UTI 13	(Manges, Dietrich, et al., 2004)
UTI PI 141	X19; isolated from a patient with pyelonephritis; coded as UTI 14	(Manges, Dietrich, et al., 2004)

UTI PI 147	Isolated from a patient with cystitis; coded as UTI 15	(Manges, Dietrich, et al., 2004)
UTI PI 192	Isolated from a patient with cystitis; coded as UTI 16	(Manges, Dietrich, et al., 2004)
UTI PI 240	Isolated from a patient with cystitis; coded as UTI 17	(Manges, Dietrich, et al., 2004)
UTI PI 247	Isolated from a patient with cystitis; coded as UTI 18	(Manges, Dietrich, et al., 2004)
UTI PI 259	Isolated from a patient with cystitis; coded as UTI 19	(Manges, Dietrich, et al., 2004)
UTI PI 268	Isolated from a patient with cystitis; coded as UTI 20	(Manges, Dietrich, et al., 2004)
UTI PI 280	Isolated from a patient with cystitis; coded as UTI 21	(Manges, Dietrich, et al., 2004)
UTI PI 374	O18; isolated from a patient with cystitis; coded as UTI 22	(Manges, Dietrich, et al., 2004)
UTI PI 20	isolated from a patient with cystitis; coded as UTI 23	(Manges, Dietrich, et al., 2004)
UTI PI 116	isolated from a patient with cystitis; coded as UTI 24	(Manges, Dietrich, et al., 2004)
W26653	O15; isolated from a patient with sepsis; coded as upper sepsis 1	(Manges et al., 2006)
W55291	O77; isolated from a patient with sepsis; coded as sepsis 2	(Manges et al., 2006)
X19714	O86; isolated from a patient with sepsis; coded as sepsis 3	(Manges et al., 2006)
X37350	O73; isolated from a patient with sepsis; coded as sepsis 4	(Manges et al., 2006)
X47726	O11; isolated from a patient with sepsis; coded as sepsis 5	(Manges et al., 2006)
S49894	O102; isolated from a patient with sepsis; coded as sepsis 6	(Manges et al., 2006)
H15	O153; isolated from a patient with sepsis;	(Manges et al., 2006)

	coded as sepsis 7	
F46700	Isolated from a patient with sepsis; coded as sepsis 8	(Manges et al., 2006)
F55268	Isolated from a patient with sepsis; coded as sepsis 9	(Manges et al., 2006)
M32569	Isolated from a patient with sepsis; coded as sepsis 10	(Manges et al., 2006)
M4026	Isolated from a patient with sepsis; coded as sepsis 11	(Manges et al., 2006)
M49611	Isolated from a patient with sepsis; coded as sepsis 12	(Manges et al., 2006)
CFT073	Uropathogenic *E. coli* O6:K2:H1	(Mobley et al., 1990)
CFT073Δ*ompT*	Uropathogenic *E. coli* O6:K2:H1 Δ*ompT*	(Brannon et al., 2013)
BL21	F^- *dcm ompT hsdS*$_B$ (r_B^- m_B^-) *gal*	Novagen
BL21(pWSK129)	BL21(DE3) containing plasmid pWSK129	This study
BL21(p*ompT*)	BL21(DE3) expressing *ompT* from pWSK*ompT*	This study
BL21(p*ompP*)	BL21(DE3) expressing *ompP* from pWSK*ompP*	This study
BL21(p*arlC*)	BL21(DE3) expressing *arlC* from pWSK*arlC*	This study
BL21(p*pla*)	BL21(DE3) expressing *Pla* from pWSK*pla*	This study
Plasmids		
pWSK129	Low-copy number plasmid (Kan^R)	(Wang & Kushner, 1991)
pWSK*arlC*	*arlC* from Cys 6 cloned into pWSK129	This study
pWSK*pla*	*pla* cloned into pWSK129	This study
pWSK*ompT*	*ompT* from isolate Cys 6 cloned into pWSK129	This study
pWSK*ompP*	*ompP* from XL1-Blue cloned into pWSK129	This study

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Table 2. Primers used in this study

Name	Sequence 5 – 3'[a]	Use	Source
iutA_f	GGCTGGACATCATGGGAACTGG	Multiplex PCR	(Johnson & Stell, 2000)
iutA_r	CGTCGGGAACGGGTAGAATCG	Multiplex PCR	(Johnson & Stell, 2000)
fimH_f	TGCAGAACGGATAAGCCGTGG	Multiplex PCR	(Johnson & Stell, 2000)
fimH_r	GCAGTCACCTGCCCTCCGGTA	Multiplex PCR	(Johnson & Stell, 2000)
papAH_f	ATGGCAGTGGTGTCTTTTGGTG	Multiplex PCR	(Johnson & Stell, 2000)
papAH_r	CGTCCCACCATACGTGCTCTTC	Multiplex PCR	(Johnson & Stell, 2000)
papC_f	GTGGCAGTATGAGTAATGACCGTTA	Multiplex PCR	(Johnson & Stell, 2000)
papC_r	ATATCCTTTCTGCAGGGATGCAATA	Multiplex PCR	(Johnson & Stell, 2000)
papEF_f	GCAACAGCAACGCTGGTTGCATCAT	Multiplex PCR	(Johnson & Stell, 2000)
papEF_r	AGAGAGAGCCACTCTTATACGGACA	Multiplex PCR	(Johnson & Stell, 2000)
sfaS_f	GTGGATACGACGATTAACTGTG	Multiplex PCR	(Johnson & Stell, 2000)
sfaS_r	CCGCCAGCATTCCCTGTATTC	Multiplex PCR	(Johnson & Stell, 2000)

fyuA_r	CGCAGTAGGCACGATGTTGTA	Multiplex PCR	(Johnson & Stell, 2000)
kpsMII_f	GCGCATTTGCTGATACTGTTG	Multiplex PCR	(Johnson & Stell, 2000)
kpsMII_r	CATCCAGACGATAAGCATGAGCA	Multiplex PCR	(Johnson & Stell, 2000)
kpsMIII_f	TCCTCTTGCTACTATTCCCCCT	Multiplex PCR	(Johnson & Stell, 2000)
kpsMIII_r	AGGCGTATCCATCCCTCCTAAC	Multiplex PCR	(Johnson & Stell, 2000)
cnf-1_f	AAGATGGAGTTTCCTATGCAGGAG	Multiplex PCR	(Johnson & Stell, 2000)
cnf-1_r	CATTCAGAGTCCTGCCCTCATTATT	Multiplex PCR	(Johnson & Stell, 2000)
hlyA_f	AACAAGGATAAGCACTGTTCTGGCT	Multiplex PCR	(Johnson & Stell, 2000)
hlyA_r	ACCATATAAGCGGTCATTCCCGTCA	Multiplex PCR	(Johnson & Stell, 2000)
ompT_mf	TTTGATGCCCCAGATATCTATCGG	Multiplex PCR	This study
ompT_mr	GGCTTTCCTGATATCCGGCCATG	Multiplex PCR	This study
arlC_mf	GATTCTTGCTACTGCACTCTCAGCTCC	Multiplex PCR	This study
arlC_mr	CTGGAGTACAGAGAAGTATCACC	Multiplex PCR	This study
ompP_mf	TGCTTCTGATTTCTTCGGCC	Multiplex PCR	This study
ompP_mr	GTAGTTTGTCTTACATAATGCTC	Multiplex PCR	This study
chuA_f	GACGAACCAACGGTCAGGAT	Phylogenetic typing	(Clermont et al., 2000)

chuA_r	TGCCGCCAGTACCAAAGACA	Phylogenetic typing	(Clermont et al., 2000)
yjaA_f	TGAAGTGTCAGGAGACGCTG	Phylogenetic typing	(Clermont et al., 2000)
yjaA_r	ATGGAGAATGCGTTCCTCAAC	Phylogenetic typing	(Clermont et al., 2000)
TSPE4.C2_f	GAGTAATGTCGGGGCATTCA	Phylogenetic typing	(Clermont et al., 2000)
TSPE4.C2_r	CGCGCCAACAAAGTATTACG	Phylogenetic typing	(Clermont et al., 2000)
ompT_cf	CATGTCTAGACCACGACTTAGAAGTTCCTAGAACG	Cloning	This study
ompT_cr	GCGAGCTCAAATCTGGTTAACTTCGTTAA	Cloning	This study
ompP_cf	GCATAGTCTAGATCCTGTAGTTGCGTCAGGCCCTCCA	Cloning	This study
ompP_cr	GCATAGCTGCAGTCCGGGTAATCCAGGTCCGCCACT	Cloning	This study
arlC_cf	CATGTCTAGACCCGGCATAAAGTGTCC	Cloning	This study
arlC_cr	CTAGGAGCTCATCGTTGAGCACATATAC	Cloning	This study
ompT_sf	ATGCGGGCGAAACTTCTGGGAATAG	Southern blot probe	This study
ompT_sr	TCCCAATTAATTGCACCTTTAATAATT	Southern blot probe	This study
arlC_sf	GATTCTTGCTACTGCACTCTCAGCTCC	Southern blot probe	This study
arlC_sr	CTAGGAGCTCATCGTTGAGCACATATAC	Southern blot probe	This study
rpoD_qf	GCTGGAAGAAGTGGGTAAAC	qPCR	This study

rpoD_qr	TAATCGTCCAGGAAGCTACG	qPCR	This study
ompT_qf	CAGCGGCTGGGTGGAAGCAT	qPCR	(Thomassin, Brannon, Gibbs, et al., 2012)
ompT_qr	ACCCGATTCCATGCGCCTTCA	qPCR	(Thomassin, Brannon, Gibbs, et al., 2012)
arlC_qf	AGGATCACCTATCGTAGCGATGT	qPCR	This study
arlC_qf	CGGTTCCATGTTCCTTCGACATAA	qPCR	This study

[a]Restriction sites are underlined

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Table 3. Phylogenetic distribution of UPEC clinical isolates.

	Phylogenetic groups				
	A	B1	B2	D	(B2 + D)/Total
Fecal (n = 12)	4	1	3	4	7/12
ABU (n = 10)	2	1	5	2	7/10
UTI (n = 24)	3	3	11	7	18/24
Sepsis (n = 12)	0	2	0	10	10/12
Total (n = 58)	9	7	19	23	42/58

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Gene	Fecal (*n* = 12)	ABU (*n* = 10)	UTI (*n* = 24)	Sepsis (*n* = 12)	*P* value*
iutA	1	6	14	12	*.0541*
fimH	12	8	24	12	*.5508*
papAH	3	6	10	10	*.4173*
papC	3	6	12	10	*.4263*
papEF	4	7	12	10	*.4550*
sfaS	1	1	4	0	*1.0000*
fyuA	9	7	23	11	***.0435***
kspMTII	7	7	14	8	*1.0000*
kspMTIII	0	0	2	0	*.5203*
cnf1	4	4	8	0	*.3641*
hylA	4	3	5	1	*.2078*
ompT	7	7	22	10	***.0418***

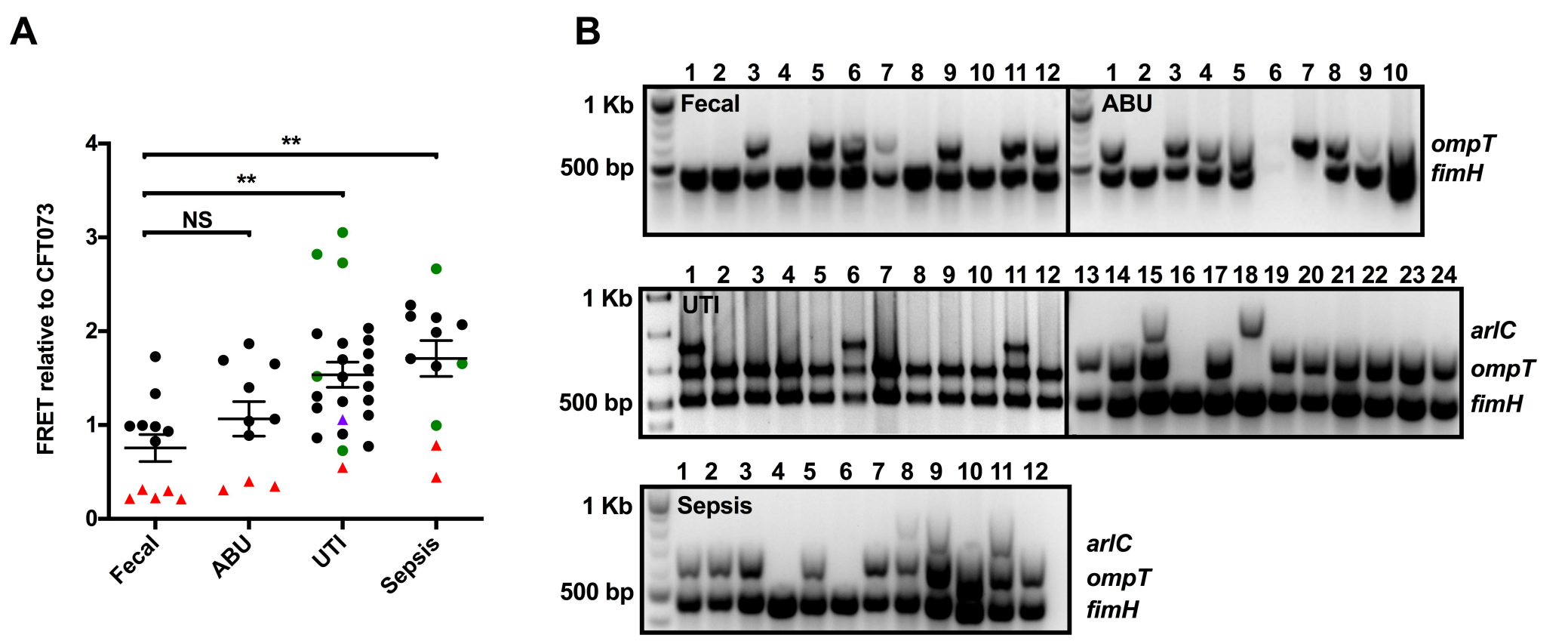
Figure 1. Omptin protease activity and distribution in clinical isolates. (A) Omptin activity was determined by monitoring fluorescence, indicative of FRET substrate cleavage, for 60 minutes. Data points indicate mean fold change in fluorescence of each isolate over the mean fold change in fluorescence of reference UPEC strain CFT073 ($\frac{\text{Area under the curve (AUC) clinical isolate}}{\text{AUC CFT073}}$) from triplicate samples. Bars represent mean ± SD fold change in fluorescence for each group. Bacteria that contain the *ompT* gene are indicated by circles and those that do not contain *ompT* are indicated by triangles. Indicated in green or purple are isolates that contain *arlC*. Statistical analysis was performed by one-way ANOVA followed by Tukey's *post hoc* test using GraphPad Prism software (NS, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$). (B) Multiplex PCR of *arlC* (852 bp), *ompT* (670 bp) and *fimH* (508 bp) from each of the clinical isolates. Amplification of *fimH* was used as a positive control. Numbers indicate isolate number for each group. Data are representative of at least three independent experiments.

Figure 2. Presence and expression of *ompT* and *arlC* among select UTI isolates. (A) Southern blot of *ompT* and *arlC* from EcoRV-treated total DNA isolated from 12 cystitis-causing isolates, as well as control strains CFT073, CFT073Δ*ompT* and plasmid DNA from pWSK*arlC*. (B) Quantitative real time PCR (qRT-PCR) of *ompT* and *arlC* from 12 clinical isolates causing cystitis, as well as from reference strain CFT073. Shown is mean ± SD of *ompT* or *arlC* expression relative to *rpoD* calculated using the $2^{-\Delta CT}$ method. Data are representative of 3 independent experiments. (C) Omptin activity of these cystitis clinical isolates was determined by monitoring cleavage of a synthetic FRET substrate for 60 minutes. Shown are mean ± SD change in fluorescence of each cystitis isolate over the change in fluorescence of reference stain CFT073 ($\frac{\text{AUC clinical isolate}}{\text{AUC CFT073}}$). Data are representative of at least three independent experiments.

Figure 3. Genomic context of *arlC* and *ompT*. Schematic representation of the genomic contexts of the *ompT* (A) and *arlC* (B) genes in cystitis isolates 1, 6 and 11. Genomic contexts of *ompT* (A) and *arlC* (B) from respective reference strains CFT073 (A) and NRG857c (B) are included for comparison. Omptin genes are indicated in dark gray, light gray indicates genes located upstream and downstream of the omptin genes, stripes indicate pseudogenes and black lines indicate intergenic space.

Figure 4. ArlC, OmpP and OmpT are functional in BL21. (A) BL21 containing empty vector (ø) or plasmids encoding *arlC*, *ompP* or *ompT* were grown until mid-log phase and normalized to OD_{595} 0.5. Proteins from whole cell preparations or isolated bacterial outer membranes were resolved by SDS-PAGE and transferred to a PVDF membrane. Omptins were detected by western blot using anti-CroP polyclonal antibodies. (B) A synthetic FRET peptide containing a dibasic motif (RK) was incubated with BL21 (open circles; control) or BL21 expressing *arlC* (filled squares; ArlC), *ompP* (filled circles; OmpP) or *ompT* (filled triangles; OmpT). Peptide cleavage, indicated by increased fluorescence, was monitored over time. Data show the mean ± SD from triplicate samples and are representative of at least three independent experiments. (C) Plasmin activation by ArlC, OmpP and OmpT. Glu-plasminogen and VLKpNA (plasmin substrate) were incubated with BL21 (open circles; control), BL21(p*pla*) (open triangles; Pla), BL21(p*arlC*) (filled squares; ArlC), BL21(p*ompP*) (filled circles; OmpP), or BL21(p*ompT*) (filled triangles; OmpT) strains. Absorbance at 405 nm was monitored over time. Data were normalized by subtracting initial absorbance from all values. Data represent mean ± SD and are representative of at least three independent experiments.

848 **Figure 5. ArlC, OmpP and OmpT cleave cathelicidins.** (A) AMP-cleavage assay. AMPs
849 were incubated with BL21 alone or BL21 expressing *arlC*, *ompP* or *ompT* for the indicated
850 times. Resulting AMP-cleavage products were separated by Tris-Tricine SDS-PAGE, fixed
851 with glutaraldehyde and visualized by coomassie staining. M indicates molecular weight
852 marker. Data are representative of three independent experiments. (B) Amino acid sequence
853 of AMPs cleaved in (A) with dibasic motifs highlighted in magenta and sequence length
854 indicated in parenthesis. (C) Far UV circular dichroism spectra (200-260 nm) of the indicated
855 peptides measured in PBS. Data were normalized by subtracting spectra from PBS alone from
856 the sample spectra. MRE indicates degree $\times$ cm^2 $\times$ $dmol^{-1}$.
857
858 **Figure 6. ArlC cleaves RNase 7.** (A) Pymol generated image of RNase 7 (Refs; PDB 2hky),
859 peptide backbone is shown in blue, cysteines and disulfide bridges are in yellow and dibasic
860 sites are coloured magenta. Numbers correspond to the following the locations of the dibasic
861 sites in the protein sequence: 1, residues 35 and 36; 2, residues 96 and 97; 3, residues 111 and
862 112. (B) Proteolytic cleavage of RNase 7. RNase 7 was incubated with BL21 containing
863 empty vector (ø) or BL21 expressing *arlC*, *ompP* or *ompT* for 60 or 90 minutes. Cleavage
864 products (arrows) were separated by SDS-PAGE and visualized by coomassie staining. M
865 indicates molecular weight marker. Data are representative of three independent experiments.
866


A
FRET relative to CFT073
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1 2 3 4 5 6 7 8 9 10 11 12
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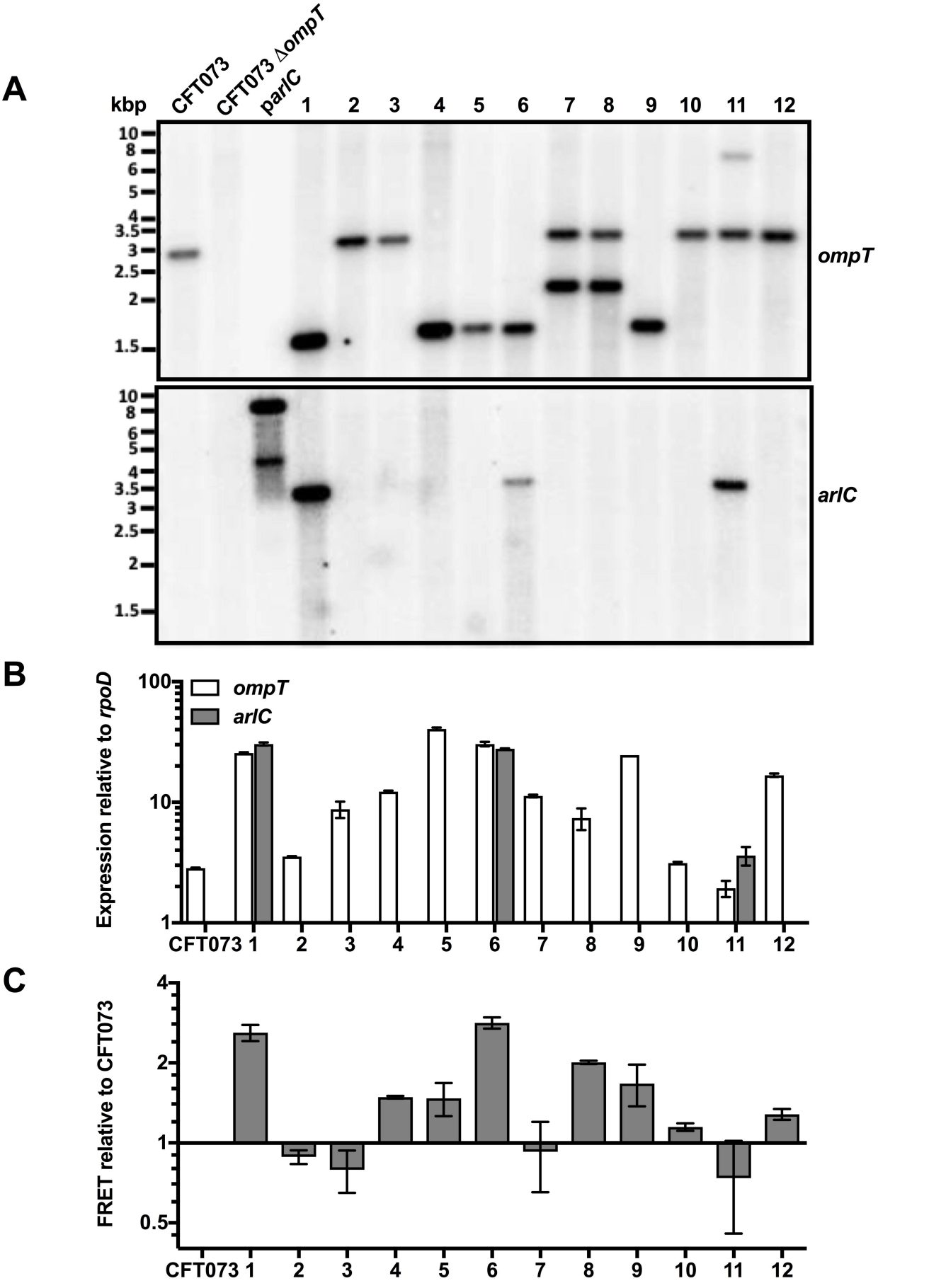
Figure 1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kbp
CFT073
CFT073 ΔompT
parlC
1
2
3
4
5
6
7
8
9
10
11
12
10
8
6
5
4
3.5
3
2.5
2
1.5
ompT
arlC
B
Expression relative to rpoD
ompT
arlC
100
10
1
CFT073
C
FRET relative to CFT073
4
2
1
0.5

Figure 2

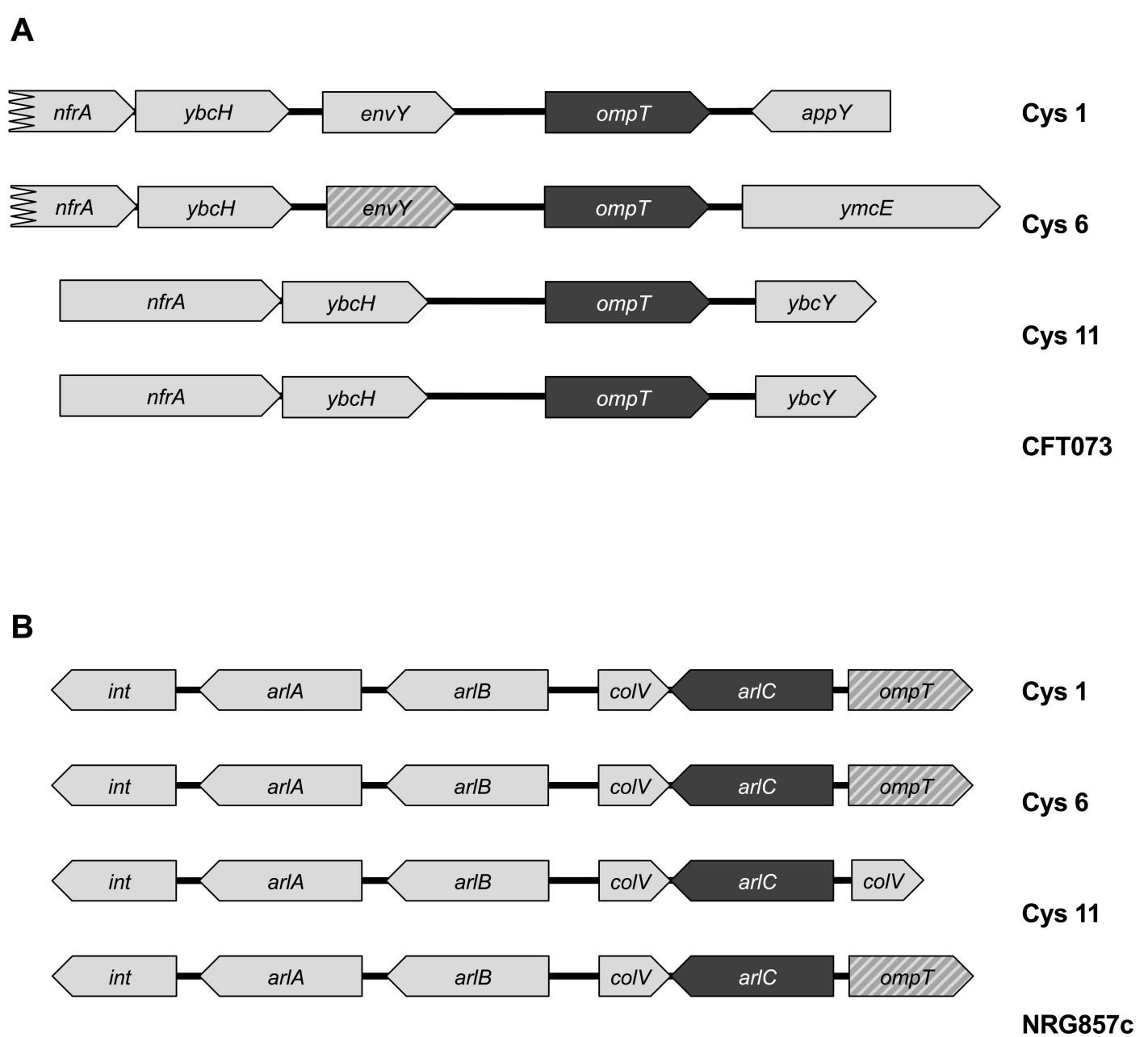

A
nfrA
ybcH
envY
ompT
appY
Cys 1
nfrA
ybcH
envY
ompT
ymcE
Cys 6
nfrA
ybcH
ompT
ybcY
Cys 11
nfrA
ybcH
ompT
ybcY
CFT073
B
int
arlA
arlB
colV
arlC
ompT
Cys 1
int
arlA
arlB
colV
arlC
ompT
Cys 6
int
arlA
arlB
colV
arlC
colV
Cys 11
int
arlA
arlB
colV
arlC
ompT
NRG857c

Figure 3

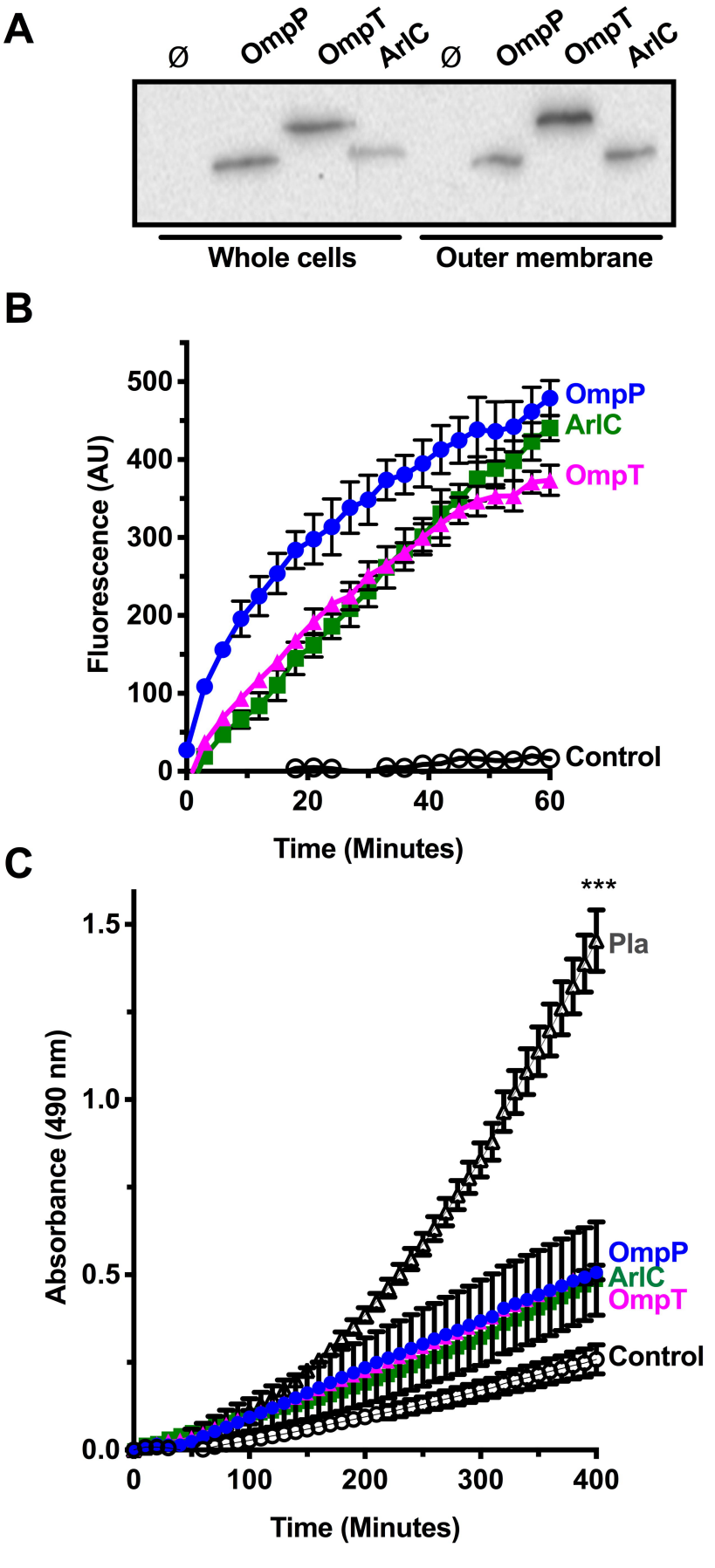

A
Ø
OmpP
OmpT
ArlC
Ø
OmpP
OmpT
ArlC
Whole cells
Outer membrane
B
Fluorescence (AU)
500
400
300
200
100
0
OmpP
ArlC
OmpT
Control
0
20
40
60
Time (Minutes)
C
***
Absorbance (490 nm)
1.5
1.0
0.5
0.0
Pla
OmpP
ArlC
OmpT
Control
0
100
200
300
400
Time (Minutes)

Figure 4

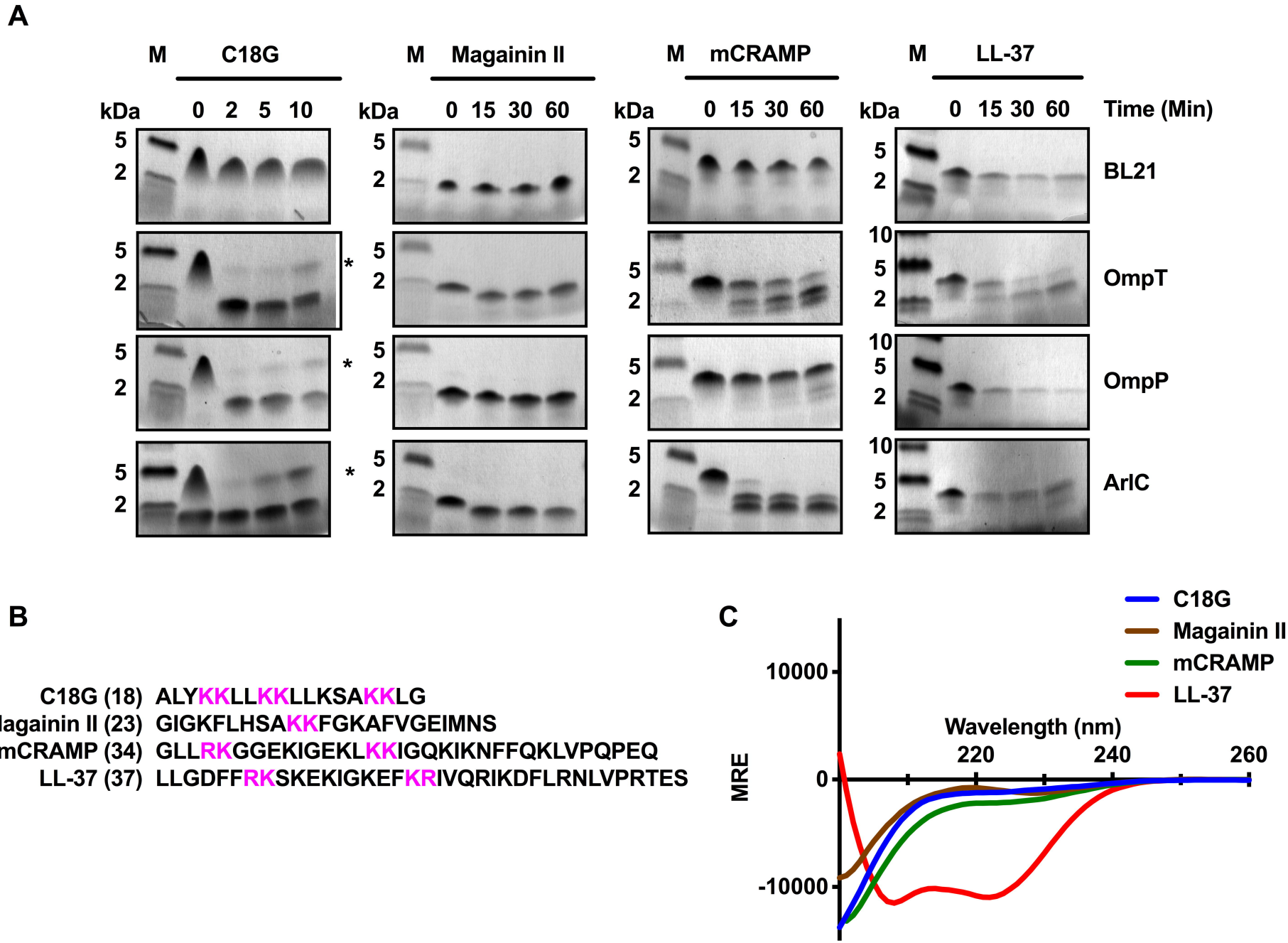
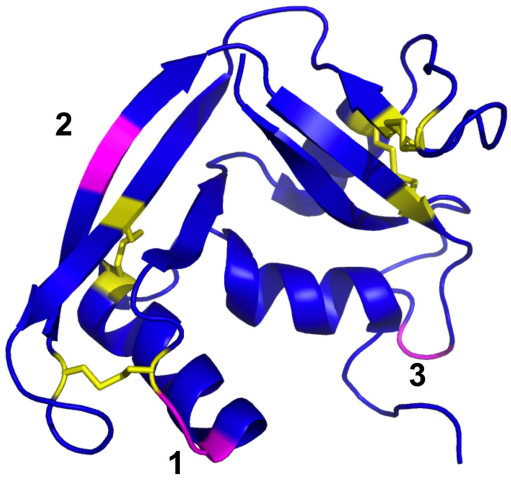

A
M
C18G
Magainin II
mCRAMP
LL-37
kDa
0 2 5 10
0 15 30 60
Time (Min)
BL21
OmpT
OmpP
ArlC
*
B
C18G (18) ALYKKLLKKLLKSAKKLG
Magainin II (23) GIGKFLHSAKKFGKAFVGEIMNS
mCRAMP (34) GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ
LL-37 (37) LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
C
C18G
Magainin II
mCRAMP
LL-37
Wavelength (nm)
220
240
260
MRE
10000
0
-10000

Figure 5

A



B

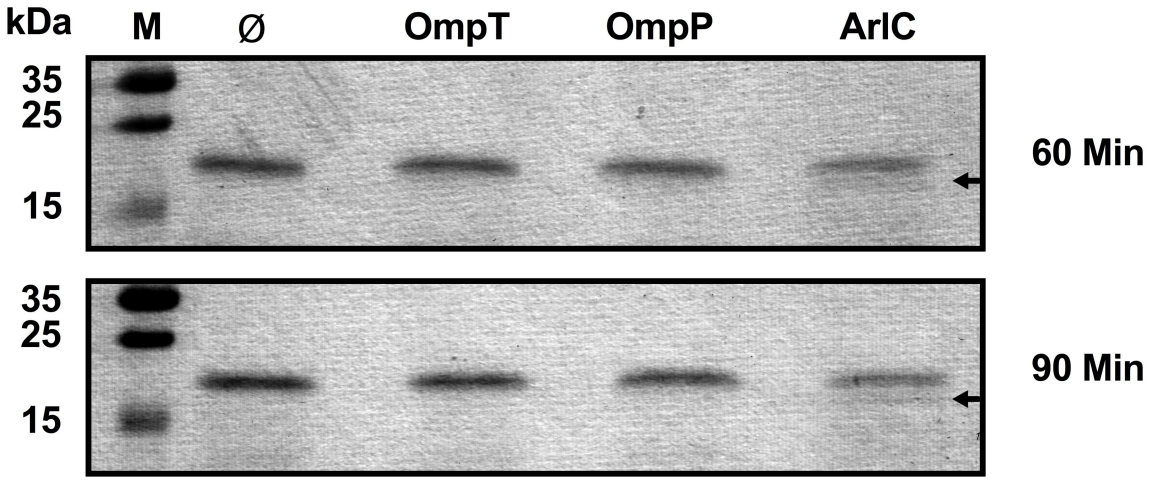


Figure 6