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PicU, a second serine protease autotransporter of uropathogenic *Escherichia coli*

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Abstract

Escherichia coli is the major ætiological agent of urinary tract infections (UTI). Like diarrhoeagenic strains of *E. coli*, uropathogenic isolates possess virulence determinants that distinguish them from commensal strains and allow them to produce the clinical manifestations associated with UTI. Several autotransporter proteins have been associated with the ability of *E. coli*, and other Gram-negative bacteria, to cause disease. Recently, we described the existence within uropathogenic *E. coli* (UPEC) strains of Sat, a toxin of the serine protease autotransporter of *Enterobacteriaceae* (SPATE) subfamily. Using features common to proteins secreted via the autotransporter pathway we have identified nine additional autotransporter proteins from the genomic sequence data of UPEC CFT073. Surprisingly, two additional members of the SPATE subfamily were identified. One protein, designated PicU, was homologous to the Pic protein identified in *Shigella flexneri* and enteroaggregative *E. coli*. The PicU protein was expressed and investigated for functional activity.

Keywords: Autotransporter; Type V secretion; Uropathogenic

1. Introduction

Urinary tract infections (UTI) are among the most common extraintestinal mucosal infections in the developed world. Individuals most susceptible to UTI include neonates, sexually active women and the elderly. Figures for 1997 reveal that UTI accounted for 8.7 million physician visits and 2.3 million hospital visits [1], indicating that UTI are the most commonly diagnosed kidney and bladder disorder in the USA.

Escherichia coli is the ætiological agent in the vast majority of UTI, causing both cystitis and pyelonephritis [2]. In cystitis, infection is limited to the bladder and is characterised by dysuria coupled with frequency and urgency of micturition. In pyelonephritis, the less common of the two diseases, infection ascends to the kidneys giving rise to the potential for bacteraemia. It remains unclear whether all uropathogenic *E. coli* (UPEC) are capable of causing cystitis and pyelonephritis or whether specific subgroups are associated with each clinical syndrome [2]. However, it appears that UPEC possess an array of virulence factors which mediate UTI and which are not normally associated with faecal strains of *E. coli*. These virulence factors include various adhesins, capsule, aerobactin, toxins and proteases [3–5]. Interestingly, half of all UPEC isolates possess none, or only one, of the virulence factors characterised thus far and, as such, it is reasonable to assume other, as yet uncharacterised, bacterial factors may be important in the pathogenesis of UTI [6].

Autotransporter proteins are a rapidly expanding family of proteins secreted via the Type V secretion pathway, the mechanism of which has been adequately reviewed else-

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Abbreviations: EAEC, enteroaggregative *Escherichia coli*; SPATE, serine protease autotransporter of *Enterobacteriaceae*; UPEC, uropathogenic *Escherichia coli*; UTI, urinary tract infection; PCR, polymerase chain reaction

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where [7–9]. Proteins secreted via this pathway may be surface located on the bacterium or released into the extracellular milieu. In every case thus far, characterised autotransporter proteins have been implicated in the ability of the bacterium to cause disease [7]. After the discovery and characterisation of Sat [29] and reports identifying multiple autotransporters in single strains of *E. coli* [10,11], in silico analyses of the *E. coli* CFT073 genome sequence were performed to identify additional uncharacterised autotransporter proteins associated with uropathogenesis. These analyses led to the identification of PicU, a homologue of the Pic serine protease autotransporter identified in *Shigella flexneri* and enteroaggregative *E. coli* (EAEC) [12]. Here we show that PicU is processed and naturally expressed in UPEC and is functionally similar to the EAEC homologue.

2. Materials and methods

2.1. In silico analyses

The genome sequence of *E. coli* strain CFT073 was searched with BLAST (http://magpie.genome.wisc.edu/cgi-bin/Authenticate.cgi/uwgp_blast.html) by using the amino acid sequences of previously characterised autotransporters from *E. coli* (Pet, antigen 43), *Helicobacter pylori* (VacA), *Serratia marcescens* (Ssph-1) and *Pseudomonas aeruginosa* (EstA), which represent an array of the evolutionary branches of the autotransporter family [13]. Open reading frames (ORFs) fitting the profile of autotransporter proteins were screened against the non-redundant GenBank databases (http://www.ncbi.nlm.gov/blast) to identify proteins with closest homology. Sequence alignments and analyses, including prediction of signal peptides, were performed using the suite of bioinformatic programmes provided by the Swiss Institute of Bioinformatics ExPASy server (http://www.expasy.ch). Promoter and Shine–Dalgarno regions were predicted from visual comparison of the nucleotide sequence with the respective canonical motifs.

2.2. Bacterial strains and plasmids

E. coli CFT073, a known uropathogenic strain, was isolated from a patient with acute pyelonephritis during the course of an antibiotic treatment study in the USA [14]. *E. coli* HB101 was used for genetic manipulations [15]. *E. coli* strains transformed with previously constructed plasmids expressing full-length Pic (pPic1) [12], a Pic serine protease mutant (pPicS258I) [12] and the homologous Pet protein (pCEFN1) [16] were used as controls. The plasmid pCR2.1-TOPO (Invitrogen) was used as a cloning vector. Strains were passed routinely on Luria–Bertani broth (L-broth) or agar with the following antibiotic supplements where appropriate: ampicillin (100 μg ml^{-1}) and tetracycline (10 μg ml^{-1}). All strains were stored at −80°C in L-broth with 25% glycerol.

To determine the prevalence of *picU* a total of 515 UTI and 200 rectal strains from various *E. coli* collections [17] were used. The first UTI group included *E. coli* isolates obtained from women aged 18–39 years, collected from the student health services of the University of Michigan and University of Texas at Austin. The second UTI group consisted of *E. coli* isolates from women in the age group 40–65 years from western Michigan and Israel. Recurring UTI isolates were *E. coli* from women at the University of Michigan student health services who presented with three or more UTI within the previous 12 months. Pyelonephritis isolates were from children in the 18–24 months age group from five hospitals in Finland. Rectal isolates were from women aged 18–39 years without UTI.

2.3. DNA manipulations

All genetic manipulations were performed by standard methods unless otherwise stated [18]. Plasmid DNA was extracted using a Plasmid Mini Kit (Qiagen). Chromosomal DNA was purified using a Genomic G/100 Tip (Qiagen) according to manufacturer's instructions. Plasmid DNA was introduced into *E. coli* HB101 by transformation of chemically competent cells according to supplier's instructions (Invitrogen).

The *picU* gene was cloned using oligonucleotide polymerase chain reaction (PCR) primers with the following sequences: upstream 5′-GGGTGATAACGTCTGGTTATC-3′ and downstream 5′-CATGGCTCATACAGAACTACC-3′. PCR amplifications were performed with 500 ng of chromosomal DNA as a template, using 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 56°C and 1 min extension by Expand (Roche) at 68°C. Products were cloned into pCR2.1-TOPO (Invitrogen) by the T-A overhang method according to the manufacturer's instructions.

DNA sequence determination was performed on an Applied Biosystems 373A automated sequencer by dye termination chemistry with Taq polymerase (Perkin-Elmer) as specified by the manufacturer: sequencing was performed by Fusion Antibodies, Belfast, Ireland.

2.4. Protein preparation and analysis

To prepare culture supernatant fractions, strains were grown overnight at 37°C in 1 l of L-broth, with shaking at 220 rpm. After centrifugation at 16 000 × *g* for 10 min, supernatant fluids were filtered (0.22 μm pore size), size fractionated and concentrated with Ultrafilters (Millipore). A 1-MDa Pellicon XL filter (Millipore) was used to remove large molecules and high molecular mass aggregates from culture supernatant fluid. The filtrates (< 1 MDa) were then concentrated to ca. 100 ml using a 30-kDa Pellicon XL cut-off filter (Millipore) and the resulting reten-

tate was finally concentrated to ca. 1 ml using a 100-kDa cut-off filter (Viva Science).

Bacterial cell envelopes were prepared by a modification of the method outlined by Caffrey and Owen [19]. Briefly, envelopes were separated from cytoplasm, following French pressure lysis of bacterial cells, by centrifugation (48 000 × *g* for 60 min at 4°C) and both fractions retained. Envelopes were resuspended in 3 ml of buffer (2% (v/v) Triton X-100, 10 mM Tris–HCl pH 7.5) and incubated at 25°C for 15 min to solubilise inner membrane components. Triton X-100-extracted envelopes were harvested by centrifugation at 48 000 × *g* for 60 min at 4°C and washed four times in 30 ml of 10 mM Tris–HCl pH 7.5. Insoluble fractions were resuspended in 1 ml 10 mM Tris–HCl pH 7.5 and stored at −20°C.

One-dimensional 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as previously described [20]. Samples were routinely heated for 5 min at 100°C in Laemmli sample buffer prior to loading. Proteins were detected by staining with Coomassie brilliant blue R-250. Western immunoblotting was performed essentially as described previously [21]. Dried skim milk (5% w/v) was used as a blocking reagent. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) was used as the localising reagent, and reacting antigens were visualised with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) as described previously [18].

To determine N-terminal amino acid sequences, proteins were separated by SDS–PAGE as before [22] and transferred to Immobilon polyvinylidene difluoride membranes (Millipore) prior to amino-terminal sequencing. Amino-terminal sequencing was performed by automated Edman degradation at Alta Bioscience, University of Birmingham, UK.

2.5. Detection of proteolytic activity

Detection of mucinolytic activity was achieved by an adaptation of the method of Colina et al. [23]. Briefly, concentrated culture supernatants were incubated for 24 h at 37°C on a medium containing 1.5% agarose, 1.0% glucose, and 0.5% bovine submaxillary mucin. Plates were then stained with 0.1% amido black in 3.5 M acetic acid for 1 h and destained with 10% acetic acid. Zones of mucin lysis were observed as discoloured halos around holes bored into the medium.

Spectrin protease activity was determined by incubating 10 μl of human spectrin solution (80 μg ml^{-1}) (Sigma) with concentrated broth culture supernatants for 4 h at 37°C. Samples were electrophoresed through 7% polyacrylamide gels and spectrin proteolysis visualised by staining with Coomassie blue R-250.

Detection of pepsin and human coagulation factor V protease activity was achieved by an adaptation of the method of Brunder et al. [24]. Briefly, 5 μl of diluted (1:5) human plasma or 60 μg of purified pepsin were mixed with equal amounts of concentrated broth culture supernatants. Reaction mixtures were adjusted to 10 μl with 150 mM phosphate-buffered saline (PBS), pH 7.2, and incubated at 37°C for 16 h. Samples were then analysed using SDS–PAGE or immunoblot analysis. Western immunoblotting was performed as described above; after blocking for 1 h, the membranes were incubated overnight with polyclonal sheep anti-human factor V antiserum, 1:5000 (Biodesign). Membranes were washed three times for 10 min each and then incubated for 2 h with alkaline phosphatase-conjugated secondary antibody: donkey anti-sheep IgG, 1:20 000 (Sigma). Bound antibody was visualised after repeated washings by incubation with NBT/BCIP as substrate in AP buffer (200 mM diethanolamine, 120 mM NaCl, 0.2 mM $MgCl_2$, pH 9.8).

2.6. Preparation and hybridisation of a picU probe

A 372-bp region of the *picU* serine protease precursor gene, that does not have homology to any of the other members of the serine protease autotransporter of *Enterobacteriaceae* (SPATE) subfamily of autotransporters, was chosen for PCR. The primers for PCR were PIC1 (5′-TCAGGCCGGTAAGAACAGCAAAAT-3′) and PIC2 (5′-ACGGTAAGAGTGTGGATGGCG-GAGTC-3′). The conditions for PCR, in a PE Biosystems 9600 thermal cycler, were as follows: 30 s at 94°C, 30 s at 55°C, 30 s at 72°C for 30 cycles, then 4 min at 72°C. The amplified *picU* serine protease precursor gene fragment was labelled with a fluorophore (ECF labeling kit, Amersham Pharmaceuticals) following manufacturer's instructions. Labeled probes were stored at −20°C. The different strains in our *E. coli* collection were spotted on nylon membranes and probed with the fluorescently labelled probe [25] using a commercially available kit (ECF random prime labeling and detection kit, Amersham) and as previously described [6].

3. Results

3.1. Identification of E. coli CFT073 autotransporter proteins

The amino acid sequences of previously characterised autotransporter proteins, including Pet [16], antigen 43 [22], VacA [26], Ssph-1 [27] and EstA [28] were used to search the genome sequence of *E. coli* CFT073. Several ORFs encoding putative autotransporter proteins were identified and further analysed for characteristics common to proteins secreted via the autotransporter pathway. Thus, 10 proteins were identified which possessed (i) an N-terminal amino acid sequence with features typical of proteins secreted via the Sec-dependent pathway, (ii) a C-terminal motif ([L/V/I/F/W/Y]-X-[W/F]) associated with

Table 1
E. coli CFT073 autotransporter proteins

Protein	ORF	Location[a]	M_r/pI[b]	Cysteine residues[c]	Signal sequence[d]	Functional motifs	Homologue (% identity)	Accession no.[e]
Sat	c3619	3 456 362–3 460 261	140 042/5.83	2 (2)	49	pfam02395 immunoglobulin A1 serine protease domain, pfam03797 autotransporter β-domain	Sat, *E. coli* vacuolating cytotoxin for bladder and kidney epithelial cells (100%)	AF289092
PicU	c0350	326 209–330 324	146 587/5.99	2 (0)	55	pfam02395 immunoglobulin A1 serine protease domain, pfam03797 autotransporter β-domain	Pic, *E. coli*/*S. flexneri* haemagglutinin mucinase (97.5%)	AF097644
YfaL	c2775	2 627 282–2 631 046	132 038/4.03	4 (4)	29	pfam03797 autotransporter β-domain	YfaL, *E. coli* K-12, putative autotransporter adhesin (82%)	D90855
Vat	c0393	371 877–376 007	148 019/5.64	2 (0)	55	pfam02395 immunoglobulin A1 serine protease domain, pfam03797 autotransporter β-domain	Tsh, *E. coli* haemagglutinin, haemoglobinase (87%)	AAA24698
UpaB	c0426	410 042–412 372	80 906/4.59	0 (0)	37	phosphopantetheine attachment site, pfam03797 autotransporter β-domain	Psyr0522, *Pseudomonas syringae* putative autotransporter adhesin (27%)	ZP_00124240
UpaC	c0478	465 478–468 465	107 003/3.92	1 (0)	27	pfam03797 autotransporter β-domain	SapA, *Salmonella typhimurium* putative autotransporter adhesion (85%)	AE008712
UpaD	c1273	1 225 454–1 228 729	113 152/6.20	2 (0)	104	RGD cell attachment sequence, ATP/GTP-binding site motif A, leucine zipper pattern, eukaryotic and viral aspartyl proteases active site, pfam03797 autotransporter β-domain	Sap, *S. flexneri* antigen 43 homologue, cell–cell aggregation and biofilm formation (92%)	AE015312
UpaE	c2895	2 748 603–2 755 766	265 217/4.72	3 (0)	56	ATP/GTP-binding site motif A, pfam03797 autotransporter β-domain	YapH, *Yersinia pestis* putative autotransporter adhesin (32%)	AJ277631
UpaF	c3655	3 491 808–3 494 936	107 190/6.07	1 (0)	54	RGD cell attachment sequence, ATP/GTP-binding site motif A, leucine zipper pattern, eukaryotic and viral aspartyl proteases active site, pfam03797 autotransporter β-domain	Sap, *S. flexneri* antigen 43 homologue, cell–cell aggregation and biofilm formation (96%)	AE015312
UpaG	c4424	4 205 984–4 211 320	177 679/4.45	0 (0)	53	COG5295 Hia, autotransporter adhesin conserved domain	SF3641, *S. flexneri* putative autotransporter adhesin (78%)	AE015372

[a]Figures denote position within the *E. coli* CFT073 genome.
[b]Figures given are predicted values.
[c]Figures in brackets represent the number of residues found in proproteins with the signal peptide removed.
[d]Figures represent the number of amino acids present in the signal sequence.
[e]Accession numbers are given for genes encoding the homologous proteins.

Table 2
Identity (%) of PicU with other members of the SPATE subfamily

Homologous protein	Precursor	PicU passenger domain	β-Domain
Pic	97.5	95.7	98.6
SepA	56.0	50.7	77.3
EatA	52.9	48.3	71.1
Orf7	52.8	46.0	80.1
EpeA	51.4	44.3	80.1
Tsh	50.0	47.1	62.1
Vat	49.2	45.8	62.1
EaaA	46.9	38.6	80.9
EaaC	46.5	38.6	80.9
EspC	44.2	35.1	79.4
EspP	41.1	31.5	80.1
Sat	40.9	31.4	79.4
Pet	40.9	31.4	80.1
SigA	39.9	30.2	79.1

integral outer membrane β-stranded proteins, (iii) few cysteine residues and (iv) a large size (>100 kDa). The characteristics of these proteins are listed in Table 1. The most notable of the autotransporter proteins identified by our in silico searches were the previously characterised SPATE protein Sat [29], and two other members of the SPATE subfamily. One putative locus demonstrated 99.9% identity with Vat, a recently described vacuolating cytotoxic SPATE of an avian septicaemic strain of *E. coli* [30]. The other locus demonstrated 96.7% identity to the previously described *pic* gene of *S. flexneri* and EAEC [12] and therefore we designated the gene encoding this locus *picU* (*Pic* of uropathogenic *E. coli*). The predicted PicU protein demonstrated between 39.9 and 97.5% identity with other members of the SPATE subfamily (see Table 2). Notably, the regions of homology were not distributed evenly across the full length of the precursor molecule, but rather the β-domains showed considerably higher identity and the passenger domains more variation.

3.2. Cloning and sequence analysis of picU

The *picU* gene is 4 275 bp long, possesses 49.95% G+C and a putative GTG initiation codon. A predicted promoter sequence was detected with a −35 site of GTGATA and a −10 site of TATGAT, beginning 50 and 35 bp upstream of the initiation codon, respectively. In addition, a Shine–Dalgarno sequence (GGAA) was detected 8 bp upstream of the initiation codon. Furthermore, an inverted repeat sequence is present downstream of the termination codon, which may form a transcription-terminating stem–loop structure consisting of an 11-bp stem and a 4-bp loop.

The *picU* gene encodes a protein comprising 1371 amino acids with a predicted p*I* of 5.99 and an M_r of 146 587 Da. A 55 amino acid signal sequence was predicted at the N-terminus of the protein. This signal sequence demonstrated homology to, and the characteristics of, an unusual

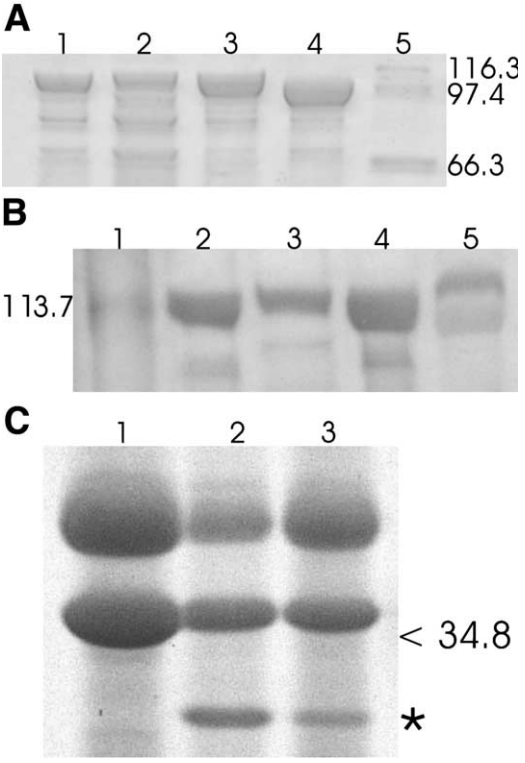

Fig. 1. SDS–PAGE analysis of PicU expression and processing. A: Analysis of concentrated culture supernatants from HB101 (pPic1) (lane 1), HB101 (pPic1S258I) (lane 2), HB101 (pPicU) (lane 3) and HB101 (pCEFN1) (lane 4). Molecular size standards are in lane 5 with masses indicated (kDa). B: Western blot analysis with anti-Pic antibodies of concentrated culture supernatants from HB101 (pPic1) (lane 2), HB101 (pPic1S258I) (lane 3), HB101 (pPicU) (lane 4) and CFT073 (lane 5). Molecular size standards are in lane 1 with masses indicated (kDa) C: Analysis of Triton X-100-insoluble fractions from HB101 (lane 1), HB101 (pPicU) (lane 2) and HB101 (pPic1) (lane 3). The position of the PicU/Pic β-subunit is indicated by an asterisk. The position of the closest molecular mass marker is shown by an arrowhead (size in kDa).

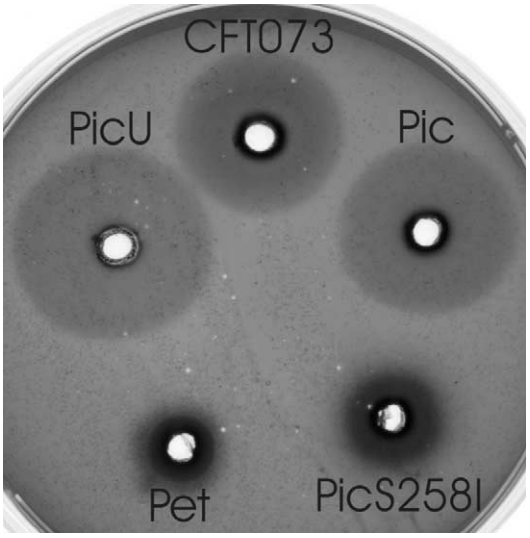

Fig. 2. Mucinolytic activity of UPEC CFT073-secreted protein. Clear zones of mucin lysis are visible around wells containing Pic, PicU and CFT073 concentrated supernatants. No lysis was observed from Pet or PicS258I.

signal sequence associated with proteins, including other members of the SPATE subfamily, which are secreted through the Srp-dependent pathway of inner membrane translocation [31]. PicU did not possess cysteine residues within the passenger domain, a facet consistent with observations for other autotransporter proteins [9]. In addition, PicU possessed a consensus serine protease motif (G^{256}DSGSP) which is characteristic of the SPATE subfamily of autotransporters, the IgA1 proteases of *Neisseria* and Hap of *Haemophilus* [32]. Indeed, examination of the predicted amino acid sequence of PicU revealed conserved aspartic acid and histidine residues (D^{158} and H^{127}, respectively) which, with the conserved serine residue of the consensus region, are presumed to form the catalytic triad of an enzyme active site reminiscent of the SA (chymotrypsin) clan of serine proteases [33].

To facilitate further analyses of PicU, the *picU* gene was amplified by PCR and cloned into pCR2.1-TOPO. The primers were located beginning 52 bp upstream and ending 105 bp downstream of the initiation and termination codons, respectively. This fragment included the predicted promoter regions. As expected, analysis of cellular compartments and culture supernatant fractions from *E. coli* HB101 containing the *picU* clone indicated the presence of a large molecular mass protein of approximately 116 kDa in the supernatant fractions and an integral membrane protein when compared to controls, suggesting that *picU* was expressed and processed (Fig. 1).

3.3. Secretion and processing of PicU

Members of the autotransporter family of secreted proteins are secreted across the outer membrane via a characteristic C-terminal domain (the β-domain) that is rich in amphipathic β-sheets and forms a pore through which the passenger domain passes to the exterior of the cell. The point at which C-terminal processing of the Pic protein occurs has not previously been described for *S. flexneri* or *E. coli*. The high identity between the C-terminal regions of Pic, PicU and other members of the SPATE family of autotransporters suggests a highly conserved cleavage site (EVN–NLN) exists between the passenger and β-domains. The point of cleavage from the passenger domain was confirmed by N-terminal amino acid sequencing, occurring between N^{1103} and N^{1104}. Such a cleavage event

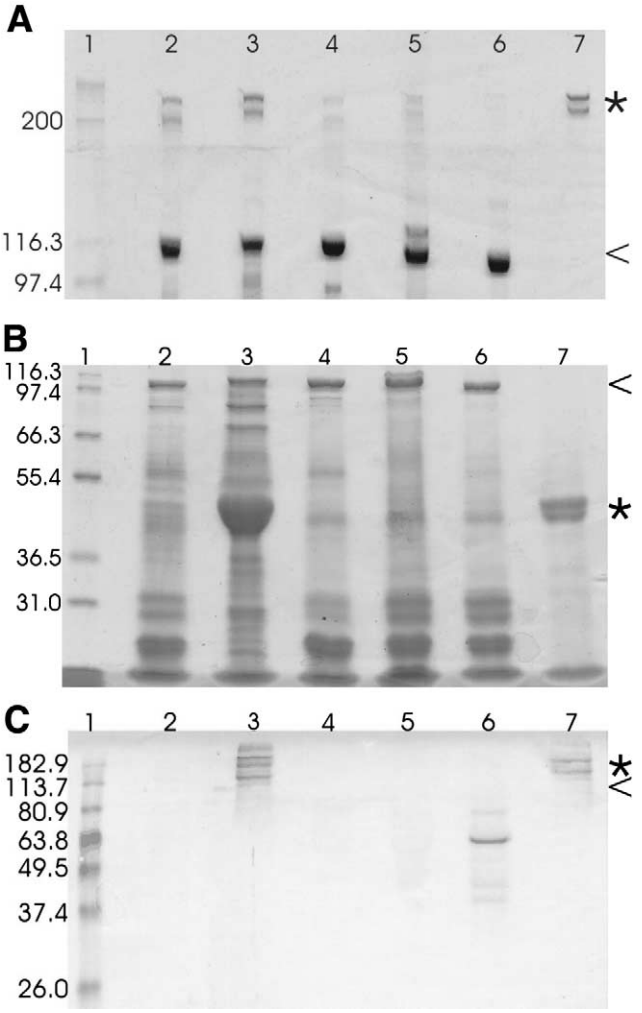

Fig. 3. Proteolytic activity of PicU. PicU and other SPATE proteins were incubated with spectrin (A), pepsin (B) and human coagulation factor V (C) and subsequently analysed by SDS–PAGE. Molecular size standards are in lane 1 with masses indicated (kDa). Lane 2 contains concentrated supernatant fractions from HB101 pPic1; lane 3, HB101 pPicS258I; lane 4, HB101 pPicU; lane 5, *E. coli* CFT073; lane 6, HB101 pCEFN1 (Pet); and lane 7 the untreated substrate protein as a negative control. Spectrin was clearly degraded by Pic, PicU, CFT073 and Pet, but not by the Pic active site mutant PicS258I. Pepsin was degraded by Pic, PicU, CFT073 and Pet, but not by the Pic active site mutant PicS258I. Western blotting was used to show that human coagulation factor V was degraded by Pic, PicU, CFT073 and Pet, but not by the Pic active site mutant PicS258I. Arrowheads indicate the approximate positions of the SPATE proteins. The positions of the native substrate proteins are designated by asterisks.

Table 3
Distribution of *picU* serine protease precursor gene by *E. coli* collection

E. coli collection	Total isolates	*picU*$^+$	(%)	Prevalence ratio	95% CI[a]
UTI					
All UTI	515	116	22.5	1.9	(1.5, 3.2)
First UTI (Michigan)	100	25	25.0	2.1	(1.2, 3.2)
UTI ages 40–65	99	22	22.2	1.9	(1.1, 3.0)
Recurring UTI	64	16	25.0	2.1	(1.2, 3.5)
Pyelonephritis	152	34	22.4	1.9	(1.1, 2.9)
First UTI (Texas)	100	19	19.0	1.6	(0.9, 2.6)
Non-UTI					
Rectal	200	24	12	1.0	Reference

[a]CI = confidence interval.

would result in a membrane-bound β-domain of 30.6 kDa, a figure which is in good agreement with the molecular mass observed by SDS–PAGE (Fig. 1C). The N-terminal amino acid sequence of the secreted PicU protein was determined, and indicated a signal peptidase cleavage event between residues A^{55} and G^{56}. These data are in agreement with that previously observed for Pic and such a cleavage event would result in a secreted protein of 109.8 kDa, a mass that agrees well with the apparent mass of 116 kDa as shown by SDS–PAGE analysis. Henderson et al. [12] showed that Pic- and PicS258I-secreted passenger domains and membrane-associated proteins were of the same size; these sizes agree well with those determined here for PicU (Fig. 1A).

3.4. Functional analysis of PicU

Previous experiments have demonstrated that Pic is a multifunctional protein which possesses mucinolytic and haemagglutinating activity in addition to its ability to confer serum resistance through interference with the complement cascade. As PicU demonstrated high levels of homology with Pic we attempted to determine if PicU possessed similar functional activities. Thus, concentrated supernatants from *E. coli* HB101 expressing PicU and UPEC CFT073 were tested for mucinolytic activity. Clear zones of mucin lysis were observed in the agarose–mucin medium around wells containing Pic, PicU and CFT073 concentrated supernatants (Fig. 2). No lysis was visible around wells containing concentrated supernatants from *E. coli* HB101 pCEFN1 (Pet) and *E. coli* HB101 pPicS258I (S^{258} to I^{258}, serine protease active site mutant) clones.

Cleavage of spectrin has been suggested to be a mechanism by which the plasmid-encoded toxin (Pet) of EAEC induces cytopathic effects [34]. Mobley et al. [14] showed that UPEC CFT073 has elevated cytotoxicity in comparison to faecal control strains, and that this was elicited by a secreted haemolysin. Hence, spectrin protease activity was determined by incubating concentrated supernatants with purified human spectrin and analysing the resultant products by SDS–PAGE and Coomassie blue R-250 staining. Spectrin was cleaved by Pic, PicU, CFT073 and Pet, but not by PicS258I (Fig. 3A), thus demonstrating the endopeptidase activity of the serine protease active site against spectrin.

EspP, a SPATE from enterohaemorrhagic *E. coli*, is a protease capable of cleaving pepsin and human coagulation factor V [24]. It has been suggested that the activity of this protein contributes to colonic infection by EHEC O157:H7. Similar activity may also be important in virulence of UPEC CFT073 in bladder/kidney infections. Pepsin and human factor V were both cleaved by Pic, PicU, CFT073 and Pet, but not by PicS258I (Fig. 3B and C, respectively), thus demonstrating the endopeptidase activities of the serine protease active site.

3.5. Distribution of picU in UTI and non-UTI isolates

We hybridised the *picU* serine protease precursor gene fragment to a total of 715 UTI and rectal *E. coli* strains. The results are shown in Table 3. The *picU* gene occurred in 22.5% of UTI *E. coli* isolates, but in only 12% of rectal *E. coli* isolates. The prevalence ratios ranged from 1.6 to 2.1 depending on the collection, and were statistically significant at the 95% confidence level for all collections except Texas first UTI isolates. There is no statistical difference between the prevalence of the *picU* in cystitis and pyelonephritis strains.

4. Discussion

The number of proteins identified in Gram-negative bacteria which are secreted via the Type V, or autotransporter, secretion pathway has grown almost exponentially since the initial description of the secretion of gonococcal IgA1 protease [35]. This expansion has been fuelled by the growing availability of microbial genome sequencing data. All of the characterised members of this family have been implicated in the pathogenesis of bacterial disease. The mechanisms by which these proteins aid in this process vary widely depending on the function of the secreted molecule which is encoded in the passenger domain of the autotransporter protein [7].

Recent studies have identified multiple autotransporter proteins encoded by single strains of bacteria [10,36]. As in other studies genes encoding multiple autotransporters were found on the *E. coli* CFT073 genome. Furthermore, the presence of 10 genes encoding putative autotransporters suggests that the Type V secretion system is the most prevalent protein secretion system in this strain. Indeed, from these data and observations from other systems it appears that the Type V system is the most common method of protein secretion among the Gram-negative bacteria [13].

Of the 10 putative autotransporter proteins identified in this study five show homology to other putative autotransporter proteins of unknown function (Table 1). The predicted proteins from two of the remaining loci (*upaD* and *upaF*) are highly homologous to antigen 43, a phase-variable protein implicated in bacterial cell–cell aggregation and biofilm formation. This protein is expressed during UTI [22,37,38]. The fact that the genes encoding UpaD and UpaF are found in different positions to the homologous gene in *E. coli* K-12, suggests that these elements are mobile. Indeed, the presence of two copies of antigen 43 has only been associated with pathogenic isolates of *E. coli*. Combined with the fact that this protein is expressed in UTI, has a complex mechanism of expression and has a role in cell–cell aggregation/biofilm formation it may be hypothesised that antigen 43 plays an important role in the pathogenesis of UTI, perhaps by contributing to persistence, a recently recognised phenomenon, and thus recurrent UTI [39].

Of the remaining three genes encoding autotransporters one was identical to the previously described SPATE autotransporter, Sat, of *E. coli* CFT073. This protein has been demonstrated to possess toxic activity against Hep-2 and Vero cells, and to cause damage to kidney cells during in vivo infection [40]. Moreover, the morphological changes induced in cells exposed to toxins from UPEC O6:K12:H6, and due to the induction of caspase-independent apoptosis of renal cells, resemble the cytopathic effects induced by Sat [41]. Surprisingly, the remaining two genes, herein designated *picU* and *vat*, also encoded members of the SPATE subfamily of autotransporters. As such the predicted proteins possess a 55 amino acid N-terminal signal sequence, a central passenger domain, and a C-terminal β-domain. The unusually long signal sequence is a feature of the SPATE subfamily and recent evidence suggests it is involved in secretion through the SRP-dependent cotranslational pathway of inner membrane translocation [31]. However, this remains controversial, as evidence from some studies appears to contradict this hypothesis [42]. Nevertheless, cleavage of the signal sequence occurs at a specific site (A^{55}–G^{56} in the case of PicU) to release the passenger and β-domains into the periplasm. The β-domain, by virtue of its amphipathic character, is involved in formation of a pore in the outer membrane through which the passenger domain is secreted. The nature of this pore remains controversial with several hypotheses abounding in the literature [35,43]. Within the SPATEs a second highly conserved cleavage site (EVN–NLN) exists between the passenger and β-domains, which allows release of the passenger domain into the extracellular milieu. Like Sat, and the other members of the SPATE subfamily, PicU possesses this conserved region and N-terminal sequencing of the cleaved β-domain demonstrated that PicU conformed to the conserved SPATE cleavage event. Similarly, PicU possesses a consensus serine protease motif and conserved aspartic acid and histidine residues which are characteristic of the SPATEs. These S, H and D residues form a catalytic triad of the enzyme active site resembling the SA chymotrypsin clan of serine proteases [33]. However, unlike Sat, PicU appears to belong to a different evolutionary branch of the SPATE subfamily [32] which do not possess toxic activity but have a variety of functions and include the proteins Tsh [44], Hbp [45], and SepA [46].

In order to ascertain the function of PicU it was necessary to clone and express the gene encoding the protein. Interestingly, every clone of *picU* obtained revealed an insert in the opposite orientation to the P_{lac} promoter of pCR2.1-TOPO, indicating that the high level expression of these proteins may be toxic to the cell, a phenomenon previously noted for other autotransporters [22,26]. However, recent evidence has demonstrated that *picU* produces mRNA transcripts during experimental infection using a mouse model, suggesting the gene encodes an expressed protein [47]. Moreover, anti-Pic antibodies recognise two species in a Western blot of concentrated *E. coli* CFT073 culture supernatants; one species reacts equally as strong as that obtained from the purified PicU and Pic proteins, whereas the other slightly smaller species is fainter and may represent a cross-reaction with Vat. Nevertheless, PicU is naturally produced by *E. coli* CFT073, such expression indicating a role for PicU in the lifestyle of this uropathogen.

Henderson et al. [12] characterised Pic as a multifunctional protease involved in enteric pathogenesis. As PicU demonstrated a high level of identity with Pic we hypothesised this protein possessed similar functional activity. However, Dutta et al. [32] compared the functional properties of seven SPATEs, including Sat and Pic, demonstrating different functional activities even for highly homologous proteins. For this reason we investigated the functional activity of PicU. We tested the proteolytic activity of PicU against mucin, pepsin, factor V and spectrin utilising Pic and Pet as controls. In each case Pic and PicU gave identical proteolytic profiles. The results of Dutta et al. [32] agree with the data presented here, in that Pic (and PicU) but not Pet had mucinolytic activity. Similarly, Pet was found to degrade pepsin, factor V and spectrin. In contrast to the previously published data, we demonstrated that Pic (and PicU) degrade pepsin and spectrin. These discrepancies might be explained by (a) too little Pic

used in the previous assay, (b) too short an incubation time to detect degradation, (c) the use of ovine rather than human spectrin in the previous assay. It is worth noting that Pic and PicU degradation of spectrin, while demonstrably repeatable, was only resolved on a low percentage SDS–PAGE gel, demonstrating a 'nicking' effect rather than the complete degradation observed with purified Pet.

We previously hypothesised that the role of Pic in mucin degradation gave intestinal pathogens a colonisation advantage by allowing the bacteria to breach the intestinal mucus barrier and interact with the underlying epithelial cells [12]. Thus, EAEC 042 Δ*pic* failed to colonise mice in competition assays with wild-type bacteria. As the urinary tract is lined with a layer of mucus, PicU may also play a role in breaching this protective layer to allow onset of disease. Indeed, previous data have shown that disruption of the urinary tract mucus layer, prior to bacterial challenge, increases the level of colonisation of the urinary tract and severity of disease [48]. Furthermore, Heimer et al. [47] demonstrated *E. coli* CFT073 *picU* mutants are less well adapted to colonising the mouse urinary tract supporting the hypothesis that PicU is involved in colonisation.

Previous epidemiological analyses of Pic demonstrated that the *pic* gene was present in 44% of EAEC isolates [49]. However, this figure may not be a true reflection of the distribution as the probe used for the hybridisation was 1200 bp in length, raising the possibility of cross-hybridisation with other members of the SPATE family and thus giving a higher positive value. It should be noted that due to the high levels of homology between *picU* and *pic*, it was not possible to design a *picU*-specific probe, thus our probe would also be expected to hybridise with the *pic* gene. Interestingly, a recent study of the ECOR collection of *E. coli* strains using a '*pic*-specific' 413-bp fragment, located in exactly the same site as our probe, only hybridised with three *E. coli* strains [50]. In the current study the *picU*/*pic* gene was present in 22.5% of UTI isolates compared to only 12% of rectal isolates (Table 3). This prevalence is similar to other known uropathogenic factors such as class III P fimbriae (*prs*) (21.2%), S fimbriae (*sfa*) (31.7%), and the toxin CNF1 (*cnf1*) (28.4%), which have prevalence ratios of similar magnitude [17]. Although the prevalence ratio for the Dr family of adhesins (*drb*) is of similar magnitude (2.1), the frequency (11.5%) is approximately half that of *picU*/*pic*. Interestingly, the frequency of *picU*/*pic* was the same for pyelonephritis and cystitis strains, suggesting that while the presence of PicU/Pic may contribute to colonisation of the urinary tract it does not contribute to progression of infection from cystitis to pyelonephritis.

In conclusion, functional analyses of PicU suggest it acts in an analogous fashion to the Pic proteins of EAEC and *S. flexneri* by degrading mucin and thus conferring a colonisation advantage. Furthermore, the higher distribution of the gene among urinary tract isolates as opposed to rectal isolates suggests that this protein enhances the ability of *E. coli* to cause UTI. Further experiments are underway to determine the exact role this protein plays in uropathogenesis.

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