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Secreted proteases: A new insight in the pathogenesis of extraintestinal pathogenic *Escherichia coli*

Rima Tapader[a], Sulagna Basu[b], Amit Pal[a,*]

[a] Division of Pathophysiology, ICMR-National Institute of Cholera and Enteric Diseases, Kolkata, West Bengal, India
[b] Division of Bacteriology, ICMR-National Institute of Cholera and Enteric Diseases, Kolkata, West Bengal, India

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ABSTRACT

Bacterial secreted proteases are the key factors that increase the virulence potential of different pathogens. Extraintestinal pathogenic *E. coli* (ExPEC) is a distinct pathotype that has unique ability to infect various body sites apart from the gastrointestinal tract causing several life-threatening diseases both in human and animals. Thus, understanding of ExPEC pathogenesis is crucial in effective management of disease caused by these pathogens. It is known that ExPEC possesses a broad spectrum of virulence factors including the secreted proteases which elude the host defence system. Recent studies have shown that high prevalence as well as the action of the secreted proteases influence the pathogenesis of ExPEC. However, literature on the secreted proteases present in ExPEC and their role in promoting virulence of ExPEC is rather limited. This review describes the distribution, characterization and the role of serine and metalloproteases secreted by diverse pathotypes of ExPEC, highlighting the significance of secreted proteases of ExPEC in pathogenesis.

1. Introduction

Microbial secreted proteases are virulence factors (VFs) that are crucially involved in the progression of diseases caused by a wide variety of pathogens. The ability to hydrolyze the peptide bonds present in proteins and peptides has endowed the proteases the ability to act as strong virulence factors. Proteases facilitate penetration of a pathogen into host tissue, target several intra and extra-cellular host molecules and subsequently play critical role in fending off the host defence (Massaoud et al., 2010; Frees et al., 2013). Several studies have documented the contribution of many such secreted proteases in pathogenesis.

E. coli that causes extraintestinal diseases such as UTI, septicaemia and meningitis is known as extraintestinal pathogenic *E. coli* or ExPEC (Poolman and Wacker, 2016). Understanding the pathogenesis of ExPEC isolates could facilitate the treatment of diseases caused by them. ExPEC is well endowed with an array of VFs including secreted proteases to combat its host and to achieve its virulence potential (Banu et al., 2011; Pitout, 2012; Bidet et al., 2012). However, there is no comprehensive review that illustrates and brings forward the importance of secreted proteases in the pathogenesis of *E. coli* causing extraintestinal infections. This review gives an in-depth analysis of extracellular proteases in pathogenesis of ExPEC, focussing on their distribution, biochemical and functional characterization and divergence.

2. Extraintestinal pathogenic *E. coli* (ExPEC)

E. coli being extremely heterogenous can remain as non-pathogenic commensal of the gut or can cause several intestinal and extraintestinal diseases (Kaper et al., 2004; Allocati et al., 2013) (Fig. 1). *E. coli* is classified on the basis of phylogroups (A, B1, B2, C, D, E and F), O:K:H serotypes and sequence types (ST) (Clermont et al., 2013; Maiden et al., 1998; Manges and Johnson, 2012; Roy et al., 2014). Commensal isolates usually lack the virulence factors (VFs) and belong to phylogroups A and B1 (Picard et al., 1999; Duriez et al., 2001; Johnson et al., 2001), whereas intestinal pathogenic *E. coli* (IPEC) isolates possess typical virulence determinants for causing diarrhoeal syndromes and generally belong to phylogroups A, B1, D or to ungrouped lineages (Pupo et al., 1997).

In contrast to commensal and intestinal pathogenic *E. coli*, ExPEC is primarily derived from phylogroup B2 and to a lesser extent from D (Picard et al., 1999; Bingen et al., 1998). Furthermore, multilocus sequence typing (MLST) of ExPEC reveals that certain lineages such as ST69, ST117 and ST131 are primarily responsible for causing extraintestinal infections in human (Manges and Johnson, 2012). ExPEC

* Corresponding author at: Division of Pathophysiology, ICMR-National Institute of Cholera and Enteric Diseases, P-33, CIT Road, Scheme-XM, Beliaghata, Kolkata, 700010, India.
E-mail address: apal7364@rediffmail.com (A. Pal).

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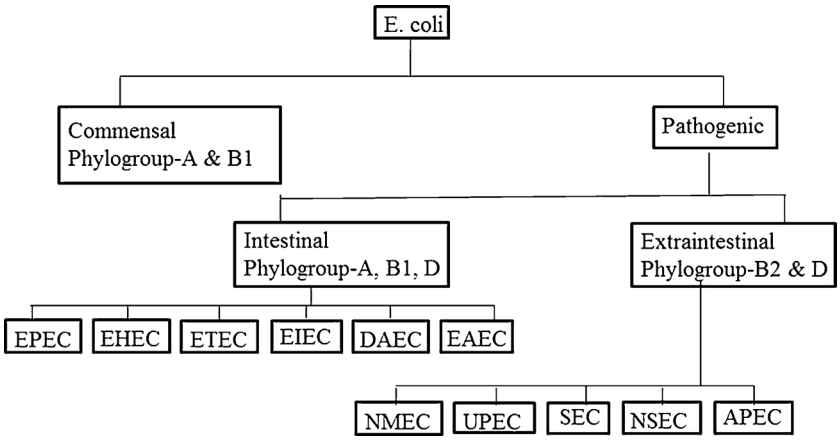

Fig. 1. Schematic representation of *E. coli* pathotypes and their corresponding phylogroups. EPEC: Enteropathogenic *E. coli*, EHEC: Enterohaemorrhagic *E. coli*, ETEC: Enterotoxigenic *E. coli*, EIEC: Enteroinvasive *E. coli*, DAEC: Diffusely adherent *E. coli*, EAEC: Enteroaggregative *E. coli*, NMEC: neonatal meningitis *E. coli*, UPEC: uropathogenic *E. coli*, SEC: septicemic *E. coli*, NSEC: neonatal septicemic *E. coli*, APEC: avian pathogenic *E. coli*.

stands out among *E. coli* as it is armed with unique VFs and is able to invade various extraintestinal tissues to cause severe disease in all age groups (Poolman and Wacker, 2016; Mellata, 2013).

The major variants of ExPEC include uropathogenic *E. coli* (UPEC), septicemic pathogenic *E. coli* (SPEC), neonatal septicemic *E. coli* (NSEC), neonatal meningitis *E. coli* (NMEC) and avian pathogenic *E. coli* (APEC) (Fig. 1). UPEC, the major variant, leads to a set of disorders ranging from asymptomatic UTI, cystitis to systemic infection like pyelonephritis. NSEC and NMEC account respectively for septicaemia and meningitis in newborns. Another predominant pathotype SPEC is implicated in community-acquired septicaemia in adults. APEC accounts for extraintestinal infection in poultry, affecting not only the respiratory tract but also the bloodstream causing massive economic loss in poultry industry (Russo and Johnson, 2003; Mellata, 2013). In addition to these, ExPEC also features in nosocomial pneumonia, cellulitis, prostatitis, peritonitis, osteomyelitis, intra-abdominal infections and infection of skin and soft tissue (Johnson and Russo, 2002; Poolman and Wacker, 2016).

3. Unique virulence factors in ExPEC

ExPEC is armed with a broad range of unique factors which not only contribute to the virulence of these isolates in multiple ways but also help them to thrive in adverse conditions (Banu et al., 2011). These include polysaccharide coating (capsule and LPS), adhesins, invasins, toxins, proteases, lipases and factors for serum resistance and iron acquisition. The details of the VFs can be found in other specific reviews (Pitout, 2012; Bidet et al., 2012). An overview of some important VFs of ExPEC is given here.

ExPEC possesses both fimbrial and afimbrial adhesins, which not only promote bacterial adhesion and invasion to host tissues but also can facilitate delivery of other bacterial toxins to host tissue and trigger host immune response (Mulvey, 2002). These include P pili (Wiles et al., 2008), S fimbriae with the subtypes like *sfaI* and *sfaII* (Parkkinen et al., 1983; Marre et al., 1990) and afimbrial adhesins (Labigne-Roussel and Falkow, 1988).

ExPEC also elaborates a number of secreted toxins such as hemolysin A (HlyA), cytotoxic necrotising factor 1 (CNF 1) and cytolethal distending toxin (CDT). HlyA forms membrane pores to lyse erythrocytes and effector immune cells (Johnson, 1991; Ostolaza and Goni,1995) and can also induce Ca^{2+} oscillations in renal epithelial cells, resulting in increased production of IL-6 and IL-8 (Uhlen et al., 2000). CNF 1 induces formation of actin stress fibers, lamellipodia, filopodia, membrane ruffle and is involved in modulation of inflammatory signalling pathways in a Rho GTPase-dependent manner (Bien et al., 2012).

In addition, ExPEC possesses some other unique parameters such as factors for serum resistance and iron acquisition, which although are not essentially linked to the causation of the disease like the toxins or invasins, are involved in the survival of the organisms in adverse conditions. Serum resistance is crucial for the isolates causing bacteremia and is mediated primarily by polysaccharide capsules and LPS (Scholl et al., 2005; Kim et al., 2003) as well as by other factors which include exopolysaccharide colonic acid (Li et al., 2005; Miajlovic et al., 2014), outer membrane protein A (OmpA) (Smith et al., 2007), TraT, an outer membrane lipoprotein (Binns et al., 1982; Pramoonjago et al., 1992), and Iss protein (Johnson, 1991). To manage the scarcity of free iron in mammalian hosts, ExPEC isolates produce certain siderophores such as aerobactin (De Lorenzo and Martinez, 1988; Zgur-Bertok et al., 1990), HPI yersiniabactin (Schubert et al., 1998; Gophna et al., 2001) and enterobactin (Johnson, 1991). These siderophores efficiently out-compete host iron-binding proteins in sequestering iron from the host environment and are readily taken up in iron bound form, by the pathogens *via* specific interactions with the bacterial cell surface receptors (Ron, 2010).

4. Secreted proteases in action in ExPEC

Proteases, the hydrolytic enzymes that degrade proteins and peptides, play essential physiological roles in both eukaryotes and prokaryotes (Hase and Finkelstein, 1993; Turk, 2006). Additionally, proteases produced and secreted by pathogenic microbes also contribute to the virulence of their producer organisms and hence in the recent times have drawn enthusiastic attention of many researchers. Secreted microbial proteases may contribute to the pathogenesis by allowing penetration of the microbes into the host tissue and subverting the host defence by exerting their effect on various intra or extra cellular targets (Massaoud et al., 2010; Frees et al., 2013).

Proteases, due to their enormous diversity of structure and function, do not fit easily into the general system of enzyme nomenclature (Jisha et al., 2013). Presently, proteases are classified based on three major criteria, nature of the functional group at the catalytic or active site, site of action on protein substrates, and their evolutionary relationships with reference to the amino acid sequence (Barett, 1994). On the basis of the nature of the functional group involved in catalysis, proteases are catalytically classified into 6 different types, metalloproteases, serine proteases, aspartic proteases, cysteine proteases, threonine proteases and glutamic proteases. The metallo, serine and aspartic proteases are the most commonly studied microbial proteases. Based on their site of action, proteases are classified into two major types, endo and exo proteases. Endopeptidases cleave internal peptide bonds of a polypeptide chain, distant from amino or carboxy termini whereas exopeptidases cleave peptide bonds proximal to the amino or carboxy termini of a polypeptide (Rawlings and Barrett, 1993; Barrett et al., 2004). This allows the exopeptidases to be further typed as aminopeptidases (site of action is at N-terminus of a polypeptide chain) and carboxypeptidases

Table 1
Classification, source, origin, substrate specificity and role in pathogenesis of different secreted proteases of ExPEC.

Protease	Classification	Source ExPEC	Origin	Substrate specificity	Role in pathogenesis
Sat (Secreted Autotransporter Toxin)	Class I SPATE	UPEC, NSEC	Chromosomal	Casein, Factor V, Spectrin	Cytopathic effects Target cytoskeletal proteins Cytoplasmic vacuolation
Vat (Vacuolating Autotransporter Toxin)	Class II SPATE	APEC, UPEC, NSEC, SPEC	Chromosomal	Still unknown	Cytoplasmic vacuolation
PicU (Protein involve in colonization)	Class II SPATE	UPEC	Chromosomal	Bovine submaxillary mucin, human spectrin, factor V	Cytopathic effects Promote adherence
Tsh (Temperature Sensitive Hemagglutinin)	Class II SPATE	APEC, UPEC	Plasmid	Mucin, Factor V	Respiratory colibacillosis Agglutination of RBCs Synergistic abcess formation
Hbp (Hemoglobin binding protein or haemoglobin Protease)	Class II SPATE	APEC	Plasmid	Haemoglobin	Intrabdominal abcess formation Degrade hemoglobin
SslE/YghJ (Secreted and surface associated lipoprotein having M60 metalloprotease domain)	M60 metalloprotease	NMEC, NSEC	Chromosomal	AAPM-pNA, Human intestinal mucins (MUC2/MUC3)	Cytotoxicity Mucinase Trigger proinflammation Promote adherence

(site of action is at C-terminus of a polypeptide chain). Based on their amino acid sequences, proteases are classified into different families and then further into different "clans" to group together sets of proteases that have diverged from a common ancestor (Rawlings and Barrett, 1993). Proteases are also classified based on their pH optima, referred to as acidic, alkaline and neutral proteases. Classification based on the active residues has been predominantly used in different literature.

ExPEC harbours a group of secreted serine proteases, the SPATEs (serine protease autotransporters of *Enterobacteriaceae)*. A particular pathotype of ExPEC, *i.e.*, neonatal septicemic *E. coli* (NSEC) however, also secretes a metalloprotease SslE (YghJ). This review provides a brief description of the different subtypes of SPATEs and SslE that have been found to be associated with diverse ExPEC. The distribution, substrate specificity and function of the secreted proteases prevalent among ExPEC are summarized in Table 1.

4.1. Serine protease autotransporters of Enterobacteriaceae (SPATEs)

As the name implies, autotransporters (ATs) are a family of proteins that mediate their own secretion through the outer membrane of Gram-negative bacteria. This secretion mechanism is also known as type V secretion, the most widespread secretion pathway for transporting proteins through outer membrane of Gram-negative bacteria. These ATs are actually quite diverse in their function and can act as lipases, esterases, adhesins and proteases. SPATE, the subfamily of autotransporters, is a group of secreted serine proteases of *Enterobacteriaceae* (Henderson and Nataro, 2001).

Provence and Curtiss described the first SPATE: the temperature-sensitive-hemagglutinin (Tsh) from an avian pathogenic *E. coli* (APEC) strain in 1994 (Provence and Curtiss, 1994). Since then, more than 20 SPATEs have been identified, secreted mostly from the pathogenic strains of *Enterobacteriaceae*. SPATEs are usually the most abundant among the secreted proteins of their producer organisms (Dautin, 2010).

4.1.1. Structure

The members that belong to the SPATE family, share conserved structural features. The secreted component and the secretion apparatus of an AT-system lie in the same polypeptide, which comprises three domains: The N-terminal signal sequence, an internal passenger domain and a C-terminal translocator or β domain (Fig. 2A). The N-terminal signal peptide is needed for targeting and export through the inner membrane. The signal sequence is essential for targeting SPATE proteins to the Sec-translocon, which catalyzes their transport through

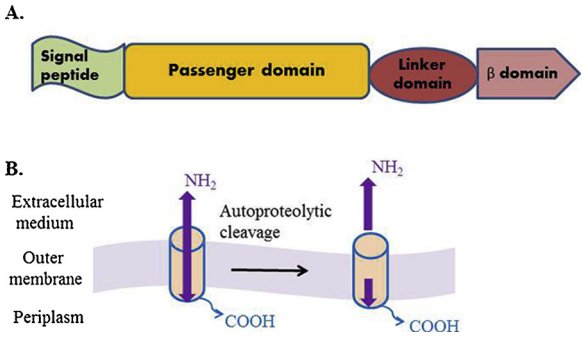

Fig. 2. General structure of SPATEs (A) and SPATEs autoproteolytic mechanism (B). (A) Structure of SPATEs shows that SPATEs comprise of an **N**-terminal signal peptide, C-terminal β domain and a passenger domain linked with β domain by a linker domain. (B) The passenger domain of SPATEs is cleaved by an autoproteolytic mechanism and is released into the extracellular medium.

inner membrane. The C-terminal domain or the translocator domain forms a β-barrel structure in the outer membrane through which the passenger domain is translocated to the cell surface. After being translocated through the outer membrane, SPATEs are autoproteolytically cleaved at a conserved cleavage site located in the β domain to release the passenger domain into the extracellular milieu (Fig. 2B) (Dautin and Bernstein, 2007; Henderson et al., 1998). The passenger domain is the functional domain which consists of an N-terminal globular domain harbouring a characteristic GDSGSP serine protease motif. The first serine of this motif forms a catalytic triad with a histidine and an asparagine residue forming a substrate binding pocket. The C-terminus of the passenger domain folds into a β-helical stalk and is thought to confer structural stability to the secreted protein. The passenger domains are variable in length (between 954 and 1050 residues). In contrast to the translocator or β-domains which are 60–99% identical among the members of SPATEs, passenger domains are only 23–50% similar by their amino acids among the members of SPATEs (Dautin, 2010). This dissimilarity among the passenger domains accounts for the distinct substrate specificities and diverse modes of action of the members of SPATEs (Dautin, 2010).

4.1.2. Classification

Multiple sequence alignment of the members of SPATE family generates different phylogenetic clusters. Phylogenetically, SPATEs are classified into two groups, class I and class II, based on the presence or absence of a domain in the globular N-terminus of the passenger

domain called domain 2. Class I SPATE proteins lack domain 2 and are cytotoxic (kill cells), whereas Class II SPATEs are positive for domain 2, possess various intra- and extracellular proteolytic targets and are cytopathic (reorganize cytoskeleton). However, in a recent study by Ruiz et al., a subset of Class II SPATEs without domain 2 has been identified, thus causing into question the use of this feature to distinguish between these two classes. Recent studies have identified two additional smaller domains in the globular N-terminus of the passenger domain, designated as domain 3 and 4. Domain 3 has a pair of cysteine residues in Class I SPATEs, but not in Class II (Ruiz-Perez and Nataro, 2014).

Members of Class I SPATEs include Pet (plasmid-encoded toxin), Sat (secreted autotransporter toxin), SigA, EspP (extracellular serine protease plasmid (p0157)-encoded), EspC (EPEC secreted protein C) and Vat (vacuolating autotransporter toxin) from *E. coli* and *Shigella flexneri*. Members of Class II SPATEs include Pic (protease involved in intestinal colonization), SepA (*Shigella* extracellular protein A), EatA (ETEC autotransporter A), Tsh (temperature-sensitive hemagglutinin) and Hbp (Hemoglobin protease) (Dutta et al., 2002; Boisen et al., 2009; Dautin, 2010) (Table 1).

Though SPATE proteins are primarily produced by the diarrheagenic pathogens like *E. coli*, *Shigella*, *Salmonella*, *Edwardsiella* and *Citrobacter* species (Boisen et al., 2009; Taddei et al., 2003; Abreu et al., 2012), some members of the family are also prevalent in *E. coli* isolates causing extraintestinal infections (Restieri et al., 2007; Parham et al., 2005; Spurbeck et al., 2012). Each subtype of SPATE family associated with ExPEC deserves individual attention to understand its role in pathogenesis.

4.1.3. Class I SPATEs in ExPEC

4.1.3.1. Sat (Secreted autotransporter toxin). Sat, a 107 kDa secreted mature protein (size of native protein is 142 kDa) was first identified in an uropathogenic *E. coli* (CFT073) isolated from blood and urine of a woman with acute pyelonephritis (Guyer et al., 2000). Subsequently, many other studies have shown significant association of Sat with UPEC, as well as with other extraintestinal *E. coli* pathotypes. Resteiri et al., examined the distribution of 13 autotransporter genes including SPATEs among UPEC, APEC, diarrheagenic *E. coli* and *E. coli* reference strains belonging to ECOR collection. Notably, Sat was found to be more significantly associated with the UPEC isolates (56%) compared to the other groups of isolates (Restieri et al., 2007). In another study, the remarkably high prevalence (75%) of Sat among the *E. coli* isolates causing UTI led the researchers to propose it as a definite diagnostic marker for molecular identification of UPEC isolates. Besides, this study also claimed that Sat can be a potential candidate as an antigen along with other determinants for the development of a new vaccine against UTI (Saraylu et al., 2012). In addition to UPEC, Sat has also been found to be highly associated with NSEC. A study to investigate the distribution of SPATEs among NSEC has identified Sat as the second most prevalent SPATE among the NSEC isolates compared to the fecal or environmental *E. coli* isolates (Tapader et al., 2014).

Sat is found to be highly homologous with other members of SPATE family, being 75% identical with Pet of enteroaggregative *E. coli* (EAEC) and 70% identical with EspP of enteropathogenic *E. coli* (EPEC).

Sat is proteolytically active against casein like Pet (Guyer et al., 2000). Sat is found to be cytotoxic, being able to induce morphological changes in kidney, bladder and other cell lines, causes detachment of cells from the monolayer and elicits immune response in mice challenged with wild type *E. coli* CFT073 (Guyer et al., 2002). Importantly, the cytotoxicity of Sat was attributed to the serine protease motif located on the passenger domain in a later study by Maroncle et al. Furthermore, this study also demonstrated that Sat targets the cytoskeletal proteins, particularly actin, fodrin and protein of β_2-integrin family of cell surface receptor (Maroncle et al., 2006). Hence, Sat is proposed as a major virulence determinant of UPEC.

4.1.3.2. Vat (Vacuolating autotransporter toxin). Vat (Vacuolating autotransporter toxin) is the least studied of all SPATEs with little information available on the characterization and function of this member of the SPATE family.

Vat, a chromosomally encoded secreted protein of 111.8 kDa (size of native protein is 148.3 kDa) was first described from a pathogenicity island (VAT-PAI) in an avian *E. coli*. VAT-PAI was found to harbour integrase genes and a dissimilar G + C content than the rest of the chromosome suggesting horizontal gene transfer of this PAI (Parreira and Gyles, 2003). After its discovery in an avian *E. coli*, Vat was subsequently found to be associated with *E. coli* isolates causing UTI, septicaemia in adults and septicaemia in neonates. Vat was found to be the most prevalent SPATE (51%) among the NSEC isolates compared to the fecal *E. coli* isolates from healthy neonates and environmental *E. coli* isolates from ground water sources (Tapader et al., 2014). Importantly, in an attempt to develop a diagnostic test to predict potential for urovirulence, *vat* was proposed as one of the four-virulence factor genes that could be used to specifically differentiate UPECs. A multiplex PCR for four genes (*vat*, *fyuA*, *chuA*, and *yfcV*) was described for an easy, rapid and prompt detection of *E. coli* isolates that can competently colonize the urinary tract (Spurbeck et al., 2012). In a recent study, Nichols et al., has found 68% prevalence of *vat* in a collection of 45 UPEC strains. Moreover, this study had investigated the regulation of Vat in UPEC. Vat was found to be highly conserved and expressed at human core body temperature. It is directly repressed by the global transcriptional regulator H-NS and is upregulated by the downstream gene *vatX* (encoding a new MarR-type transcriptional regulator). Because of these, Vat has been proposed to be highly conserved and tightly regulated in urosepsis (Nichols et al., 2016).

Vat differs from other members of SPATEs at the active site. It has alteration in the first two amino acids (ATSGSPL) in the conserved serine protease motif (GDSGSPL), which is present in other subtypes of SPATEs. These changes of residues at the active site are proposed to be responsible for the inactivity of Vat against casein (Parreira and Gyles, 2003). Notably, no specific substrate has been identified for Vat to date. The closest homologs of Vat are Tsh (the member of SPATE having hemagglutination activity) and Hbp (the haemoglobin binding SPATE). However, no vacuolating activity for Tsh and Hbp have been reported yet (Dautin, 2010). On the other hand, Vat shares only 36% homology with Sat (Parreira and Gyles, 2003) and both of these members have vacuolating activity on different cell lines.

The study by Parreira et al. showed that Vat can induce vacuolation in chicken embryo fibroblasts. Furthermore, a *vat* mutant of *E. coli,* isolated from a septicemic chicken turned out to be non-invasive and non-pathogenic in both the respiratory septicemic and cellulitis models of the disease (Parreira and Gyles, 2003), thus establishing the pathogenic potential of the vacuolating toxin Vat.

4.1.4. Class II SPATEs in ExPEC

4.1.4.1. PicU (Protein involved in colonization). After the identification and characterization of Sat from UPEC*, in silico* analysis of UPEC isolate CFT073 genome was performed to identify further uncharacterized autotransporters associated with uropathogenesis. This effort led to the identification of another secreted serine protease of UPEC known as PicU, a chromosomally encoded secreted protease of 116 kDa (size of native protein is of 146.5 kDa) (Henderson et al., 1999).

PicU is a variant of Pic, a multifunctional protease of the SPATE family and is known to be involved in enteric pathogenesis of EAEC and *Shigella flexneri* 2a. PicU exhibits 96.7% homology to the previously characterized Pic and shares between 39.9 and 97.5% identity with the other members of SPATEs with considerable variations in the passenger domain (Parham et al., 2004).

PicU possesses protease activity against a broad range of substrates such as mucin, spectrin, pepsin and coagulation factor V reflecting its virulence potential. PicU shows mucinase activity against bovine submaxillary mucin like Pic (Parham et al., 2004; Dutta et al., 2002). Of note, mucinases are known virulence determinants which aid their

producer enteric pathogens in promoting colonization. The ability of PicU to degrade the mucus layer lined in the urinary tract helps the urinary tract pathogens in breaching the mucosal barrier thereby initiating an infection (Parham et al., 2004). It is noteworthy that previous studies had already shown that rupture of mucus layer of the urinary tract prior to a bacterial infection, not only increases the level of colonization but also enhances the severity of the disease (Cornish et al., 1988). In agreement with these observations, Heimer et al. has reported that *picU* mutant of *E. coli* CFT073 shows lower degree of colonization in mouse urinary tract (Heimer et al., 2004). Apart from mucin, PicU also cleaves human spectrin like its closest homolog Pic, and another member of this family of secreted autotransporter serine protease Pet (Parham et al., 2004). The ability of PicU to cleave spectrin actually potentiates its cytopathic activity. PicU has also been demonstrated to have protease activity against pepsin and the coagulation factor V (Parham et al., 2004) like the EspP of EHEC (Brunder et al., 1997) and this could be crucial for UPEC to aid in the infection of kidney or bladder (Parham et al., 2004). With these significant functional divergences, PicU can be proposed as one of the key mediators in the pathogenesis of UPEC.

4.1.4.2. Tsh (Temperature sensitive hemagglutinin). Tsh was the first SPATE member to be discovered in which hemagglutinin or lectin-like properties was speculated. Bacterial lectins or hemagglutinins are the factors responsible for bacterial adhesion to specific carbohydrate receptors on host tissues (Alam et al., 1996; Sasmal et al., 2002). Tsh was first identified in an avian pathogenic *E. coli* isolate and was found to confer mannose-resistant hemagglutinating property in *E. coli* K12 when expressed in it (Provence and Curtiss, 1994). The association of Tsh with the lethality of avian *E. coli* isolates was determined in a later study. Tsh was found to be greatly associated with APEC isolates of high lethality group. Around sixty one percent of these isolates were *tsh* positive whereas only 30% and 9.8% of isolates belonging to low-lethal and non-lethal groups, respectively, were positive for *tsh* (Dozois et al., 2000). The association of Tsh with the virulence of APEC isolates in causing respiratory colibacillosis was further reinforced when *tsh* mutant was found to exhibit fewer and less pronounced lesions compared to wild type, in the air sacs of chicken. The mutants regained their virulence potential on complementation with *tsh,* pointing to the clear role of Tsh in the pathogenesis of APEC (Dozois et al., 2000).

The closest member of Tsh is Hbp which is almost identical to Tsh and differ from it by only two amino acid residues in the passenger domain (Dautin, 2010). Both of these are close to Vat, as already mentioned. Tsh and Hbp, being almost identical, share several functional similarities. Likewise the Hbp-producing strain, a Tsh-producing strain was able to induce a synergistic interaction with *B. fragilis* in a mouse model for intra-abdominal abscess formation (Kostakioti and Stathopoulos, 2004), suggesting that Tsh can degrade haemoglobin and can transfer heme, the breakdown product of haemoglobin to *B. fragilis*.

In addition to haemoglobin, Tsh can also bind to extracellular matrix proteins such as collagen IV and fibronectin, indicating its role in adhesion of the producer organism (Kostakioti and Stathopoulos, 2004). However, the ability of Tsh to agglutinate red blood cells or to bind other proteins does not depend on its protease activity as has been confirmed by mutation analysis of the active serine protease site.

Although discovered in APEC, a significantly higher prevalence of *tsh* has also been found among the UPEC isolates causing pyelonephritis (61%) and cystitis (65%) compared to the fecal isolates (33%) in a later study. However, the exact mode of pathogenesis of Tsh in the isolates causing UTI has not been explored till date (Heimer et al., 2004).

4.1.4.3. Hbp (Haemoglobin binding protein or haemoglobin protease). Hbp was identified in an *E. coli* strain (EB1) isolated from a patient with wound infection (Otto et al., 1998). As the name implies, Hbp can bind haemoglobin like Tsh. However, in contrast to Tsh, the ability of Hbp to degrade haemoglobin depends on its serine protease activity (Otto et al., 2002). Although Hbp and Tsh differ by only two amino acids in their passenger domains, Hbp does not possess any mannose-resistant haemagglutinating activity like Tsh (Otto et al., 1998). It needs to be mentioned, however, that like Tsh, Hbp could also synergistically induce abscess formation in a mouse model when coinfected with *B. fragilis* (Van Diemen et al., 2005). These appear to suggest that the two amino acid difference perhaps has a role to play in the haemagglutinating property of these two members of SPATE family, but are not necessarily involved in the abscess formation.

Interestingly, though Hbp has binding affinity for both heme and haemoglobin, it binds haemoglobin with a greater affinity. The proteolytic activity of Hbp against haemoglobin and its ability to bind free heme actually reflects its role in iron acquisition. There are two proposed mechanisms by which Hbp is thought to acquire iron for its producer organism. In the first one, it is conjectured that degradation of haemoglobin by Hbp releases free heme molecules in the environment which are subsequently taken up by heme receptors such as chuA present on bacterial surface. Now this is only possible if release of heme by Hbp takes place after the conversion of all Hbp molecules to holo-Hbp because Hbp itself can bind heme with a higher affinity. In the alternate mechanism it is suggested that, Hbp itself can act as a siderophore by degrading haemoglobin and then scavenging the released heme, to deliver it to bacterial cell. However, it is to be noted that no receptor has yet been recognized on the surface of bacteria for binding of Hbp (Otto et al., 2005, 2002).

The role of Hbp in the synergistic abscess formation of *E. coli* with *B. fragilis* was investigated. The ability of the culture supernatant of *E. coli* EB1 in promoting growth of *B. fragilis* in a heme-limiting environment could perhaps provide an explanation for synergy of these two organisms during intra-abdominal infection. To establish the contribution of Hbp as a hemophore, a special medium was developed with heme as growth-restricting factor for the growth-promoting studies. *B. fragilis* which was unable to grow in this medium started to grow when holo-Hbp was added. In a mouse infection model, Hbp was shown to contribute to the pathogenic synergy of abscess formation; Hbp immunized mice were found to be protected against mixed infections and did not develop intra-abdominal lesions. Moreover, a non-pathogenic *E. coli* when transformed with plasmid carrying *hbp* was found to promote increased growth of *B. fragilis* in a mixed infection besides inducing the formation of clear lesions (Otto et al., 2002).

4.2. SslE (YghJ), the cell associated and secreted lipoprotein has metalloprotease domain

SslE belongs to a large and diverse family of eukaryotic and prokaryotic proteins containing a putative metalloprotease domain, known as M60-like pfam 13402 as it is most closely related to M60-enhancin Zn metalloprotease (Nakjang et al., 2012). Proteins containing this domain are predicted to possess a signal peptide and one or more transmembrane domains or a bacterial lipoprotein motif, suggesting that these proteins are either secreted or anchored to the surface of microbial cells and hence can act on extracellular targets. Different biochemical analyses have revealed that M60-like/PF13402 containing proteins possess a characteristic HEXXH motif within the M60 metallopeptidase domain (Nakjang et al., 2012). This motif, also known as zincin motif, universally represents a broad range of well characterized Zn dependent metallopeptidases which have two histidine residues to act as ligands for Zn++ and a glutamate residue as a single catalytic amino acid for the active site (Hooper, 1994). Some Zn++ dependent metallopeptidases also possess an additional glutamate, generating the pattern HEXXHX (Marion and Guille´n, 2006; Cone et al., 2005) E (Nakjang et al., 2012) which is characteristic of gluzincin-like family of Zn-metallopeptidases, where the second glutamate acts as a potential third proteous Zn++ ligand (Hooper, 1994). Most of the M60-like/PF13402 containing proteins also possess an additional prototype Pfam

domain which is hypothesized to be involved in glycan binding and in the adhesion of the producer organism (Nakjang et al., 2012). SslE being a member of this family also has mucinase activity (Luo et al., 2014).

SslE shares closest homology with the *V. cholerae* colonization factor AcfD which also has mucinase activity. However, SslE differs both structurally and functionally from other well documented mucinases such as Pic from *S. flexneri* and EAEC (Luo et al., 2014).

The importance of SslE in ExPEC not only lies in its ability to promote virulence but also in acting as a potent immunogenic candidate for diverse ExPEC. In search of a novel vaccine candidate for ExPEC, Moriel et al., identified ECOK1-3385 as one of the potential antigens for ExPEC by "substractive reverse vaccinology" approach and showed it as the most protective antigen that provided nearly complete protection from bacteremia and mortality in a mouse model of sepsis (Moriel et al., 2010, 2012). This ECOK1-3385 has been described as SslE in a later study (Nesta et al., 2014). Furthermore, immunization with SslE was also found to protect mice against UTI and this study proposes SslE as a broadly protective vaccine against ExPEC (Nesta et al., 2014).

SslE is known to be secreted *via* type II secretion system (T2SS), a known exporting apparatus of Gram-negative bacteria to deliver various proteins including diverse virulence determinants (Korotkov et al., 2012). Among the two T2SSs of *E. coli*, T2SSβ operon is composed of three genes *yghJ*, *pppA* and *yghG*. *yghJ* encodes the SslE protein (Strozen et al., 2012), and because of this SslE was formerly named as YghJ (Iguchi et al., 2009; Yang et al., 2007). We had designated it as YghJ in our earlier studies (Tapader et al., 2016, 2017).

In our search for novel proteases from neonatal septicemic *E. coli* (NSEC), we identified SslE from the culture supernatant of a clinical NSEC isolate which we later cloned, expressed in *E. coli* TOP10 and characterized (Tapader et al., 2016). SslE was found to be proteolytically active against methoxysuccinyl Ala-Ala-Pro-Met-p-nitroanilide oligopeptide substrate, but not against casein and gelatin. SslE exhibited cytotoxicity to Int407, HT-29 and HEK293 cells, causing clear cell rounding and distortion of cellular morphology (Tapader et al., 2016). SslE stimulates the production of an array of diverse proinflammatory cytokines such as TNF-α, IL-1α and IL-1β in murine macrophages through TLR2/TLR1 heterodimer with the subsequent involvement of MyD88 to finally activate both NFκB and MAPK signalling pathways (Fig. 3) (Tapader et al., 2018). Such proinflammatory cytokines are critically implicated as one of the key mediators in pathogenesis of sepsis and are therefore considered to be important as diagnostic markers for early detection of neonatal sepsis (Netea et al., 2003; Sikora et al., 2001; Schulte et al., 2013). Of note is the fact that no virulence factor other than LPS (Ulevitch and Tobias, 1995; Poltorak et al., 1998; Fattori et al., 1994) has yet been identified for the stimulation of proinflammatory cytokines in sepsis. SslE was also found to cause substantial tissue haemorrhage in mice ileum in a dose dependent manner which could be inhibited by 1, 10 phenanthroline, a specific Zn ++ dependent metalloprotease inhibitor. This established the involvement of the M60 metalloprotease domain of SslE in causing *in vivo* tissue damage (Tapader et al., 2017). All these actually delineate the role of SslE in promoting virulence of pathogenic *E. coli* causing sepsis in neonates.

Since it is proposed that SslE is a potent vaccine candidate for ExPEC, a detailed molecular mechanism of SslE-induced activation of innate immune defence during an NSEC infection was investigated. In addition to proinflammatory cytokines, SslE was found to induce the production of other proinflammatory hallmarks such as reactive nitrogen and oxygen species and also proinflammatory chemokines such as MIP-1α, MIP-1β and RANTES in mouse macrophages. Additionally, SslE was found to overexpress MHC-II and other co-stimulatory molecules such as CD80 and CD86 on mouse macrophages (Fig. 3) (Tapader et al., 2018). All these essentially indicate that SslE of NSEC activates and polarizes macrophages toward the M1 type, crucial in framing host's innate immune response to this protein, and suggests it as a probable immunotherapeutic target against *E. coli* sepsis (Tapader et al., 2018).

The distribution of SslE among both pathogenic and non-pathogenic *E. coli* has been studied by different investigators. SslE was found to be widely distributed among the commensal isolates. However, the incidence was higher in case of intestinal and extraintestinal pathogens compared to the commensal isolates (Moriel et al., 2010; Tapader et al., 2016). Moreover, Moriel et al. have found that SslE is expressed but is not secreted by the commensal isolates (Moriel et al., 2010). In contrast, our study revealed that SslE is both expressed and secreted from fecal *E. coli* isolates, however, the expression and secretion was significantly lower compared to those from the septicemic isolates (89% vs 33%) (Tapader et al., 2016).

5. Secreted proteases in intestinal pathogenic *E. coli* (IPEC)

Although in this review we have concentrated on the secreted proteases from ExPEC in the context of distribution, characterization and function with particular emphasis on the serine and metalloproteases, a brief description of the secreted proteases of IPEC was also thought to be in order and is presented here. IPEC has six distinct pathotypes based on the virulence profile and clinical implications. These include enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), enteroaggregative (EAEC), shiga-toxin producing (STEC) and diffusely adherent *E. coli* (DAEC) (Kaper et al., 2004).

IPEC is mostly equipped with members of SPATEs and often with multiple subtypes of the SPATE family. Interestingly, the two class II SPATEs, Vat and Sat which are predominant among various pathotypes of ExPEC as already discussed are less common among IPEC. Though Sat has been reported to be present among DAEC isolates in a study by Taddei et al. (Taddei et al., 2003), there is no report on the presence of Vat among the diarrheagenic isolates. Conversely, IPEC is associated with other members of SPATE family that are generally less common among ExPEC, such as EspP (Extracellular serine protease-plasmid encoded), EspC (EPEC secreted protein C), Pet (Plasmid-encoded toxin), EspC (EPEC secreted protein C), Pic (protein involved in colonization), EatA (ETEC autotransporter A), SepA (*Shigella* extracellular protein A) and SigA.

EspP is secreted by several serogroups of EHEC, STEC and atypical EPEC strains (Brunder et al., 1997; Brockmeyer et al., 2007; Hernandes et al., 2009). Another class I SPATE, EspC, is produced by EPEC. Pet is found to be associated with EAEC (Eslava et al., 1998). Pic and SepA are found to be secreted by EAEC. A very recent study has reported the presence of SPATEs, specifically, Pic, in both typical and atypical subgroups of EAEC (Andrade et al., 2017). Isolates of ETEC are reported to produce a plasmid encoded class II SPATE, EatA.

These subtypes of SPATEs accomplish a number of pivotal functions in the pathogenesis of their producer IPEC. For instance, EspP, secreted from EHEC, STEC and atypical EPEC isolates cleaves human coagulation factor V, an important factor in blood clotting (Brunder et al., 1997). EspP also disrupts actin cytoskeleton and opens cell-cell junction, disrupts tight junction integrity in Vero cells and causes cell detachment and rounding, (Djafari et al., 1997). EspC, elaborated by EPEC exhibits enterotoxicity as detected on rat jejunal tissue mounted in Ussing chambers (Mellies et al., 2001) as well as cytotoxicity on epithelial cells *in vitro* (Navarro-Garcia et al., 1998). EspC is also able to cleave another cytoskeletal protein fodrin. Pet, another member of SPATE family has been implicated in mucosal toxicity and exfoliation of intestinal cells in EAEC induced diarrhea (Henderson et al., 1999). Pet showed enterotoxic activity on rat jejunal tissue mounted in Ussing chambers (Eslava et al., 1998; Navarro-Garcia et al., 1998). Pet exhibits cytotoxicity on HEp-2 and HT-29 cells and cleaves fodrin along with redistribution of focal adhesion complex through cleavage of FAK (focal adhesion kinase), ultimately leading to cytoskeletal disruption and cell exfoliation (Eslava et al., 1998; Dutta et al., 2002). The closest homolog of PicU, *i.e.* Pic was identified in EAEC chromosome (Henderson et al.,

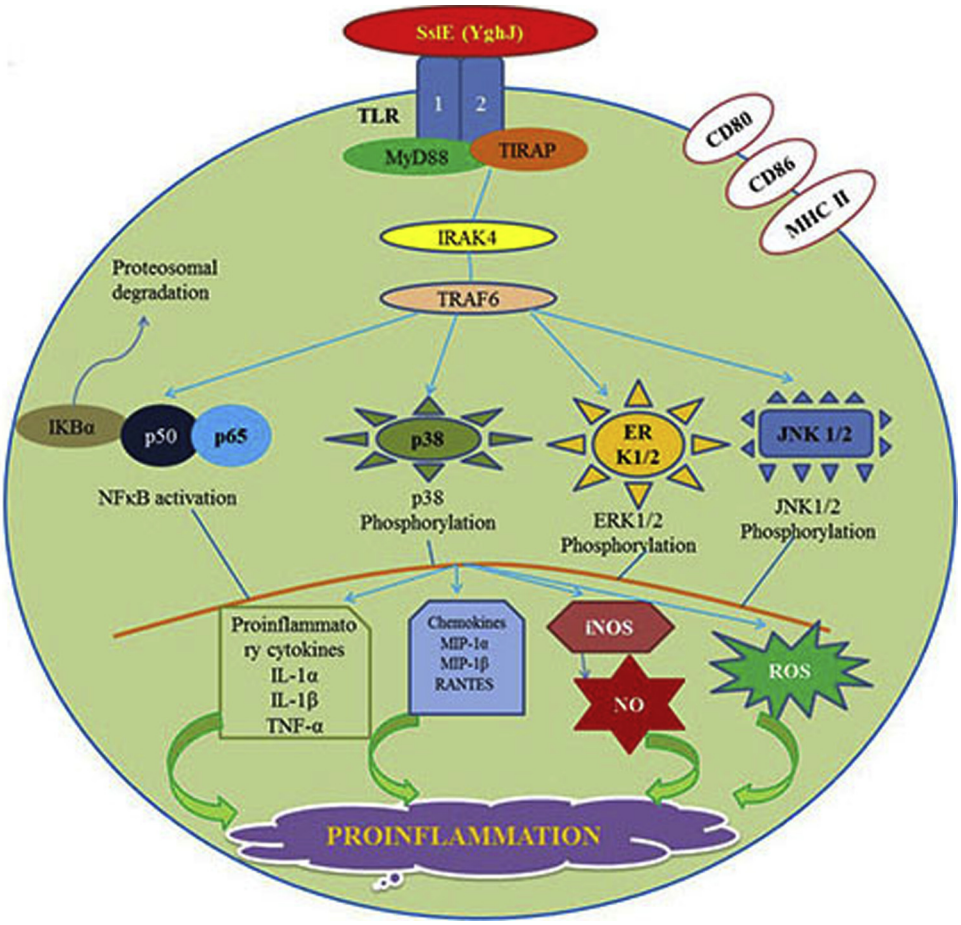

Fig. 3. Schematic representation of SslE (YghJ) induced TLR1/2 mediated proinflammation and macrophage activation with the involvement of NFκB and MAP kinase signaling pathways. SslE activates TLR1/2 heterodimer and recruits subsequent adaptors MyD88, TIRAP and TRAF6. This leads to the IκB proteosomal degradation followed by nuclear translocation of p50 and p65 and phosphorylation of p38, ERK1/2 and JNK1/2. The activation of NFκB and MAP kinase signaling pathways leads to the secretion of various proinflammatory cytokines such as TNF-α, IL-1α, IL-1β, different M1 chemokines such as MIP-1α, MIP-1β, RANTES and also other inflammatory hallmarks such as cellular ROS and NO. SslE also stimulates surface overexpression of MHC class II and co-stimulatory molecules (CD80 and CD86), leading to proinflammation and macrophage activation.

1999) and was shown to confer serum resistance. Although the exact mechanism of how this happens is not clear, it is hypothesized that Pic probably achieves this by degrading one of the components of the complement classical pathway of activation (Henderson et al., 1999). Pic also possesses mucinase activity which is evidenced by its ability to cleave the glycoproteins CD43, CD45, CD44 and PDGL1 expressed on hematopoietic cells (Ruiz-Perez and Nataro, 2014). Isolates of ETEC possess another plasmid encoded SPATE, EatA which is known to display enterotoxicity in rabbit ileal loops like the other members of SPATE. Moreover, EatA along with EtpA, an extracellular adhesin, were found to confer significant protection against ETEC infection upon co-immunization of mice (Luo et al., 2014).

Similar to serine proteases, the metalloprotease SslE has also been documented to be secreted from various IPEC isolates including ETEC and EPEC. In EPEC, SslE is required for biofilm formation and appears to be involved in pathogenesis (Baldi et al., 2012). SslE has been shown to facilitate intestinal colonization of ETEC by degrading the major mucins of the small intestine, MUC2 and MUC3. In addition, this study has shown that SslE is required for the optimal delivery of ETEC heat labile toxin (LT) (Luo et al., 2014; Valeri et al., 2015). Importantly, SslE not only contributes to the virulence of IPEC, but also is one of the potent antigens that trigger immune response in IPEC infection. In a study by Luo et al., SslE was found to be secreted by 89% of ETEC isolates distributed over diverse phylogenetic lineages and was shown to be expressed during the course of ETEC infection. This study proposed that SslE is one of the key proteins to trigger immune response during ETEC infection (Luo et al., 2015). Furthermore, both in patients infected with ETEC or mice repeatedly exposed to ETEC, SslE was identified as an immunogen along with other proteins (Roy et al., 2010) and hence needs to be given attention also for the development of ETEC vaccine.

6. Conclusion

Secreted proteases are one of the essential weapons in aggravating the virulence of pathogenic bacteria (Massaoud et al., 2010; Frees et al., 2013). Several studies on proteases have proven their importance in the pathogenesis of ExPEC (Tapader et al., 2014; Restieri et al., 2007; Parham et al., 2005). Serine proteases, specifically SPATEs, are more common among ExPEC than the metalloproteases. Several members of the SPATE family such as Sat, Vat, PicU, Tsh and Hbp are found to be secreted from variety of ExPEC and in most cases multiple subtypes are also present. The two most prevalent SPATEs among ExPEC are Sat and Vat. Although the subtypes of SPATEs share similar structure, they show widely different functional properties. The diversity in function of these serine proteases of ExPEC family establishes them as crucial factors for pathogenesis. Apart from serine proteases, metalloproteases are also secreted from ExPEC, SslE being the prime example.

Increasing antibiotic resistance among pathogenic *E. coli*, specifically the emergence of multi-drug resistant *E. coli* sequence type ST131 clone, presents a critical challenge for prevention and management of infection due to ExPEC (Rogers et al., 2011; Olesen et al., 2013; Mathers et al., 2015; Abdullah and Lakshmidevi, 2016). In this regard a prophylactic vaccine may open a new avenue for the treatment of extraintestinal infection by *E. coli*. In order to generate a potent vaccine against ExPEC, the identification and characterization of novel VFs must be considered. Secreted proteases could be a probable candidate as they contribute largely to the virulence of ExPEC. In this context, it is worthy to state that some antibodies that neutralize proteases such as SslE, can provide almost complete protection against sepsis and therefore SslE represents novel candidate vaccine target against *E. coli* sepsis.

Another major challenge in treatment of extraintestinal infection is confirmation of the disease particularly in case of sepsis. The presently

available methods for proper diagnosis of sepsis are not at all satisfactory. In this respect, secreted proteases can serve as biomarkers for rapid and easy diagnosis of UTI, sepsis or meningitis.

Pathogens possess multiple mechanisms to evade the host defences. We can benefit human health by specifically targeting these very mechanisms, and by using them as target for vaccines or for early detection to control these infections.

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