The Autotransporter Protease EspP: Crystal Structure of the Passenger Domain and Relation to Clot Formation and Stability in Human Blood

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Medical Biophysics
University of Toronto

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Abstract

Autotransporters represent a large superfamily of known and putative virulence factors produced by Gram-negative bacteria. They consist of an N-terminal “passenger domain” responsible for the specific effector functions of the molecule and a C-terminal “β domain” responsible for translocation of the passenger across the bacterial outer membrane. The serine protease autotransporters of Enterobacteriaceae (SPATEs) represent those autotransporters produced by Enterobacteriaceae where, as the name suggests, the passenger domain functions as a serine protease. Members of this family of autotransporters include among others the extracellular serine protease EspP produced by enterohemorrhagic Escherichia coli (EHEC) O157:H7.

EHEC, especially those of serotype O157:H7, have been implicated as causative agents of hemorrhagic colitis and hemolytic-uremic syndrome, both of which include disruption of the normal processes in human blood responsible for maintaining good health. EspP has previously
been shown to cleave human coagulation factors V and VIII and has been hypothesized to possibly contribute to the mucosal hemorrhage in patients infected with EHEC.

This thesis aims to better understand the functional significance of EspP in EHEC pathogenesis by analyzing the crystallographic structure of the mature passenger domain of EspP and by investigating, *in vitro*, its effects on the coagulation and fibrinolytic processes in human blood.

Like the previously determined autotransporter passenger domains, the EspP passenger domain is found to contain an extended right-handed parallel β-helix preceded by an N-terminal globular domain housing the catalytic function of the protease. Of note, however, is the absence of a second globular domain protruding from this β-helix. Furthermore, EspP is found to alter hemostasis *in vitro* by drastically decreasing the activities of human blood coagulation factors V, VII, VIII and XII, by enhancing platelet-fibrin clot formation, and by accelerating fibrinolysis. These results provide compelling evidence for a pathogenic role played by EspP during EHEC infection.
Acknowledgments

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Many thanks also to Fred Pluthero and Hong Wang for their assistance in collecting blood from my original two volunteers and analysing that blood by thrombelastography, to the staff at the Blood Transfusion Clinic at Sunnybrook for their assistance in collecting blood from my subsequent seven volunteers, and to the staff at the Special Coagulation Laboratory, especially Elena Brnjac, for their assistance, support, and training in analyzing this second set of blood samples. Thank you to Vanessa Speers for use of the mobile thrombelastograph analyzer, and to Nancy Pennell for generously donating bench space and lab equipment to use that mobile thrombelastograph analyzer. Many thanks as well to the volunteers who generously donated their blood for my research – your generosity of spirit in helping others pursue knowledge makes the advancement of science possible.

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Finally, I thank my parents, my sisters Lina, Rooya, and Leeda, and my brother Shuayb, as well as my extended family, for their unconditional love and support, and for giving me the opportunities and freedom to pursue this degree – I could not have done it without you.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Å</td>
<td>Ångström, $1 \times 10^{-10}$ m</td>
</tr>
<tr>
<td>AC</td>
<td>Autochaperone</td>
</tr>
<tr>
<td>ACL TOP</td>
<td>Automated Coagulation Laboratory Total Operational Performance</td>
</tr>
<tr>
<td>aPTT</td>
<td>Activated Partial Thromboplastin Time</td>
</tr>
<tr>
<td>ASU</td>
<td>Asymmetric Unit</td>
</tr>
<tr>
<td>β-ME</td>
<td>β-Mercaptoethanol</td>
</tr>
<tr>
<td>BPTI</td>
<td>Bovine Pancreatic Trypsin Inhibitor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>EHEC</td>
<td>Enterohemorrhagic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>ECLT</td>
<td>Euglobulin Clot Lysis Time</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionization</td>
</tr>
<tr>
<td>EspP</td>
<td>Extracellular Serine Protease, Plasmid-Encoded</td>
</tr>
<tr>
<td>FFP</td>
<td>Fresh Frozen Plasma</td>
</tr>
<tr>
<td>Fib-C</td>
<td>Fibrinogen Concentration</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>Hbp</td>
<td>Hemoglobin Protease</td>
</tr>
<tr>
<td>HBS-G</td>
<td>HEPES Buffered Saline Containing 30% (v/v) Glycerol</td>
</tr>
<tr>
<td>HC</td>
<td>Hemorrhagic Colitis</td>
</tr>
<tr>
<td>HUS</td>
<td>Hemolytic-Uremic Syndrome</td>
</tr>
<tr>
<td>IgAP</td>
<td>Immunoglobulin Protease</td>
</tr>
<tr>
<td>K</td>
<td>Thrombus Time, As Measured By TEG</td>
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LB  Lysogeny Broth
LY30  Percent Clot Lysis 30 Minutes After Reaching MA, As Measured By TEG
MA  Maximum Amplitude, As Measured By TEG
MALDI-TOF  Matrix-Assisted Laser Desorption/Ionization-Time of Flight
MOPS  3-(N-Morpholino)propanesulfonic Acid
MR  Molecular Replacement
PAGE  Polyacrylamide Gel Electrophoresis
PBS  Phosphate Buffered Saline
PBS-G  PBS Containing 25% (v/v) Glycerol
PCR  Polymerase Chain Reaction
PDB  Protein Data Bank
PEG  Polyethylene Glycol
PMSF  Phenylmethanesulfonyl Fluoride
PT  Prothrombin Time
R  Reaction Time, As Measured By TEG
R_free  Crystallographic R-Factor, Free Set
R_work  Crystallographic R-Factor, Working Set
R_merge  R-Factor, Intensity Merging
RBC  Red Blood Cell
RMSD  Root Mean Square Deviation
S263A  Ser263Ala Mutant Form of EspP
SAD  Single-Wavelength Anomalous Diffraction
SDS  Sodium Dodecyl Sulphate
SPATE  Serine Protease Autotransporters of the Enterobacteriaceae
STEC  Shiga Toxin Producing Escherichia coli
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>TCEP</td>
<td>tris(2-Carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TEG</td>
<td>Thrombelastograph</td>
</tr>
<tr>
<td>TLS</td>
<td>Translation / Libration / Screw</td>
</tr>
<tr>
<td>TT</td>
<td>Thrombin Time</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-Type</td>
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Chapter 1:

Introduction
The ability of organisms to perceive and correctly respond to their microenvironment is of critical importance to the development, maintenance and propagation of life. Consequently, biological systems have developed very intricate pathways to accomplish these critical functions. The focus of my research has been on the secreted protease EspP (extracellular serine protease, plasmid-encoded) from the Gram-negative bacterium *E. coli* O157:H7 and its effects on the processes in human blood responsible for maintaining homeostasis. In this first chapter I will begin with an introduction to the various secretion mechanisms utilized by Gram-negative bacteria for transport of protein molecules to the extracellular milieu. I will then describe in greater detail the type V secretion system, also known as the autotransporter system, of which EspP is a member. The focus will then shift to enterohemorrhagic *E. coli* and the roles it plays in human health and disease. Finally, I will give a brief introduction to human blood physiology and the various mechanisms within it responsible for maintaining good health.

### 1.1 Protein secretion in Gram-negative bacteria

Using the staining method originally developed by Danish pharmacologist and physician Hans Christian Joachim Gram, bacteria can be differentiated by their ability or inability to retain the stain Gentain violet and are thus termed Gram-positive or Gram-negative, respectively (Holt *et al.*, 1994; Desvaux *et al.*, 2009). This difference in staining is related to a profound divergence in the structural organization of the bacterial cell envelope (Desvaux *et al.*, 2009). Whereas Gram-positive bacteria are delimited by a single phospholipid bilayer, termed the cytoplasmic membrane, surrounded by a thick peptidoglycan layer, Gram-negative bacteria contain only a thin peptidoglycan layer and are further enclosed within a second bilayer, termed the outer membrane, with the space encapsulated between the two membranes termed the periplasmic space. Molecular analysis of the protein secretion pathways of Gram-negative bacteria has revealed the existence of at least three differing mechanisms for protein export across their cytoplasmic membrane (Desvaux *et al.*, 2009). These have been termed the ‘Sec’ (secretion), ‘Tat’ (twin-arginine translocation) and ‘holin’ (hole forming) pathways (Pugsley, 1993; Chaddock *et al.*, 1995; Mori and Ito, 2001; Sargent *et al.*, 2006; Desvaux *et al.*, 2009).
The type I secretion system is widespread in Gram-negative bacteria and is notable for its apparent simplicity (Masi and Wandersman, 2010). It is composed of only three proteins – an ABC (ATP binding cassette) protein that spans the cytoplasmic membrane, an adapter protein or membrane fusion protein that spans the periplasmic space and an outer membrane protein that spans the outer membrane – and is predominantly but not exclusively involved in the secretion of toxins, proteases and lipases into the extracellular milieu (Delepelaire, 2004). In contrast, the type III secretion system is composed of approximately 30 different proteins,
making it one of the most complex secretion systems identified to date (Cornelis, 2006). Together with the type IV and type VI secretion systems, the type III secretion system is most notable for its ability to deliver protein (or additionally in the case of the type IV secretion system, protein-protein and protein-DNA complexes) directly from the bacterial cytoplasm to the cytoplasm of target bacterial and eukaryotic cells (Fronzes et al., 2009; Beeckman and Vanrompay, 2010). This, however, requires direct cell-to-cell contact with the host. The type VI secretion system is further capable of mediating secretion of protein molecules into the extracellular milieu when direct cell-to-cell contact is not present.

Whereas the types I, III, IV and VI secretion systems translocate effector molecules in a single energy-coupled step across the bacterial double-membrane cell envelope and thereby bypass the periplasm, the types II, V, VII and VIII secretion systems function in a two-step process that does not bypass the periplasm. Here, substrates are synthesized with a consensus N-terminal signal peptide that first targets them to the Sec or Tat translocons to cross the inner membrane (Masi and Wandersman, 2010). Once in the periplasm, they are then translocated across the outer membrane by the dedicated machinery of the type II, V, VII or VIII secretion systems. Of these, the type II secretion system, forming a multimeric pore complex composed of between 12-16 protein components, is by far the most complex (Filloux, 2004). The types V (autotransporter/two-partner secretion), VII (chaperone-usher) and VIII (extracellular nucleation-precipitation) secretion systems on the other hand are composed of between one and three copies of a single protein component that forms a pore in the outer membrane through which substrate molecules are translocated from the periplasm to the extracellular milieu (Desvaux et al., 2009). The type VIII secretion system further contains a periplasmic chaperone that facilitates folding of the translocating substrates and targets them to the outer-membrane pore (Dodson et al., 1993). The type IV secretion system, while able to effect translocation in a single energy-coupled step across the bacterial cell envelope, is further capable of secreting effector molecules in a two-step process following their translocation into the periplasmic space via the Sec pathway.
1.2 Autotransporters

The type V secretion system was first proposed by Pohlner et al. to describe the very peculiar secretion characteristics of the exoprotein IgA1 protease from the Gram-negative bacterium *Neisseria gonorrhoeae* (Pohlner et al., 1987). These included, among others, the observations that (i) while the mature extracellular enzyme had a relative molecular mass of ~106,000, the DNA sequence producing the enzyme contained a single gene (the iga gene) that coded for a precursor of ~169,000 Da, (ii) gonococcal IgA protease was secreted not only from the original host system but also from *E. coli* strains transformed with a plasmid containing the iga gene, (iii) secretion of the mature IgA protease in the non-native host was not due to a general leakiness of the outer membrane, and (iv) 3’-terminal deletion mutants expressed in the *E. coli* host produced a complete lack of IgA protease activity in the extracellular environment but not in the periplasm. No energy coupling or accessory factors were known to be required for the secretion across the outer membrane. The term ‘autotransporter’ was soon thereafter proposed to describe the self-promoted secretion of proteins following this pathway (Jose et al., 1995).

To date over a thousand different examples have been reported of proteins that share overall similarities in structural organization and mode of translocation across the bacterial outer membrane, making the autotransporter superfamily the largest protein family in pathogenic Gram-negative bacteria (Kajava and Steven, 2006; Benz and Schmidt, 2011). However, sufficient heterogeneity exists that secretion via this pathway has been divided into three subgroups. These have been termed the type Va or classic autotransport, Vb or two-partner and Vc or trimeric autotransport secretion systems (Henderson et al., 2004).

The classic autotransport secretion system, to which the IgA1 protease from *N. gonorrhoeae* belongs, utilizes three functional domains present within a single polypeptide chain to effect secretion: an N-terminal signal sequence, a passenger domain and a C-terminal β-domain. The N-terminal signal sequence is usually about 20-50 amino acid residues in length and targets these proteins to the Sec transport machinery of the cytoplasmic membrane (Pohlner et al., 1987; Henderson et al., 1998). Translocation across this membrane then proceeds via the Sec pathway utilizing ATP as an energy source and culminates with the loss of the signal peptide. From
within the periplasm, the β-domain, also referred to as the translocator domain, then integrated into the outer membrane and forms a pore through which the rest of the protein, termed the passenger domain, translocates from the C to the N terminus onto the bacterial cell surface (Henderson et al., 2004; Ieva and Bernstein, 2009); (Figure 1.2, left panel). At this point the passenger domain can remain associated with the outer membrane, via its covalent attachment to the β-domain or in a noncovalent manner following cleavage from the β-domain. Alternatively, as is the case with many autotransporters, the passenger domain can be released free into the extracellular milieu following cleavage from the β-domain (Benz and Schmidt, 2011).

In the two-partner secretion system, the passenger and the translocator domains are not encoded within a single polypeptide chain but are instead produced as two separate molecules. Here, both the passenger, termed the TpsA, and the translocator, termed the TpsB, components contain an N-terminal signal sequence that mediates their individual translocation across the inner membrane by the Sec machinery. Once within the periplasm, specific recognition between the two components is achieved by further secretion signals present within a conserved N-terminal 250-residue-long domain, termed the two-partner secretion domain, of TpsA proteins (Hodak and Jacob-Dubuisson, 2007). Translocation of TpsA then proceeds across the outer membrane from the N to the C terminus through the TpsB (Thanassi et al., 2005) (Figure 1.2, centre panel).

Trimeric autotransporters, like classical autotransporters, are synthesized as a single polypeptide chain harbouring an N-terminal signal sequence that mediates their export across the inner membrane via the Sec machinery, a passenger domain, and a C-terminal translocator domain. Here, however, the translocator domain is generally about 70-100 amino acid residues in length and does not function as a monomer (Wells et al., 2010). Instead, it inserts into the outer membrane as a trimer to form a single pore with each monomer contributing equally to its formation (Surana et al., 2004; Wells et al., 2010). Translocation of all three passenger domains across the outer membrane then proceeds through this pore (Figure 1.2, right panel).

Sequence analysis of the translocator domains of autotransporters has shown a high propensity for formation of structures rich in β-strand elements, leading to the prediction that these domains insert into the outer membrane as β-barrels (Loveless and Saier, 1997). To date the
Figure 1.2  Schematic overview of the type V secretion system.

The secretion pathway of the classic autotransporter proteins (type Va) is depicted at the left of the diagram, the two-partner system (type Vb) in the centre and the trimeric autotransporters (type Vc) on the right. Passage through the inner membrane (IM) is initiated by the signal sequence and occurs via the Sec machinery. Passage through the outer membrane (OM) proceeds through a pore formed by the β-domain (translocator domain). Figure adapted from Thanassi et al. (Thanassi et al., 2005) with modifications.

crystal structures of six autotransporter translocator domains have been reported; these include the β-domains of the classic autotransporters NalP from Neisseria meningitides, PDB ID 1UYN (Oomen et al., 2004), EspP from Escherichia coli, PDB ID 2QOM (Barnard et al., 2007), EstA from Pseudomonas aeruginosa, PDB ID 3KVN (van den Berg, 2010), Hbp from Escherichia coli, PDB ID 3AEH (to be published) and BrkA from Dordetella pertussis, PDB ID 3QQ2 (Saurí et
al., 2011) as well as the β-domain of the trimeric autotransporter Hia from *Haemophilus influenzae*, PDB ID 2GR7 (Meng et al., 2006). Indeed all six β-domains were revealed to adopt a β-barrel architecture composed of 12 β-strands arranged in an anti-parallel fashion. However, there remains much confusion and disagreement regarding the precise mechanism by which the passenger domains are translocated through the periplasm and across the bacterial outer membrane.

Two alternative models to that discussed above and depicted in Figure 1.2 have been proposed for the translocation mechanism of the autotransporter passenger domains. The first, termed the multimer model of autotransport secretion and proposed by Veiga et al. (Veiga et al., 2002), posits that rather than translocating through the pore formed by a single β-domain, multiple β-domains first oligomerize to form a large central channel within the outer membrane; the autotransporter passenger domains are then transported through this central channel. As experimental support for this model Veiga et al. present biophysical and electron microscopic results showing that the translocator domain (45 kDa) of the classical autotransporter IgA protease from *N. gonorrhoeae* forms an oligomeric complex of ~500 kDa with a ring-like structure containing a central cavity ~2 nm in diameter. They further show this channel to have clear porin activity when embedded in artificial liposomes and to be hydrophilic in nature, suggesting, together with the large pore size, that the autotransporter passenger domains may traverse the central channel in a folded state (Veiga et al., 2002). This model, however, seems to be incompatible with the more recent findings that most translocator domains are monomeric (or trimerize to form a single β-barrel), suggesting that this mechanism would be used by, at most, only a subset of autotransporters (Bernstein, 2007).

The second, termed the Omp85 (outer membrane protein 85)-mediated model, has been proposed by HD Bernstein (Bernstein, 2007) and, taking into account the recent structural, biochemical and molecular genetic studies, posits that rather than acting alone, the autotransporter β-domain acts only to target the protein to the extracellular membrane and to serve as an anchor there; integration of the β-domain into and translocation of the passenger domain across the bacterial outer membrane are mediated by exogenous factors such as the Omp85/YaeT complex (also known as the Bam complex) involved in the assembly of other
bacterial outer membrane proteins (Voulhoux et al., 2003; Wu et al., 2005; Bernstein, 2007; Jain and Goldberg, 2007; Tommassen, 2010).

1.3 The serine protease autotransporters of Enterobacteriaceae (SPATEs)

The autotransporter β-domains are functionally limited. They serve only to target these proteins to the bacterial outer membrane and facilitate translocation of the autotransporter passenger domain across that membrane. The specific effector functions of autotransporters reside within their passenger domains, and are highly varied. The serine protease autotransporters of Enterobacteriaceae (SPATEs) represent those autotransporters produced by Enterobacteriaceae where, as the name suggests, the passenger domain functions as a serine protease (Dautin, 2010) (Figure 1.3). Members of this family include, among others, hemoglobin protease (Hbp) (Otto et al., 1998; Otto et al., 2005) and EPEC secreted protein C (EspC) (Stein et al., 1996) from enteropathogenic E. coli (EPEC), the serine protease isoforms of the immunoglobulin A1 proteases (IgAPs) produced by a number of different bacteria (Mistry and Stockley, 2006; Johnson et al., 2009), vacuolating autotransporter toxin (Vat) (Salvadori et al., 2001) and temperature-sensitive hemagglutinin (Tsh) (Provence and Curtiss, 1994) from avian pathogenic E. coli (APEC) as well as EHEC plasmid-encoded autotransporter (EpeA) (Leyton et al., 2003) and extracellular serine protease, plasmid encoded, (EspP) from enterohemorrhagic E. coli (EHEC) (Brunder et al., 1997).

The precise contributions of SPATEs to the fitness of their parent bacterium are unclear. However, although SPATE genes have been detected in non-pathogenic strains of E. coli (Sandt and Hill, 2000), they seem to be more strongly associated with bacterial species that are pathogenic to humans or animals (Dautin, 2010). Moreover, SPATEs are usually among the most abundant proteins secreted by these organisms, at least under laboratory conditions (Henderson and Nataro, 2001). These proteins are therefore thought to play an important role in pathogenesis. However, most SPATEs are produced by pathogenic species for which relevant animal models of infection do not exist, thus making a clear determination of their contribution
Figure 1.3 Phyllogenetic tree of autotransporter family proteins.

The tree was created in MEGA5 (Tamura et al., 2011) using the Maximum Likelihood method based on a ClustalW multiple sequence alignment (Larkin et al., 2007) of the full-length autotransporter genes. Listed are protein names, species abbreviations, and GenBank accession numbers.

to disease difficult (Dautin, 2010). Still, the study of these proteins in vitro has provided valuable information about their potential role in pathogenesis (Henderson and Nataro, 2001; Dutta et al., 2002; Dautin, 2010; Ruiz-Perez et al., 2011).

1.4 Enterohemorrhagic Escherichia coli

*Escherichia coli* are Gram-negative rod-shaped bacteria in the family Enterobacteriaceae. They encompass a large population that exhibit a very high degree of both genetic and phenotypic diversity. Most are normal commensals found in the intestinal tract of humans and other warm-blooded organisms. Some strains, however, are pathogenic and capable of causing mild to severe
disease. These strains are distinguished from the normal commensals by their possession of virulence factors such as exotoxins. The specific collection of virulence factors produced together with the type of disease has been used to separate these organisms into different pathotypes for better classification (CFSPH, 2009). These include the enteroadherent *E. coli* (EAEC), enteroaggregative *E. coli* (EAggEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enteroenterotoxigenic *E. coli* (ETEC) and enterohemorrhagic *E. coli* (EHEC). Of these, EHEC is associated with the most severe disease.

1.4.1 Serotypes

As with all *E. coli*, EHEC are classified into serotypes based on their O (somatic lipopolysaccharide), H (flagellar) and K (capsular) antigens. EHEC serotypes associated with disease include members of O26, O91, O103, O104, O111, O113, O117, O118, O121, O128, O145 and O157. Of these, infection with serotype O157:H7 is most common, is associated with the most severe disease and is easily differentiated biochemically from other *E. coli* serotypes. Consequently, most available information on enterohemorrhagic *E. coli* relate to this serotype.

1.4.2 Sources and transmission

Ruminants, such as cattle and sheep are the most important reservoir hosts for EHEC O157:H7, which are primarily transmitted by the fecal-oral route. In addition, other animals including deer, horses, rabbits and birds that are not normal reservoir hosts for these bacteria can serve as secondary reservoirs after contact with ruminants. Transmission to humans is primarily through consumption of contaminated foods derived from these sources, such as raw or undercooked ground meat products and unpasteurized milk and cheese. However, faecal contamination of water and other foods, as well as cross-contamination during food preparation will also lead to infection (WHO, 2011). An increasing number of outbreaks are also associated with the consumption of fruits and vegetables including lettuce, tomatoes, spinach, sprouts, and unpasteurized cider. Here, contamination may be due to contact with faeces from domestic or wild animals at some stage during cultivation or handling of these foods. Infection has also been
associated with water sources, including ponds, streams, wells, water troughs, recreational waters
and municipal water supplies, again usually as a result of contact with faeces from domestic or
wild animals.

EHEC O157:H7 can remain viable for long periods in many food products. It can survive for
many months in ground beef stored frozen. It is also tolerant to low pH conditions and remains
infectious for many weeks in acidic foods such as mayonnaise, sausage, apple cider and cheddar
at refrigeration temperatures. Furthermore, the infection dose for humans is estimated to be
under 100 organisms, and might be as few as 10 organisms (CFSPH, 2009), meaning that even
the smallest amount of contamination may be sufficient to cause infection.

1.4.3 Typical clinical course

The incubation period for disease caused by EHEC O157:H7 ranges between 2 days and 12
days, but is on average generally about 3 days (Bell et al., 1994; Tarr et al., 2005). Some
individuals remain asymptomatic throughout the course of infection, or develop only very mild
symptoms that resolve without treatment in approximately a week. Most individuals however,
following this initial incubation period, develop hemorrhagic colitis. Hemorrhagic colitis is
characterized by watery and bloody diarrhoea accompanied with mucosal oedema or hemorrhage
and sever abdominal cramps. Some patients have a low-grade fever while in other fever is
absent. Nausea and vomiting is also typically present leading to the possibility of dehydration.
Many instances of hemorrhagic colitis are self-limiting and resolve in about a week. In about
15% of infected individuals, however, hemorrhagic colitis progresses to hemolytic-uremic
syndrome (HUS).

HUS is characterized, to varying degrees among patients, by hemolytic anemia, consumptive
thrombocytopenia and thrombosis in the body’s microvasculature and affects primarily but not
exclusively the kidneys, gastrointestinal tract, brain and pancreas (Ruggenenti et al., 2001).
Acute renal failure resulting from damage to the glomerular endothelium of the kidneys is
common. A subset of HUS patients further develop central nervous system deteriorations
marked by lethargy, irritability, seizures, stroke, cerebral edema or coma. Approximately 75% of
patients recover without permanent damage. However, long-term complications including
hypertension, renal insufficiency and end-stage renal failure also occur (CFSPH, 2009). Left untreated, HUS is fatal in about 5-7% of infected individuals.

1.4.4 Virulence factors

The primary characteristics of EHEC that have been linked to the organism’s virulence are their production of Shiga toxins (Brunder et al., 1997). This family of protein toxins consist of two major types, Stx1 and Stx2, with the latter being more closely associated with severe disease (Law, 2000). These toxins act on vascular endothelial cells of small blood vessels, such as found in the digestive tract, kidneys and lungs, to cause damage and to facilitate uptake into the host cell (Römer et al., 2007). The causal role of Shiga toxins in HUS has been inferred from numerous findings, including the observation that Shiga toxin production is common to all HUS-associated E. coli isolates, regardless of serotype, that HUS patients develop immune responses to Shiga toxins produced by the infecting strain, and that HUS-like disease can be induced in laboratory animals intravenously injected with purified Shiga toxin, which is prevented by Shiga toxin neutralizing antibodies (Karch, 2001). However, severe clinical outcomes have also been associated with E. coli strains that do not possess Shiga toxins indicating that additional virulence factors do contribute to the pathogenesis of HUS and HC (Schmidt, Scheef, et al., 1999; Mellmann et al., 2005; Brockmeyer et al., 2007).

A further key characteristic found in EHEC but which is not exclusive to these organisms is their ability to cause attaching and effacing (A/E) lesions on human intestinal epithelium. These lesions are characterized by close attachment of the bacterium to the epithelial cell membrane and the destruction of brush border microvilli at the site of adherence (Frankel and Phillips, 2008; Zoja et al., 2010). A pathogenicity island termed the locus of enterocyte effacement (LEE) and present within the genome of these bacteria has been found to be crucial to the formation of the A/E lesions. It contains genes that encode and/or support the translocation of bacterial effector proteins into the host cell via a type III secretion system (Zoja et al., 2010).

Almost all clinical EHEC isolates also possess a large plasmid approximately 75 to 100 kb in size which encodes determinants that may serve as additional virulence factors important in EHEC pathogenicity (Karch et al., 1987; Brunder et al., 1997; Karch, 2001). The large plasmid of
EHEC O157:H7, termed pO157 and about 90 kb in size, carries genes encoding for EHEC hemolysin, which acts as a pore-forming cytolsin and is cytotoxic to human and bovine cell lines (Schmidt et al., 1996), the bifunctional periplasmic catalase-peroxidase KatP, which is though to help protect these pathogens from oxidative damage caused by reactive oxygen molecules produced by phagocytes or other host cells during the infection process (Brunder et al., 1996), the etp gene cluster that encodes a type II secretion system (Schmidt et al., 1997), and the serine protease EspP, a member of the SPATE family of autotransporters (Brunder et al., 1997; Karch, 2001).

1.5 The autotransporter EspP

EspP (extracellular serine protease, plasmid-encoded) was first identified as a secreted protein encoded by the large plasmid (pO157) of enterohemorrhagic E. coli (EHEC) O157:H7 (Brunder et al., 1997). Like other SPATEs, EspP is produced as a precursor, here 1300 amino acid residues in length, composed of an N-terminal signal sequence (55 residues), a passenger domain (968 residues and housing the serine protease function of the protein) and a C-terminal translocator domain (277 residues). Following its translocation onto the bacterial cell surface, the EspP passenger domain is separated from the translocator domain by an autoproteolytic cleavage reaction mediated by amino acid residue lining the barrel pore, and released free into the extracellular milieu.

1.5.1 Functional roles

In their initial characterization of this protease, Brunder et al. showed EspP to cleave, in vitro at pH 7.2, both porcine pepsin A and human coagulation factor V (Brunder et al., 1997). Pepsin A is a digestive enzyme present in the highly acidic environment of the stomach. When incubated at the more physiologically relevant pH of 3, EspP was no longer able to cleave porcine pepsin A. In fact, at pH 3 porcine pepsin A, itself a protease active at this low pH, was observed to break down EspP (Brunder et al., 1997). While the authors did not explicitly test cleavage of human pepsin, and while EspP may come in contact with pepsin during the course of EHEC
colonization owing to the latter’s fecal–oral route of transmission, these results nevertheless suggest that cleavage of pepsin A by EspP is not a biologically significant process. Human coagulation factor V, on the other hand, is a 330 kDa single-chain glycoprotein present in human plasma (Kane and Davie, 1986) and is a critical component of the coagulation cascade in blood. Reduced factor V activity, as is the case in patients with hereditary deficiencies of this protein, while rarely fatal, is associated with prolonged bleeding (Brunder et al., 1997; Duckers et al., 2009). The authors hypothesize that local degradation of factor V by EspP, secreted by EHEC attached to the gastrointestinal mucosa, could result in a decreased coagulation reaction leading to increased hemorrhage into the gastrointestinal tract (Brunder et al., 1997). EspP could therefore be an additional virulence factor of EHEC and contribute to the mucosal hemorrhage observed in patients with E. coli O157:H7 infection (Brunder et al., 1997).

Almost at the same time, Djafari et al., describing this protease as PssA (protease secreted by Shiga-toxin-producing E. coli), showed it to be cytotoxic to Vero cells (Djafari et al., 1997). While the exact mechanisms for this cytotoxicity remain unclear, PssA induced cytoskeletal damage to these cells, with loss of stress fibres, disruption of the actin cytoskeleton, cell detachment and rounding, and opening of the cell–cell junctions (Djafari et al., 1997). The authors suggest that this protease may therefore play an important part in processes leading to tissue destruction in the gut that are commonly associated with EHEC infections (Djafari et al., 1997).

To date, genes highly similar to the one encoding EspP in E. coli O157:H7 have been identified in a multitude of non–O157:H7 E. coli serogroups (Table 1.1), with the most distantly related of these (espP of E. coli serogroups O84:H4 and O156:NM) having 97.7% sequence identity to the espP of E. coli O157:H7. Based on their secretion and proteolytic activity, the resultant proteins have been classified as EspP and distinguished into four subtypes: EspPα, EspPβ, EspPγ and EspPδ (Brockmeyer et al., 2007). Of these, EspPα and EspPγ are functional as serine proteases while EspPβ and EspPδ are either secretion-incompetent or proteolytically inactive (Brockmeyer et al., 2007; Khan et al., 2009). Furthermore, EspPα, to which the EspP produced by E. coli O157:H7 belongs, is predominantly associated with highly pathogenic serogroups (Brockmeyer et al., 2007; Khan et al., 2009; Brockmeyer et al., 2011).
Table 1.1 List of EspP-producing serogroups and EspP subtypes identified to date.

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>EspP subtype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Known highly pathogenic serogroups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O157:H7/H-/NM</td>
<td>α</td>
<td>(Brunder et al., 1997; Djafari et al., 1997; Brockmeyer et al., 2007; Khan et al., 2009)</td>
</tr>
<tr>
<td>O26:H2/H11/H-/NM</td>
<td>α</td>
<td>(Brunder et al., 1997; Schmidt, Geitz, et al., 1999; Brockmeyer et al., 2007) (Schmidt, Geitz, et al., 1999; Brockmeyer et al., 2007; Khan et al., 2009)</td>
</tr>
<tr>
<td>O111:H8/NM</td>
<td>α</td>
<td>(Brockmeyer et al., 2007)</td>
</tr>
<tr>
<td>O145:H25/H28/NM</td>
<td>α</td>
<td>(Brockmeyer et al., 2007; Khan et al., 2009)</td>
</tr>
<tr>
<td>Less pathogenic serogroups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O1:HNT</td>
<td>β</td>
<td>(Brockmeyer et al., 2007)</td>
</tr>
<tr>
<td>O5:NM</td>
<td>γ</td>
<td>(Khan et al., 2009)</td>
</tr>
<tr>
<td>O6:NM/HNT</td>
<td>β</td>
<td>(Brockmeyer et al., 2007)</td>
</tr>
<tr>
<td>O7:H18</td>
<td>β</td>
<td>(Brockmeyer et al., 2007)</td>
</tr>
<tr>
<td>O8:NM</td>
<td>β</td>
<td>(Brockmeyer et al., 2007)</td>
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<tr>
<td>O17:H45</td>
<td>β</td>
<td>(Khan et al., 2009)</td>
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<tr>
<td>O22:H8/NM</td>
<td>β</td>
<td>(Brockmeyer et al., 2007; Khan et al., 2009)</td>
</tr>
<tr>
<td>O41:NM</td>
<td>γ</td>
<td>(Brockmeyer et al., 2007)</td>
</tr>
<tr>
<td>O55:H7/HNT</td>
<td>γ</td>
<td>(Schmidt, Geitz, et al., 1999; Brockmeyer et al., 2007)</td>
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<tr>
<td>O69:H11</td>
<td>α</td>
<td>(Brockmeyer et al., 2007)</td>
</tr>
<tr>
<td>O77:H18</td>
<td>γ</td>
<td>(Brockmeyer et al., 2007)</td>
</tr>
<tr>
<td>O77:H45</td>
<td>β</td>
<td>(Brockmeyer et al., 2007)</td>
</tr>
<tr>
<td>O82:H11</td>
<td>β</td>
<td>(Khan et al., 2009)</td>
</tr>
<tr>
<td>O82:H8</td>
<td>δ</td>
<td>(Brockmeyer et al., 2007)</td>
</tr>
<tr>
<td>O84:H2/NM</td>
<td>γ</td>
<td>(Brockmeyer et al., 2007; Khan et al., 2009)</td>
</tr>
<tr>
<td>O84:H4/HNT</td>
<td>δ</td>
<td>(Brockmeyer et al., 2007)</td>
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<tr>
<td>O91:H21/NM</td>
<td>β</td>
<td>(Brockmeyer et al., 2007; Khan et al., 2009)</td>
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<tr>
<td>O92:H33</td>
<td>α</td>
<td>(Brockmeyer et al., 2007)</td>
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<tr>
<td>O98:NM</td>
<td>γ</td>
<td>(Brockmeyer et al., 2007)</td>
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<tr>
<td>O106:H18</td>
<td>γ</td>
<td>(Brockmeyer et al., 2007)</td>
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<tr>
<td>O107:HNT</td>
<td>γ</td>
<td>(Brockmeyer et al., 2007)</td>
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<tr>
<td>O111:NM</td>
<td>α</td>
<td>(Brockmeyer et al., 2007)</td>
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<tr>
<td>O113:H4</td>
<td>γ</td>
<td>(Khan et al., 2009)</td>
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<tr>
<td>O113:H21</td>
<td>β</td>
<td>(Brockmeyer et al., 2007)</td>
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<tr>
<td>O114:H18</td>
<td>γ</td>
<td>(Khan et al., 2009)</td>
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<tr>
<td>O115:NM</td>
<td>γ</td>
<td>(Brockmeyer et al., 2007)</td>
</tr>
</tbody>
</table>
Since its initial characterization, many further functional roles have been described for EspP: it cleaves human apolipoprotein A-I (Schmidt et al., 2001), impairs complement activation by cleaving human complement factors C3/C3b and C5 (Orth et al., 2010), cleaves EHEC hemolysin (Brockmeyer et al., 2011), forms rope-like fibres with cytopathic and adhesive properties (Xicohtencatl-Cortes et al., 2010), and mediates intestinal colonization of EHEC.
O157:H7 in a calf model as well as adherence to primary bovine intestinal epithelial cells (Dziva et al., 2007).

Apolipoprotein A-I (apoA-I) is the primary protein component of high-density lipoprotein (HDL) present in human blood and plays a key role in lipid transport and metabolism (Moore et al., 2003). Reduced levels of apoA-I have been associated with an increased risk for coronary heart disease in humans and with atherosclerosis in mice (Francis and Frohlich, 2001; Moore et al., 2003). Interestingly, apoA-I also acts as a stabilizing factor of prostacyclin (PGI$_2$) in human serum (Yui et al., 1988). PGI$_2$ is an eicosanoid lipid molecule and a natural anticoagulant; it inhibits platelet activation and prevents the formation of the platelet plug involved in the formation of blood clots (Davenport, 2010). Inactivation of apoA-I by EspP may therefore present a further mechanism whereby EspP may contribute to the bleeding observed in patients with *E. coli* O157:H7 infection.

The complement system consists of over 30 proteins in plasma and on the host cells and is an important part of innate immunity (Speth et al., 2008). Activation of the complement system following infection is critical for a wide variety of functions including generation of the initial inflammatory response to the infection, prevention of spread of the infection from its initial site to other areas of the body, and clearance of the infecting organism from the blood stream (Winkelstein and Sullivan, 2010). Complement factors C3/C3b and C5 in particular are important in the opsonic, anaphylatoxic, chemotactic and bacteriolytic activities of the activated complement system (Winkelstein and Sullivan, 2010). Downregulation of complement by EspP may therefore influence the colonization of EHEC bacteria in the gut or disease severity of EHEC infection (Orth et al., 2010).

EHEC hemolysin (EHEC-Hly) is a 107 kDa exoprotein of enterohemorrhagic *E. coli*. Like other hemolysins, EHEC-Hly functions as a pore-forming cytolsin and is active on various cells including lymphocytes, granulocytes, erythrocytes and renal tubular cells (Schmidt et al., 1995). Through these functions, hemolysins in general and EHEC-Hly in particular are thought to provide a mechanism for bacteria to obtain better access to nutrients from their host organisms. Moreover, EHEC-Hly injures microvascular endothelial cells and is thought to play a possible role in the pathogenesis of hemolytic colitis and HUS during EHEC infection (Aldick et al.,
In investigating the interplay between EHEC-Hly and EspP, Brockmeyer et al. observed that EspP cleaves and inactivates EHEC-Hly \textit{in vitro} (Brockmeyer et al., 2011). While the significance of this cleavage during the course of infection by EHEC remains unknown, the authors propose that such a cleavage event can represent a further mechanism whereby pathogens in general, and EHEC in particular, can regulate their virulence phenotypes (Brockmeyer et al., 2011).

That EspP is an important virulence factor of EHEC is further supported by observations that patients with EHEC infection have antibodies to EspP, suggesting that this protease makes it into the blood stream (Brunder et al., 1997; Djafari et al., 1997; Law, 2000). Moreover, this protease promotes intestinal colonization of calves and adherence to bovine primary intestinal epithelial cells (Dziva et al., 2007) and is important for EHEC adherence to T84 intestinal epithelial cells, suggesting a role in tissue interactions \textit{in vivo}: espP-knockout derivatives of EHEC O157:H7, when compared with the parental strain, were deficient in adherence to T84 colonic adenocarcinoma cells \textit{in vitro} (Puttamreddy et al., 2010). Interestingly however, EHEC O157:H7 cured of the large plasmid pO157 (which encoded EspP among the many other known and putative virulence factors), is still infectious in gnotobiotic piglets, suggesting that neither EspP nor the multitude of other factors encoded by pO157 are essential for expression of virulence by \textit{E. coli} O157:H7, at least in gnotobiotic piglets (Tzipori et al., 1987).

In addition to cleavage of human coagulation factor V, EspP has been observed to cleave human coagulation factor VIII (unpublished work from our lab; Figure 1.4). Like factor V, factor VIII is a critical cofactor within the coagulation cascade responsible for formation of blood clots following internal or external vascular injury. That EspP cleaves human coagulation factor VIII provides further support for an important role for EspP in the mucosal hemorrhage observed in patients with \textit{E. coli} O157:H7 infection.

The crystallographic structure of the EspP β-domain has been described (Barnard et al., 2007). Like other members of the autotransporter family, it forms a 12-stranded β-barrel structure. As well, though a high-resolution structure of the EspP passenger domain has not yet been determined, the crystallographic structures of the native passenger domains of the EspP-related SPATEs Hbp (Otto et al., 2005) from enteropathogenic \textit{E. coli} and IgAP (Johnson et al., 2009)
Figure 1.4 Cleavage of human coagulation factor VIII by the EspP passenger domain.

Purified human coagulation factor VIII (10 µg) was incubated for 16 hours at 37 °C with buffer alone (50 mM TEA, pH 7.4 and 500 mM NaCl; lane 1) or with 0.1 µg WT EspP (lane 2). WT EspP incubated with buffer alone is loaded in lane 3. Molecular weight markers are loaded in lane M. FVIII is a highly glycosylated heterodimer composed of a ~200 kDa heavy chain and a ~80 kDa light chain.

from *Haemophilus influenzae* have been described. Like the known or predicted structures of passenger domains of most autotransporters (Emsley et al., 1996; Gangwer et al., 2007), they contain an extended right-handed β-helical structure that forms a spine to which various other structural features are appended: in Hbp and IgAP, this includes a globular subdomain reminiscent of the chymotrypsin family of serine proteases, which carries the proteolytic function of the passenger, and a second subdomain (termed domain 2) that adopts a chitinase b-like fold but for which a functional role has not yet been identified (Nishimura et al., 2010). Also not well defined is the functional role of the β-helical spine of autotransporters in general. The β-helical structure in autotransporters, however, through its sequential folding on the bacterial exterior, is hypothesized to provide a mechanism for translocation of the passenger across the bacterial outer membrane (Klauser et al., 1992; Henderson et al., 2004; Dautin and Bernstein, 2007). Owing to its high degree of sequence similarity to Hbp and IgAP, the secreted EspP passenger domain may therefore function in a similar manner.
1.6 Hemostasis and the fibrinolytic system

The human body contains within it an intricate network of vasculature and other tissues (the cardiovascular system) that circulates blood throughout the body and is responsible for distributing to the cells of the body oxygen from the lungs, various products of metabolism and substances absorbed from the digestive tract, CO$_2$ to the lungs and other waste products to the kidneys. It also acts as a communication channel for hormonal control, plays a key role in regulating body temperature and pH, and helps to fight disease (Leaning et al., 1983). The cardiovascular system is further responsible for its own repair when internal or external forces disrupt its integrity. The physiological processes that are responsible for this endogenous repair are the hemostatic and fibrinolytic systems.

The hemostatic system is composed of three primary mechanisms through which loss of blood from the cardiovascular system is controlled. These include vascular spasm, platelet plug formation and coagulation. Upon injury to the cardiovascular system, smooth muscle surrounding the damaged vessel undergoes a rapid contraction and reduces blood flow to the site of injury. Concurrently, platelet cells normally circulating freely in the vasculature become exposed to tissue collagen released as a result of the damage and become adherent, sticking to the exposed endothelium and to each other and forming a platelet plug. These platelets further release various signals that themselves increase platelet adhesiveness and result in positive feedback favouring the recruitment of more platelets and formation of a much larger platelet plug. If the damage to the vasculature is not too extensive, this platelet plug can then stop bleeding by occluding the opening at the site of damage. The platelet plug, however, is an insecure and temporary structure. Coagulation is the final step of the hemostatic system and results in the formation of a cross-linked fibrin clot that anchors the platelet plug in place and further strengthens it, allowing the body time to repair the damaged endothelium.

1.6.1 The coagulation pathway

Coagulation is the most complex of the hemostatic processes. It utilizes a family of plasma proteins termed coagulation factors that circulate in blood in an inactive form and has
traditionally been classified as proceeding through two distinct activation pathways termed the
intrinsic and extrinsic pathways (Figure 1.5). The intrinsic pathway, also referred to as the
contact pathway, begins with the conversion of coagulation factor XII (FXII) to its active form
(FXIIa; the lower case ‘a’ denotes an active clotting factor). This is achieved through a series of
reactions termed contact activation reactions that are initiated when blood comes into contact
with a negatively charged surface (Colman et al., 2001; Gailani and Renné, 2007; Renné and
Gailani, 2007). In the human body such negative surfaces include the phospholipids (primarily
phosphatidylethanolamine) of circulating lipoprotein particles such as chylomicrons, very-low-
density lipoproteins, and oxidized low-density lipoproteins. Contact activation of the intrinsic
pathway can also occur on the surface of bacteria, and through interaction with urate crystals,
protoporphyrin, amyloid β-peptides, sulfatide, and the platelet-derived inorganic polymer
polyphosphate (Maas et al., 2011). Once activated, FXIIa cleaves and activates FXI, which in
turn converts FIX to FIXa. Activated FIX then forms a complex with FVIIIa, binds to the
phospholipid-rich surface of platelets and converts FX to FXa. The extrinsic pathway, also
referred to as the tissue factor pathway, begins when blood is exposed to tissue factor (TF). TF
is an integral membrane glycoprotein constitutively expressed on subendothelial cells of blood
vessels and is generally barricaded from the blood by the endothelial barrier of the vasculature
(Mackman et al., 2007). Its exposure to blood therefore is a sign of vasculature tissue damage.
Once tissue factor is exposed to the blood, it forms a complex with circulating FVII and
promotes the autoactivation of FVII to FVIIa, resulting in a TF/FVIIa complex. The TF/FVIIa
complex, in the presence of the phospholipid-rich surface of activated platelets, then proceeds to
activate FX to FXa.

Upon activation of FX, the intrinsic and extrinsic pathways converge to form the common
pathway of blood coagulation. Here, FXa forms a complex, termed the prothrombinase
complex, with its activated cofactor FVa and converts prothrombin to thrombin (Mann et al.,
2003). Thrombin is a master regulator of the coagulation cascade. Once activated, it further
amplifies its own creation through positive feedback by catalyzing the conversion of FVII to
FVIIa, FXI to FXIa, FVIII to FVIIIa and FV to FVa. This results in an explosive increase in
thrombin concentrations at the site of vessel injury and promotes the rapid development of a clot.
To create the clot, thrombin converts fibrinogen to soluble fibrin monomers and FXIII to
Figure 1.5 Simplified schematic of the coagulation system in human blood.

The intrinsic coagulation pathway starts with the activation of factor XII (FXII) while the extrinsic pathway begins with the activation of factor VII (FVII). Both pathways lead to the conversion of prothrombin to thrombin and culminate with the generation of a fibrin clot. Activated forms of the coagulation factors are denoted by ‘a’ while orange lines indicate reactions catalyzed by thrombin.

FXIIIa. The soluble fibrin monomers subsequently self-associate in a non-covalent manner to form an insoluble loose fibrin polymer mesh. FXIIIa in turn catalyzes the formation of covalent intermolecular crosslinks between the fibrin molecules in this mesh and promotes the formation of a strong fibrin polymer that affords structural integrity to the clot.

1.6.2 Fibrinolysis

The positive feedback mechanism associated with coagulation, if left unchecked, could lead to excessive generation of clots that may prove detrimental to the body. Moreover, it is imperative that the coagulation cascade remains localized to the site of damage. The crosslinked fibrin polymer provides one mechanism to keep coagulation in check. It has the ability to absorb and inactivate up to 90% of the thrombin generated from prothrombin and in doing so can help to
keep coagulation limited and localized. The circulating blood provides another mechanism for localizing coagulation. It both helps to carry clotting factors away in the blood thereby reducing their local concentration and provides molecules that act as antagonists to the clotting factors, such as tissue factor pathway inhibitor (TFPI), antithrombin, heparin, protein C, protein S and thrombomodulin, among others (Rosenberg and Rosenberg, 1984; van ’t Veer and Mann, 1997). A third mechanism for regulating coagulation is provided by the fibrinolytic system.

The fibrinolytic system counter-balances coagulation through the coordinated destruction of fibrin by plasmin, a process termed fibrinolysis (Figure 1.6). During the course of clot formation, plasminogen, a glycoprotein present in plasma (De Marco et al., 1982), becomes anchored and entrapped within the clot. Concurrently, the damaged endothelium of the vasculature at the site of injury produces, at a very slow rate, a protease termed tissue plasminogen activator (TPA). TPA and, to a lesser extent, a second protease termed urokinase plasminogen activator (UPA) convert the entrapped plasminogen into plasmin (Collen and Lijnen, 1991). Once activated, plasmin cleaves the fibrin molecules present within the clot into many smaller fragments until they are no longer able to maintain their crosslinked state. The fibrin degradation products are then cleared by the actions of phagocytic cells and of other proteases.

The slow production of TPA by the damaged endothelium ensures that plasminogen activation, and subsequently clot breakdown, do not occur too rapidly, before the damaged tissue vasculature has been repaired. TPA and UPA are further inhibited by the plasma proteins plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2). Other inhibitors of the fibrinolytic system include α2-antiplasmin and α2-macroglobulin, each of which reduces fibrinolysis by inhibiting the proteolytic functions of plasmin, and thrombin-activatable fibrinolysis inhibitor (TAFI), which inhibits the activation of plasminogen to plasmin (Boffa et al., 1999). Finally, factors XIa and XIIa as well as kallikrein involved in the intrinsic pathway of coagulation are also able catalyze the activation of plasminogen into plasmin.
Figure 1.6 Simplified schematic of fibrinolysis.
Fibrinolysis is initiated by the conversion of plasminogen to plasmin and results in the degradation of the clot. Blue arrows denote stimulation and red arrows inhibition.

1.6.3 Laboratory assessment of coagulation and fibrinolysis

Laboratory methods are available to assess various aspects of coagulation and fibrinolysis. Prothrombin time (PT) is an in vitro measure used to monitor the proper functioning of the extrinsic and common pathways of the coagulation system (Rodgers, 2004). It quantitates time to clot formation following the reconstitution of calcium-citrate blood plasma with calcium, which is a critical component for many of the coagulation reactions, and tissue factor. In contrast, activated partial thromboplastin time (aPTT) evaluates the effectiveness of the intrinsic and common pathways of coagulation (Rodgers, 2004). It records the time to clot formation following reconstitution of calcium-citrate plasma with calcium, an activator such as silica, celite, kaolin or ellagic acid, and phospholipids. A deficiency in factor VII would result in an increased PT while deficiencies in factors VIII, IX, XI or XII would result in an increased aPTT (Rodgers, 2004). Both PT and aPTT would be prolonged if there are deficiencies in the activities of factors V or X, prothrombin or fibrinogen. Finally, thrombin time (TT) is used to assess the conversion of fibrinogen to insoluble fibrin (Rodgers, 2004). This is accomplished by
the addition of an excess of thrombin to the calcium-citrate blood plasma and in doing so bypassing the extrinsic, intrinsic and some of the common pathways of the coagulation system. More specialized assays of the coagulation system are also available whereby the specific activity of each coagulation factor, including but not limited to factors V, VII, VIII, IX, X, XI, and XII as well as thrombin, can be individually determined (Dahlbäck, 2000; Milos et al., 2009).

While the assays mentioned above are very good indicators of proper functioning of the coagulation system, they are performed in vitro and, owing to technical limitations, using blood plasma as opposed to whole blood samples. Plasma is a subfraction of whole blood. And while it contains all the molecular components necessary for coagulation and fibrinolysis, it is devoid of all cellular components. In vivo, hemostasis and fibrinolysis are complex processes that involve both cellular and non-cellular components of blood. Thrombelastography (TEG) is an in vitro approach that enables a more complete evaluation of coagulation and fibrinolysis. Here, the production and, if monitored for long enough, the dissolution of a blood clot is monitored by mechanical means in a sample of whole blood and therefore in the presence of the cellular components of blood (Hartert, 1948; Mallett and Cox, 1992; Ganter and Hofer, 2008). Finally, the euglobulin clot lysis time (ECLT) assay is an in vitro assay that offers an alternate method to assess plasma fibrinolytic activity. Here, fibrinolysis is measured within the euglobulin fraction of blood, which is devoid of cellular components but also of the natural inhibitors of the fibrinolytic system, and therefore allows a more rapid measurement of the fibrinolysis-promoting non-cellular components of blood (MacFarlane and Biggs, 1948; Kowalski et al., 1959; Smith, Jacobson, et al., 2003) – ECLT is a particularly good measure of the actions of plasminogen activators and of plasmin.

1.7 Thesis rationale

Enterohemorrhagic E. coli O157:H7 is a causative agent of hemolytic anemia and hemolytic-uremic syndrome. This organism also produces the autotransporter EspP whose passenger domain is secreted and functions as a serine protease. Previous work has shown human coagulation factors V and VIII, essential cofactors in normal hemostasis, to be substrates of
EspP. Beyond this, the effects of EspP on hemostasis and fibrinolysis in humans are not known. It is possible that EspP may also act on coagulation factors other than factors V and VIII. EspP could therefore be a contributing factor in the pathogenesis of EHEC-induced hemolytic anemia and hemolytic-uremic syndrome. This thesis aims to provide a better understanding of the functional roles of the EspP passenger domain. To this end, the crystallographic structure of the EspP passenger domain will be determined and analyzed. As well, the effects of the EspP passenger domain on the coagulation and fibrinolytic processes in human blood will be examined. Results from this study may provide both an explanation for and the means to formulating a treatment for the pathological conditions resulting from infection with enterohemorrhagic *E. coli*.
Chapter 2:

Materials and Methods
2.1 Expression and purification of the EspP passenger domain from bacterial culture supernatants

*Escherichia coli* BL21 (DE3) cells were transformed with recombinant plasmid pB9-5 harbouring the wild-type *espP* gene from *E. coli* O157:H7 EDL933 (Brunder *et al.*, 1997) or pB9-5-S263A harbouring the point-mutant gene *espP*<sup>S263A</sup>. A single transformed colony was inoculated in 5 mL Lysogeny Broth (LB) medium (Bertani, 1951; Bertani, 2004) supplemented with 50 mg/L kanamycin and grown for 16 hours at 37 °C. LB (4 L) was inoculated with 4 mL of this primary culture and grown for 12 hours at 37 °C. Selenomethionine-labelled protein was expressed similarly but in *E. coli* B834 (DE3) and in 1 L of M9 minimal media (Sambrook *et al.*, 1989) supplemented with 40 mg/L selenomethionine. The culture was centrifuged at 10,000 g relative centrifugal force for 20 minutes at 4 °C and supernatant was collected. The supernatant was then supplemented with HEPES, pH 7.4, to 20 mM and NaCl to 1.6 M, filtered through a 0.22-µm membrane, applied onto a butyl-Sepharose hydrophobic interaction chromatography column (GE Healthcare, Pittsburgh, PA), and eluted with a decreasing linear gradient of NaCl in 20 mM HEPES, pH 7.4. Fractions corresponding to the expected molecular weight were pooled after analyzing for purity on SDS-PAGE, concentrated to 4 mL and applied onto a Superdex-200 gel filtration column (GE Healthcare, Pittsburgh, PA) pre-equilibrated with 20 mM HEPES, pH 7.4, and 300 mM NaCl. Fractions corresponding to the expected molecular weight were again pooled after analyzing for purity on SDS-PAGE, concentrated to 10 mg/mL and stored at 4 °C or supplemented with 25% (v/v) glycerol and stored at -70 °C. This procedure typically yields ~15 mg of EspP passenger domain per litre of culture.

2.2 Crystallization and data collection

The S263A mutant of the EspP passenger domain (10 mg/mL in 20 mM HEPES pH 7.4, and 300 mM NaCl) was crystallized using 18–22% polyethylene glycol 4000, 0.1 M HEPES pH 7.5 and 0.05–0.15 M tri-lithium citrate. Crystals were grown using the hanging-drop vapour diffusion method at 298 K, with a starting volume of 4 µL in the drops, composed of 2 µL of
protein solution and 2 µL of reservoir solution, and 1 mL of reservoir solution in the well. Large tetragonal crystals belonging to space group \( P4_32_12 \) with unit cell dimensions \( a = b = 88.3 \, \text{Å} \), \( c = 311.5 \, \text{Å} \), grew within 1-2 weeks. Crystals were harvested from the drop on a cryoloop, cryoprotected by passing through a 4:1 mixture of Paratone-N and paraffin oil and flash frozen in liquid nitrogen. Both native and selenomethionine-substituted S263A crystals were prepared in this manner. X-ray diffraction data were collected at the Advanced Photon Source (APS). A 2.5-Å data set was collected from native crystals on beamline 14-BM-C, and a 2.9-Å data set, from selenomethionine-derivative crystals on beamline 17-ID-B. All data were integrated and scaled with the computational program package HKL2000 (Otwinowski and Minor, 1997).

### 2.3 Structure determination and refinement

The structure of the EspP passenger domain was determined by the SAD (single-wavelength anomalous diffraction) method (Wang, 1985) using selenomethionine-containing protein. Initial phase determination and refinement were performed using the Se-SAD data and the computational programs SHARP and autoSHARP (Vonrhein et al., 2007). Out of 13 expected Se sites, 12 were located. The 3.8-Å resolution map obtained from autoSHARP was of sufficient quality to permit an almost complete unambiguous tracing of the polypeptide backbone, using the program COOT (Emsley and Cowtan, 2004), without further refinement of the heavy atom sites. This model was subsequently used to determine a molecular replacement solution in the 2.5-Å native data set using the program PHASER (McCoy et al., 2007). The model was further built and refined using an iterative process involving COOT and REFMAC5 (Murshudov et al., 1997; Winn et al., 2003) in the CCP4 suite (Collaborative Computational Project, 1994; Potterton et al., 2003) using the 2.50-Å resolution native data set. A final round of TLS (translation/libration/screw) (Winn et al., 2003) refinement was performed in REFMAC5 in the CCP4 suite using three groups determined manually per protein molecule: Group 1 consisted of residues 56-313 and 569-629, corresponding to the globular subdomain of the EspP passenger; Group 2 consisted of residues 314-568 and 630-670, corresponding roughly to the N-terminal half of the \( \beta \)-helical stalk subdomain; and Group 3 consisted of residues 630-
670 and 698-1023, corresponding roughly to the C-terminal half of the β-helical stalk subdomain. Solvent molecules were placed at positions where spherical electron density peaks were found above 1.5 σ in the 2Fo–Fc map and where stereochemically reasonable hydrogen bonds were allowed.

Unless otherwise indicated, images were rendered using the program PyMOL (Schrodinger, 2010). Electrostatic surface potentials were calculated using the APBS Tools2 plugin for PyMOL (Baker et al., 2001; Lerner and Carlson, 2006) and rendered at ±7 kT/e.

### 2.4 Analysis of proteolytic activity of the EspP passenger domain

Ten micrograms of purified WT EspP or S263A were incubated (15 h at 37 ºC) with 100 µg of porcine pepsin A (Sigma-Aldrich, Oakville, ON) or a 2 mM solution of the N-succinyl- and para-nitroaniline-conjugated oligopeptide substrate alanine–alanine–proline–leucine (N-Suc-Ala-Ala-Pro-Leu-pNA) (Sigma-Aldrich, Oakville, ON) in 10 mM HEPES buffer with 150 mM NaCl, pH 7.4, and a total reaction volume of 100 µL (Brockmeyer et al., 2009). Incubations with buffer alone served as negative control. Substrate cleavage was determined for the oligopeptide by measurement of the absorbance at 405 nm and for pepsin A by SDS-PAGE analysis followed by Coomassie staining. Masses of the oligopeptide substrate and cleaved products were then determined by matrix assisted laser desorption ionization – time of flight (MALDI-TOF) mass spectrometry (Advanced Protein Technology Centre, the Hospital for Sick Children, Toronto, ON). Pepsin A cleavage products were analyzed by Edman degradation to determine proteolytic cleavage sites.

For the analysis of proteolytic activity against coagulation factor V, 0.1 µg of WT EspP was incubated (5–15 hours at 37 ºC) with 4.2 µg of purified human coagulation factor Va (BioPur AG, Budendorf, Switzerland) in 20 mM HEPES buffer with 150 mM NaCl, pH 7.4, in a total reaction volume of 20 µL. Incubations with buffer alone served as the negative control. Cleavage products were analyzed by SDS-PAGE followed by silver staining.
2.5 Polyacrylamide gel electrophoresis and protein staining

Polyacrylamide gel electrophoresis (PAGE) was performed in a mini-gel apparatus (Bio-Rad Laboratories, Richmond, CA) using pre-cast NuPAGE Novex Bis-Tris gradient gels (4–12% acrylamide; Invitrogen, Carlsbad, CA) run in MOPS (3-(N-morpholino)propanesulfonic acid) SDS (sodium dodecyl sulphate) buffer according to the manufacturer’s instructions, or home-made non-gradient gels (3% stacking on 10% or 15% separating) run in SDS buffer according to the method of Laemmli (Laemmli, 1970). Protein bands were visualized either by Coomassie Brilliant Blue R-250 (Sigma-Aldrich, Oakville, ON), silver staining, or SYPRO Orange (Molecular Probes, Eugene, OR). Gels to be stained with Sypro Orange were incubated overnight in the dark with 1:5,000 Sypro Orange dye in 7.5% acetic acid, rinsed according to the manufacturer’s instructions, and visualized using a standard UV transilluminator together with a gelatin Sypro photographic filter.

2.6 Blood Sampling

2.6.1 Donors R01 and R02

Two healthy adult donors who had not taken any medications known to interfere with hemostasis were recruited for blood collection at the Hospital for Sick Children (Toronto, ON). Informed consent was obtained from each volunteer under Hospital Research Ethics Board approval. Venous blood samples were collected from the donors by venipuncture at the antecubital fossa into syringes using a 21-gauge needle by a phlebotomy-certified technologist. To minimize the effects of venous endothelial damage on sample quality, the first 5 mL of blood was discarded. Blood was transferred to Vacutainers (BD and Co., Franklin Lakes, NJ) containing 0.3 mL of 3.2 % trisodium citrate as anticoagulant for each 2.7 mL of blood. About 25 mL of blood was routinely collected from these donors as needed, but not more frequently than once each month. These samples were used immediately and any remaining unused blood was discarded.
2.6.2 Donors H01 to H07

Seven hemochromatosis patients undergoing regular therapeutic phlebotomies at Sunnybrook Health Sciences Centre (Toronto, ON) were recruited for blood collection. Informed consent was obtained from each donor under Hospital Research Ethics Board approval. Venous blood samples were collected from each donor by venipuncture at the antecubital fossa using a 16-gauge needle into a whole blood collection bag containing citrate phosphate double dextrose anticoagulant by a phlebotomy registered nurse. To minimize the effects of venous endothelial damage on sample quality, the first 5 mL of blood was discarded. About 500 mL of blood was routinely collected from these donors as needed and as available. These samples were used immediately; any remaining blood was centrifuged (10 minutes, 4 °C) at 1,000g relative centrifugal force to obtain the plasma fraction, transferred to sterile 50-mL conical tubes, frozen in a dry ice/ethanol bath and stored at -70 °C. In the remainder of this thesis this frozen plasma obtained from fresh citrated whole blood will be referred to as fresh frozen plasma (FFP). Blood samples from donor H05 were not used in any assays as this donor was later determined to be on medications that are known to interfere with laboratory assays of hemostasis.

2.7 Flow cytometry analysis of binding

WT EspP and S263A were fluorescently labeled with either the thiol-reactive Alexa Fluor 488 C5 maleimide (Sigma-Aldrich, Oakville, ON) or the amine-reactive fluorescein isothiocyanate (FITC) isomer I (Sigma-Aldrich, Oakville, ON). For labeling with Alexa Fluor 488, 0.5 mL of dye (1 mM in water) was incubated with 1 mL of protein solution (5 mg/mL in PBS at pH 7.0 and containing 1 mM tris(2-carboxyethyl)phosphine (TCEP) as a reducing agent) at 25 °C for 2 hours in the dark. Excess thiol-reactive dye was then consumed by addition of β-mercaptoethanol (β-ME) to 10 mM. For labeling with FITC, 250 µL of dye (2.5 mM in 0.1 M sodium carbonate buffer, pH 9.0) was incubated with 1 mL of protein solution (5 mg/mL in 0.1 M sodium carbonate buffer, pH 9.0) at 25 °C for 2 hours in the dark. Excess amine-reactive dye was then consumed by addition of ammonium chloride to 50 mM. After fluorolabeling, the protein-dye conjugates were purified and extensively buffer-exchanged into 0.2 N NaCl, 20 mM
HEPES, pH 7.4 using Amicon Ultra centrifugal filter units having 10,000 nominal molecular weight limit (Millipore, Billerica, MA). The resulting protein solutions were then analyzed by optical spectroscopy to determine the degree of labeling (calculated to be on average 2 molecules of fluorophore bound per molecule of EspP passenger domain), adjusted to a concentration of 5 mg/mL in HBS-G (14 mM HEPES, pH 7.4, 140 mM NaCl, and 30% (v/v) glycerol) and stored in the dark at -70 °C until use.

For cell binding assays 10 µL of FITC-labeled WT EspP, FITC-labeled S263A, Alexa Fluor 488-labeled WT EspP, or Alexa Fluor 488-labeled S263A, was incubated with 40 µL of red blood cell (RBC) suspension (10⁷ cells/mL, in PBS) or platelet cells (10⁷ cells/mL in PBS) at room temperature for 45 minutes in the dark – fractionated packed RBCs and pooled platelet cells were obtained from the University Health Network Blood Transfusion Service (Toronto, ON) under Hospital Research Ethics Board approval, and resuspended in PBS as appropriate. In the control tubes, cells were incubated with PBS alone. Cells were then washed twice in PBS to remove unbound label, diluted in 5 mL PBS, and fixed at 25 °C for 10 minutes with 1.6% paraformaldehyde. Finally, the samples were examined with a flow cytometry analyzer (LSR II, BD Biosciences, San Jose, CA) to determine protein binding, using a 488-nm light from an argon-ion laser for excitation and 530/30-nm bandpass emission filter for collection. Dead cells were excluded by forward and side scatter gating (Schreiber et al., 1981; Wang et al., 2001). Typically 10,000 gated events were acquired for each sample. Data analysis was performed using the software package FLOWJO (Tree Star, Ashland, OR).

2.8 Incubation of blood samples for coagulation assays

2.8.1 Time course analysis

2.8.1.1 Donors R01 and R02

To each of three Vacutainers containing 3.0 mL of fresh citrated whole blood was added 125 µL of WT EspP passenger domain (10 mg/mL in PBS-G). A second and a third set of three
Vacutainers incubated with PBS-G alone or with S263A (125 μL of 10 mg/mL in PBS-G) served as negative controls. Samples were immediately mixed by gentle inversion and stored on a pre-warmed heating block at 37 °C for 0.5, 2 or 4 hours. At each time point, one Vacutainer from each set was removed from the heating block and the clotting behaviour of its content was immediately analyzed by thrombelastography.

2.8.1.2 Donors H01 to H07

On the day that fresh citrated whole blood was obtained from a donor, the following two procedures were followed.

Procedure 1 – To 8.1 mL of fresh citrated whole blood was added 900 μL of PBS-G containing 10 mg/mL WT EspP passenger domain to produce 9.0 mL of reaction mixture. The mixture was immediately mixed by gentle inversion, separated into three 3-mL aliquotes and stored in a pre-warmed incubation chamber at 37 °C with occasional agitation for 0.5, 2 or 4 hours. Simultaneous incubations with PBS-G alone, or PBS-G containing 10 mg/mL BSA or S263A served as negative controls. At each time point, one 3-mL aliquot from each set was removed from the incubation chamber. 0.34 mL from each aliquot was immediately assayed in a thrombelastograph for a global analysis of its clotting behaviour. The remaining 2.66 mL from each aliquot was centrifuged (10 minutes, 4 °C) at 1,000g relative centrifugal force to recover the plasma fraction, flash frozen on dry ice, and stored at -70 °C. At a later date, this plasma fraction was thawed (5 minutes) in a 37 °C water bath and assayed in an automated hemostasis analyzer to further characterize its clotting behaviour.

Procedure 2 – Forty millilitres of fresh citrated whole blood were centrifuged (10 minutes, 25 °C) at 1,000g relative centrifugal force to recover the plasma fraction. To 2.7 mL of the recovered plasma was added 300 μL of PBS-G containing 10 mg/mL WT EspP passenger domain. The resulting mixture was immediately mixed by gentle inversion, separated into three 1-mL aliquotes and stored in a pre-warmed incubation chamber at 37 °C with occasional agitation for 0.5, 2 or 4 hours. Simultaneous incubations with PBS-G alone, or PBS-G containing 10 mg/mL BSA or S263A served as negative controls. At each time point, one 1-mL aliquot from each set was removed from the incubation chamber, flash frozen on dry ice, and stored at -70 °C.
At a later date, this plasma fraction was thawed (5 minutes) in a 37 °C water bath and assayed in an automated hemostasis analyzer to characterize its clotting behaviour.

2.8.2 Concentration assay

Eighteen hundred microlitres of fresh frozen plasma (FFP) were incubated by itself, with 200 µL of PBS-G alone, with 200 µL of PBS-G containing a concentration range of BSA, with 200 µL of PBS-G containing a concentration range of S263A, or with 200 µL of PBS-G containing a concentration range of WT EspP passenger domain, at 37 °C in a water bath for 4 hours with occasional agitation. The concentration ranges of protein (BSA, S263A, or WT EspP passenger domain) in PBS-G were 0.1, 0.5, 1.0, 2.5, 5.0, 7.5 and 10.0 mg/mL. The final concentrations of protein in the reaction mixture were therefore 0.01, 0.05, 0.10, 0.25, 0.50, 0.75 and 1.0 mg/mL. Following this incubation, samples were either immediately analyzed in an automated hemostasis analyser to characterize their clotting behaviour, or flash frozen on dry ice, stored at -70 °C, and thawed and analyzed at a later date.

2.9 Thrombelastography

Thrombelastographic assays of whole blood were performed, according to the manufacturer’s guidelines, using a Computerized Thrombelastograph Hemostasis Analyzer (TEG) Model 5000 (Haemoscope Corp, Niles, IL). 0.34 mL of citrated fresh blood reaction mixture, prepared as described in section 2.8.1, was placed into a TEG sample cup, recalcified by addition of 20 µL of 0.2 M CaCl₂, and monitored for 1 hour in the thrombelastograph to generate a TEG tracing. The following parameters were determined from these tracings: R (reaction time), the latency between placing the recalcified blood sample into the thrombelastograph and when the first fibrin strands form; K, a measure of the kinetics of clot formation; α, also a measure of the kinetics of clot formation; MA (maximum amplitude), a measure of the maximum strength of the clot; and LY30, a measure of the percentage of clot lysis 30 minutes after MA is reached. Quality control on the instrumentation was performed according to the manufacturer’s
guidelines once each day before sample analysis to ensure proper functioning of the TEG analyzer. A schematic of a typical TEG tracing is presented in Figure 2.1 with R, K, α, MA and LY30 indicated.

Figure 2.1 Schematic of a typical TEG tracing.
A schematic of a typical TEG tracing is shown with the five measured parameters labeled. Figure adapted from Gurbel et al. (2008).

2.10 Routine coagulation assays performed on the ACL TOP

One millilitre of plasma reaction mixture, prepared as describe in section 2.8, was placed into an appropriate sample cup and loaded into an Automated Coagulation Laboratory (ACL) Total Operational Performance (TOP) hemostasis analyzer (Instrumentation Laboratory, Lexington, MA) located in the Special Coagulation Laboratory of the Sunnybrook Health Sciences Centre (Toronto, ON). Samples were then analyzed according to the Sunnybrook Health Sciences Centre Standard Operative Procedures, which are based on the ACL TOP manufacturer’s guidelines, to determine thrombin time, prothrombin time, activated partial thromboplastin time, activities of coagulation factors II, V, VII, VIII, IX, X, XI and XII, and fibrinogen and D-dimer concentrations. All assays were performed in the Single Test Mode. Quality control on
the instrumentation was performed daily before sample analysis to ensure proper functioning of
the ACL TOP.

2.11 Euglobulin clot lysis time assays

Euglobulin clot lysis time (ECLT) assays were performed according to the method described by
Smith et al. (2003), but with some modifications.

2.11.1 Method 1

Four hundred and fifty microlitres of fresh frozen plasma from donor H02 was incubated by
itself, with 50 µL of PBS-G alone, with 50 µL of PBS-G containing a concentration range of
BSA, with 50 µL of PBS-G containing a concentration range of S263A, or with 50 µL of PBS-
G containing a concentration range of WT, in a waterbath for 4 hours at 37 °C with occasional
agitation. The concentrations of protein (BSA, S263A, or WT) in PBS-G were 1.0, 5.0 or 10.0
mg/mL. The final concentration of protein in the reaction mixture was therefore 0.1, 0.5 or 1.0
mg/mL.

Following this incubation the samples were immediately processed to produce the euglobulin
fraction. The euglobulin fraction was prepared essentially by the method described by Smith et
al. (2003), which itself is a modified version of the assay described by Copley at al. (1959), but
with the following modifications. 350 µL of incubated reaction mixture was added to 6.3 mL of
2.8 mM acetic acid in a glass test tube, immediately mixed by gentle inversion, and stored on ice
for 15 minutes. Following this incubation, the sample was centrifuged at 250g relative
centrifugal force at 4 °C for 5 minutes, decanted, and freed of residual excess liquid using a
cotton swab. The euglobulin fraction was then resuspended in 350 µL of borate buffer (2.6 mM
sodium tetraborate, 154 mM NaCl, pH 9.0) at 25 °C. For each sample, 150 µL of sample was
pipetted into each of two wells of a Corning 3641 96-well non-binding surface polystyrene flat-
bottom microtiter plate (Corning, Corning, NY) and incubated for 5 minutes at 37 °C in a
Spectramax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA). 150 µL of 0.025
M CaCl$_2$ was then added to one well of each sample. The other well served as an individual blank control. The plate was immediately placed back into the spectrophotometer and the absorbance in each well was monitored at 405 nm every minute for 600 minutes, maintaining the temperature in the reader at 37 °C throughout. The plate was shaken in the reader for 10 seconds before the first reading but was not shaken thereafter. The resulting data file was analyzed using the computational program Excel (Microsoft, Redmond, WA).

Figure 2.2a depicts an idealized schematic of a typical spectrophotometric tracing of euglobulin clot formation and lysis. In their analysis of such tracings, Smith et al. (2003) defined the euglobulin clot lysis time simply as the time at which the spectrophotometric tracing, corrected for the sample's individual blank, reached an absorbance of 0.05 or less. They did not explicitly define a euglobulin clot formation time. The actual experimental spectrophotometric tracings obtained during the course of my thesis work, however, while following the general trend of the tracings described by Smith et al., had much greater variability in absorbance during the course of clot formation and lysis – a sample experimental tracing from my work is shown in Figure 2.2b, with more tracings shown in Figures 6.7 and 6.8. Many of these tracings had secondary peaks (around 4.5 hours in Figure 2.2b), had much lower or higher maximum absorbance as compared to the idealized tracing, and were very noisy towards the completion of clot lysis. In an attempt to define both a robust clot formation time and a more robust clot lysis time than that described by Smith et al., the experimental spectroscopic tracings of clot formation and lysis, corrected for each sample's individual blank control, were analyzed as follows. For each sample, $t_0$ was defined as the time at which CaCl$_2$ was added to the well and hence coagulation allowed to proceed, and $Abs_0$ as the absorbance, corrected for the sample's individual blank, at this time $t_0$. $Abs_1$ was defined as the maximum absorbance reached within the first 120 minutes following $t_0$, and $t_1$ as the time to reach this maximum absorbance. $Abs_2$ was limited to the first 120 minutes following $t_0$ because the tracings were quite smooth during this initial 120 minutes and because all secondary peaks seemed to be limited to later timepoints. Similarly, $Abs_2$ was defined as the minimum absorbance reached between $t_1$ and the end of data collection, and $t_2$ as the time from $t_0$ to reach this minimum absorbance. Clot formation time was then defined as the time from $t_0$ to reach an absorbance of $0.2 \times Abs_0 + 0.8 \times Abs_1$, and clot lysis time as the time in minutes from
but $t_1$, to reach an absorbance of $0.2 \times \text{Abs}_1 + 0.8 \times \text{Abs}_2$. These various parameters as
they relate to the sample tracing presented in Figure 2.2b, are labeled therein.

![Figure 2.2 Spectrophotometric tracing of euglobulin clot formation and lysis.](image)

(a) Idealized schematic of a typical spectrophotometric tracing of euglobulin clot formation and lysis, with the points of maximum absorbance and complete clot lysis indicated. (b) Typical experimental spectrophotometric tracing of euglobulin clot formation and lysis obtained during the course of this thesis work, with the various measured parameters labeled. Tracing in panel (a) is adapted from Smith et al. (2003). Tracing is panel (b) represents the spectrophotometric tracing of clot formation and lysis in the euglobulin fraction of FFP, from donor H02, treated (4 hours at 37 °C) with 1.0 mg/mL BSA.

2.11.2 Method 2

Euglobulin clot lysis time assays were performed exactly as described for Method 1 above but with two modifications. Firstly, whereas in Method 1 FFP from donor H02 was used, here FFP was from donor H07. Secondly, whereas in Method 1 FFP was first incubated for 4 hours under the test conditions and subsequently fractionated to produce the euglobulin fraction, here the euglobulin fraction was prepared first from FFP, and subsequently the euglobulin fraction was mixed with the test conditions, incubated for 4 hours, and assayed for clot lysis. This method ensures that BSA, S263A and WT are present in the euglobulin fraction during clot formation and lysis to exert their effects and are not unknowingly removed from the euglobulin fraction during the acid precipitation step of Method 1.
2.11.3 Method 3

Clot lysis time assays were performed exactly as described in Method 1 but with two modifications. Firstly, whereas in Method 1 FFP from donor H02 was used, here FFP was from donor H07. Secondly, after incubation with the test conditions, the euglobulin fraction was not prepared. Instead, the incubated FFP samples were loaded as is directly into the microtiter plate and clot formation and lysis were monitored in unfractionated FFP. This method ensures that all inhibitors of the fibrinolytic system that are removed from plasma in preparing the euglobulin fraction are present throughout the course of clot formation and lysis and serves to determine the effects of EspP on these inhibitors.

2.12 Statistical analyses

Data are expressed as means ± SD where three or more measurements were made. Where two or fewer measurements were made, only the mean of the samples is reported. All comparisons were made using a paired t-test and the computational program GraphPad Prism (GraphPad Software, San Diego, CA) to determine the significance of differences.
Chapter 3:

Crystal structure of the EspP passenger domain

To study the structural and functional characteristics of the EspP passenger domain, I purified WT EspP passenger (968 residues, numbered 56-1023) from *E. coli* BL21 (DE3) cells transformed with plasmid pB9-5 containing the unmodified native gene espP (Brunder *et al.*, 1997). SDS-PAGE, analytical size-exclusion chromatography and mass spectrometry revealed the purified passenger domain to be monomeric (Figure 3.1). Functional activity of this domain was confirmed by its cleavage of porcine pepsin A and of the tetrapeptide N-Suc-Ala-Ala-Pro-Leu-pNA, in agreement with earlier reports (Brunder *et al.*, 1997; Brockmeyer *et al.*, 2007). The protein, however, was found to be unstable under normal storage conditions. WT EspP degraded from a native molecular weight of about 105 kDa to two lower weight products, one around 84 kDa and a second around 21 kDa, during the course of a few days at 25 °C as determined by SDS-PAGE. This degradation was inhibited by addition to 2 mM of the serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF). Crystallization attempts of WT EspP with and without PMSF were, however, unsuccessful as well diffracting crystals were not obtained. We therefore generated a point mutant form of EspP, termed S263A, where the serine residue at position 263 of the molecule has been mutated to an alanine. The S263A passenger domain has abrogated serine protease activity due to removal of the critical hydroxyl moiety from Ser-263, thought to be the catalytic serine responsible for proteolytic activity of EspP (Brunder *et al.*, 1997). S263A was purified in a manner identical to that for WT EspP, determined to be monomeric, lacked serine protease activity, and remained stable in solution over the course of many months as determined by SDS-PAGE. Crystallization trials employing S263A yielded protein crystals of sufficient quality to determine the structure of this passenger domain (Figure 3.2).
Figure 3.1 Purification of the EspP passenger domain.

The EspP passenger domain was (a) expressed as a ~105 kDa secreted protein in bacterial culture supernatant and (b) first captured by hydrophobic interaction chromatography using a butyl-Sepharose column. (c) Typically, elution fractions 30-62 were pooled, concentrated, and (d) further...
purified by gel filtration chromatography using a Superdex-200 column. (e) Here, typically, fractions 64-75 were pooled and further concentrated for structural studies. (f) Coomassie-stained SDS-PAGE and (g) ESI mass spectrometric analysis of the purified EspP passenger domain. The location of the EspP passenger domain in each panel is indicated by an arrowhead.

Figure 3.2 Crystals and diffraction pattern of the EspP passenger domain.

Left panel: Crystals of S263A grown by the hanging-drop method in 20% (w/v) PEG 2000, 100 mM sodium citrate, 100 mM HEPES pH 7.4. Right panel: Diffraction pattern of an S263A crystal, cryoprotected by passing through a 4:1 mixture of Paratone-N and paraffin oil and collected at beamline 14-BM-C at APS.

3.1 Overall structure

The mature EspP passenger domain contains 968 residues, numbered 56-1023. The structure of this full-length passenger domain was determined by selenium single-wavelength anomalous diffraction phasing and refined to 2.50 Å resolution with good statistics. The final model extends from residues 56-1023 of the passenger domain. However, residues 581-593 and 1000-1011 are disordered and therefore invisible in the electron density map. The EspP passenger domain can be divided into two major structural subdomains: a globular subdomain containing residues 56-313 and 569-630 and a β-helical stalk subdomain spanning the majority of the
remaining 648 residues (Figure 3.3). The final model has 85.1% of the residues in the most favourable region, 14.0% in additional allowed regions and 0.2% in disallowed regions of the Ramachandran plot (Ramachandran et al., 1963). The final statistics for data collection and refinement are listed in Table 3.1.

Table 3.1 Data collection and refinement statistics.

<table>
<thead>
<tr>
<th></th>
<th>Se-SAD&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Native&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>X-ray wavelength (Å)</td>
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<td>Unit-cell parameters (Å)</td>
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<td>88.34</td>
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<tr>
<td>c</td>
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<td>311.53</td>
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<td>No. of unique reflections</td>
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<td>Completeness (%)</td>
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<td>Redundancy</td>
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<td>I / σ(I)</td>
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<td>10.9 (48.6)</td>
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<tr>
<td><strong>Model refinement</strong></td>
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<td></td>
</tr>
<tr>
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<td>0.188 / 0.231</td>
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<td>Disallowed (%)</td>
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</table>

<sup>a</sup> Values in parenthesis are for the outer shell.

<sup>b</sup> R<sub>merge</sub> = Σ<sub>hkl</sub>Σ<sub>i</sub> |I<sub>obs</sub> | (I<sub>obs</sub> − I(hkl))| / Σ<sub>hkl</sub>Σ<sub>i</sub> I(hkl), where I(hkl) is the intensity of reflection hkl, Σ<sub>hkl</sub> is the sum over all reflections and Σ<sub>i</sub> is the sum over i measurements of reflection hkl.

<sup>c</sup> R = Σ<sub>hkl</sub> |F<sub>obs</sub>| – |F<sub>calc</sub>| / Σ<sub>hkl</sub>|F<sub>obs</sub>|, where F<sub>obs</sub> and F<sub>calc</sub> are the observed and calculated structure-factor amplitudes, respectively. R<sub>free</sub> is calculated for a randomly chosen 5% of reflections that were not used for structure refinement, and R<sub>work</sub> is calculated for the remaining reflections.
The overall fold of the EspP passenger domain displays high similarity to the folds of the passenger domains of *Escherichia coli* hemoglobin protease (Hbp; PDB ID 1WXR; Otto *et al.*, 2005) and *Haemophilus influenzae* immunoglobulin A1 protease (IgAP; PDB ID 3H09; Johnson *et al.*, 2009), the two other members of the SPATE family of autotransporters for which high resolution structures of their passenger domain have been determined. Like Hbp and IgAP, the EspP passenger domain contains an extended β-helical stalk subdomain forming a spine from which protrudes a globular subdomain (termed domain 1 in Hbp) with a fold reminiscent of that of the chymotrypsin family of serine proteases (Figure 3.3). Unlike Hbp and IgAP however, the EspP passenger does not contain a second chitinase β-like subdomain (domain 2 in Hbp and IgAP) protruding from the β-helical spine. Instead, a single residue in EspP, namely Ala517, bridges the region occupied by the 77-residue subdomain 2 in Hbp and the 89-residue subdomain 2 in IgAP. If subdomain 2 is indeed involved in substrate recognition by these autotransporters as suggested by Johnson *et al.* (Johnson *et al.*, 2009), our results show that, unlike Hbp and IgAP, EspP does not utilize such distal structures within the protein fold for substrate recognition; like other members of the chymotrypsin family, substrate recognition by EspP is likely mediated only by local structural features within and surrounding the active site cleft of the EspP globular subdomain.
Figure 3.3 Crystal structure of the EspP passenger domain.

(a) Linear schematic representation of the EspP primary sequence, with the globular subdomain coloured red (subdomain 1 or SD1, residues 56-313) and green (subdomain 3 or SD3, residues 569-630), subdomain 4 (or SD4, residues 671-699) coloured yellow and the β-helical stalk subdomain coloured blue. The signal sequence and the translocator domain (β-domain) are coloured gray. For ease of comparison with the other structurally known SPATE autotransporter passenger domains, the established nomenclature for subdomains is used, although EspP does not contain the equivalent of subdomain 2. (b) Wall-eyed stereo ribbon diagram of the EspP passenger coloured as in (a).
Encircled in light blue and rendered as stick models are the catalytic triad residues (His127, Asp156 and Ser263Ala) of the passenger. (c) Ribbon diagram of the EspP passenger rotated 90° clockwise about a vertical axis relative to (b). Represented as dotted loops are residues 581-593 (green) and 1000-1011 (blue); these residues are disordered in the crystal and not visible in the electron density map. (d) Ribbon diagram of the passenger domain of the SPATE autotransporter Hbp (PDB ID: 1WXR) shown in the same orientation and to the same scale as the EspP passenger domain in (c). Subdomain 2 of the Hbp passenger is labeled and coloured purple. (e) Wall-eyed stereo representation of the electrostatic surface of the EspP passenger oriented as in (b), with red indicating electronegative potential and blue indicating electropositive potential. Note the patch of negative charge at the bottom of the groove where the globular and the β-helical domains meet.

3.2 The globular subdomain

The N-terminal 258 amino acid residues (residues 56-313; subdomain 1) of the EspP passenger, together with an additional 62 residues (residues 569-630; subdomain 3) further downstream in the primary structure of the passenger, coalesce together to form the globular subdomain of the EspP passenger. However, as with Hbp and IgAP, these two regions of primary structure seem to fold independently and interact over one mostly planar surface with the smaller 62-residue subdomain 3, which forms a helix-turn-helix motif that rests on one face of the structure formed by the larger 258-residue subdomain 1 (Figure 3.4). Analysis of this interface using the PISA web tool (Krissinel and Henrick, 2007) reveals a buried surface area of 945 Å² and corresponding solvation free energy of -6.4 kcal/mol, not including the effects of satisfied hydrogen bonds and salt bridges across the interface. This interface is further stabilized by 13 hydrogen bonding interactions and 6 salt bridges. Of note is the presence of a tetramethionine cluster, formed by Met58, Met69 and Met229 from subdomain 1 and Met600 from subdomain 3, which lies at the interface between these two subdomains. According to the PISA web tool, at -3.4 kcal/mol, this cluster accounts for over half of the solvation free energy gain of the interface formation. In contrast, this interface covers an area of 610 Å² in Hbp and 602 Å² in IgAP, lacks any such sidechain clustering, and has a solvation free energy of -4.2 kcal/mol and -2.2 kcal/mol, respectively, indicative of a much weaker interaction between subdomains 1 and 3 in Hbp and IgAP.
Figure 3.4 Globular subdomain architecture of the EspP passenger.
(a) Subdomain 3 (green) forms a helix-turn-helix motif that rests on one face of subdomain 1 (red). Residues 581-593 within subdomain 3 are disordered in the crystal structure and are presented here as a green dotted loop. (b) A tetramethionine cluster lies at the interface of subdomains 1 and 3, likely providing further structural stability to the globular subdomain. Interatomic distances between the sulphur atoms of the methionine cluster are indicated by yellow dashed lines and marked in Ångström units.

3.2.1 Subdomain 3

At 68 amino acid residues in size, subdomain 3 of the EspP passenger is much bulkier than the equivalent region in Hbp (37 residues) and IgAP (34 residues; Figure 3.5). However, like in Hbp and IgAP, this region remains devoid of β-strand secondary structure elements, and contains only 20 residues that form α-helical elements in comparison to 10 residues in Hbp and none in IgAP. Consequently, a search of the PDB using the DALI server (Holm and Rosenström, 2010) does not produce any matches between subdomain 3 of these autotransporter passengers and other structures within the PDB.

A further point of difference between subdomain 3 of the EspP passenger and that of Hbp and IgAP is the presence of a disordered loop within subdomain 3 of the EspP passenger, comprising residues Ser581-Gly593. This sequence contains one methionine residue (Met588), from the total of 13 methionines found in the EspP passenger domain, and, interestingly, the two cysteine residues (Cys582 and Cys592), the only cysteines in the entire EspP passenger domain. The
oxidation state of these amino acids has been the subject of a number of studies aimed at elucidating the autotransport mechanism – specifically, at determining whether the passenger is translocated across the bacterial outer membrane in an unfolded or in a partially folded state (Skillman et al., 2005). The mobility of the loop obviously makes it impossible to assign the oxidation state of Cys582 and Cys592. The closest residues represented by interpretable electron density are Phe580 and Ser594; the relatively short distance of ~8.2 Å between their respective Cα-atoms certainly allows for Cys582 and Cys592 to be placed at positions (distance of ~6 Å between Cα-atoms) typical for disulphide bond formation (Goldblum and Rein, 1987).

![Figure 3.5](image)

**Figure 3.5** **Comparison of subdomain 3 of EspP with those of Hbp and IgAP.** Subdomains 3 of (a) EspP, (b) Hbp and (c) IgAP are presented as cartoons and coloured green with the remainder of each molecule rendered as a faded-gray molecular surface.

### 3.2.2 Subdomain 1

Subdomain 1 of the EspP passenger, composed of the N-terminal 258 residues of the protein, shares 27% sequence identity and 39% sequence similarity with the equivalent region (256 residues) in Hbp and 27% sequence identity and 38% sequence similarity with the equivalent region (312 residues) in IgAP. This same region in IgAP shares 31% sequence identity and 43% sequence similarity with the equivalent region in Hbp, and adopts a fold very similar to that in Hbp (Johnson et al., 2009). Not surprisingly then, subdomain 1 of the EspP passenger, too, adopts a fold very similar to those of Hbp and IgAP.

Subdomain 1 in all the three structurally characterized SPATE passenger domains adopts a fold reminiscent of the chymotrypsin family of serine proteases. A search of the PDB for structural
homologs using the DALI server revealed, other than Hbp and IgAP, that subdomain 1 of the EspP passenger is most similar to bovine pancreatic δ-chymotrypsin (PDB ID: 1DLK), yielding a Z-score of 17.3 (Figure 3.6). The 178 residues that matched shows 12% sequence identity to subdomain 1 of the EspP passenger and could be fitted with a root mean square deviation (RMSD) of 2.5 Å over the main chain. In contrast, subdomain 1 of the EspP passenger yields a Z-score of 25.2 with Hbp, matching on 234 residues with 30% sequence identity and an RMSD of 1.9 Å, and 26.8 with IgAP, matching on 238 residues with 28% sequence identity and an RMSD of 2.1, when analyzed by the DALI server.

Superposition of the structures of δ-chymotrypsin, Hbp, and IgAP with that of EspP produced an excellent match between the catalytic triad of the former three structures with His127, Ser263Ala and Asp156 of EspP, in agreement with earlier predictions (Brunder et al., 1997; Brockmeyer et al., 2009; Orth et al., 2010) that these three residues constitute the catalytic triad of EspP. Closer examination of the region surrounding these residues reveals that EspP is more similar to Hbp than to either IgAP or δ-chymotrypsin. As discussed in the context of the structure of IgAP, the active site cleft of IgAP, which lies at the side opposite to the β-helix, is more occluded compared to that in Hbp and other members of the chymotrypsin family; utilizing the loop nomenclature of the chymotrypsin family, IgAP has a 17-residue insert in loop C and a 14-residue insert in loop D relative to Hbp, resulting in the formation of two large pillars on either side of the active site cleft of IgAP that are not present in Hbp (Johnson et al., 2009). In contrast, these same loops are each one residue shorter in EspP than in Hbp. Similarly, loop E, situated at the end of the binding cleft C-terminal to the substrate scissile bond, is 18 residues longer in IgAP, and yet is two residues shorter in EspP, relative to Hbp. These results demonstrate that the active site cleft in EspP is slightly more exposed than that of Hbp but much more exposed than that of IgAP and suggest that like Hbp, and unlike IgAP, EspP may be more suited to interacting with a larger surface on its substrate molecules or possibly larger substrates altogether.

Extensive attempts to co-crystallize EspP with bound substrate or inhibitor were unsuccessful. To gain a better insight into substrate specificity of EspP from its crystal structure, the structure of the active site of EspP was compared to that of bovine chymotrypsin in complex with the
Figure 3.6 Comparison of subdomain 1 of EspP with that of Hbp, IgAP and the structure of δ-chymotrypsin.

Subdomains 1 of (a) EspP, (b) Hbp and (c) IgAP and the structure of (d) δ-chymotrypsin are presented as cartoons, with α-helices coloured red and β-strands coloured dark blue. The side chains of the serine, histidine and aspartate residues forming the catalytic triad of each protease are presented as stick models and coloured with carbon atoms in silver, nitrogen atoms in blue and oxygen atoms in red. The bound inhibitor present in the crystal structure of δ-chymotrypsin is presented as a stick model and coloured purple. All images are rendered from the same view. To orient the reader, I present subdomain 1 of the EspP passenger in the context of subdomains 3 (green cartoon) and 4 (yellow surface) and the β-helical spine subdomain (light blue surface) of EspP.

Lys15Leu mutant variant of bovine pancreatic trypsin inhibitor (BPTI; PDB ID 1P2N; Figure 3.7). Using the nomenclature originally proposed by Schechter and Berger (Schechter and Berger, 1967) and the scientific knowledge accumulated since then, we know that serine proteases bind to a linear sequence of amino acids on the substrate designated NH$_3^+$-P$_n$ … P$_2$, P$_1$, … P$_n$H$_2$O.
\( P_1', P_2' \ldots P_n'-\text{COO}^- \) and hydrolyze the peptide bond located between residues \( P_1 \) and \( P_1' \). The enzymes in turn have a series of binding pocket subsites designated \( S_n \ldots S_2, S_1, S_1', S_2' \ldots S_n' \) each of which accommodates one amino acid residue of the polypeptide substrate, with the \( P_i \)th substrate residue being accommodated by the \( S_i \)th enzyme subsite. Work by Hedstrom \textit{et al.} and others (Hedstrom \textit{et al.}, 1992; Perona and Craik, 1995; Perona \textit{et al.}, 1995; Perona and Craik, 1997; Helland \textit{et al.}, 2003) has also demonstrated that while binding and substrate recognition is a cooperative event involving all enzyme subsites together with the global protein scaffold, subsite \( S_1 \) serves as a primary determinant and in at least some cases provides over half of the total association energy for binding and cleavage. With this in mind, and appreciating the limitations of interpreting homology models when we lack direct experimental evidence in the form of an EspP-substrate or EspP-inhibitor complex crystal structure, I have limited my analysis of the EspP binding subsites to subsite \( S_1 \).

Comparison of the \( S_1 \) subsite of EspP to that of bovine chymotrypsin reveals the former to be both narrower and shallower (Figure 3.7). This is in large part due to the presence of Ile286 in EspP filling up the otherwise deeper channel found in bovine chymotrypsin, which has Gly216 at this equivalent position. Furthermore, the \( S_1 \) subsite of EspP is slightly more hydrophobic than that of chymotrypsin; Ser189 present at the bottom of the \( S_1 \) subsite in chymotrypsin is substituted with Ile286 in the EspP \( S_1 \) subsite, Ala258 in EspP replaces Ser190 in chymotrypsin, and the slightly more protruding Ala296 in EspP replaces Gly226 in chymotrypsin. The position of the oxyanion hole, formed by contributions from the backbone amine atoms of Cyc191, Gly193, Asp194 and Ser195 in bovine chymotrypsin, is well conserved in EspP through the positioning of Ser259, Gly261, Asp262 and Ser263Ala backbone amine atoms. Taken together, these results suggest that EspP is more suited to cleaving the peptide bond following amino acid residues with smaller and more hydrophobic sidechains than those preferred by chymotrypsin – the \( S_1 \) subsite of chymotrypsin is optimized for the large hydrophobic residues tyrosine, tryptophan, phenylalanine, leucine and methionine at the substrate \( P_1 \) position. Indeed, the literature suggests that EspP preferentially cleaves the peptide bond immediately N-terminal to leucine (Brunder \textit{et al.}, 1997; Orth \textit{et al.}, 2010; Brockmeyer \textit{et al.}, 2011), and less frequently threonine (Orth \textit{et al.}, 2010), residues. In contrast, the \( S_1 \) subsite of EspP is slightly wider and much deeper than that found in Hbp (specificity unknown) and IgAP (optimized for proline
Figure 3.7 Comparison of the putative S1 binding pocket subsite of EspP with the S1 subsite of bovine chymotrypsin, Hbp and IgAP.

(a) Wall-eyed stereo view of the S1 binding pocket subsite of bovine chymotrypsin in complex with bovine pancreatic trypsin inhibitor (BPTI; PDB ID 1P2N). (b) Bovine chymotrypsin was matched onto EspP by least-squares fitting residues 56-58, 101-103, 137-139, 183-196 and 212-229 in bovine chymotrypsin with residues 126-128, 155-157, 202-204, 247-264 and 282-299 in EspP.
This match produces an RMSD of 1.5 Å over all backbone atoms. The position of BPTI from its complex with bovine chymotrypsin was then transposed onto the structure of EspP to highlight the putative S1 binding pocket of EspP. This panel is a wall-eyed stereo view of BPTI as bound in bovine trypsin superimposed onto the putative S1 binding pocket of EspP. (a and b) BPTI (green) is depicted from positions P4-P4’ (Gly12-Ile19) with the residue at position P1 (Leu15) together with the backbone atoms spanning positions P2-P2’ (Cys14-Arg17) rendered as a stick models. The catalytic triad of bovine chymotrypsin and EspP are also rendered as stick models and labeled. Protein residues surrounding the inhibitor position P1 (Leu15) are rendered as line models and labeled. (c-f) Electrostatic surface representation of the substrate binding pocket of (c) bovine chymotrypsin from its complex with BPTI [BPTI rendered as in (a) and (b)], (d) EspP, (e) Hbp and (f) IgAP, with red indicating electronegative potential and blue indicating electropositive potential. The S1 binding pocket subsites in (c) to (f) are highlighted by yellow circles.

residues at the substrate P₁ position), clearly indicative of differing substrate P₁ residue preferences between these SPATE autotransporters.

3.3 The β-helical stalk subdomain

As with Hbp and IgAP, the most striking structural feature of the EspP passenger is the presence of an extended right-handed β-helix, acting as a spine to which subdomains 1, 3, and 4 as well as various other smaller surface loops are adjoined. In EspP, the prominence of this β-helix is further underscored due to this passenger’s lack of a subdomain 2, so that the only major protrusions are subdomains 1, 3 and 4 forming a single globular domain at the N-terminal end of the β-helix.

The EspP β-helix is comprised of 60 β-strands, ranging from as few as 2 residues to 12 residues in length. They are joined together by numerous loops into a right-handed spiral structure with 23 turns and about 23 amino acid residues per turn (Figure 3.8a). A large majority of these turns contain three β-strands that themselves hydrogen bond with β-strands in neighbouring turns to form three imperfect parallel β-sheets. The resultant β-helix is reminiscent of a slightly spiralling and slightly kinked imperfect triangular rod (Figure 3.8a) and contains a core that is largely but not exclusively filled with aliphatic side chains. Both the N- and C-termini of this helix are capped by a single β-hairpin each that serve to protect the hydrophobic core of the helix.
from solvent (Figure 3.8 b and c). The β-helix of the EspP passenger is one turn shorter than the one found in the Hbp passenger. Sequence and structural alignment indicates that the extra turn in the Hbp β-helix encompasses Hbp residues Lys713-Asp732 and assumes the position equivalent to the junction between rungs 13 and 14, or residues Glu701 and Thr702, of the EspP β-helix. The N-terminal 13 rungs of the Hbp and EspP β-helices, composed of 310 amino acid residues between Asp257-Gly682 in Hbp and 292 amino acid residues between Ser314-Gly668 in EspP, despite having a sequence identity of only 36%, are structurally very similar. They can be superposed on 292 amino acid residues with a DALI Z-score of 25.4 and a root mean square deviation of only 1.6Å. Similarly, the C-terminal 10 rungs of the β-helix in these two autotransporter passengers, spanning 316 residues between Ile733-Asn1048 in Hbp and 322 residues between Thr702-Asn1023 in EspP, have a sequence identity of only 34% but superpose with a DALI Z-score of 33.2 and a root mean square deviation of only 1.6Å on 310 residues. When compared with the IgAP passenger, the β-helix of the EspP passenger is found to be 4+⅓ turns longer. The IgAP passenger β-helix is best described as lacking the equivalent of rungs 16-19⅓, corresponding to amino acids Gly741-Asn860, of the EspP passenger β-helix. The N-terminal 15 rungs of the EspP and IgAP β-helices, spanning 331 residues between Ser314-Asn740 in EspP and 362 residues between Thr338-Glu856 in IgAP, despite having a sequence identity of only 30%, are structurally very similar and superpose on 320 amino acid residues with a DALI Z-score of 35.6 and a root mean square deviation of only 1.7Å. Similarly, the C-terminal 3+⅔ rungs of the β-helix in these two autotransporter passengers, spanning 163 residues between Asn861-Asn1023 in EspP and 158 residues between Asn857-Pro1014 in IgAP have a sequence identity of only 22% but superpose with a DALI Z-score of 11.5 and a root mean square deviation of only 1.9Å on 121 residues.

A further conserved feature of all SPATE autotransporters, as well as many others, is the presence of a region spanning the last ~100 amino acid residues of the passenger C-terminus. Termed the linker, or the autochaperone domain (AC), this region is believed to initiate folding of the passenger after translocation across the bacterial outer membrane (Ohnishi et al., 1994; Oliver et al., 2003; Kühnel and Diezmann, 2011). The autochaperone region of EspP, with a sequence identity of 43% over 87 amino acid residues when compared to the AC region of Hbp but only 12% over 59 amino acid residues to the AC region of IgAP, adopts a rather similar fold
Figure 3.8 The β-helical stalk subdomain of EspP.

(a) The β-helical stalk subdomain of EspP is presented as a ribbon diagram with α-helices coloured yellow and β-strands coloured blue. Subdomains 1, 3 and 4 are presented as faded-gray molecular surfaces. Dotted loops represent residues disordered in the crystal structure: In EspP these are residues 581-593 (faded gray) and 1000-1011 (orange). The whole autochaperone (AC) region of EspP (residues 925-1023) is coloured orange. Every fifth turn of the β-helix along with the first (1) and last (23) turns are marked by green dots and numbered at the far left. (b) Close-up view of the EspP AC region and comparison to the same region of Hbp (PDB ID: 1WXR) and IgAP (PDB ID: 3H09). One turn of the β-helix immediately preceding the AC region in each autotransporter is shown for reference. Residues 938-940 (light blue dotted loop) and 1025-1030 (orange dotted loop) in Hbp and residues 990-1014 (orange dotted loop) in IgAP are not present in the respective models. Shown in green with sidechains rendered as sticks are those residues that are absolutely conserved between the AC regions of EspP, Hbp and IgAP. (c) Close-up of the EspP β-helical stalk N-terminal cap and comparison to that of Hbp and IgAP. Shown are the first three turns of the β-helix in each protein, with α-helices coloured yellow and β-strands coloured blue. In white are the last four to five residues of subdomain 1 of each protein leading into the β-helix.

and forms the C-terminal cap on the β-helix of the passenger (Figure 3.8 a and b). As with Hbp and IgAP, the AC region of EspP is rich in hydrophobic residues. Gly928, Ala954, Val935, Leu951, Leu992 and Tyr995, numbered according to their positions in EspP, are the only
structurally conserved amino acid residues within the folded AC region of these three autotransporters. The latter four of these residues all cluster within one side of the AC, and all six residues with the exception of Tyr995 point into the hydrophobic core of the AC, where they could form the ‘folding nucleus’ to start the folding process for the whole passenger domain. Indeed, the importance of the hydrophobic residues within the AC and the necessity of proper folding of the AC for passenger domain translocation across the outer membrane has been previously described (Velarde and Nataro, 2004; Renn and Clark, 2008; Peterson et al., 2010).

Within the surface of the EspP passenger, a concave structure, formed by the passenger’s globular subdomain on one side and the right-handed β-helix on the other, generates a large groove, which runs at a ~35 degree angle along the β-helix. It measures ~30 Å wide and runs diagonally across the β-helical stalk for ~60 Å. It contains a cluster of acidic amino acid residues right at the junction between the globular and the β-helical stalk subdomains (Figure 3.9).

3.4 Discussion and conclusions

Whereas the function of the globular subdomain of EspP, and indeed its equivalents in other SPATE autotransporters, is known – they serve as serine endopeptidases (Otto et al., 2005; Johnson et al., 2009; Dautin, 2010) – the function of the β-helical stalk subdomain within these autotransporters remains unresolved. Based on sequence alignments and amino acid propensity analysis, >97% of all autotransporters are predicted to contain a β-helical stalk subdomain (Junker et al., 2006). Furthermore, while autotransporter passengers traverse the inner cellular membrane of their Gram-negative hosts utilizing the Sec machinery and ATP as an energy source (Henderson et al., 2004; Thanassi et al., 2005), the energy source allowing for their translocation across the outer cellular membrane remains unknown. Taken together, these observations have lead to the proposition that the β-helical stalk serves a structural role and may provide a mechanism to translocate the passenger domain across the bacterial outer membrane by stabilizing the passenger in the extracellular space following its translocation. Specifically, while the early stages of outer membrane translocation remain elusive, Brownian motion of the polypeptide chain is suggested to feed the passenger polypeptide chain, C-terminus first, across
the outer membrane through a pore formed by the autotransporter β-domain (Klauser et al., 1992). Concurrently, on the extracellular surface of the outer membrane, the passenger autochaperone region is proposed to serve as a nucleation point to initiate formation of the β-helical stalk subdomain (Velarde and Nataro, 2004; Renn and Clark, 2008; Peterson et al., 2010). Recent results suggest that the Bam complex, too, may be involved in the insertion of the β-domain into and translocation of the passenger domain C-terminal region across the bacterial outer membrane (Ieva and Bernstein, 2009; Peterson et al., 2010).

The stiff structural properties of the parallel β-helix together with the hydrogen bonding requirements of the β-strands along the β-helix and its ability to form continuously one β-strand at a time are thought to supply the energy to drive secretion of the polypeptide chain across the bacterial outer membrane. Accordingly, the primary function of the β-helical stalk domain would be to provide a mechanism for translocation of the passenger across the outer membrane.

While convincing arguments have been presented that the β-helical stalk subdomain performs such a function, it certainly cannot be the only role it plays in the process. There exist naturally occurring autotransporters lacking a β-helical stalk subdomain but that nevertheless are fully functional in presenting their native globular passenger domain onto the extracellular surface. The monomeric autotransporter EstA from Pseudomonas aeruginosa is an excellent example (Wilhelm et al., 1999; van den Berg, 2010). While the EstA translocator domain is a typical 12-stranded β-barrel that inserts into the bacterial outer membrane, the EstA passenger domain is dramatically different when compared with the structures of other known passengers; the EstA passenger has a globular fold that predominantly contains α-helices and loops and is covalently attached directly to the translocator domain (van den Berg, 2010). The EstA autotransporter has no β-helical structure within it yet is fully functional in translocating the passenger to the bacterial outer membrane. In addition, numerous studies have demonstrated that engineered autotransporters with their passenger domain replaced in its entirety by nonnative and non-β-helical globular surrogates are fully capable of translocating these surrogates to the extracellular space in a functional form (Jose and Meyer, 2007). In another example also of particular interest to my work, the natural passenger domains of five different autotransporters (EstA, EspP, YpjA, IgAP and AIDA-I) were replaced with small single-chain Anticalin molecules (artificial proteins
with a mass of about 20 kDa and derived from human lipocalins (Binder et al., 2010). The Anticalins were successfully translocated across the bacterial outer membrane and were fully functional on the cell exterior. This shows that the entire native passenger domain of EspP up to and including Val998 – in effect removing the entire β-helical stalk of the EspP passenger and most of its autochaperone region (His925-Asn1023) – could be replaced by Anticalins without abrogating translocation of the surrogate passenger to the extracellular surface. The last 25 amino acid residues (Ser999-Asn1024) of the native EspP passenger were left in place to serve as a linker between the Anticalins and the EspP translocator domain thereby ensuring steric accessibility of the surrogate passenger onto the cell surface (Binder et al., 2010). These results clearly demonstrate that a β-helical stalk structure is not an absolute requirement for translocation of autotransporter passengers across the outer membrane.

On the other hand, while no such function has yet been observed for SPATE autotransporters, many other protein molecules with a parallel β-helical fold are known to be involved in both the recognition and, in some cases, also cleavage of various substrate molecules (Petersen et al., 1997; Jenkins and Pickersgill, 2001). One example is found in Salmonella sp. bacteriophage P22 tailspike proteins (Steinbacher et al., 1994). Surface features within parallel β-helices of these homotrimeric proteins have been identified by x-ray crystallographic analysis and mutational data to directly bind to and cleave O-antigenic repeating units of the bacterial outer membrane lipopolysaccharides (Baxa et al., 1996; Steinbacher et al., 1996; Steinbacher et al., 1997). A second example is found in the antifreeze proteins produced by many organisms that inhabit ice-laden environments. These proteins adsorb to the surface of ice crystals and prevent their growth (Raymond and DeVries, 1977; Garnham et al., 2011). Structural data over the last 20 years have determined that a significant subset of these proteins adopt a parallel β-helical fold and that surface features within these parallel β-helices mediate adsorption to ice (Graether et al., 2000; Garnham et al., 2011). A third example is found in the class of enzymes known as pectinas, including polygalacturonases (PGases) and rhamnogalacturonases (RGases) among others, which are implicated in the degradation of pectic plant cell wall polysaccharides. Many of these enzymes adopt a parallel β-helical fold with a groove almost perpendicular to the helical axis mediating polysaccharide binding and cleavage (Petersen et al., 1997; Pickersgill et al., 1998; Scavetta et al., 1999; Cho et al., 2001; Fries et al., 2007; Abbott and Boraston, 2008). In parallel
Figure 3.9 Proposed binding cleft within the EspP passenger domain.

The EspP passenger molecular surface is presented with the same colour scheme as in Figure 3.3a (left and centre panels) and as an electrostatic surface (right panel). Encircled in light blue is the location of the catalytic triad of the passenger. The proposed binding cleft within the EspP passenger is marked with broken lines.

with these findings, I suggest that the β-helical subdomain of EspP, and indeed those of other autotransporters in general, while being involved in the efficient translocation of passengers across the bacterial outer membrane, serves an additional, more specialized role as a mediator of binding to select polymeric structures of the host-organism. When looking at the three-dimensional structure of the EspP passenger, it is tempting to interpret the features of the large groove formed at the junction between the globular and the β-helical stalk subdomains as a surface very well suited to serve as a contact site for an as-yet unidentified molecular binding partner.

In conclusion, I have determined the crystallographic structure of the protease passenger of EspP from the enterohemorrhagic pathogen *E. coli* O157:H7, the first naturally occurring SPATE autotransporter whose fold does not contain a chitinase b-like subdomain (labelled subdomain 2 in Hbp and IgAP). The serine protease function of this protein resides within the globular subdomain of EspP, and the catalytic site is at a distance and pointing away from the parallel β-helical stalk domain of this passenger. The precise function of this parallel β-helical stalk
subdomain remains unclear but I propose that one of its roles may be in binding to select cellular structures of the host organism. A large cleft formed at the junction between the globular and the β-helical subdomains seems like an excellent candidate for such an interaction, which in turn may play a role in EHEC pathogenesis.
Chapter 4:

Functional characterization of the EspP passenger domain
4.1 Analysis of proteolytic activity of the EspP passenger domain

4.1.1 Cleavage of pepsin and autoproteolysis

In order to verify that my purified EspP passenger domain preparation was functionally active, I first performed a proteolytic digestion assay with purified WT EspP using porcine pepsin A as a substrate. Porcine pepsin A is known to be cleaved by EspP at the peptide bond between Leu226 and Gly227, numbered according to the amino acid sequence of the pepsinogen precursor from which pepsin is derived (Tsukagoshi et al., 1988; Brunder et al., 1997). Indeed, incubation of pepsin with WT EspP resulted in the disappearance of a band with an apparent molecular mass of ~40 kDa on SDS-PAGE and appearance of three new bands of apparent molecular masses ~21 kDa, ~18 kDa and ~17 kDa, (Figure 4.1a). Incubation of pepsin with buffer alone or with S263A (data not shown) did not produce any degradation products. To determine the identity of these degradation products, the ~21 kDa, ~18 kDa and ~17 kDa bands were analyzed by Edman degradation. For the ~18 kDa fragment I obtained the amino acid sequence IGDEP, corresponding to the N-terminus of mature porcine pepsin. The ~17 kDa fragment produced the sequence GGIDSS, corresponding to amino acid residues 227-232 of mature pepsin. These results suggest that EspP cleaves porcine pepsin between amino acid residues Leu226 and Gly227 (Figure 4.1b), in agreement with earlier reports by Brunder et al. (Brunder et al., 1997), and confirm that my WT EspP passenger domain preparation is functionally active.

N-terminal amino acid sequencing of the ~21 kDa band, however, produced the sequence ALGLLLG. This sequence is not found anywhere within mature pepsin or the pepsinogen precursor. This sequence is found within the EspP passenger domain, and corresponds to cleavage of the peptide bond between amino acid residues Leu834 and Ala835. A ~21 kDa band was also observed when purified WT EspP passenger domain was incubated with buffer alone, suggesting that it is a degradation product of the EspP passenger domain. Furthermore, when purified WT EspP passenger domain was incubated with buffer alone or with pepsin the passenger domain was observed to degrade from a native molecular mass of ~105 kDa to a
slightly lower mass product of around 84 kDa. Degradation of the EspP passenger from ~105 kDa to the two smaller products was inhibited by addition of the serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF), or when S263A passenger domain (having abrogated serine protease activity) was used, suggesting that cleavage of the EspP passenger domain N-terminal to Leu834 is a result of autoproteolysis.

![Image of gel and amino acid sequences](image)

**Figure 4.1 Mapping of pepsin cleavage sites.**

(a) Pepsin (10 µg) was incubated with buffer alone (lane 3) or WT EspP (1 µg; lane 2) for 15 hours at 37 °C. WT EspP incubated with buffer alone is loaded into lane 1. The three cleavage products indicated by asterisks were then analyzed by Edman degradation. This identified the ~21 kDa fragment (experimental N-terminal sequence ALGLLG) as an EspP degradation product and the ~18 kDa (experimental N-terminal sequence IGDEP) and ~17 kDa (experimental N-terminal sequence GGIDSS) fragments as pepsin degradation products. Molecular weight markers are loaded in lane M. (b) The single EspP cleavage site (indicated by an arrow) producing the ~18 kDa (N-terminal fragment) and ~17 kDa (C-terminal fragment) pepsin degradation fragments is mapped onto the primary structure of pepsin.

To further characterize the EspP passenger domain degradation products the ~83 kDa breakdown fragment was analyzed by positive ion electrospray ionization (ESI) mass spectrometry. Molecular mass profiling of the resultant mass-to-charge ratio spectrum yielded three peaks corresponding to fragments of molecular mass 83,531 kDa, 83,859 kDa and 83,909 kDa (Figure 4.2). The third peak could not be placed with certainty to cleavage of the EspP passenger domain about Leu834. The first and second peaks, however, agree well with the expected molecular masses of 83,858 kDa and 83,529 kDa for EspP residues Ser56–Leu826 and
Figure 4.2 Mapping of the EspP passenger domain self cleavage sites.

Analysis of wild-type EspP breakdown products by Edman degradation identified the site at position a (experimental N-terminal sequence ALGLLG) as an autoproteolytic site. Analysis by ESI mass spectrometry (top panel) identified two further autoproteolytic sites at positions b (experimental mass of 83,531 Da) and c (experimental mass of 83,859 Da). Positions a, b and c are indicated here (bottom panel) by arrows mapped onto the primary structure of the EspP passenger domain, coloured as in Figure 3.3.

Ser56-Leu823, respectively, suggesting that, further to the peptide bond between amino acid residues Leu834 and Ala835, the peptide bonds between residues Leu826 and Ser827 and between residues Leu823 and Ser824 are also cleaved by autoproteolytic activity of the EspP passenger domain. Interestingly, autoproteolysed WT EspP passenger domain, while migrating on SDS-PAGE as two separate bands of molecular weights ~83 kDa and ~21 kDa, elutes in size-exclusion chromatography as a single peak and at the same volume as the intact passenger domain (data not shown), suggesting that autoproteolysis does not lead to dissociation of the
EspP passenger domain tertiary structure. This finding is further supported by analyzing the autoproteolytic cleavage sites of the EspP passenger domain in the context of the latter’s crystallographically determined tertiary structure as presented in the previous chapter. EspP passenger domain autoproteolytic sites a, b and c (Figure 4.2) are located on a single surface loop protruding from the parallel β-helix of the passenger domain and encompassing amino acid residues His816-Asp843. Being a surface loop, this region does not play a critical role in maintaining the tertiary structure of the EspP passenger domain. Cleavage at autoproteolytic sites a, b and c therefore does not lead to disruption of the β-sheets forming the parallel β-helix. The regions of the passenger domain N-terminal and C-terminal to the three cleavage sites therefore remain bound to one another, thereby leaving the overall tertiary structure of the passenger domain intact.

4.1.2 Cleavage of an oligopeptide substrate

In order to develop a simpler assay of EspP passenger domain proteolytic activity, I analyzed the N-succinyl- and para-nitroaniline-conjugated oligopeptide alanine-alanine-proline-leucine (N-Suc-Ala-Ala-Pro-Leu-pNA) as a potential substrate for the EspP passenger domain. Such oligopeptides are known to serve as substrates for serine proteases in general. The oligopeptide Ala-Ala-Pro-Leu-pNA (lacking the N-succinyl conjugation) has even been reported to be a suitable substrate for the EspP passenger domain (Brockmeyer et al., 2007). Indeed, when I incubated (15 h at 37 °C) N-Suc-Ala-Ala-Pro-Leu-pNA with purified WT EspP passenger domain the reaction mixture turned markedly yellow, indicating release of free para-nitroaniline from its conjugation with the oligopeptide and suggesting cleavage of the oligopeptide N-terminal to the leucine residue. To confirm that cleavage of the oligopeptide was N-terminal to the leucine residue, I analyzed the oligopeptide degradation products by matrix-assisted laser desorption ionization - time of flight (MADLI-TOF) mass spectrometry (Figure 4.3). The results confirmed that WT EspP passenger domain does cleave the oligopeptide N-Suc-Ala-Ala-Pro-Leu-pNA and that this cleavage occurs at the peptide bond between leucine and para-nitroaniline.
4.1.3 Cleavage of human coagulation factor V

Human coagulation factor V (FV) was first reported by Brunder et al. to be a cleavage substrate of the EspP passenger domain (Brunder et al., 1997). They demonstrated that WT EspP passenger domain-producing bacterial culture supernatants, but not culture supernatants of controls, when incubated with human blood plasma, resulted in the loss of a band of approximately 300 kDa. The authors identified this band as FV by immunoblot analysis using antiserum raised against human factor V (coagulation factor V is an approximately 330 kDa single-chain glycoprotein found in human plasma present at a concentrations of about 12-40 nmol/L (Tracy et al., 1982; Undas et al., 2001)). The authors, however, did not analyze any of the FV degradation products due to the complex nature of whole blood plasma. In the interim between then and now numerous other publications have confirmed human FV as a substrate of EspP going so far as to use purified proteins and direct staining for the analysis. They too, however, for reasons that remain unclear, have not presented or analyzed FV degradation products. In an attempt to identify human coagulation factor V cleavage sites, I performed a
proteolytic digest of commercially obtained purified human coagulation factor Va (FVa) using purified WT EspP passenger domain (Figure 4.4).

![Image of SDS-PAGE gel showing cleavage of FVa](image)

Figure 4.4 Cleavage of human coagulation factor Va by the EspP passenger domain.
Purified human coagulation factor Va (FVa, 4.2 µg) was incubated with buffer alone (5 hours at 37 °C, lane 1) or WT EspP (0.1 µg; 5 hours at 37 °C, lane 2; 15 hours at 37 °C, lane 3). WT EspP incubated with buffer alone (5 hours at 37 °C) is loaded in lane 4. Molecular weight markers are loaded in lane M. EspP is a ~105 kDa monomer that breaks down into a ~83 kDa heavy chain and ~21 kDa light chain due to autoproteolysis during the course of this incubation. FVa is a highly glycosylated heterodimer composed of a ~105 kDa heavy chain and a ~74 kDa light chain, and electrophoresis as multiple bands in this experiment.

Coagulation factor Va is the activated form of factor V. In humans it is produced by cleavage of the latter at the three peptide bonds between amino acid residues Arg709 and Ser710, Arg1018 and Thr1019, and Arg1545 and Ser1546 by thrombin (Kane and Davie, 1988). Factor Va then contains a heavy chain (having an apparent molecular mass on SDS-PAGE of ~105 kDa due to glycosylation) encompassing FV amino acid residues Ala1-Arg709 and a light chain (having an apparent molecular mass on SDS-PAGE of ~74 kDa due to glycosylation) encompassing FV residues Ser1546-Tyr2196 (Kane and Davie, 1988). Factor V amino acid residues Ser710-Arg1545 are not present in FVa. My results demonstrate that indeed the EspP passenger domain does cleave human FVa, as evidenced by the many lower molecular weight degradation product bands observed in lanes 2 and 3 of Figure 4.4 relative to lane 1. Furthermore, these
results demonstrate that cleavage of human FVa by the EspP passenger domain does not seem to be limited to one or a few sites. Instead, the EspP passenger domain seems to cleave human FVa at many sites distributed over the entire length of the latter. Due to the glycosylated nature of FV, the low abundance of degradation fragments and the monetary costs associated with obtaining larger quantities of human factor V, further attempts to identify FV cleavage sites were not pursued.

4.2 Cellular binding assay

As discussed in Chapter 3 section 3.4, a precise function for the β-helical stalk subdomain of the EspP passenger has not yet been determined. Furthermore, as presented in Figure 3.9, I propose that the β-helical stalk subdomain of the EspP passenger contains a binding cleft that may mediate interaction of the passenger with select host cellular structures and play a role in EHEC pathogenesis. Such an interacting partner for the EspP passenger would need to be physically available to the secreted passenger during the life cycle of EHEC within host species. Possible candidates would include secreted or cell surface components of EHEC itself, secreted or cell surface components of host commensal species, and secreted, cell surface or cell interior components of the host species harbouring EHEC. From the myriad of choices available for potential EspP passenger domain binding partners and the multitude of experimental tools available to probe for a potential interaction, I elected to use flow cytometry to determine if the EspP passenger domain binds to human peripheral blood cells.

Human platelet cells and, separately, human red blood cells (RBCs) were incubated in PBS with either a thiol-reactive Alexa Fluor 488-conjugated or amine-reactive FITC-conjugated WT EspP or S263A passenger domain. Cells were then washed free of unbound protein, fixed, and analyzed by flow cytometry for bound EspP (Figure 4.5). The results show that while the EspP passenger does not bind to human RBCs under these test conditions, the EspP passenger domain does bind to human platelet cells, as evidenced by an increased fluorescence of platelet cells incubated with fluoro-conjugated EspP passenger domain in comparison to platelet cells incubated with buffer control. These results, however, do not conclusively support the idea that
Within the EspP passenger domain, a structural feature could be involved in mediating its binding. This binding comes about from a specific interaction mediated by the proposed binding cleft within the β-helical stalk subdomain of the EspP passenger. It remains possible that any structural feature within the EspP passenger domain could be involved in mediating its binding.

Platelet cells (Platelets) and, separately, red blood cells (RBCs) were incubated with either a thiol-reactive Alexa Flour 488-conjugated or amine-reactive FITC-conjugated wild-type EspP (WT) or S263A. Cells were then washed free of unbound protein, fixed, and analyzed by flow cytometry for bound EspP.

**Figure 4.5** Binding of the EspP passenger domain to platelet cells and red blood cells.

Platelet cells (Platelets) and, separately, red blood cells (RBCs) were incubated with either a thiol-reactive Alexa Flour 488-conjugated or amine-reactive FITC-conjugated wild-type EspP (WT) or S263A. Cells were then washed free of unbound protein, fixed, and analyzed by flow cytometry for bound EspP.

this binding comes about from a specific interaction mediated by the proposed binding cleft within the β-helical stalk subdomain of the EspP passenger. It remains possible that any structural feature within the EspP passenger domain could be involved in mediating its binding.
to human platelet cells. Furthermore, platelet cells are known to be inherently sticky, and become more so upon activation when they undergo a transition from a discoid structure to one that is more spherical and containing extended filamentous structures (Wurzinger, 1990). It therefore remains possible that the EspP passenger does not solicit the binding to platelet cells observed in my experiment, but that instead the binding results from the sticky nature of platelet cells themselves, particularly if the platelet cells have become artifactually activated prior to or during their incubation with the EspP passenger – even with careful preparation, low levels of artifactual platelet activation can be difficult to avoid (BD Biosciences technical manual; Shattil et al., 1987). Nevertheless, the EspP passenger domain does bind to human platelet cells, leading to the possibility of further roles for this virulence factor in EHEC pathogenesis. Attempts to further identify sites and molecular features on platelet cells and the EspP passenger domain which mediate the observed binding were not pursued.

4.3 Conclusions

In this chapter, the proteolytic activity of purified WT EspP passenger domain against porcine pepsin A, human coagulation factor V and the oligonucleotide N-Suc-Ala-Ala-Pro-Leu-pNA was confirmed. Cleavage sites within FV could not be identified. Cleavage sites within pepsin and the tetrapeptide were confirmed to be immediately N-terminal to leucine residues, suggesting that the EspP passenger domain has a strong preference for leucine residues at the substrate P$_1$ position. Furthermore, WT EspP passenger domain was found to undergo autoproteolytic degradation at three sites located on a single surface loop protruding from the parallel $\beta$-helix of the protease. All three of these sites were immediately N-terminal to leucine residues providing further support for this amino acid as the preferred residue by the EspP passenger at the substrate P$_1$ position. The one exception to this residue preference comes from work by Orth et al. (2010) who observed through Edman degradation analysis that the EspP passenger domain, in addition to Leu967, cleaves human complement C3b immediately N-terminal to a threonine residue at position 1333 of this protein’s amino acid sequence. The EspP passenger domain was also found to bind to human platelet cells, but was not observed to bind to
human RBCs. Further work is required to determine whether this binding is specific, to identify both the cellular components and the EspP passenger structural features involved, and to probe the functional significance of this binding to EHEC pathogenesis.
Chapter 5:

Global analysis of the effects of the EspP passenger domain on clot formation and stability in human blood

The material presented in this chapter has been obtained through collaboration with Dr. M. L. Rand, Division of Hematology, The Hospital for Sick Children, Dr. K. H. M. Kuo, Department of Medical Oncology and Hematology, University Health Network, Ms. E. Brnjac, Department of Clinical Pathology, Sunnybrook Health Sciences Centre, and Dr. A. E. Chesney, Department of Clinical Pathology, Sunnybrook Health Sciences Centre, Toronto, Canada.
As presented in Chapter 1, the extracellular serine protease EspP is one of a myriad of established or putative virulence factors produced by enterohemorrhagic *E. coli* (EHEC) O157:H7. EHEC O157:H7 is one of the known causative agents of hemorrhagic colitis and hemolytic-uremic syndrome. In their initial publication describing this protease, Brunder *et al.* reported that EspP cleaves, amongst other proteins, human coagulation factor V. Factor V is a critical component for the normal hemostatic functions of human blood and the authors therefore hypothesized that cleavage of this factor may contribute to the mucosal hemorrhage observed in patients with EHEC infection. Cleavage of factor V by EspP has since been reproduced in many labs as well as by myself as described in the previous chapter. It remains unclear, however, where specifically in the protein sequence these cleavages occur or whether the cleavages are activating or permanently inactivating events for factor V. Our lab has further shown (unpublished data; Figure 1.4) that the EspP passenger domain also cleaves human coagulation factor VIII. Factor VIII too is a critical component of normal hemostasis and again where these cleavage sites are and whether they are activating or permanently inactivating cleavages is not known. The mechanism by which EHEC induces hemorrhagic colitis and hemolytic-uremic syndrome has not been fully elucidated and to date the interaction of EspP with the various processes in human blood responsible for maintaining homeostasis has not been further investigated. In this chapter, the interaction of EspP with human whole blood is investigated *in vitro* in a global test of hemostasis as allowed for by thrombelastography (TEG) instrumentation. Fresh whole blood from eight human donors is analyzed and the effect of the EspP passenger domain on the coagulation and fibrinolytic pathways is examined.

### 5.1 Donor demographics and blood sampling

Two donors, R01 and R02, were initially recruited and their blood sampled a total of six times (twice for R01 and four times for R02) in a span of six months. These donors were male, aged between 20–40 years and healthy. A further seven donors, H01–H07, were then recruited for blood sampling. Donor H04 and H05 were the only females in this group. These seven donors were all hemochromatosis patients undergoing regular therapeutic phlebotomies but were
otherwise healthy, and aged between 30-65 years. Blood samples from these donors were collected, as part of their regular therapeutic phlebotomies, a total of eight times (once each for donors H01 and H03-H07, twice for donor H02) in a span of four months. Blood samples from donor H05 were not used in any of the assays as this donor was later determined to have taken, within eight days preceding blood sampling, medications that are known to interfere with laboratory assays of hemostasis.

### 5.2 EspP induces accelerated clot formation

In Figure 5.1 is presented TEG tracings of fresh citrated whole blood from two donors (R01 and R02) incubated *in vitro* with 0.4 mg/mL WT EspP passenger domain. The resulting clot formation parameters derived from these tracings is presented in Figure 5.2. These data show that when fresh citrated blood from donor R01 was incubated with WT EspP passenger domain reaction time (R-time) and clot formation time (K-time) were decreased while clot kinetics (α-angle) was increased when compared to incubations with buffer control (Figure 5.2, left panels). This pattern was observed at each of three incubation times tested (0.5, 2 and 4 hours) and on both of two blood sampling and analysis days (10 weeks separated). Although modest in effect (following the longest incubation time, R-time decreased on average by 2.8 minutes or 31%, K-time decreased on average by 0.5 minutes or 23% and α-angle increased on average by 6° or 10%, relative to buffer control) and statistically not significant (blood from donor R01 was analyzed only twice), this nevertheless does indicate an acceleration, *in vitro*, of platelet-fibrin clot formation and suggests that the EspP passenger domain can potentially alter the clotting behaviour of human blood at a global level.

When fresh citrated whole blood from donor R02 was incubated with WT EspP passenger domain (0.4 mg/mL) and subsequently analyzed by TEG, as with donor R01 R-time was decreased when compared to incubations with buffer control (Figure 5.2, top-right panel); following a 0.5-hr incubation R-time was reduced by 2.7 minutes (95% CI 1.4-4.0, P=0.007) or 29% (95% CI 14-44, P=0.008), following a 2-hr incubation R-time was reduced by 3.0 minutes (95% CI 1.8-4.1, P=0.008) or 30% (95% CI 25-35, P=0.002) and following a 4-hr incubation R-
Figure 5.1 Thrombelastograph tracings of blood from two donors treated with WT EspP passenger domain.

Fresh citrated whole blood from donors R01 and R02 were incubated with buffer alone or with WT EspP passenger domain (0.4 mg/mL) for 0.5, 2 or 4 hours, then analyzed by TEG. This procedure was performed twice (on separate days) using blood from donor R01 and four times (on separate days) using blood from donor R02. Whole blood from donor R02 was also incubated with S263A (0.4 mg/mL) in addition to buffer or to WT EspP passenger domain for two of these four runs. Shown are the TEG tracings of blood samples monitored for a duration of 70 minutes following reconstitution with calcium chloride. All tracings are plotted to the same scale as shown in the lower left panel. Tracings obtained of blood samples treated for 4 hours with WT EspP are marked with an asterisk (*).

Time was reduced by 2.1 minutes (95% CI 0.2-3.9, P=0.038) or 23% (95% CI 3-42, P=0.033). Here statistical significance could be determined because blood samples from donor R02 were drawn and analyzed a total of four times (spanning 12 weeks). K-time and α-angle however were only significantly altered by WT EspP passenger domain treatment relative to buffer controls following the 0.5-hour incubation period (Figure 5.2, middle-right and bottom-right panels); K-time was reduced by 1.1 minutes (95% CI 0.5-1.6, P=0.008) or 37% (95% CI 25-49, P=0.002) and α-angle was increased by 21° (95% CI 1-41, P=0.046) or 56% (95% CI 17-130, P=0.093). K-time and α-angle were not significantly altered by WT EspP passenger domain treatment relative to buffer controls following the 2-hr or 4-hr incubation periods. Owing to the reduced R-time, these results indicate an acceleration, in vitro, of platelet-fibrin clot formation and provides further support that the WT EspP passenger domain can alter the clotting behaviour of human blood on a global level.

In order to differentiate the proteolytic effects of the EspP passenger domain, owing to its serine
Figure 5.2 The EspP passenger domain enhances platelet-fibrin clot formation in both of two initial donors.

Fresh citrated whole blood from donors R01 and R02 were incubated with buffer alone (PBS-G) or with WT EspP passenger domain (0.4 mg/mL) for 0.5, 2 or 4 hours, then analyzed by TEG. This procedure was performed twice (on separate days) using blood from donor R01 and four times (on separate days) using blood form donor R02. Whole blood from donor R02 was also incubated with S263A (0.4 mg/mL) in addition to buffer or to WT EspP passenger domain for two of these four runs. The reaction time (R-time), clot formation time (K-time) and α-angle observed during each
run are plotted here. P-values indicated are for paired two-tailed t-tests. A more extensive listing of P-values is presented in Table 5.1.

Table 5.1 Changes in clot formation parameters when 0.4 mg/mL WT EspP passenger domain was incubated with whole blood from two initial donors.

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Change relative to PBS-G</th>
<th>Donor R01</th>
<th></th>
<th></th>
<th>Change relative to PBS-G</th>
<th>Donor R02</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute value</td>
<td>Normalized percentage (%)</td>
<td>Mean</td>
<td>95% CI</td>
<td>P value</td>
<td>Absolute value</td>
<td>Normalized percentage (%)</td>
<td>Mean</td>
</tr>
<tr>
<td>R-time (min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>-3.1</td>
<td>-25.2</td>
<td>-3.1</td>
<td>-28.9</td>
<td>0.007</td>
<td>-2.7</td>
<td>-4.0 to -1.4</td>
<td>0.007</td>
</tr>
<tr>
<td>2.0</td>
<td>-2.4</td>
<td>-24.6</td>
<td>-3.0</td>
<td>-4.1 to -1.8</td>
<td>0.008</td>
<td>-3.0</td>
<td>-4.1 to -1.8</td>
<td>0.008</td>
</tr>
<tr>
<td>4.0</td>
<td>-2.8</td>
<td>-31.0</td>
<td>-2.1</td>
<td>-3.9 to -0.2</td>
<td>0.038</td>
<td>-2.1</td>
<td>-3.9 to -0.2</td>
<td>0.038</td>
</tr>
<tr>
<td>K-time (min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>-0.3</td>
<td>-12.8</td>
<td>-1.1</td>
<td>-1.6 to -0.5</td>
<td>0.008</td>
<td>-1.1</td>
<td>-1.6 to -0.5</td>
<td>0.008</td>
</tr>
<tr>
<td>2.0</td>
<td>-0.8</td>
<td>-31.7</td>
<td>-0.5</td>
<td>-1.8 to 0.7</td>
<td>0.208</td>
<td>-0.5</td>
<td>-1.8 to 0.7</td>
<td>0.208</td>
</tr>
<tr>
<td>4.0</td>
<td>-0.5</td>
<td>-23.0</td>
<td>0.05</td>
<td>-1.3 to 0.2</td>
<td>0.119</td>
<td>0.05</td>
<td>-1.3 to 0.2</td>
<td>0.119</td>
</tr>
<tr>
<td>α-angle (°)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>3.7</td>
<td>6.0</td>
<td>21.1</td>
<td>0.8 to 41.4</td>
<td>0.046</td>
<td>56.2</td>
<td>-17.2 to 129.5</td>
<td>0.093</td>
</tr>
<tr>
<td>2.0</td>
<td>10.2</td>
<td>18.2</td>
<td>9.3</td>
<td>-3.4 to 22.0</td>
<td>0.088</td>
<td>17.1</td>
<td>-3.9 to 38.1</td>
<td>0.073</td>
</tr>
<tr>
<td>4.0</td>
<td>6.1</td>
<td>10.0</td>
<td>4.1</td>
<td>-4.2 to 12.4</td>
<td>0.213</td>
<td>8.1</td>
<td>-7.4 to 23.5</td>
<td>0.196</td>
</tr>
</tbody>
</table>

P-values and 95% confidence intervals listed are for paired two-tailed t-tests. A dash (-) indicates those measurements where fewer than three measurements were recorded and therefore a P-value and 95% confidence interval could not be calculated.

protease activity, on any potential effects observed during TEG analysis from any other roles this passenger domain may play, in addition to incubations with buffer control and with WT EspP passenger domain, blood samples from donor R02 were also incubated with S263A (0.4 mg/mL). When fresh citrated whole blood from donor R02 was incubated with the S263A passenger domain, following the longest incubation period R-time was reduced by 2.8 minutes or 32%, K-time was reduced by 0.7 minutes or 21% and α-angle was increased by 5° or 10% relative to incubations with buffer control (Figure 5.2, right panels). Relative to incubations with
the WT EspP passenger domain, R-time was reduced by 1.3 minutes or 18% while K-time and α-angle remained unchanged (K-time was increased by 0.1 minutes or 3% and α-angle was reduced by 1° or 2%). Incubations with S263A, however, were only included in two of the four blood sampling and analysis sessions performed using donor R02 and so these observed differences in clot formation parameters could not be statistically validated. Nevertheless, these results imply that the observed acceleration in vitro of platelet-fibrin clot formation caused by the EspP passenger domain relative to buffer control may not be a specific result of proteolytic activity of the EspP passenger domain and suggests that other features of the passenger domain may be at work.

In order to determine whether the EspP passenger domain induces an accelerated rate of platelet-fibrin clot formation in the wider population, blood samples from an additional six donors were analyzed (Figure 5.3). As a further control here incubations with BSA were also included. However, due to instrumentation limitations, samples incubated with BSA were only analyzed for four (donors H02, H03, H06 and H07) of the six donors and samples incubated with buffer control were only analyzed for two donors (donors H02 and H04). Furthermore, whereas incubations for donors R01 and R02 were performed at a protein (WT or S263A EspP passenger domain) concentration of 0.4 mg/mL, incubations for donors H01-H07 were performed at a 150% higher protein (BSA, WT EspP passenger domain or S263A passenger domain) concentration of 1.0 mg/mL, to make more pronounced any potential effects of these proteins on the blood samples. When blood samples from donors H01-H07 were incubated with WT EspP passenger domain (1.0 mg/mL) for 0.5 hours or 2 hours and subsequently analyzed by TEG, R-time, K-time and α-angle were not significantly altered (P>0.05) in comparison to incubations with BSA over the same time periods (Figure 5.4). When samples were analyzed after a 4-hr incubation with WT EspP passenger domain, R-time decreased by 7.0 minutes (95% CI 3.7-10.3, P=0.007) or 61% (95% CI 52-69, P<0.001), K-time decreased by 0.9 minutes (95% CI 0.2-1.5, P=0.023) or 24% (95% CI 3-44, P=0.034) and α-angle increased by 9.5° (95% CI 8.3-10.8, P<0.001) or 21% (95% CI 15-28, P=0.002) relative to incubations with BSA, suggesting an accelerated rate of platelet-fibrin clot formation in WT EspP passenger domain-treated samples relative to BSA controls. When compared to incubations with S263A, WT EspP passenger domain-treated samples had a significantly reduced R-time (by 3.5 minutes
Figure 5.3 Thrombelastograph tracings of blood from six additional donors treated with WT EspP.

Fresh citrated whole blood from donors H01-H04 and H06-H07 were incubated with WT EspP passenger domain (1.0 mg/mL) or with S263A (1.0 mg/mL) for 0.5, 2 or 4 hours, then analyzed by TEG. Blood from donors H02 and H04 were additionally incubated with buffer alone as a negative control. Blood from donors H02-H03 and H06-H07 were additionally incubated with BSA (1.0 mg/mL) as a negative control. Shown are the TEG tracings of blood samples monitored for a duration of 70 minutes following reconstitution with calcium chloride. All tracings are plotted to the same scale as shown in the lower left panel. Marked are TEG tracings of blood samples treated for 4 hours with WT EspP (*), BSA (+) and S263A (x).

[95% CI 0.6-6.3, P=0.028] or 20% [95% CI 5-35, P=0.018]) when analyzed after 0.5 hours, but did not have significantly altered R-time at the longer incubation times of 2 and 4 hours, did not
Figure 5.4 The EspP passenger domain enhances platelet-fibrin clot formation in six additional donors.

Fresh citrated whole blood from donors H01-H04 and H06-H07 were incubated with WT EspP passenger domain (1.0 mg/mL) or with S263A (1.0 mg/mL) for 0.5, 2 or 4 hours, then analyzed by TEG. Blood from donors H02 and H04 were additionally incubated with buffer alone (PBS-G) as a negative control. Blood from donors H02-H03 and H06-H07 were additionally incubated with BSA (1.0 mg/mL) as a negative control. The reaction time (R-time), clot formation time (K-time) and α-angle observed during each run are plotted here. P-values indicated are for paired two-tailed t-tests. A more extensive listing of P-values is presented in Table 5.2.
Table 5.2 Changes in clot formation parameters when 1.0 mg/mL WT EspP passenger domain was incubated with whole blood from six additional donors.

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Change relative to PBS-G</th>
<th>Change relative to BSA</th>
<th>Change relative to S263A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute value</td>
<td>Normalized percentage (%)</td>
<td>Absolute value</td>
</tr>
<tr>
<td></td>
<td>mean 95% CI P value</td>
<td>mean 95% CI P value</td>
<td>mean 95% CI P value</td>
</tr>
<tr>
<td>R-time</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 hr</td>
<td>-5.9 -34.9 -</td>
<td>-12.5 -68.7 to 43.7 0.529</td>
<td>-12.5 -68.7 to 43.7 0.529</td>
</tr>
<tr>
<td>(min)</td>
<td>-31.9 -51.9 -</td>
<td>0.369 -12.5 -68.7 to 43.7 0.529</td>
<td>-31.9 -51.9 -</td>
</tr>
<tr>
<td>2.0 hr</td>
<td>-5.1 -35.0 -</td>
<td>0.199 -25.8 77.2 to 25.5 0.208</td>
<td>-25.8 77.2 to 25.5 0.208</td>
</tr>
<tr>
<td>4.0 hr</td>
<td>-11.3 -71.0 -</td>
<td>0.007 -60.8 -69.2 to 52.4 &lt;0.001</td>
<td>-60.8 -69.2 to 52.4 &lt;0.001</td>
</tr>
<tr>
<td>K-time</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 hr</td>
<td>-2.7 -41.2 -</td>
<td>0.345 -19.5 -102.5 to 63.5 0.509</td>
<td>-19.5 -102.5 to 63.5 0.509</td>
</tr>
<tr>
<td>(min)</td>
<td>-5.7 -5.3 -</td>
<td>0.185 -33.7 -82.6 to 15.2 0.116</td>
<td>-33.7 -82.6 to 15.2 0.116</td>
</tr>
<tr>
<td>2.0 hr</td>
<td>-5.2 -60.9 -</td>
<td>0.023 -24.0 -43.6 to -3.4 0.034</td>
<td>-24.0 -43.6 to -3.4 0.034</td>
</tr>
<tr>
<td>4.0 hr</td>
<td>-2.9 -53.8 -</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α-angle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 hr</td>
<td>15.8 -53.5 -</td>
<td>0.167 -36.4 -36.5 to 109.3 0.211</td>
<td>-36.4 -36.5 to 109.3 0.211</td>
</tr>
<tr>
<td>(°)</td>
<td>16.9 -40.0 -</td>
<td>0.167 -36.4 -36.5 to 109.3 0.211</td>
<td>-36.4 -36.5 to 109.3 0.211</td>
</tr>
<tr>
<td>4.0 hr</td>
<td>20.8 -57.4 -</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

P-values and 95% confidence intervals listed are for paired two-tailed t-tests. A dash (-) indicates those measurements where fewer than three measurements were recorded and therefore a P-value and 95% confidence interval could not be calculated.

have a significantly reduced K-time following any of the three incubation times tested, and had a significantly altered α-angle only following the 4-hr incubations – α-angle was reduced by 5.3° (95% CI 0.1-10.5, P=0.046) or 8% (95% CI 1-17, P=0.069) following the 4-hr incubations.
Taken together, the results presented in this section clearly demonstrate that the EspP passenger domain accelerates platelet-fibrin clot formation \textit{in vitro} in human blood. However, this acceleration seems to be independent of proteolytic activity of the passenger domain as evidenced by the ability of the S263A mutant to produce a similar result.

5.3 EspP induces accelerated fibrinolysis

Two further parameters generated by TEG analysis are maximum amplitude of clot (MA) and percent clot lysis (LY30). MA is a measure of the maximum strength of clot and depends both on the rate of clot formation and the rate of clot breakdown in the blood sample being analyzed. LY30 is a measure of clot lysis 30 minutes after MA is reached and provides a powerful parameter to monitor the rate of fibrinolysis in the sample. Analysis of blood samples from donor R01 did not yield a marked difference in MA or LY30 between WT EspP passenger domain-treated samples and those incubated with buffer control for any of the three incubation times tested (Figure 5.5 left panels). Interestingly, when blood samples from donor R02 were analyzed, treatment with WT EspP passenger domain for 4 hours reduced MA by 16.4 mm (95% CI 6.2–26.6, $P=0.014$) or 26.5% (95% CI 10.0–42.9, $P=0.014$) and increased LY30 by 42.8% (95% CI 31.9–53.7, $P=0.001$) from less than 1% relative to incubations with buffer control (Figure 5.5 right panels). Incubations with WT EspP passenger domain for the shorter period of 2 hours also produced a reduced MA and increased LY30 relative to buffer controls but these were not found to be statistically significant with the available number of measurements. Incubations with S263A did not produce a marked difference in MA or LY30 relative to buffer controls. This too, however, could not be statistically validated due to an insufficient number of observations. These results indicate that the EspP passenger domain induces, \textit{in vitro}, an accelerated rate of fibrinolysis in blood samples from donor R02 but not in blood samples from donor R01 and suggest that this enhancement is dependant on the proteolytic activity of the EspP passenger domain facilitated by its active site serine residue.

When whole blood from the second group of donors (donors H01–H07) was analyzed, a similar effect on fibrinolysis was observed. Here, treatment with the WT EspP passenger domain, but
Figure 5.5 The EspP passenger domain accelerates fibrinolysis in one (donor R02) but not the other (donor R01) of two initial donors.

Fresh citrated whole blood from donors R01 and R02 were incubated with buffer alone or with WT EspP passenger domain (0.4 mg/mL) for 0.5, 2 or 4 hours, then analyzed by TEG. This procedure was performed twice (on separate days) using blood from donor R01 and four times (on separate days) using blood from donor R02. Whole blood from donor R02 was also incubated with S263A (0.4 mg/mL) in addition to buffer or to WT EspP passenger domain for two of these four runs. The maximum amplitude of clot (MA) and percent clot lysis (LY30) observed during each run are plotted here. P-values indicated are for paired two-tailed (MA) or paired one-tailed (LY30) t-tests. A more extensive listing of P-values is presented in Table 5.3.

not S263A, induced a reduced MA and increased LY30 relative to BSA or buffer control in five (H02–H04, H06 and H07) of the six donors whose blood were analyzed, but not in the sixth (H01) donor (Figure 5.6). Of the five donors for whom an enhanced fibrinolytic pattern was observed, treatment with WT EspP passenger domain decreased MA, and increased LY30, by 21.9 mm (95% CI 10.8–33.0, P=0.008) and 40.5% (95% CI 18.6–62.3, P=0.005), respectively, relative to incubations with BSA and 29.1 mm (95% CI 16.6–41.5, P=0.002) and 34.5% (95% CI 14.2–54.9, P=0.004), respectively, relative to incubations with S263A, when samples were
incubated for 2 hours and by 25.9 mm (95% CI 11.0-40.8, P=0.012) and 27.1% (95% CI 17.0-71.6, P=0.072), respectively, relative to incubations with BSA and 36.3 mm (95% CI 23.6-48.9, P=0.001) and 27.2% (95% CI -1.1 to 55.4, P=0.028), respectively, relative to incubations with S263A, when samples were incubated for 4 hours. The observed inability of the EspP passenger domain to induce enhanced fibrinolysis in blood samples from donor H01, however, is based on measurements from a single blood sample on a single day. It therefore cannot be conclusively determined from the available data that this individual offers resistance to the fibrinolytic effects of the EspP passenger domain.

Table 5.3 Changes in clot stability parameters when 0.4 mg/mL WT EspP passenger domain was incubated with whole blood from two initial donors.

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Donor R01</th>
<th>Change relative to PBS-G</th>
<th>Donor R02</th>
<th>Change relative to PBS-G</th>
<th>Change relative to S263A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute value</td>
<td>Normalized percentage (%)</td>
<td>Absolute value</td>
<td>Normalized percentage (%)</td>
<td>Absolute value</td>
</tr>
<tr>
<td></td>
<td>mean</td>
<td>95% CI</td>
<td>P value</td>
<td>mean</td>
<td>95% CI</td>
</tr>
<tr>
<td>MA (mm)</td>
<td>0.5 hr</td>
<td>2.5</td>
<td>-</td>
<td>3.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2.0 hr</td>
<td>4.5</td>
<td>-</td>
<td>6.7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4.0 hr</td>
<td>3.3</td>
<td>-</td>
<td>4.9</td>
<td>-</td>
</tr>
<tr>
<td>LY30 (%)</td>
<td>0.5 hr</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>2.0 hr</td>
<td>-0.1</td>
<td>-</td>
<td>-</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td>4.0 hr</td>
<td>0.7</td>
<td>-</td>
<td>-</td>
<td>42.8</td>
</tr>
<tr>
<td>Incubation time</td>
<td>0.5 hr</td>
<td>4.0</td>
<td>-</td>
<td>-</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>2.0 hr</td>
<td>2.0</td>
<td>-</td>
<td>-</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>4.0 hr</td>
<td>-19.3</td>
<td>-</td>
<td>-</td>
<td>-29.8</td>
</tr>
<tr>
<td>LY30 (%)</td>
<td>0.5 hr</td>
<td>0.4</td>
<td>-</td>
<td>-</td>
<td>68.3</td>
</tr>
<tr>
<td></td>
<td>2.0 hr</td>
<td>7.7</td>
<td>-</td>
<td>-</td>
<td>2350.0</td>
</tr>
<tr>
<td></td>
<td>4.0 hr</td>
<td>38.0</td>
<td>-</td>
<td>-</td>
<td>9035.4</td>
</tr>
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</table>

P-values and 95% confidence intervals listed are for paired two-tailed (MA) or paired one-tailed (LY30) t-tests. A dash (-) indicates those measurements where fewer than three measurements were available and therefore a P-value and 95% confidence interval could not be calculated.
Figure 5.6 The EspP passenger domain accelerates fibrinolysis in five of six additional donors.

Fresh citrated whole blood from donors H01-H04 and H06-H07 were incubated with WT EspP passenger domain (1.0 mg/mL) or with S263A (1.0 mg/mL) for 0.5, 2 or 4 hours, then analyzed by TEG. Blood from donors H02 and H04 were additionally incubated with buffer alone (PBS-G) as a negative control. Blood from donors H02-H03 and H06-H07 were additionally incubated with BSA (1.0 mg/mL) as a negative control. The maximum amplitude of clot (MA) and percent clot lysis (LY30) observed during each run are plotted here. In red are the MA and LY30 values obtained for donor H01, who did not show an increased LY30 upon incubation with WT EspP passenger domain. P-values indicated are for paired two-tailed (MA) or paired one-tailed (LY30) t-tests. A more extensive listing of P-values is presented in Table 5.4.
Table 5.4 Changes in clot stability parameters when 1.0 mg/mL of WT EspP passenger domain was incubated with whole blood from six additional donors.

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Change relative to PBS-G</th>
<th>Change relative to BSA</th>
<th>Change relative to S263A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute value</td>
<td>Normalized percentage (%)</td>
<td>Absolute value</td>
</tr>
<tr>
<td></td>
<td>mean 95% CI P value</td>
<td>mean 95% CI P value</td>
<td>mean 95% CI P value</td>
</tr>
<tr>
<td>MA (mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 hr</td>
<td>-8.7 -13.0</td>
<td>-</td>
<td>-45.1</td>
</tr>
<tr>
<td>2.0 hr</td>
<td>-21.4 -35.4</td>
<td>-</td>
<td>-44.5 - 71.5 to -17.4</td>
</tr>
<tr>
<td>4.0 hr</td>
<td>-26.2 -45.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LY30 (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 hr</td>
<td>12.1 -</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.0 hr</td>
<td>37.5 -</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4.0 hr</td>
<td>49.7 -</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P-values and 95% confidence intervals listed are for paired two-tailed (MA) or paired one-tailed (LY30) t-tests. A dash (-) indicates those measurements where fewer than three measurements were available and therefore a P-value and 95% confidence interval could not be calculated.

5.4 Conclusions

In this chapter, the effects of purified EspP passenger domain on the clotting and fibrinolytic behaviour of human whole blood was assayed in vitro. The EspP passenger domain was found to alter hemostasis in all individuals (n=8) by enhancing platelet-fibrin clot formation. Furthermore, the EspP passenger domain was found to accelerate fibrinolysis but only in a subset of individuals (n=6 of n=8). These results provide compelling evidence for a role for the EspP
passenger domain in the clino-pathological manifestations of EHEC infection. Furthermore, if these results can be generalized to the entire population and if the observation that only a subset of the population – here represented by donors R02, H01 to H04, H06 and H07 – is susceptible to an EspP-induced enhanced fibrinolysis can be validated, it may be possible to utilize this behaviour in vitro as an a priori means to predict and perhaps even manipulate the clino-pathological outcomes of EHEC infection. In the next chapter, blood samples from donors H01-H07 are analyzed in greater detail to better elucidate the mechanism by which the EspP passenger domain induces the observed changes in hemostatic behaviour.
Chapter 6:

Further characterization of the effects of the EspP passenger domain on clot formation and stability in human blood

The material presented in this chapter has been obtained through collaboration with Dr. K. H. M. Kuo, Department of Medical Oncology and Hematology, University Health Network, Ms. E. Brnjac, Department of Clinical Pathology, Sunnybrook Health Sciences Centre, and Dr. A. E. Chesney, Department of Clinical Pathology, Sunnybrook Health Sciences Centre, Toronto, Canada.
In chapter 5, the interaction of EspP with human whole blood was investigated \textit{in vitro} using a global test of hemostasis. The EspP passenger domain was found to enhance platelet-fibrin clot formation in all donors and accelerate fibrinolysis in a majority of the donors. In this chapter, the interaction of the EspP passenger domain with human blood is analyzed using more specific assays of clot formation and clot breakdown in an attempt to better understand the mechanisms by which EspP induces this altered hemostasis. Specifically, using incubations with buffer alone and with BSA as controls, the effect of WT EspP passenger domain and S263A on prothrombin time, activated partial thromboplastin time, thrombin time, activities of coagulation factors II, V, VII, VIII, IX, X, XI and XII and fibrinogen and D-dimer levels is investigated \textit{in vitro} using blood samples from the six donors with hemochromatosis (donors H01-H04, H06 and H07) described in chapter 5. Finally, clot formation and lysis times in plasma and the euglobulin fraction of plasma is investigated using blood from donors H02 or H07 – as presented in chapter 5, whole blood samples from both donors H02 and H07 displayed accelerated fibrinolysis following treatment with WT EspP passenger domain.

### 6.1 Analysis of general clotting times

When fresh-frozen plasma from donor H02 was incubated (37 °C, 4 hrs) with WT EspP passenger domain and subsequently analyzed, prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT) were all prolonged in a concentration-dependent manner relative to incubations with buffer alone, with BSA or with S263A (Figure 6.1). At the highest incubation concentration of 1.0 mg/mL, WT EspP passenger domain prolonged PT by 23.7 seconds (95% CI 22.2-25.2, P<0.001) or 179.3\% (95% CI 169.9-188.7, P<0.001), aPTT by 76.0 seconds (95% CI 61.3-90.7, P<0.001) or 205.3\% (95% CI 147.4-263.3, P=0.004) and TT by 9.1 seconds (95% CI 8.3-9.9, P<0.001) or 48.5\% (95% CI 38.0-59.0, P=0.003) relative to BSA. The results were similarly pronounced and statistically significant when compared to incubations with buffer alone or with S263A. Incubations with S263A did not produce a marked difference relative to incubations with buffer alone or with BSA: while
some of these differences were statistically significant, they all remained within 16% of the PBS-G-incubated values.

In order to determine whether these results could be extended to the larger population, FFP from all six donors was incubated (37 °C) with 1.0 mg/mL of WT EspP passenger domain or S263A for 0.5, 2.0 or 4.0 hours and subsequently analyzed. Incubations with buffer alone or with BSA served as controls. PT, aPTT and TT were indeed prolonged for all six donors, now in an incubation time-dependent manner, when FFP samples were treated by WT EspP passenger domain, and not when treated with buffer control, BSA or S263A (Figure 6.2, left panels). Following the longest incubation time of 4.0 hours, WT EspP passenger domain prolonged PT by 22.7 seconds (95% CI 17.7-27.6, P<0.001) or 174.9% (95% CI 131.4-218.5, P<0.001), aPTT by 40.1 seconds (95% CI 29.6-50.6, P=0.001) or 128.8% (95% CI 95.5-162.1, P<0.001) and TT by 6.0 seconds (95% CI 5.5-6.5, P<0.001) or 28.9% (95% CI 25.8-32.0, P<0.001) relative to BSA. Again, these results were similarly pronounced and statistically significant when compared to incubations with buffer alone or with S263A.

To determine whether the prolongation of PT, aPTT and TT following incubation with WT EspP passenger domain is dependent on the cellular components of blood, the above experiment was repeated with one modification; whereas previously fresh citrated whole blood from each donor was first fractionated to produce FFP and subsequently incubated with WT EspP passenger domain, now fresh citrated whole blood from the same six donors is first incubated with WT EspP passenger domain and then fractionated to produce FFP. The final step in both procedures remains unchanged; analysis of the plasma samples to determine clotting times. PT, aPTT and TT were again found to be prolonged for all six donors when whole blood samples were treated with WT EspP passenger domain and not when treated with buffer alone, BSA or S263A (Figure 6.2, right panels). Following the longest incubation time of 4.0 hours, WT EspP passenger domain prolonged PT by 32.7 seconds (95% CI 26.7-38.8, P<0.001) or 256.7% (95% CI 202.1-311.5, P<0.001), aPTT by 77.5 seconds (95% CI 56.4-98.6, P=0.001) or 241.8% (95% CI 178.6-304.9, P<0.001) and TT by 10.2 seconds (95% CI 9.2-11.1, P<0.001) or 45.1% (95% CI 39.5-50.8, P<0.001) relative to BSA. These results were similarly pronounced and statistically significant when compared to incubations with buffer alone or with S263A. The
Figure 6.1 Effect of EspP concentration on PT, aPTT and TT, measured for a single donor.

Fresh-frozen plasma from donor H02 was incubated (37 °C, 4 hrs) with buffer alone (PBS-G) or with 0.1, 0.5 or 1.0 mg/mL of BSA, WT EspP or S263A. Prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT) were then determined for each sample. Shown are mean ± SD values derived from three parallel experiments. Note that the error bars are shorter than the height of the data point markers for the majority of the data points. A statistical analysis of the observed differences following incubation with the highest concentration of protein (1.0 mg/mL) is presented in Table 6.1.

slightly more exaggerated prolongation of clotting times here may be due to the increased processing time necessary to prepare the plasma fraction from EspP-treated whole blood samples and may not represent a real difference in clotting times between incubation of whole blood and
of plasma alone.

Table 6.1 Changes in PT, aPTT and TT when 1.0 mg/mL WT EspP passenger domain was incubated for 4 hours with fresh-frozen plasma from a single donor.

<table>
<thead>
<tr>
<th>Plasma incubations</th>
<th>Change relative to PBS-G</th>
<th>Change relative to BSA</th>
<th>Change relative to S263A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute value</td>
<td>Normalized percentage (%)</td>
<td>Absolute value</td>
</tr>
<tr>
<td></td>
<td>mean 95% CI P value</td>
<td>mean 95% CI P value</td>
<td>mean 95% CI P value</td>
</tr>
<tr>
<td>PT (s)</td>
<td>23.4 21.8 to 24.9 &lt;0.001</td>
<td>171.9 145.1 to 198.7 0.001</td>
<td>23.7 22.2 to 25.2 &lt;0.001</td>
</tr>
<tr>
<td>aPTT (s)</td>
<td>75.7 60.9 to 90.5 &lt;0.001</td>
<td>203.3 127.3 to 279.4 0.008</td>
<td>76.0 61.3 to 90.7 &lt;0.001</td>
</tr>
<tr>
<td>TT (s)</td>
<td>7.8 7.0 to 8.7 &lt;0.001</td>
<td>39.1 32.0 to 46.1 0.002</td>
<td>9.1 8.3 to 9.9 &lt;0.001</td>
</tr>
</tbody>
</table>

For changes in absolute values, P-values and 95% confidence intervals listed are derived from unpaired two-tailed t-tests. For changes in normalized percentage values, P-values and 95% confidence intervals listed are derived from one-sample two-tailed t-tests comparing the mean differences to a theoretical value of zero.
Figure 6.2 Effect of EspP on PT, aPTT and TT, measured for six donors.

Blood plasma (left panels), or whole blood (right panels) from donors H01-H04 and H06-H07 was incubated (37 °C) with buffer alone (PBS-G) or with 1.0 mg/mL of BSA, WT EspP or S263A for 0.5, 2 or 4 hours. Prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT) were then determined for each sample. Shown are values obtained from single measurements. A statistical analysis of the observed differences following incubation for the longest duration (4 hours) is presented in Table 6.2.
Table 6.2 Changes in PT, aPTT and TT when 1.0 mg/mL WT EspP passenger domain was incubated for 4 hours with blood plasma or whole blood from a further six donors.

<table>
<thead>
<tr>
<th></th>
<th>Plasma incubations</th>
<th>Whole blood incubations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Change relative to PBS-G</td>
<td>Change relative to PBS-G</td>
</tr>
<tr>
<td></td>
<td>Absolute value</td>
<td>Normalized percentage (%)</td>
</tr>
<tr>
<td></td>
<td>mean</td>
<td>95% CI</td>
</tr>
<tr>
<td>PT (s)</td>
<td>22.2</td>
<td>17.9 to 26.5</td>
</tr>
<tr>
<td>aPTT (s)</td>
<td>40.4</td>
<td>32.3 to 48.5</td>
</tr>
<tr>
<td>TT (s)</td>
<td>6.5</td>
<td>3.8 to 9.1</td>
</tr>
</tbody>
</table>

Change relative to S263A

|                   | Absolute value | Normalized percentage (%) | Absolute value | Normalized percentage (%) | Absolute value | Normalized percentage (%) |
|-------------------| mean | 95% CI | P value | mean | 95% CI | P value | mean | 95% CI | P value |
| PT (s)            | 22.7 | 17.7 to 27.6 | <0.001 | 174.9 | 131.4 to 218.5 | <0.001 | 32.7 | 26.7 to 38.8 | <0.001 | 256.7 | 202.1 to 311.3 | <0.001 |
| aPTT (s)          | 40.1 | 29.6 to 50.6 | 0.001  | 128.8 | 95.5 to 162.1 | <0.001 | 77.5 | 56.4 to 98.6 | 0.001  | 241.8 | 178.6 to 304.9 | <0.001 |
| TT (s)            | 6.0  | 5.5 to 6.5  | <0.001  | 28.9  | 25.8 to 32.0  | <0.001  | 10.2 | 9.2 to 11.1  | <0.001  | 45.1  | 39.5 to 50.8  | <0.001  |

Change relative to BSA

|                   | Absolute value | Normalized percentage (%) | Absolute value | Normalized percentage (%) | Absolute value | Normalized percentage (%) |
|-------------------| mean | 95% CI | P value | mean | 95% CI | P value | mean | 95% CI | P value |
| PT (s)            | 21.0 | 16.6 to 25.4 | <0.001  | 153.9 | 115.0 to 192.9 | <0.001  | 30.0 | 24.9 to 35.1  | <0.001  | 209.3 | 170.8 to 247.8 | <0.001  |
| aPTT (s)          | 39.7 | 31.5 to 48.0 | <0.001  | 122.6 | 97.3 to 148.0 | <0.001  | 77.2 | 6.0 to 93.9  | <0.001  | 230.3 | 179.2 to 281.3 | <0.001  |
| TT (s)            | 4.1  | 3.3 to 4.9  | <0.001  | 18.3  | 14.0 to 22.6  | <0.001  | 7.8  | 6.7 to 8.9   | <0.001  | 31.5  | 25.1 to 38.0  | <0.001  |

P-values and 95% confidence intervals listed are derived from paired two-tailed t-tests.

6.2 Analysis of coagulation factor activities

To investigate the effect of the EspP passenger domain on the various coagulation factors present in plasma, enzymatic activity of factors II, V, VII, VIII, IX, X, XI and XII in plasma from donor H02 was measured subsequent to incubation (37 °C, 4 hrs) of FFP from this donor with 0.1, 0.25, 0.50, 0.75 or 1.00 mg/mL WT EspP passenger domain or S263A. Incubations with buffer alone and with BSA served as controls. I found that WT EspP passenger domain, but not S263A, reduced the residual activities of coagulation factors II, V, VII, VIII, and XII, but not of factors IX, X, or XI, in a concentration-dependent manner relative to incubations with BSA (Figure 6.5). At the highest incubation concentration of 1.0 mg/mL, WT EspP passenger domain reduced the residual activity of coagulation factor V by 0.45 U/mL (95% CI 0.41-0.48, P<0.001) or 70.7% (95% CI 65.1-76.3, P<0.001), VII by 0.60 U/mL (95% CI 0.56-0.64, P<0.001) or 90.6% (95% CI 89.7-91.5, P<0.001), VIII by 0.33 U/mL (95% CI 0.30-0.36, P<0.001) or 57.5% (95% CI 54.4-60.5, P<0.001) and XII by 0.66 U/mL (95% CI 0.61-0.72,
P<0.001) or 65.7% (95% CI 57.7-73.8, P=0.001) relative to incubations with BSA. Incubation with 1.0 mg/mL WT EspP passenger domain reduced the activity of coagulation factor II by 0.10 U/mL (95% CI 0.01-0.19, P=0.042) or 10.9% (95% CI 5.2-16.7, P=0.015) and did not significantly alter the activities of factors IX, X or XI relative to incubations with BSA. Similar results were obtained when WT EspP passenger domain-treated samples were compared to incubations with buffer alone or with S263A. Incubations with S263A and BSA did not produce a marked difference relative to incubations with buffer alone.

To determine the effect of the EspP passenger domain on coagulation factor activities in the larger population, and, separately, to determine its dependence on the cellular components of blood, FFP or fresh citrated whole blood from donors H01, H03, H04, H06 and H07 was incubated (37 °C) with 1.0 mg/mL WT EspP passenger domain or S263A for 0.5, 2.0 or 4.0 hours and subsequently analyzed to determine coagulation factor activities – activity of coagulation factors II, IX, X, XI and XII were analyzed only for samples from donors H03, H04, H06 and H07. As with donor H02 but now in a time-dependent manner, incubation with WT EspP passenger domain, but not S263A, dramatically reduced the residual activities of factors V, VII, VIII and XII but not of factors II, IX, X or XI, relative to incubations with BSA (Figure 6.4). Following the longest incubation time of 4 hours, WT EspP passenger domain reduced the residual activity of coagulation factor V by 0.68 U/mL (95% CI 0.58-0.79, P<0.001) or 70.6% (95% CI 63.7-77.5, P<0.001), of VII by 0.79 U/mL (95% CI 0.41-1.17, P=0.005) or 90.5% (95% CI 88.1-92.8, P<0.001), of VIII by 0.54 U/mL (95% CI 0.20-0.88, P=0.011) or 54.4% (95% CI 46.9-61.9, P<0.001), and of XII by 0.36 (95% CI 0.22-0.49, P=0.004) or 37.9% (95% CI 26.9-48.9, P=0.002) in FFP relative to incubations with BSA. Over this same period, WT EspP passenger domain reduced the activity of factor II by 0.18 U/mL (95% CI 0.00-0.35, P=0.047) or 18.0% (95% CI 4.3-31.6, P=0.025) and did not significantly alter the activities of factors IX, X or XI relative to BSA. Similar results were obtained when compared to incubations with buffer alone or with S263A and when fresh citrated whole blood samples were incubated in place of FFP. Incubations with S263A did not result in a marked difference in coagulation factor activities relative to incubations with BSA or buffer alone.
Figure 6.3 Effect of EspP concentration on coagulation factor activities, measured for a single donor.

Fresh-frozen plasma from donor H02 was incubated (37 °C, 4 hrs) with buffer alone (PBS-G) or with 0.1, 0.5 or 1.0 mg/mL of BSA, WT EspP or S263A. Residual activities of coagulations factors
II, V, VII, VIII, IX, X, XI and XII were then determined for each sample. Shown are mean ± SD values derived from three parallel experiments. Note that for many of the data points these error bars are shorter than the height of the data point markers. A statistical analysis of the observed differences following incubation with the highest concentration of protein (1.0 mg/mL) is presented in Table 6.3.

Table 6.3 Changes in coagulation factor activities when 1.0 mg/mL WT EspP passenger domain was incubated for 4 hours with fresh-frozen plasma from a single donor.

<table>
<thead>
<tr>
<th>Plasma incubations</th>
<th>Change relative to PBS-G</th>
<th>Change relative to BSA</th>
<th>Change relative to S263A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute value</td>
<td>Normalized percentage (%)</td>
<td>Absolute value</td>
</tr>
<tr>
<td></td>
<td>mean</td>
<td>95% CI</td>
<td>P value</td>
</tr>
<tr>
<td>Factor II (U/mL)</td>
<td>-0.12</td>
<td>-0.19 to -0.05</td>
<td>0.008</td>
</tr>
<tr>
<td>Factor V (U/mL)</td>
<td>-0.44</td>
<td>-0.52 to -0.36</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Factor VII (U/mL)</td>
<td>-0.61</td>
<td>-0.67 to -0.55</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Factor VIII (U/mL)</td>
<td>-0.35</td>
<td>-0.40 to -0.30</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Factor IX (U/mL)</td>
<td>-0.03</td>
<td>-0.11 to 0.05</td>
<td>0.311</td>
</tr>
<tr>
<td>Factor X (U/mL)</td>
<td>0.05</td>
<td>-0.04 to 0.13</td>
<td>0.189</td>
</tr>
<tr>
<td>Factor XI (U/mL)</td>
<td>0.02</td>
<td>-0.03 to 0.08</td>
<td>0.327</td>
</tr>
<tr>
<td>Factor XII (U/mL)</td>
<td>-0.69</td>
<td>-0.73 to -0.64</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

For changes in absolute values, P-values and 95% confidence intervals listed are derived from unpaired two-tailed t-tests. For changes in normalized percentage values, P-values and 95% confidence intervals listed are derived from one-sample two-tailed t-tests comparing the mean differences to a theoretical value of zero.
Plasma incubations

Whole blood incubations

(Figure 6.4, continued on next page)
Figure 6.4 Effect of EspP on coagulation factor activities, measured for a further five donors.

Blood plasma (left panels) or whole blood (right panels) from donors H01, H03-H04 and H06-H07 was incubated (37 °C) with buffer alone (PBS-G) or with 1.0 mg/mL of BSA, WT EspP or S263A
for 0.5, 2 or 4 hours. Residual activities of coagulation factors II, V, VII, VIII, IX, X, XI and XII were then determined for each sample. Shown are values obtained from single measurements.

Blood samples from donor H01 were analyzed only for residual activity of factors V, VII and VIII. Blood samples from donor H02 were not analyzed for any factor activity. A statistical analysis of the observed differences following incubation for the longest duration (4 hours) is presented in Table 6.4.

Table 6.4 Changes in coagulation factor activities when 1.0 mg/mL WT EspP passenger domain was incubated for 4 hours with blood plasma or whole blood from a further five donors.

<table>
<thead>
<tr>
<th></th>
<th>Plasma incubations</th>
<th>Whole blood incubations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Change relative to PBS-G</td>
<td>Change relative to PBS-G</td>
</tr>
<tr>
<td></td>
<td>Absolute value</td>
<td>Normalized percentage (%)</td>
</tr>
<tr>
<td></td>
<td>mean 95% CI P value</td>
<td>mean 95% CI P value</td>
</tr>
<tr>
<td>Factor II (U/mL)</td>
<td>-0.16 -0.31 to -0.01 0.040</td>
<td>-16.6 -29.3 to -3.8 0.026</td>
</tr>
<tr>
<td>Factor V (U/mL)</td>
<td>-0.66 -0.73 to -0.54 &lt;0.001</td>
<td>-68.9 -76.7 to -83.1 &lt;0.001</td>
</tr>
<tr>
<td>Factor VII (U/mL)</td>
<td>-0.77 -1.16 to -0.37 0.006</td>
<td>-90.2 -92.6 to -87.8 &lt;0.001</td>
</tr>
<tr>
<td>Factor VIII (U/mL)</td>
<td>-0.48 -0.72 to -0.24 0.005</td>
<td>-52.3 -56.5 to -48.0 &lt;0.001</td>
</tr>
<tr>
<td>Factor IX (U/mL)</td>
<td>0.01 -0.09 to -0.10 0.803</td>
<td>0.8 -8.1 to 9.6 0.805</td>
</tr>
<tr>
<td>Factor X (U/mL)</td>
<td>-0.05 -0.17 to 0.07 0.279</td>
<td>-4.5 -18.6 to 9.6 0.383</td>
</tr>
<tr>
<td>Factor XI (U/mL)</td>
<td>-0.01 -0.06 to 0.04 0.576</td>
<td>-1.1 -6.1 to 3.9 0.533</td>
</tr>
<tr>
<td>Factor XII (U/mL)</td>
<td>-0.35 -0.51 to -0.18 0.008</td>
<td>-36.6 -44.2 to -29.0 0.001</td>
</tr>
</tbody>
</table>

|                          | Change relative to BSA | Change relative to BSA |
|                          | Absolute value               | Normalized percentage (%) | Absolute value               | Normalized percentage (%) |
|                          | mean 95% CI P value       | mean 95% CI P value       | mean 95% CI P value       | mean 95% CI P value       |
| Factor II (U/mL)         | -0.18 -0.35 to 0.00 0.047 | -18.0 -31.6 to -4.3 0.025 | -0.17 -0.24 to -0.10 0.005 | -19.2 -25.9 to -12.5 0.003 |
| Factor V (U/mL)          | -0.68 -0.79 to -0.58 <0.001 | -70.6 -77.5 to -63.7 <0.001 | -0.66 -0.78 to -0.54 0.000 | -75.5 -81.1 to -69.8 <0.001 |
| Factor VII (U/mL)        | -0.79 -1.17 to -0.41 0.005 | -90.5 -92.8 to -88.1 <0.001 | -0.73 -1.07 to -0.40 0.004 | -94.7 -97.0 to -92.4 <0.001 |
| Factor VIII (U/mL)       | -0.54 -0.88 to -0.20 0.111 | -54.4 -61.9 to -46.9 <0.001 | -0.58 -0.80 to -0.37 0.002 | -67.7 -71.7 to -63.7 <0.001 |
| Factor IX (U/mL)         | -0.02 -0.10 to 0.07 0.587 | -1.5 -8.9 to 5.9 0.572 | 0.06 -0.05 to 0.16 0.206 | 5.3 -5.1 to 15.8 0.203 |
| Factor X (U/mL)          | -0.04 -0.14 to 0.06 0.274 | -4.8 -16.2 to 6.6 0.273 | -0.04 -0.06 to 0.02 0.009 | -5.4 -8.5 to -2.4 0.011 |
| Factor XI (U/mL)         | -0.03 -0.13 to 0.453      | -2.9 -13.7 to 7.8 0.449 | 0.03 -0.08 to 0.14 0.429 | 3.4 -8.5 to 15.4 0.428 |
| Factor XII (U/mL)        | -0.36 -0.49 to -0.22 0.004 | -37.9 -48.9 to -26.9 0.002 | -0.46 -0.66 to -0.27 0.005 | -50.6 -61.3 to -40.0 0.001 |

|                          | Change relative to S263A | Change relative to S263A |
|                          | Absolute value               | Normalized percentage (%) | Absolute value               | Normalized percentage (%) |
|                          | mean 95% CI P value       | mean 95% CI P value       | mean 95% CI P value       | mean 95% CI P value       |
| Factor II (U/mL)         | -0.12 -0.23 to -0.02 0.032 | -13.4 -22.6 to -4.2 0.019 | -0.11 -0.20 to -0.02 0.034 | -12.5 -20.5 to -4.6 0.015 |
| Factor V (U/mL)          | -0.64 -0.75 to -0.53 <0.001 | -69.4 -74.7 to -64.0 <0.001 | -0.61 -0.71 to -0.52 <0.001 | -74.3 -78.9 to -69.8 <0.001 |
| Factor VII (U/mL)        | -0.79 -1.17 to -0.40 0.005 | -90.4 -92.9 to -88.0 <0.001 | -0.74 -1.10 to -0.38 0.005 | -94.7 -96.9 to -92.4 <0.001 |
| Factor VIII (U/mL)       | -0.51 -0.81 to -0.21 0.009 | -53.2 -60.0 to -46.3 <0.001 | -0.55 -0.75 to -0.35 0.002 | -66.3 -72.0 to -60.7 <0.001 |
| Factor IX (U/mL)         | -0.01 -0.10 to 0.08 0.646 | -1.4 -9.9 to 7.0 0.625 | 0.08 -0.01 to 0.18 0.061 | 8.6 0.2 to 17.1 0.048 |
| Factor X (U/mL)          | -0.04 -0.14 to 0.06 0.259 | -3.7 -16.4 to 6.9 0.417 | 0.00 -0.04 to 0.03 0.023 | 0.0 -5.6 to 5.6 >0.999 |
| Factor XI (U/mL)         | -0.06 -0.17 to 0.14 0.044 | -6.6 -17.7 to 4.2 0.144 | 0.00 -0.10 to 0.11 0.005 | 0.2 -11.8 to 12.3 0.955 |
| Factor XII (U/mL)        | -0.37 -0.53 to -0.21 0.005 | -38.5 -44.5 to -32.5 <0.001 | -0.48 -0.71 to -0.25 0.007 | -51.1 -61.4 to -40.8 0.001 |

P-values and 95% confidence intervals listed are derived from paired two-tailed t-tests.
6.3 Analysis of fibrinogen and D-dimer levels

To determine the effect of EspP on fibrinogen stability, fibrinogen and D-dimer concentrations were measured firstly in FFP from donor H02 incubated (37 °C) with 0.1, 0.25, 0.50, 0.75 or 1.00 mg/mL WT EspP passenger domain or S263 for 4.0 hours (Figure 6.5), secondly in blood plasma from donors H01-H04, H06 and H07 incubated (37 °C) with 1.0 mg/mL WT EspP passenger domain or S263A for 0.5, 2.0 or 4.0 hours (Figure 6.6, left panels), and thirdly in fresh citrated whole blood from donors H01-H04, H06 and H07 incubated (37 °C) with 1.0 mg/mL WT EspP passenger domain or S263A for 0.5, 2.0 or 4.0 hours (Figure 6.6, right panels). Samples incubated with buffer alone or with BSA served as controls. Samples derived from donor H03 were not analyzed for D-dimer concentration. For donor H01, D-dimer concentration was only analyzed in samples treated as described in the third scenario; blood plasma-incubated samples from donor H01, prepared as described in the second scenario, were not analyzed for D-dimer concentration.

Incubation with WT EspP passenger domain or S263A did not significantly alter the fibrinogen level in blood samples from these donors relative to incubations with buffer alone or with BSA (top panels of Figure 6.5 and Figure 6.6). The response of D-dimer level to incubation with the EspP passenger domain, however, was mixed. When FFP from donor H02 was incubated (37 °C, 4 hrs) with 0.10, 0.25, 0.50, 0.75 or 1.00 mg/mL WT EspP passenger domain or S263A D-dimer level increased in a concentration-dependent manner relative to incubations with buffer alone or with BSA (Figure 6.5, bottom panel). At the highest concentration of 1.0 mg/mL WT EspP passenger domain D-dimer level increased by 323 ng/mL (95% CI 271-375, P<0.001) or 53.8% (95% CI 36.2-71.4, P=0.006) relative to incubations with buffer alone, by 477 ng/mL (95% CI 426-528, P<0.001) or 106.9% (95% CI 80.8-133.0, P=0.003) relative to incubations with BSA and by 151 ng/mL (95% CI 92-209, P=0.002) or 19.6% (95% CI 2.5-36.8, P=0.039) relative to incubations with S263A. When data from H01, H02, H04, H05 and H07 were analyzed together, incubation of blood samples with WT EspP passenger domain or S263A did not significantly alter D-dimer level relative to incubations with buffer alone or with BSA (Figure 6.6, bottom panels).
Figure 6.5 Effect of EspP concentration on Fib-C and D-dimer, measured for a single donor.

Fresh-frozen plasma from donor H02 was incubated (37 °C, 4 hrs) with buffer alone (PBS-G) or with 0.1, 0.5 or 1.0 mg/mL of BSA, WT EspP or S263A. Fibrinogen concentration by the Clauss method (Fib-C) and D-dimer concentration were then determined for each sample. Shown are mean ± SD values derived from three parallel experiments. Note that these error bars are shorter than the height of the data point markers for many of the data points. A statistical analysis of the observed differences following incubation with the highest concentration of protein (1.0 mg/mL) is presented in Table 6.5.
Table 6.5 Changes in fibrinogen and D-dimer concentrations when 1.0 mg/mL WT EspP passenger domain was incubated for 4 hours with fresh-frozen plasma from a single donor.

<table>
<thead>
<tr>
<th></th>
<th>Plasma incubations</th>
<th>Change relative to PBS-G</th>
<th>Change relative to S263A</th>
<th>Change relative to BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute value</td>
<td>Normalized percentage (%)</td>
<td>Absolute value</td>
<td>Normalized percentage (%)</td>
</tr>
<tr>
<td>Fib-C (g/L)</td>
<td>mean</td>
<td>95% CI</td>
<td>P value</td>
<td>mean</td>
</tr>
<tr>
<td></td>
<td>-0.27</td>
<td>-0.41 to -0.13</td>
<td>&lt;0.001</td>
<td>-10.9</td>
</tr>
<tr>
<td>D-Dimer (ng/mL)</td>
<td>323</td>
<td>271 to 375</td>
<td>&lt;0.001</td>
<td>53.8</td>
</tr>
</tbody>
</table>

For changes in absolute values, P-values and 95% confidence intervals listed are derived from unpaired two-tailed t-tests. For changes in normalized percentage values, P-values and 95% confidence intervals listed are derived from one-sample two-tailed t-tests comparing the mean differences to a theoretical value of zero.
Figure 6.6 Effect of EspP on Fib-C and D-Dimer, measured for five and six donors, respectively.

Fresh-frozen plasma (left panels), or whole blood (right panels) from donors H01-H04 and H06-H07 was incubated (37 °C) with buffer alone (PBS-G) or with 1.0 mg/mL of BSA, WT EspP or S263A for 0.5, 2 or 4 hours. Fibrinogen concentration by the Clauss method (Fib-C) and D-dimer concentration were then determined for each sample. Shown are values obtained from single measurements. Whole blood- and FFP-incubated samples from donor H03 as well as FFP-incubated samples from donor H01 were not analyzed for D-dimer concentration. A statistical analysis of the observed differences following incubation for the longest duration (4 hours) is presented in Table 6.6.
Table 6.6 Changes in fibrinogen and D-dimer concentrations when 1.0 mg/mL WT EspP passenger domain was incubated for 4 hours with blood plasma or whole blood from a further six donors.

<table>
<thead>
<tr>
<th></th>
<th>Plasma incubations</th>
<th></th>
<th>Whole blood incubations</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Change relative to PBS-G</td>
<td>Change relative to PBS-G</td>
<td>Change relative to BSA</td>
<td>Change relative to BSA</td>
</tr>
<tr>
<td></td>
<td>Absolute value mean</td>
<td>Normalized percentage (%) mean</td>
<td>P value</td>
<td>Absolute value mean</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>-0.02</td>
<td>-0.14 to 0.10</td>
<td>0.730</td>
<td>0.7</td>
</tr>
<tr>
<td>D-Dimer (ng/mL)</td>
<td>194</td>
<td>-158 to 547</td>
<td>0.178</td>
<td>0.178</td>
</tr>
</tbody>
</table>

P-values and 95% confidence intervals listed are derived from paired two-tailed t-tests. A dash (-) indicates those measurements where fewer than three measurements were available and therefore a P-value and 95% confidence interval could not be calculated.

6.4 Analysis of clot formation and lysis of unfractionated plasma and of the euglobulin fraction of plasma

Finally, to determine the effect of the EspP passenger domain on clot lysis times in the absence of the cellular components of blood, a standard euglobulin clot lysis time as described by Smith et al. (2003), as well as a modified version of this assay wherein unfractionated plasma is used in place of the euglobulin fraction of plasma, were performed. Such measurements of clot lysis times further allow measurements of clot formation times. This method therefore also allowed measurement of the effect of the EspP passenger domain on clot formation times in the euglobulin fraction of blood. Moreover, it allowed a further method to measure clot formation times in unfractionated plasma.

When FFP from donor H02 was incubated (37 °C, 4 hrs) with WT EspP passenger domain, processed to produce the euglobulin fraction and analyzed, clot formation time was prolonged in
a concentration-dependent manner relative to incubations with buffer alone, with BSA or with S263A (Figure 6.7, left panels). At the highest concentration of 1.0 mg/mL, incubation of FFP from this donor with WT EspP passenger domain prolonged clot formation time of the euglobulin fraction by 7.5 minutes or 150% relative to incubations with BSA. A similar increase in clot formation time was observed when compared to incubations with buffer alone or with S263A. Clot formation times of the euglobulin fraction of S263A-treated FFP from this donor was not markedly altered relative to those observed in the euglobulin fractions of buffer alone- or BSA-treated FFP. Incubation of FFP from donor H02 with WT EspP passenger domain or S263A did not markedly alter clot lysis times of the euglobulin fraction relative to incubations with buffer alone or with BSA (Figure 6.7, right panels). For logistical reasons, this experiment was performed with FFP from donor H02 only and only twice. The results therefore cannot be statistically validated.

To preclude the possibility of the EspP passenger domain, PBS-G buffer components or BSA possibly being removed from the euglobulin fraction as a consequence of preparation of the euglobulin fraction such that these component are not present in the sample mixture during the course of clot formation and lysis, the above experiment was repeated with one modification; whereas previously FFP was first incubated with buffer alone, with BSA, with WT EspP passenger domain or with S263A and subsequently processed to produce the euglobulin fraction, now the euglobulin fraction was first prepared from FFP and then incubated with buffer alone, with BSA, with WT EspP passenger domain or with S263A. The final step in both procedures remains unchanged; analysis of the euglobulin fraction to determine clot formation and clot lysis times. For logistical reasons, here only blood samples from donor H07 were analysed. Furthermore, this experiment was performed only once and so the results cannot be statistically validated. When the euglobulin fraction of blood from donor H07 was incubated (37 °C, 4 hrs) with WT EspP passenger domain and subsequently analyzed, clot formation time was reduced relative to incubation with buffer alone, with BSA and with S263A (Figure 6.8a, left panel). At the highest concentration of 1.0 mg/mL, incubation with WT EspP passenger domain reduced the clot formation time of the euglobulin fraction by 3 minutes or 27% relative to incubation with buffer alone or with BSA and by 4 minutes or 33% relative to incubations with S263A. Incubation of the euglobulin fraction with S263A prolonged clot formation times,
Figure 6.7 Effect of EspP on euglobulin clot formation and lysis times, measured for donor H02.

Fresh-frozen plasma from donor H02 was incubated (37 °C, 4 hrs) by itself (untreated), with buffer alone (PBS-G), or with 0.1, 0.5 or 1.0 mg/mL of each of BSA, WT EspP or S263A. The euglobulin fraction was then prepared and assayed as described in chapter 2, section 2.11.1. Plotted in the top panels are the resultant experimental spectrophotometric tracings of clot formation and lysis from two identical experiments performed at different times. Clot formation times and clot lysis times determined from these tracings are presented in the middle and bottom panels, respectively.
by 1 minute or 9% at the highest concentration of 1.0 mg/mL, relative to incubations with buffer alone or BSA. Incubation of the euglobulin fraction of blood from donor H07 with WT EspP passenger domain or with S263A did not markedly alter clot lysis time relative to incubation with buffer alone or with BSA (Figure 6.8a, right panel).

Lastly, to determine the effect of the EspP passenger domain on clot lysis of unfractionated plasma, the above experiment was repeated with one further modification; whereas previously the euglobulin fraction of plasma was incubated with buffer alone, with BSA, with WT EspP passenger domain or with S263A and subsequently analyzed, now unfractionated FFP was incubated and analyzed directly. As before, this experiment was performed only once and only using blood samples from donor H07. The results therefore cannot be statistically validated. When FFP from donor H07 was incubated (37 °C, 4 hrs) with WT EspP Passenger domain, clot formation time was reduced relative to incubations with buffer alone, with BSA or with S263A (Figure 6.8b, left panel). At the highest concentration of 1.0 mg/mL, incubation with WT EspP passenger domain reduced clot formation time by 19 minutes or 73% relative to incubations with buffer alone, by 17 minutes or 71% relative to incubation with BSA and by 14 minutes or 67% relative to incubations with S263A. Incubation with 1.0 mg/mL S263A reduced clot formation time by 5 minutes or 19% relative to incubation with buffer alone and by 3 minutes of 13% relative to incubations with BSA. A clear concentration-dependent prolongation of clot formation time, however, was not observed for either treatment. When FFP from donor H07 was incubated with WT EspP passenger domain or with S263A, clot lysis times were well in excess of 8 hours, as were clot lysis times of samples incubated with buffer alone or with BSA. The only exception to this was a clot lysis time of 6.4 hours observed when FFP from this donor was incubated with 0.5 mg/mL of S263A. However, again here a clear concentration-dependent shortening of clot lysis time was not observed for any treatment.

6.5 Conclusions

In this chapter, the effect of purified EspP passenger domain on the coagulation cascade and on clot stability was analyzed *in vitro* using human plasma and the euglobulin fraction of plasma.
Figure 6.8 Effect of EspP on clot formation and lysis times, measured for donor H07.

The euglobulin fraction prepared from FFP from donor H07 (left panels) or unfractionated FFP from donor H07 (right panels) was incubated (37 °C, 4 hrs) by itself (untreated), with buffer alone (PBS-G), or with 0.1, 0.5 or 1.0 mg/mL of each of BSA, WT EspP or S263A. Samples were then assayed as described in chapter 2, section 2.11.1. Plotted in the top panels are the resultant experimental spectrophotometric tracings of clot formation and lysis. Clot formation times and clot lysis times determined from these tracings are presented in the middle and bottom panels, respectively. Clot lysis times greater than 10 hours are indicated by (++) on the vertical axis.
The EspP passenger domain was found to inhibit the coagulation cascade in human plasma – prothrombin time, activated partial thromboplastin time and thrombin time were all prolonged – by reducing the activities of coagulation factors II, V, VII, VIII and XII, the later four to well below their normal levels. This inhibition was found to be wholly dependent on the serine protease activity of the EspP passenger domain – the S263A passenger domain did not produce a similar result – and was not affected by the cellular components of blood. Activities of coagulation factors IX, X and XI were not altered by the EspP passenger domain. The EspP passenger domain also did not alter fibrinogen concentration in blood samples suggesting that it does not act to directly cleave the fibrinogen stores of human blood. Preliminary results obtained using the euglobulin fraction of plasma from two donors suggest that while the EspP passenger domain does indeed alter clot formation times in a manner dependent on its serine protease activity, it does not alter clot lysis times. This suggests that the EspP passenger domain does not act to directly cleave the fibrin clot. Lastly, preliminary results obtained using plasma from a single donor indicates that the EspP passenger domain does not alter clot lysis times in unfractionated plasma, suggesting that this protease also does not act on any inhibitors of the fibrinolytic cascade. Taken together, these results provide further evidence for a role for the EspP passenger domain in the clino-pathological manifestation of EHEC infection and shed some light on the mechanisms by which this protease functions. Further work is required to better understand the precise mechanisms involved.
Chapter 7:

Effects of the EspP passenger domain on clot formation and stability in human blood – a general discussion
In their initial characterization of this protease, Brunder et al. showed EspP to cleave human coagulation factor V and, owing to the critical role of factor V in normal hemostasis, hypothesized that EspP may inactivate factor V via this proteolytic cleavage and thereby contribute to the mucosal hemorrhage observed in patients with *E. coli* O157:H7 infection (Brunder et al., 1997). This hypothesis was further supported by previous observations in our lab that EspP also cleaves human coagulation factor VIII, a second critical cofactor involved in normal hemostasis. The data presented in this thesis demonstrate that indeed WT EspP induces a time- and concentration-dependent reduction in the activities of both factor V and factor VIII *in vitro* in human blood. The S263A mutant variant of EspP, which has abrogated proteolytic activity, did not alter the activities of factors V and VIII, indicating that the catalytic activity of the proteolytic subdomain of the EspP passenger is responsible for the inactivation of these coagulation factors. In addition, the results presented in this thesis further demonstrate that WT EspP, but not S263A, reduces the activities of coagulation factors VII and XII as well as prothrombin in a time- and concentration-dependent manner. EspP is found not to have a statistically significant effect on the activities of coagulation factors IX, X or XI. Moreover, similar results were obtained irrespective of whether incubations with EspP were performed in whole blood or in blood plasma, suggesting that the cellular components of blood (i.e. platelets, erythrocytes and leukocytes) do not play a significant role in the reduction of coagulation factor activities by WT EspP.

Perhaps unsurprisingly, the results presented in this thesis also demonstrate substantial variability in the ability of EspP to reduce the activities of coagulation factors V, VII, VIII and XII as well as prothrombin. EspP was found to have the largest effect on the activity of factor VII, reducing it on average by about 0.80 U/mL or about 90% relative to incubations with controls (Chapter 6, Table 6.4), followed by factors V, VIII and XII. In contrast, EspP had the smallest effect on prothrombin activity: prothrombin activity, although statistically significantly reduced, was reduced by only about 15 U/mL or about 15% relative to incubations with controls. That EspP reduced the activities of five of the eight coagulation factors tested, and that these reductions were substantially variable in degree suggests that EspP may be acting somewhat non-discriminately on altering coagulation factor activity.
To determine if these reductions in coagulation factor activities translate to an overall change in the ability of human blood to coagulate, the effects of EspP on prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT) were determined. PT is a measure of the proper functioning of the extrinsic and common pathways of the coagulation system and is therefore sensitive to changes in the activities of coagulation factors V, VII and X as well as prothrombin (Rodgers, 2004). Indeed, WT EspP, but not S263A, was found to significantly prolong PT in a time- and concentration dependent manner in agreement with the observations that EspP reduces the activities of coagulation factors V and VII as well as of prothrombin. Likewise, in agreement with the observations that EspP reduces the activities of coagulation factors V, VIII and XII as well as of prothrombin, WT EspP, but not S263A, was found to significantly prolong aPTT; aPTT evaluates the effectiveness of the intrinsic and common pathways of coagulation and is therefore sensitive to changes in the activities of coagulation factors V, VIII, IX, X, XI and XII as well as of prothrombin (Rodgers, 2004). Surprisingly, however, treatment with WT EspP, but not S263A, was found to also prolong TT, but to a much lower extent than PT or aPTT. TT is a measure of the conversion of fibrinogen to insoluble fibrin and records the time to the formation of insoluble fibrin from fibrinogen following the addition to calcium-citrate blood plasma samples of an excess of thrombin (Rodgers, 2004). In so doing, TT bypasses the extrinsic, intrinsic and some of the common pathways of the coagulation system and is therefore not expected to be sensitive to changes in the activities of any of coagulation factors V-XII or to prothrombin. TT is sensitive to decreased fibrinogen levels in blood, as well as to elevated levels of fibrin degradation products (Chandler, 2009). However, EspP was found not to alter the concentrations of fibrinogen or D-dimers (the most notable form of fibrin degradation products).

To further test the hypothesis that EspP may be a contributing factor to the mucosal hemorrhage observed in patients with *E. coli* O157:H7 infection, the effects of EspP on clot formation and stability in human blood were assayed by thrombelastography (TEG). TEG offers a more global assay of clot formation and stability and provides statistics to monitor both the rates of platelet-fibrin clot formation and of fibrinolysis in unfractionated whole blood. Unexpectedly, incubation of whole blood with WT EspP resulted in a time-dependent acceleration of the rate of platelet-fibrin clot formation, as suggested by the reduced reaction
time (R-time) and clot formation time (K-time) and increased clot kinetics (α-angle). Moreover, a similar effect was observed following incubation with the S263A mutant form of EspP, but not with buffer alone or with BSA negative controls. This suggests that EspP, but not its serine protease activity, is responsible for the accelerated rate of platelet-fibrin clot formation. This is in stark contrast to the coagulation results wherein the WT EspP led to a reduction in the activities of multiple coagulation factors and a prolongation in PT, aPTT and TT.

The precise differences giving rise to these seemingly contradictory results between the accelerated rate of platelet-fibrin clot formation observed in the TEG assays on the one hand and the prolonged PT, aPTT and TT, together with the reduced activities of multiple coagulation factor, observed in the other hand, remains unclear. There exist only three differences between the measurements of PT, aPTT and TT, together with the measurements of specific coagulation factor activities, and those of TEG that may account for the observed differences in clot formation behaviour: first, while TEG measures platelet-fibrin clot formation in whole blood, PT, aPTT, TT and the specific coagulation factor activities are measured in blood plasma that is devoid of cellular components; second, while TEG measures platelet-fibrin clot formation via mechanical perturbations, albeit small, of the blood sample, PT, aPTT, TT and the specific coagulation factor activities are measured by optical observations that do not perturb the system; third, while platelet-fibrin clot formation in TEG proceeds over the course of about 5-15 minutes, clot formation for measurements of PT, aPTT, TT and the specific coagulation factor activities typically take no longer than about 2 minutes.

It is unlikely that the observed reversal in the effect of EspP on clot formation is due to the slight difference in the clot formation time between the two measurement systems: EspP is a very slow-acting protease as evidenced by the minor differences of its effects observed following the 0.5 hour and 2 hour incubation times presented in this thesis and is therefore unlikely to produce such a drastic change owing to its proteolytic activity over the course of only a few minutes. It is possible that the observed reversal in the effect of EspP on clot formation is due to the mechanical versus optical observation system employed in the two measurement systems: Perhaps the mechanical perturbation introduced into the system during TEG analysis is sufficient to cause clot formation to be accelerated in EspP-treated samples while these same
samples monitored optically and undisturbed take a longer time to form a clot. However, it is most likely that the presence, or lack thereof, of the cellular components of blood in the two measurement systems is responsible for the observed reversal in the effect of EspP on clot formation times. Here, EspP would indeed be acting, via its proteolytic activity, to reduce the activities of the various coagulation factors as well as to prolong PT, aPTT and TT. However, concomitantly, EspP would also be active to activate the cellular components of blood, particularly to activate platelet cells, perhaps via EspP’s extended β-helical stalk domain. And while EspP is inactivating the various coagulation factors, this inactivation, under the assay times and concentrations used in this thesis, is not complete: enough residual coagulation factor activities remain to allow sufficient activation of prothrombin to thrombin and the beginnings of a fibrin mesh. The activated platelets would then sequester onto this initial fibrin mesh and lead to a much more accelerated rate of platelet-fibrin clot formation.

Along with the accelerated rate of platelet-fibrin clot formation, EspP was found to promote enhanced fibrinolysis, as evidenced by a decreased MA and increased LY30. This enhanced fibrinolysis was only observed for samples incubated with WT EspP but not S263A, suggesting that the enzymatic activity of the protease is responsible for the effect. MA is a measure of the maximum clot strength and is dependent on both the rate of clot formation and lysis while LY30 is a measure of clot lysis 30 minutes after MA is reached and provides a marker for fibrinolysis. However, of the eight individuals whose whole blood was assayed by TEG, WT EspP-induced enhanced fibrinolysis was observed only for six: Blood samples from donors R01 and H01 were resistant to this EspP-induced enhanced fibrinolysis. And while blood samples from donor H01 were analysed from a single donation, blood samples from donor R01 were analyzed from two donations separated by about two months, suggesting that this observed resistance to WT EspP-induced enhanced fibrinolysis may not be an outlier for this patients. These results suggest the tantalizing possibility that there may be a subset of individuals in the population who are resistant to the fibrinolysis-enhancing effects of EspP. However, additional studies with a larger sample size are required to address this hypothesis.

Finally, to better understand the mechanism whereby EspP enhances fibrinolysis in blood samples from the majority of the donors, the effect of EspP on the fibrinolytic system was
assayed via measurements of clot lysis times in the euglobulin fractions of blood for two of the donors for whom EspP-induced enhanced fibrinolysis has been observed. The euglobulin fraction of plasma is devoid of cellular components but also of the natural inhibitors of the fibrinolytic system. ECLT is a particularly good measure of the actions of plasminogen activators and of plasmin. EspP was found unable to alter clot lysis times within the euglobulin fraction of blood samples from donors H02 and H07, suggesting that EspP acts neither directly to cleave the cross-linked fibrin polymer within blood clots nor directly to enhance the activities of the natural promoters of fibrinolysis, including plasmin, tissue plasminogen activator and urokinase plasminogen activator. EspP was further unable to alter clot lysis times within unfractionated blood plasma, suggesting that it also does not act directly to inhibit the actions of the normal inhibitors of the fibrinolytic system, including plasminogen activator inhibitors 1 and 2, α2-antiplasmin, α2-macroglobulin and thrombin-activatable fibrinolysis inhibitor (a schematic of the fibrinolytic system is provided in Chapter 1, Figure 1.6). The EspP-induced enhanced fibrinolysis observed in TEG analysis of whole blood is therefore likely a result of the interplay of EspP with the cellular components of blood. However, the precise mode whereby EspP enhances fibrinolysis and does so only in a subset of the donors remains unclear.

The observed fibrinolysis and accelerated platelet-fibrin clot formation seem to support a pathogenic role for EspP in the development of hemorrhagic colitis and hemolytic-uremic syndrome (HUS). Evidence of increased thrombin generation has been reported in patients with HUS during the course of illness (Nevard et al., 1997; Van Geet et al., 1998) and in patients diagnosed with E. coli O157:H7 infection that subsequently developed HUS (Chandler et al., 2002). However, evidence for enhanced fibrinolysis in HUS is more ambiguous. Van Geet et al. reported an elevation in markers of fibrinolysis (tissue plasminogen activator, urokinase plasminogen activator and D-dimer) but found no difference in PT, aPTT or factor VIII activity level in an analysis of 24 patients diagnosed with HUS and compared to 15 patients with acute renal failure as controls (Van Geet et al., 1998). A number of studies also reported elevated levels of plasminogen-activator inhibitor type 1, an inhibitor of fibrinolysis, in patients with HUS (Bergstein et al., 1992; Menzel et al., 1994; van de Kar et al., 1994; Nevard et al., 1997). The current prevailing thought in the pathogenesis of hemorrhagic colitis is that Shiga toxins produced by the invading EHEC induces apoptosis of intestinal epithelial cells thereby leading
to the destruction of the gastrointestinal mucosal membrane (Smith, Kane, et al., 2003; Schüller et al., 2004). Given that patients with EHEC infection have antibodies to EspP, suggesting direct contact between EspP and the bloodstream of the infected individuals (Brunder et al., 1997; Djafari et al., 1997; Law, 2000), one possible role for the effects of EspP to alter clot formation and stability may be to facilitate the invasion of Shiga toxins into the circulatory system by maintaining a hemorrhagic state at the site of the damaged intestinal epithelium.

Other evidence that support a pathogenic role for EspP in EHEC infection include cleavage of human apolipoprotein A-I (Schmidt et al., 2001), which acts as a stabilizing factor for prostacyclin (PGL2) (Yui et al., 1988), itself an inhibitor of platelet activation (Davenport, 2010); downregulation of complement activation by cleavage of C3/C3b and C5, which may facilitate bacterial adherence and colonization in the gut by protecting the bacterium from opsonization and complement-mediated lysis (Dziva et al., 2007; Orth et al., 2010), as well as the formation of rope-like fibres with cytopathic and adhesive properties (Xicohtencatl-Cortes et al., 2010). Paradoxically, EspP has also been shown to cleave and inactivate EHEC hemolysin, a pore-forming cytolysin that damages microvascular endothelial cells and is thought to play a role in the pathogenesis of hemorrhagic colitis and HUS (Aldick et al., 2007; Brockmeyer et al., 2011).

Therefore, the results presented in this thesis together with results from other studies suggest that EspP may have a role in the pathogenesis of hemorrhagic colitis and HUS, but is neither a necessary nor sufficient virulence factor in the genesis of the disease. It is most likely that EspP may act through many different mechanisms and in concert with other virulence factors like Shiga toxin, EHEC hemolysin, and subtilase cytotoxin to enhance the virulence of EHEC in the formation of hemorrhagic colitis and HUS.
Chapter 8:

Final remarks and future directions
8.1 Final remarks

This thesis aimed to better understand the functional significance of EspP in enterohemorrhagic *E. coli* pathogenesis by analyzing the crystallographic structure of the mature passenger domain of EspP and by investigating, *in vitro*, its effects on the coagulation and fibrinolytic processes in human blood. The crystallographic structure of the EspP passenger domain was determined to 2.5 Å resolution. As presented in chapter 3, this revealed the EspP passenger domain to be, in many regards, similar in structure to the passenger domains of the *E. coli* autotransporter Hbp and *H. influenza* autotransporter IgA1 protease, the two other SPATE autotransporters for which a high resolution structure has been determined; each contains a globular subdomain housing the serine protease function of the protein and a right-handed β-helical stalk subdomain whose function to date has not been strictly determined but which is thought to provide a mechanism for translocation of the passenger domain across the bacterial outer membrane (Klauser *et al.*, 1992; Otto *et al.*, 2005; Johnson *et al.*, 2009). The EspP passenger domain, however, lacks a chitinase b-like subdomain. The function of this subdomain is unknown but at least in the structure of the IgA1 protease, is proposed to be involved in substrate recognition. The structure of the EspP passenger domain further revealed a large cleft formed at the junction between the globular and the β-helical stalk subdomains. I proposed that one of the roles of this cleft may be in binding to select cellular structures of the host organism and in so doing may play a role in EHEC pathogenesis.

As presented in chapter 4, cellular binding studies using human peripheral blood cells and fluoro-labelled EspP passenger domain revealed that while this passenger domain does not bind to human erythrocytes, at least under the experimental test conditions, it does bind to human platelet cells. And while the specific mechanism and consequences of this binding were not pursued, it does provide support for the possibility that this binding may be mediated by the β-helical stalk subdomain of the EspP passenger and involved in altering platelet functions, and in so doing may play a role in EHEC pathogenesis.

Finally, in chapters 5 and 6, the significance of EspP in EHEC pathogenesis was examined by studying, *in vitro*, the effects of this protease on the coagulation and fibrinolytic systems in
human blood. Interestingly, the EspP passenger domain was found to accelerate clot formation in whole blood but inhibit clot formation in blood plasma, suggesting that this protease has an inhibitory effect on the coagulation system but is a stimulant of platelet functions. Indeed, in specific assays of coagulation factor activities, EspP was found to reduce the activities of human coagulation factors II, V, VII, VIII and XII. More interestingly still, EspP was found to enhance fibrinolysis in whole blood, but only in a subset (6 of 8) of volunteers. Enhanced fibrinolysis, however, was not observed in the euglobulin fraction of blood, suggesting that EspP may promote fibrinolysis in whole blood by interfering with the actions of inhibitors of the fibrinolytic system. These results provide compelling evidence for a pathogenic role played by EspP during EHEC infection.

8.2 Future directions

8.2.1 Further structural studies of the EspP passenger domain

With the high-resolution structure of the EspP passenger domain, as well as those of the passenger domains of the related autotransporters Hbp and IgAP, determined, we now have a much clearer understanding of the architectural arrangements of these proteins and their functions as serine proteases. However, much still remains unclear. These include an understanding of the precise mode of substrate recognition employed by these proteases, the functional significance of the chitinase b-like subdomain within these autotransporters (a subdomain that is not present within EspP), and the role of their β-helical stalk subdomain. Cocrystallization of these proteases with one or more of their macromolecular substrates would be very useful in identifying the precise mode utilized by these proteases to recognize and bind to their substrates. Such a structure determination may possibly also identify a role for the chitinase b-like subdomain if the autotransporter within the cocrystal structure were one that contains a chitinase b-like subdomain and if this subdomain were indeed found to be involved in substrate recognition as suggested by Johnson et al. (2009). Cocrystallization of EspP with one or more of its substrates, including human coagulation factors II, V, VII, VIII or XII, would further provide
invaluable information as to how substrate recognition by this autotransporter differs from those of the other SPATEs. Coagulation factors II, V, VII, VIII and XII are commercially available. As well, numerous recombinant expression and purification methods have been described for these proteins in the literature (Ortel et al., 1992; Citarella et al., 1996; Soukharev et al., 2002; Halabian et al., 2009; Masroori et al., 2010; Mirzaahmadi et al., 2011). However, these sources are presently either too expensive or too time-consuming to yield sufficient quantities of protein for crystallization screening. Cocrystallization of EspP with porcine pepsin, a much more readily available substrate of EspP, would therefore be an alternative method to gain insight into the mode of substrate recognition by EspP.

To determine the functional significance of the β-helical stalk subdomain within these autotransporters, it would be useful to first determine if this subdomain plays a critical role in secretion of the passenger domain, the globular subdomain of which carries the proteolytic activity of the macromolecule. Here a good first attempt would be to generate a simple deletion mutant of the autotransporter wherein the β-helical stalk subdomain is removed, and monitor the effect of this deletion on secretion and folding of the resultant passenger domain. For EspP in particular, the following two deletion mutants seem reasonable first candidates: EspPΔ314-998 wherein amino acid residues Ser314-Val998 are removed and EspPΔ631-998 wherein amino acid residues Thr631-Val998 are removed. Both mutants leave amino acid residues Ala999-Asn1023 of the EspP passenger domain intact. These residues form part of the autochaperone region of EspP and are known to be important for proper secretion of the EspP passenger domain (Velarde and Nataro, 2004). Work by Binder et al. has also demonstrated that these residues are sufficient for the translocator domain of EspP to transport non-native (Anticalin) passenger domains (Binder et al., 2010).

The work presented in this thesis further suggests that the EspP passenger domain binds to and possibly accentuates the function of platelet cells. It would be prudent to identify which, if any, specific cellular structures are involved in this binding, whether this binding is limited to platelet cells or extends to other cells types, and whether the β-helical stalk subdomain is involved in the binding. Here, colocalization experiments employing fluorolabeled EspP together with fluorolabeled antibodies directed at specific cellular components of the platelets, monitored using
confocal microscopy, would be informative. At the same time, cellular binding assays, monitored by flow cytometry or confocal microscopy, using fluorolabelled EspP together with other human cell types with which EHEC and EspP may come in contact with during the course of an infection, such as human intestinal epithelial cells, would be very useful. The EspP deletion mutants lacking the β-helical stalk subdomain described above would also be of utility here; if the β-helical stalk subdomain is indeed involved in mediating this binding, the mutants lacking this subdomain would then be expected to loose their binding ability.

8.2.2 Further elucidating the role of EspP in EHEC pathogenesis

As presented in Chapters 5 and 6, blood samples incubated with EspP on the one hand have a coagulation system with inhibited function, as determined by measurements of PT, aPTT and TT and owing to the degradation by this protease of coagulation factors V, VII, VIII and XII, while on the other hand they display an enhanced rate of platelet-fibrin clot formation, as determined by TEG. These seemingly contradictory outcomes may be due to activation of platelets by EspP leading to the enhanced rate of clot formation observed in TEG. Alternatively, they may be due to other as yet unidentified effects of EspP on whole blood. Further experiments to better understand these differences should include TEG measurements of clot formation using EspP-treated platelet-poor and EspP-treated platelet-rich plasma samples. As well, if EspP is indeed enhancing activation of platelets, measurements of PT, aPTT and TT using EspP-treated platelet-rich plasma may prove very informative.

Concurrently, direct measurements of platelet function in EspP-treated whole blood or EspP-treated platelet-rich plasma should be performed. Here, measurements of platelet-related hemostasis using the Platelet Function Analyzer (PFA-100) (Kundu et al., 1995) system or the Cone and Platelet Analyzer (CPA) (Shenkman et al., 2000) system, could be employed, as well as flow cytometry to assess the state of platelet activation by monitoring the expression levels of specific markers of platelet activation on the cellular surface of platelet cells, such as glycoproteins Ib, IIb or IIIa, among others (Rand et al., 2003).

It would also be interesting to determine the effects of EspP on other molecular components of blood involved in hemostasis, such as vWF (von Willebrand factor) and ADAMTS13 (a
disintegrin and metalloprotease with thrombospordin-1-like domain repeats, family member 13), abnormal levels of which are thought to play a role in HUS coagulopathy (Motto et al., 2005; Goldwater and Bettelheim, 2012). Furthermore, EspP is known to interact with human physiology beyond the hemostatic systems (Orth et al., 2010). Future studies should focus on better understanding these interactions. Only with the knowledge obtained from all these sources will we have a complete understanding of EspP and the many ways by which we may be able to manipulate this autotransporter to promote human health.

8.2.3 Design of diagnostics and therapeutics

As presented in Chapters 5 and 6, of the eight donors whose blood samples were analyzed, treatment with EspP resulted in an enhanced rate of fibrinolysis, as monitored by TEG using whole blood samples, in six of the donors while blood samples from the remaining two donors were resistant to this EspP-induced enhanced fibrinolysis. The exact mechanism wherein EspP enhances the rate of fibrinolysis in the first group remains unclear, as do the differences between the blood samples in the two groups that afford the second group their resistance to this EspP-induced enhanced fibrinolysis. Moreover, given that only a subset of patients develop HUS following infection with EHEC, and that the mechanistic differences between patients that determines their susceptibility to EHEC-induced HUS remains unknown, it becomes very tempting to speculate that susceptibility to EspP-induced enhanced fibrinolysis as measured by TEG analysis of a patient’s blood sample may be a predictor of HUS susceptibility following infection with EHEC. Future studies will need to address the mechanisms whereby EspP produces an enhanced rate of fibrinolysis in blood samples from some donors, the mechanistic differences between donors that afford others a resistance to this EspP-induced enhanced fibrinolysis, and whether these differences in EspP-induced enhanced fibrinolysis correlate with susceptibility to HUS following infection with EHEC. To address this later possibility, ideally donors who have survived infection with EHEC and for whom disease outcome, including development of HUS, is known would be recruited. Blood samples from these donors would then be analyzed by TEG and the observed susceptibility to EspP-induced enhanced fibrinolysis would be compared to the known disease outcomes of the donors. Were a clear correlation to be observed between EspP-induced enhanced fibrinolysis and known disease outcome, this would
provide very compelling evidence to alter future clinical diagnostics and procedures for managing patients presenting with EHEC infection.
References


