Characterization of OmpT: An outer membrane vesicle protease from *Escherichia coli* that attenuates blood coagulation

Submitted by

Veena Premjani

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CERTIFICATE OF APPROVAL

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The undersigned certify that the student has presented her thesis, that the thesis is acceptable in form and content and that a satisfactory knowledge of the field covered by the thesis was demonstrated by the candidate through an oral examination. They recommend this thesis to the Office of Graduate Studies for acceptance.

Examining Committee

Dr. Jean-Paul Desaulniers
Chair of Examining Committee

Dr. Holly Jones-Taggart
External Examiner

Dr. John Samis
Research Supervisor

Dr. Ayush Kumar
Examining Committee Member

Dr. Julia Green-Johnson
Examining Committee Member

As research supervisor for the above student, I certify that I have read and approved changes required by the final examiners and recommend the thesis for acceptance:

Dr. John Samis
Research Supervisor

Abstract

Escherichia coli O157:H7 is an emerging food-borne and water-borne pathogen that belongs to Enterohemorrhaghic pathogenic group of bacteria. *E. coli* O157:H7 colonizes the gastrointestinal tract and is responsible for hemorrahagic colitis, renal failure, bloody diarrhoea, hemolytic uremic syndrome, thrombocytopenia, hemolytic anemia and other systemic problems. *E. coli* O157:H7 is responsible for outbreaks of Enterohemorrhaghic *E. coli* infections and causes significant mortality and morbidity globally. Factor V (Mr-330,00Da) is a procofactor that upon activation to FVa profoundly enhances thrombin generation and fibrin clot formation as part of *prothrombinase*. The fibrin clot immobolizes the pathogen and allows the immune system to target and destroy the bacterium. Our research has shown that pathogenic *Escherichia coli* O86a:K61 secretes the protease OmpT as part of outer membrane vesicles (OMVs), which inactivates coagulation Factor V and in so doing, induces a hemorrhagic state. To further characterize the effect of OmpT on the coagulation, this study has employed a genetic approach using wild type, an OmpT deletion mutant, and an over-expressing OmpT construct in pathogenic *E. coli* O157:H7. Although the growth of the three strains in liquid culture was not significantly different over time in nutrient rich media, OmpT over expression retarded cell growth in nutrient deficient media ($p<0.05$). Wild type cells and OmpT over expressing cells produced significantly larger numbers of different size ranges of outer membrane vesicles than OmpT deletion mutant ($p<0.05$). Wild type cells and outer membrane vesicles prolonged both the prothrombin time and activated partial thromboplastin time in normal human plasma, and this effect was enhanced with OmpT over expressing cells and abolished in the OmpT deletion mutant. Wild type cells and outer membrane vesicles inactivated Factor V, but not other factors (fibrinogen, FII and FX), in normal human plasma to a 250kDa product and the effect was

increased in OmpT overexpressing strain and abolished in the OmpT deletion mutant. The effect of OmpT was direct and did not involve the host plasminogen system. In summary, this research indicates that OmpT has a role in OMV biogenesis and composition, and disrupting the coagulation process and fibrin barrier formation during the host innate immune response and in doing so may permit enhanced bacterial survival within host environments. The research will also lead to a greater understanding of the mechanism of action of *E. coli* virulence factors and positively impact healthcare environments through the development of novel and robust vaccines, antimicrobial drugs, diagnostic methods, and medical treatments.

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LIST OF ABBREVIATIONS

A1PI Alpha 1-Protease Inhibitor

Amp Ampicillin

APTT Activated Partial Thromboplastin Time

APC Activated Protein C

AT Antithrombin

B-gal β-galactosidase

BCA Bicinchoninic Acid

bla gene encoding β-lactamase

BLAST Basic Local Alignment Search Tool

bp base pair

BSA Bovine Serum Albumin

CBB Coomassie Brilliant Blue

CFF Centrifugal Flow Filtration

cfu Colony Forming Unit

Cro-P Omptin of *Citrobacter rodentium*

Δ Deletion

DIC Disseminated Intravascular Coagulation

DNA Deoxyribonucleic acid

dNTP deoxyribonucleoside triphosphate

DTT Dithiothreitol

E. coli *Escherichia coli*

ECL Enhanced Chemiluminescence

EDTA Ethylenediaminetetraacetic acid

EHEC Enterohaemorrhagic *Escherichia coli*

EPEC Enteropathogenic *Escherichia coli*

EspC Enteropathogenic *Escherichia coli* protease C

EspP Extracellular Serine Protease Plasmid-Protease

ETEC Enterotoxigenic *Escherichia coli*

ExPEC Extracellular Pathogenic *Escherichia coli*

F Factor

FDPs Fibrin Degradation Products

HBS HEPES Buffered Saline

Hbp Hemoglobin Protease

HMWK High Molecular Weight Kininogen

IgG Immunoglobulin G

IL Interleukin

IPTG Isopropyl-β-D-Thiogalactoside

Kbp Kilobase pair

kDa Kilodalton

LB Luria Bertani

LD Loading dye

LPS Lipopolysaccharide

MA Minimal A

min minute

µL microliter

ml millilitre

µM micromolar

MWCO Molecular Weight Cut Off

ng nanogram

NHP Normal Human Plasma

OD600 Optical Density at 600nm

OmpC Outer Membrane Protein C

OmpF Outer Membrane Protein F

OmpN Outer Membrane Protein N

OmpT Outer Membrane Protein Temperature-regulated

Δ**OmpT-** OmpT deletion mutant

OMV Outer Membrane Vesicle

PBS Phosphate Buffered Saline

PBS_{TW} Phosphate Buffered Saline Tween-20

PCR Polymerase chain reaction

pEHOmpT- OmpT overexpressing strain

Pet Plasmid-Encoded Toxin

Pic Protease Involved in Intestinal Colonization

PK Plasma Kallikrein

Pla Plasminogen Activator

PLG-deficient plasma Plaminogen deficient plasma

PMSF Phenylmethanesulfonylfluoride

PT Prothrombin time

pUC18 Plasmid of University of California 18

PVDF Polyvinylidene Fluoride

RNA Ribonucleic acid

Sat Secreted Autotransporter Toxin

SDS Sodium Dodecyl Sulfate

SDS-PAGE Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SPATE Serine Protease Autotransporter of Enterobacteriaceae

spp Species

STEC Shiga Toxin-Producing *Escherichia coli*

TEM Transmission Electron Microscope

TF Tissue factor

TFF Tangential Flow Filtration

TM Thrombomodulin

TNF Tumor Necrosis Factor

tPA Tissue Plasminogen Activator

uPA Urokinase-Type Plasminogen Activator

UPEC Uropathogenic *Escherichia coli*

X-gal 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1

Introduction

1.1 Blood Coagulation Cascade

Hemostasis (Haemo from Latin - blood, stasis from Greek- stop) is a property of the circulatory system where blood is maintained in a liquid state within a vessel in the uninjured state and the ability of the system to form a fibrin clot, in response to injury to prevent excessive blood loss. Hemostasis is maintained within the body in three stages. Primary hemostasis is maintained by platelets; secondary hemostasis by plasma clotting factors which leads to fibrin clot formation and tertiary hemostasis by plasmin formation that leads to the fibrin breakdown. The delicate physiological balance of hemostasis is maintained by fibrinolysis and coagulation. The blood coagulation process is initiated by two important pathways: the intrinsic pathway that is triggered by a negatively charged surface (such as glass), and the extrinsic pathway that is stimulated by sub-endothelial membrane protein, tissue factor released from the site of injury. These pathways in turn activate a rapid, sequential and tightly regulated common pathway that converts soluble fibrinogen to an insoluble fibrin clot. Figure 1.1A represents the blood coagulation cascade in detail.

Experimental evidence indicates that the intrinsic pathway may have less hemostatic significance than the extrinsic pathway (Davie *et al.,* 1991). The intrinsic pathway is activated by contact through damaged negatively charged endothelium and subendothelium that activates Factor XII (FXII) to Factor XIIa (FXIIa), which in turn activates prekallikrein to kallikrein (Riddel *et al.*, 2007). High molecular weight kininogen (HMWK) anchors these proteins to the blood vessel. Kallikrein also activates FXII to FXIIa by a positive feedback mechanism (Riddel *et al.*, 2007). FXIIa along with kallikrein (attached to HMWK) proteolytically cleaves Factor XI (FXI) to Factor XIa (FXIa). FXIa bound to a charged surface with HMWK activates Factor IX (FIX) to

Figure 1.1A The blood coagulation cascade. The coagulation cascade is divided into extrinsic (represented by the blue panel) and intrinsic pathways (represented by the cream panel), which leads to the common pathway (represented by the green panel). The inactive proteases are represented in red, active proteases in green, phospholipid membrane by highlighted yellow line and calcium is represented by a pink circle. The arrows represent the proteolytic conversion of inactive to active form of protease. Adapted from Riddel, J., Aouizerat, B., Miaskowski, C., & Lillicrap, D. (2007). Theories of Blood coagulation. *Journal of Pediatric Oncology Nursing*, 24, 123-131.

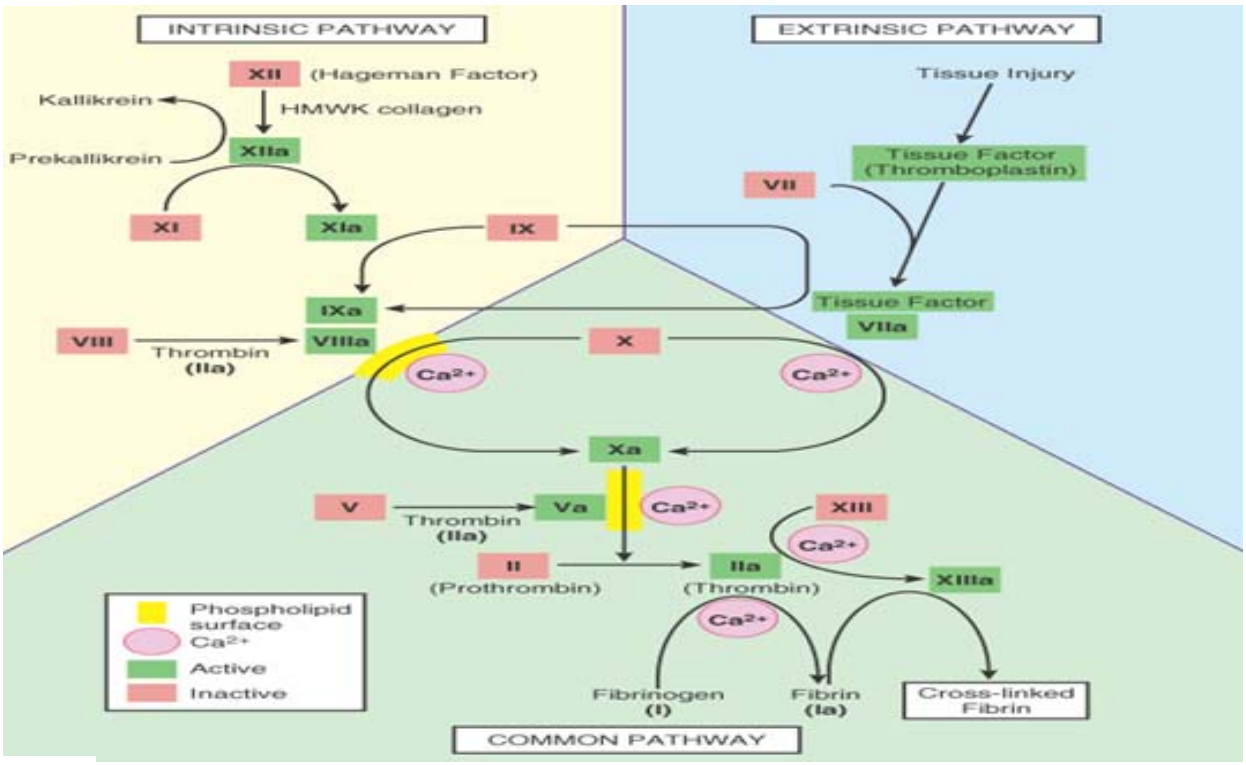
Figure 1.1B Regulation of coagulation and fibrinolysis. The blood coagulation process is initiated by tissue factor (TF) and mediated by the protease thrombin. Fibrinolysis is affected by the protease plasmin that is acitivated by tissue plasminogen activator (t-PA). Plasmin further degrades fibrin to D-Dimer. The fibrinolysis process is also controlled by α2-antiplasmin and plasminogen activator inhibitor (PAI-1). The inhibitors of blood coagulation cascade are tissue factor pathway inhibitor (TFPI), Antithrombin (AT III), Activated protein C (Act C) and protein S (S). Adapted from McGraw-Hill. (2005). Normal Hemostasis. Hematology in Clinical practice 4th edition (Chapter 27).

Retrieved from:

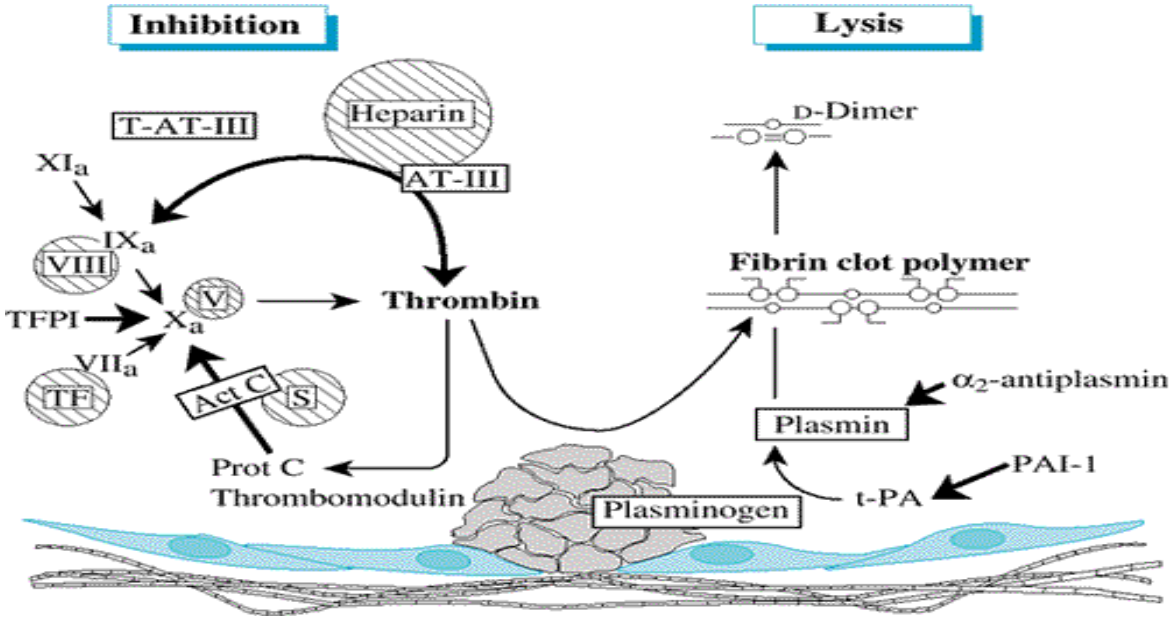
http://www.drugswell.com/winow/+%20b20/Hematology%20in%20Clinical%20practice-2005/III%20-%20Disorders%20of%20Hemostasis/27%20-%20Normal%20Hemostasis.htm

Figure 1.1

A


INTRINSIC PATHWAY
EXTRINSIC PATHWAY
XII (Hageman Factor)
Kallikrein
HMWK collagen
Prekallikrein
XIIa
XI
XIa
IX
Tissue Injury
Tissue Factor (Thromboplastin)
VII
IXa
VIII
Thrombin (IIa)
VIIIa
X
Tissue Factor
VIIa
Ca2+
Xa
V
Va
XIII
II
(Prothrombin)
IIa
(Thrombin)
XIIIa
Fibrinogen (I)
Fibrin (Ia)
Cross-linked Fibrin
Phospholipid surface
Active
Inactive
COMMON PATHWAY

B


Inhibition
Lysis
T-AT-III
Heparin
AT-III
XIa
IXa
VIII
V
TFPI
Xa
VIIa
TF
Act C
S
Thrombin
D-Dimer
Fibrin clot polymer
α2-antiplasmin
Plasmin
PAI-1
t-PA
Prot C
Thrombomodulin
Plasminogen

Factor IXa (FIXa). The two proteases from the downstream cascade, Factor Xa (FXa) and thrombin activate Factor VIII (FVIII) to Factor VIIIa (FVIIIa) and Factor V (FV) to activated Factor Va (FVa) by positive feedback reactions. FVIIIa along with FIXa in the presence of Ca^{2+} on a negatively charged phospholipid provided by damaged cell membranes forms the *tenase* complex, which is comprised of FXIa, FX and FVIIIa, and then converts FX to FXa. FXa activity proceeds along the common pathway (Riddel *et al.*, 2007).

The extrinsic pathway is stimulated by tissue factor (TF) released from subendothelial cells upon endothelial damage. In addition, activated monocytes and smooth muscle cells are also able to express TF in a process regulated by cytokines and inflammatory mediators (Delvaeye & Conway, 2009). Experimental evidence now indicates that the extrinsic pathway is a more significant pathway for fibrin clot formation than the intrinsic pathway (Delvaeye & Conway, 2009). This is based on the notion that humans deficient in contact factors, FXII, FXI, kininogen or kallikrein, do not suffer from severe bleeding (Monroe and Hoffmann, 2005). FVIIa, when bound to TF on a cell membrane, is a potent activator of the common pathway, which can then activate FX to FXa. Conversely, FVIII/FVIIIa and FV/FVa in the uncomplexed or free form have no activity alone.

The common pathway begins with protease FXa from either the intrinsic, extrinsic or both pathways. Initially, FX is capable of converting prothrombin (FII) to thrombin (FIIa). Once a small amount of thrombin is generated, it further activates FV and FVIII by positive feedback reactions in order to amplify its own generation. FXa together with FVa as a co-factor, Ca^{2+}, and an anionic phospholipid membrane forms the *prothrombinase* enzyme complex, which converts prothrombin to thrombin. FVa alone increases the rate of conversion of prothrombin to thrombin by 1000-fold primarily by increasing the catalytic efficiency or Kcat of FXa (Mann & Kalafatis,

2003). Thrombin catalyzes the proteolysis of soluble fibrinogen to insoluble fibrin monomers that trap red blood cells and platelets. At the same time, thrombin also activates FXIII to FXIIIa which crosslinks fibrin monomers to form a mesh-like and more stable fibrin clot.

1.2 Regulation of Blood Coagulation

The regulation of blood coagulation has been described previously by Norris (Norris L A., 2003). Hemostasis involves a balance of blood coagulation and fibrinolysis. If either of these process is out of balance, it may lead to either excessive vascular thrombosis or a bleeding disorder. The coagulation cascade, as described above, is regulated at different steps by anticoagulant proteins and co-factors such as tissue factor plasminogen inhibitor (TFPI), antithrombin (AT-III), and activated protein C (Act C). TFPI, released from the endothelial layer, inhibits the tissue factor-FVIIa complex of the extrinsic pathway. Antithrombin (AT-III) irreversibly binds to and inactivates proteases the FIXa, FXa, TF-VIIa. Antithrombin binds to thrombin forming thrombin-Antithrombin (T-AT-III) complex in a process accelerated by heparin, which is rapidly cleared from the circulation. Protein C (Prot C) is activated by the thrombin-thrombomodulin complex to activated protein C (Act C), which in presence of protein S (S) then inhibits FVIIIa and FVa, respectively (Norris, L.A., 2003). Fibrinolysis is mediated by the protease plasmin derived from plasminogen by urokinase plasminogen activator (uPA) or tissue plasminogen activator (tPA). Plasmin converts fibrin to fibrin degradation products (FDPs)/ D-Dimer, which halt the coagulation process by interfering with thrombin generation and platelet aggregation. Fibrinolysis is regulated and controlled by α_2-antiplasmin and to some extent by α_{2-} macroglobulin, antithrombin, α_1 antitrypsin, and C1-inactivator. Figure 1.1B describes the regulation of blood coagulation.

1.3 Coagulation Factor V

FV was discovered by the Norwegian haematologist, Paul Owren, in 1943 during World War II (Owren, 1947). After a long controversy, FV was purified and isolated from bovine plasma in 1979 (Nesheim *et al.*, 1979). Isolation and characterization of FV led to the paradigm of a membrane-bound clotting process (Kane *et al.*, 1987). FV deficiency results into the haemorrhagic condition while the overactiviation of FVa (FV Leiden), associated with the common genetic risk factor, that results into the venous thrombosis (Mann & Kalafatis, 2003). Approximately 80% of FV in humans is found in human plasma at a concentration of 20-40nM with a Mr of 330,000Da and about 20% of FV is present in platelet α-granules. The liver is the main site of synthesis of FV (Tracy *et al.*, 1982; Mann *et al.*, 1981). The gene for FV spans 80kbp and is located on chromosome 1 at q21-25 and contains 25 exons (Cripe *et al.*, 1992). A 6.8kb mRNA is transcribed to give rise to a 2224 amino acid product having a 28 amino acid signal sequence. The mature FV protein is comprised of three domains organised as A1-A2-B-A3-C1-C2 as described in Figure 1.2A. The A domains are homologous to FVIII and the copper binding protein, ceruloplasmin (Mann *et al.*, 1984) while the C domain is similar to the slime mold protein, discoidin (Jenny *et al.*, 1987). The B domain of FV has a Mr 150,000Da and is poorly conserved amongst species. The inactive procofactor is activated to FVa upon cleavage by thrombin at Arg709, Arg1018 and Arg1545 (Krishnaswamy *et al.*, 1989). FVa is comprised of a heavy chain (A1-A2), having Mr 105,000Da and a light chain (A3-C1-C2) having Mr 74,000Da. After dissociation from the B domain, both the heavy and light chains are held together non-covalently and stabilised by Ca^{2+}, which in turn penetrates the phospholipid membrane of the damaged endothelium or the surface of activated platelets via the C2 domain

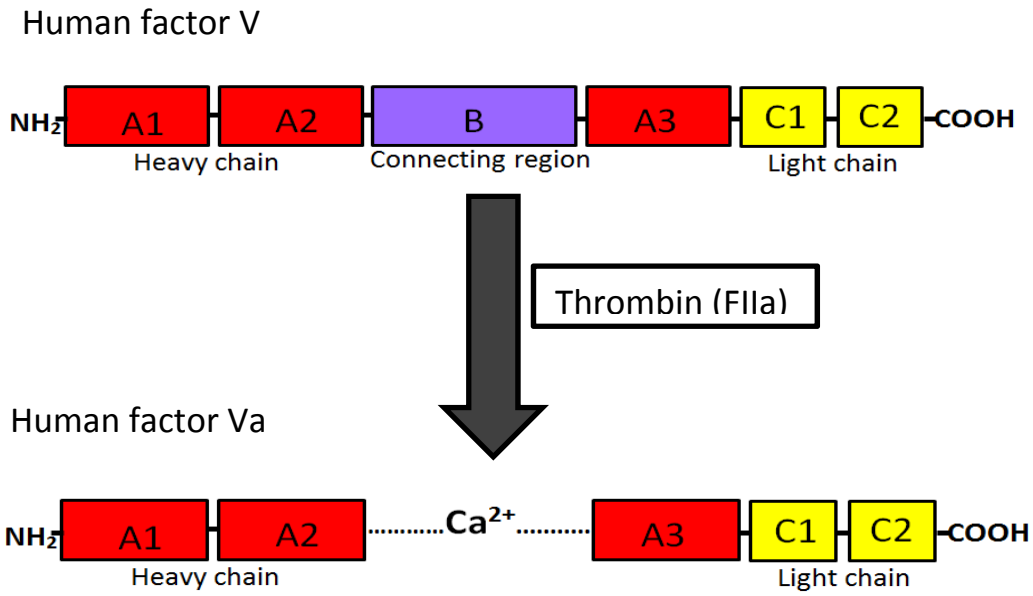
Figure 1.2A Factor (F) V. FV is comprised of an A domain represented in red; B domain in purple; and C domain in yellow. Thrombin highlighted in white box converts inactive FV to active FV (FVa). After activation, the B domain of FVa is replaced by calcium represented as Ca^{2+}. The N- terminal (NH_2) is represented by the heavy chain (left side) and the C-terminal (COOH) by the to light chain (right side) of FV and FVa.

Figure 1.2B FVarole in *prothrombinase* complex. FVa has a light chain represented in the small yellow circle and a heavy chain in the large red circle. FVa, bound to the phospholipid membrane, is represented in pink by a light chain. FVa acts as a cofactor and brings together the FII (substrate), represented in light blue, and FXa (protease) represented in green, via heavy chain. The complex is referred to as *prothrombinase*. Factor II (II) is converted to its active form Factor IIa (IIa) (thrombin) and dissociates from the *prothrombinase* as represented in panel B.

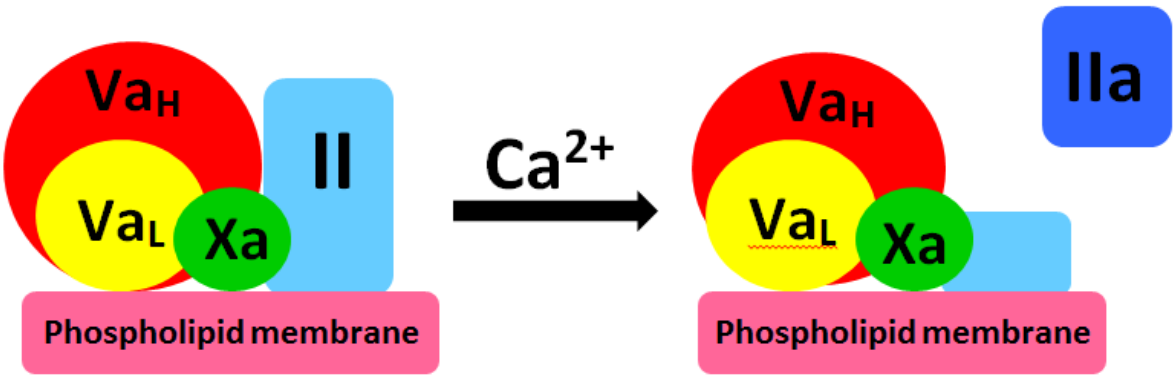
Modified from Mann, K., & Kalafatis, M. (2003). FactorV: a combination of Dr. Jekyll and Mr Hyde. *The American Society of Haematology*, 101, 20-30.

Figure 1.2

A


Human factor V
NH2
A1
A2
B
A3
C1
C2
COOH
Heavy chain
Connecting region
Light chain
Thrombin (FIIa)
Human factor Va
NH2
A1
A2
Ca2+
A3
C1
C2
COOH
Heavy chain
Light chain

B


VaH
VaL
Xa
II
Phospholipid membrane
Ca2+
VaH
VaL
Xa
IIa
Phospholipid membrane

(Kim *et al.*, 2000) and associates with FXa to form the *prothrombinase* complex, described in Figure 1.2B. After thrombin activation of FV to FVa, the Km for prothrombin activation decreases by 2 orders of magnitude and the Kcat of FXa increases by 3 orders of magnitude, resulting in an overall enhancement of prothrombin activation of prothrombin by 5 orders of magnitude (Mann & Kalafatis, 2003) compared with FXa alone. FV possesses both anticoagulant and procoagulant properties. Down regulation of FV/FVa and FVIII/FVIIIa is accomplished by activated protein C (APC), protein S (PS) and thrombomodulin. APC inactivates FVa by cleavage at Arg 506, Arg 306 and Arg 679. APC cleavage of FVa at Arg 506 causes partial inactivation and subsequent cleavage at Arg 306 results in complete inactivation. Plasma FV can also be degraded by APC when bound to phospholipid membrane by cleaving at Arg 306, Arg 506, Arg679 and Lys994 (Mann & Kalafatis, 2003). FV and FVa can act as a cofactor during the inactivation of FVIIIa by APC. Plasmin also cleaves and inactivates FV at Lys 309, Lys310, Arg 313 and Arg 348 (Mann & Kalafatis, 2001).

1.4 Coagulation and the Innate Immune System

The coagulation process and immune system are co-regulated as indicated by comparative studies of invertebrate and vertebrate defense systems. The horse shoe crab has coagulation factors C and G which are serine protease zymogens, converted to coagulin after activation, they also possess bacterial agglutination and microbicidal activities. Hence, the vertebrate blood coagulation process has evolved as part of innate immune system as underscored by this defense mechanism in horseshoe crab which is the oldest living arthropod fossil (Krem & Di Cera, 2002). In addition, *in vivo* and *in vitro* evidence has shown study showing that the FXIIIa in the common pathway cross-links bacteria to fibrin and provides additional time for the immune system to clear bacteria from the site of infection in humans (Loof *et al.*, 2011). Thus, bacterial

infection activates the coagulation pathway and the fibrin clot has a 'barrier function' by restricting pathogen growth, survival and transmission (Levi *et al.*, 2004).

Thrombin plays a central role in mediating the interations between pathogen recognition and its destruction as a part of the innate immune system. Thrombin induces the host inflammatory response through endothelial, mural, epithelial, adipocyte and immune cells (Delvaeye& Conway, 2009). Thrombin also directly affects the complement pathway by activating C3, C5 and the lytic membrane complex (C5b-9), and recruits activated leukocytes to destroy the invading pathogen (Delvaeye& Conway, 2009).

Gram negative bacteria have been associated with sepsis, during which endotoxin/LPS from bacterial cells binds to toll-like receptor 4 (TLR4) of macrophages and dendritic cells leading to the overproduction of proinflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-8 (Park *et al.*, 2010). These cytokines induce mononuclear cells to express tissue factor and this result in widespread blood clotting, ischemia and multiple organ dysfunction syndromes (Levi *et al.*, 2004). With improper regulation, an inflammatory response derived from complement activation propagates more blood coagulation, which in turn promotes more inflammation.

The propogation of inflammation leads to the excessive coagulation and organ damage. In the final stage, this process may cycle out of control and results in cell apoptosis or necrosis, which and may contribute to immunoparalysis and superinfection (Faix J, 2013). This immuosuppressed condition is often difficult to treat and is associated with disseminated intravascular coagulation (DIC), multiple organ failure, and a high mortality rate (Annane *et al.*, 2005).

1.5 Bacteria and Coagulation

Besides minimizing blood loss and maintaining hemostasis, the coagulation process protects the body against bacterial infection by immobilizing and trapping the pathogen within the fibrin clot which permits targeted immune cell-mediated destruction (Levi *et al.*, 2004). Hence, the coagulation system plays a significant role in host-pathogen interactions. Local thrombus formation serves as a first line of defence against microbial invasion. Sepsis is caused by bacterial infection of the blood, while the severe and uncontrolled septic condition results into multiple organ failure, low blood pressure, rapid heart rate and altered mental state of septic shock (Reinhart *et al.*, 2013). During sepsis or septic shock, the coagulant/anticoagulant balance is disturbed leading to excessive thrombosis or bleeding. The main pathogens responsible for septic shock are gram negative bacteria (25-30%), gram positive bacteria (30-50%), fungi (1-3%), and parasites (1-3%) and Viruses (2-4%) (Tsiotou *et al.*, 2005). Viruses promote secondary bacterial infections. It has been shown recently that in response to bacterial sepsis, neutrophils secrete micro-vesicle (MVs), derived from opsonized particles which decreases bacterial survival and limits pathogen transmission (Timár *et al.*, 2012). Studies have recently shown that blood coagulation, which is a part of innate immune response, immobilises and kill *Streptococcus pyogenes* within the fibrin matrix mediated by FXIIIa, a highly conserved transglutaminase (Loof *et al.*, 2011). This is also underscored by the fact that FXIII deficient mice show the higher indicies of infections from bacteria compared to wild type animals (Loof *et al.*, 2011).

In view of the above described information, bacteria have evolved to express and release proteases which degrade not only fibrin, but other coagulation factors that mediate clot formation (Delvaeye & Conway 2009). For instance, *Streptocooccus pyrogenes, Staphylococcus aureus, Yersinia pestis, Haemophilus influenza, Salmonella typhimurium, Neisseria meningitidis*, and

Borrelia burgdorferi rely on host plasminogen (tPA/uPA) to activate plasmin which dissolves the fibrin clot barrier and causes more infection at sites distant from the site of infection. Wild-type mice have a greater mortality rate from *Yersinia pestis* infection compared to plasminogen-deficient mice (Levi *et al.*, 2004). Eventually, this process allows the pathogen to remove the fibrin barrier surrounding the local site of infection and thereby facilitates pathogen dissemination and survival. Other studies have demonstrated that OmpT from *E. coli*, PgtE from *Salmonella enterica*, Pla from *Yersinia pestis* degrade antimicrobial pepides (AMPs) during the host immune response. OmpT is 50-70% similar at the amino acid level to Pla and PgtE of the Omptin family (Hritonenko & Stathopoulos, 2007). Pla and PgtE have also been associated with cleavage of serum components and affect complement activation (Ramu *et al.*, 2007; Riley *et al.*, 1983). OmpT from enterohemorrhagic (EHEC) cleaves and inactivates human LL-37, the precursor of a human cationic antimicrobial peptide (Thomassin *et al.*, 2011). Also EspP from EHEC strain has been shown to cleave and inactivate C3/C3b and C5 of the complement pathway (Orth *et al.*, 2010).

1.6 Outer Membrane Vesicles

Outer membrane vesicle (OMV) production is conserved among prokaryotes and eukaryotes (Rompikuntal. P K, MD Dissertation, 2012). It was recently shown that eukaryotic human cells secrete microvesicles (MVs) in response by invasion of gram negative bacteria. Gram negative bacteria such as *Escherichia coli* secrete OMVs (Annane *et al.*, 2005) which are enriched with lipopolysaccharide (LPS) and outer membrane proteins and are potent stimulators of production of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β (Kuehn *et al.*, 2005).

OMVs are round, 20-200nM in diameter and are comprised of LPS, protein, lipid, DNA, and RNA (Kuehn & Kesty, 2005). OMV production follows a "Type IV" mechanism of gram negative pathogens to release a large cargo of lipid and proteins into the extracellular environment. OMVs harbour outer membrane proteins but lack inner membrane proteins (Kuehn *et al.* 2005).

E. coli secrete OMVs as a means of communication among the bacterial community to exchange protein, plasmid, antibiotics resistant protein, phage DNA, toxins, and other virulent factors. OMVs modulate the host immune response, survival, colonization and growth within the competitive environment to facilitate host cell destruction (Park *et al.*, 2010). OMVs were also shown to protect hyper-vesiculating *E. coli* from destruction within the host by nullifying the effect of antimicrobial peptides (AMPs) such as polymyxin-B and colistin and T4 bacteriophage infections; which are usually outer membrane stressors to gram negative bacteria (Manning and Kuehn, 2011). OMVs contain outer membrane proteins which assist in adhesion of bacterial cells to host epithelial cells and manipulate the host immune response (Kuehn & Kesty, 2005). OMVs have been referred to as "bacterial bombs" that penetrate deep inside host tissues to sites that are not easily accessible by larger cells (Kuehn & Kesty, 2005). OMVs from *E. coli* injected intraperitoneally into mice cause 95% mortality with 25µg of OMV protein in 24 hours and 100% mortality with 50µg of OMV protein in 36 hours (Park *et al.*, 2010).

OMVs are produced more commonly in pathogenic bacteria than non-pathogenic cells (Horstman & Kuehn, 2002). Enterotoxigenic *E. coli* (ETEC) associated traveller diseases in developing countries produce 10-fold more OMVs in the presence of antibiotics (such as polymixinB and colistin) than a *E. coli* K12 laboratory strain (Manning and Kuehn, 2011). LPS serotype, oxygen stress, availability of iron, and presence of antibiotics within the host

environment impact production, composition and toxicity of OMVs. *E. coli, V.cholerae,* and *B.melitensis* have a maximum rate of vesicle production at the end of log growth phase and OMVs are abundant at the site of cell division (Chatterjee & Das 1967, Hoekstra *et al.*, 1976; Gamazo & Moriyn, 1987). As shown in Figure 1.3, the link between the peptidoglycan layer and the outer membrane may serve as the site of vesiculation and these sites are influenced by cell division. There are two other pathways of OMV production as described elsewhere (Rompikuntal P K, MD Dissertation, 2012) where either peptidoglycan generates fragments which are ultimately exported out of the cell by turgor pressure or by a quorum sensing molecule, described for *Pseudomonas aeruginosa*, on the outer membrane which interacts with Mg^{2+} and disrupts salt bridges that leads to vesicle budding, as described in Figure 1.3.

OMVs induce the host response of pattern recognition receptor (PRR) expression and other toll-tike receptors through a nucleotide binding domain containing protein (NOD)-like receptor (Rompikuntal P K, MD Dissertation, 2012). This in turn activates various cytokines (TNF, IL-1B, IL-6) and chemokines (MCP-1 and IL-8) in response to infections (Faix. J., 2013). These processes trigger tissue factor expression and eventually lead to an enhanced high inflammatory response (Levi *et al.*, 2004). The imbalance in blood coagulation and fibrinolysis blocks blood flow and vessels and then results in wide spread blood clotting which then finally leads to organ necrosis, multiple organ failure and finally death (Zeerleder *et al.*, 2005). OMV production is cytotoxic, induces bacterial survival and promotes invasion within the host cell for colonization. It was recently shown that *Vibrio cholerae* has *VrrA* gene which regulates OMV production by controlling the expression of OmpA. Interestingly, OmpA mutant secretes a large number of OMVs (Rompikuntal. P K, MD Dissertation, 2012). However, the overall mechanisms underlying OMV production in *E. coli* are poorly understood.

Figure 1.3 Model of Outer Membrane Vesicles Biogenesis. There are three different proposed models for OMV production in gram negative bacteria.

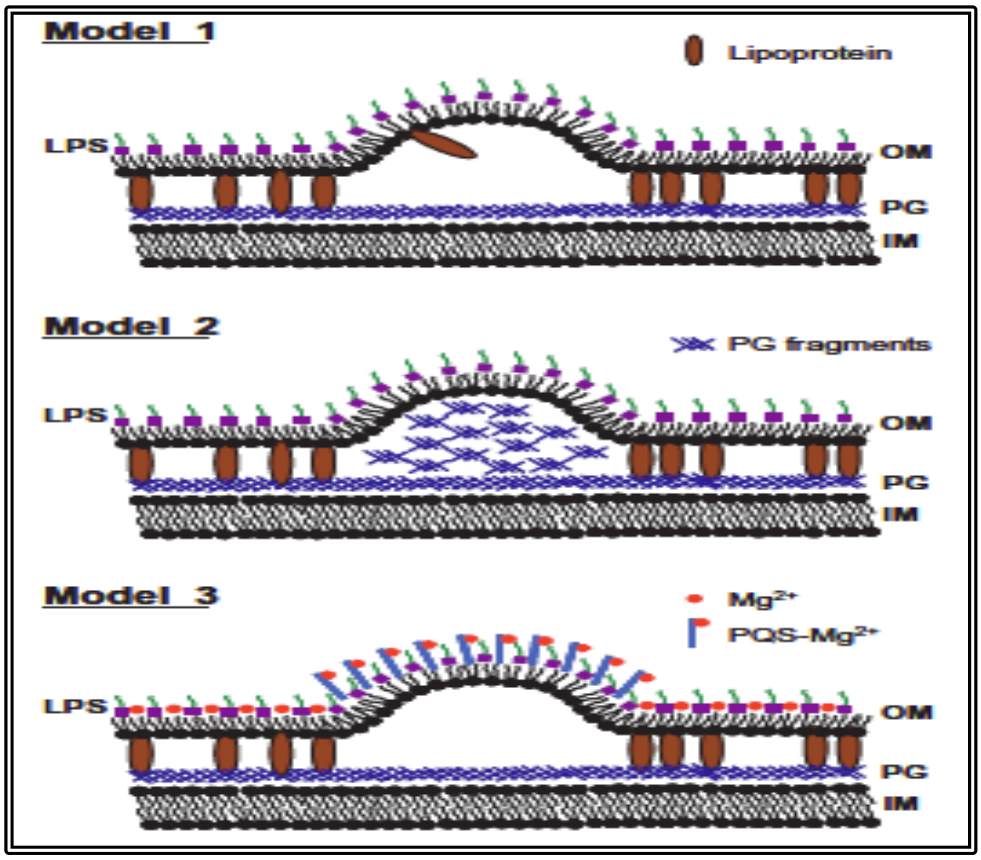
Model 1: OMVs are produced when a linkage between the peptidoglycan layer (In blue) and outer membrane is absent.

Model 2: The peptidoglycan layer generates excessive fragments (Represented by blue particles), which are not transported back to the cytoplasm but are secreted as OMVs.

Model 3: The outer membrane has quorum sensing molecules (Represented by a purple line) which interact with Mg^{2+} ions (Represented by a pink dot) and forms OMVs by ionic repulsion in *Pseudomonas aeruginosa*.

Adapted from Mashburn-Warren, L.M., and Whiteley, M. (2006). Special delivery: vesicle trafficking in prokaryotes. *Molecular Microbiology*. *61*, 839-846.

Figure 1.3


Model 1
Lipoprotein
LPS
OM
PG
IM
Model 2
PG fragments
LPS
OM
PG
IM
Model 3
Mg2+
PQS-Mg2+
LPS
OM
PG
IM

E. coli secrete OMVs in association with Shiga toxin (Stx) 1 and 2 (Yokoyama *et al.*, 2000), Cytolysin A (ClyA) (Wai *et al.*, 2003), heat-labile enterotoxin (LT) (Horstman &Kuehn, 2000), α-haemolysin (Balasalobre *et al.* 2003) and LPS, which is cytotoxic to human cells. *E. coli* 0157:H7 secrete OMVs with Stx 1 which is 98% identical at the amino acid level to Shiga toxin from *Shigella dysenteriae* and Stx 2 which is 56% identical at the amino acid level to Stx1 (Kim *et al.*, 2010). Stx 1 and 2 are transported through a Twin arginine translocator (Tat) to the periplasmic space and then sorted or packaged by an unknown (protein) factor (Choi & Lee, 2004).

OMV associated proteases OmpT (37kDa) and/or EatA (110kDa), as shown in Figure 1.5B, have recently been purified, and identified by LC mass spectrometry (Tilley D, MSc. Thesis, 2011) as being responsible for inactivating FV upon addition to normal human plasma. These proteases from *E. coli* reduced FV activity by up to 80% in normal human plasma.

OMVs can be used as a vaccine and provide a promising future for treating different bacterial diseases (Rompikuntal P K, MD Dissertation, 2012; Kim *et al.*, 2013).

1.7 Bacterial Outer membrane protease T

The outer membrane protein temperature regulated protease (OmpT) is an outer membrane associated protease that is temperature regulated with protein expression higher at 37^{o}C than 25^{o}C (Manning & Reeves, 1976). OmpT is found in both pathogenic and non-pathogenic strains of *E. coli*. OmpT has a vase-shaped structure embedded in the outer membrane in a 10 stranded antiparallel β-barrel structure and is 70Å in length (Fig 1.4B). OmpT has an absolute requirement for rough LPS as a co-factor for its proteolytic activity (Yun *et al.*, 2008; Kukkonen *et al.*, 2004; Kramer *et al.*, 2002) which is present in the outer membrane. The LPS binding sites are present

at Arg 175, Arg 138, Lys 226, Glu136 and Tyr134 (Kramer *et al.*, 2001). The detailed structure of OmpT is described in Figures 1.4A and 1.4B. OmpT has five negatively charged surface loops (L1, L2, L3, L4 and L5) exposed to the extracellular space and four short intracellular periplasmic turns (T1, T2, T3 and T4). OmpT generally cleaves its protein substrates between dibasic residues. OmpT has specific cleavage site at P1 position for positive charged amino acid (Arg and Lys) and is less stringent at P1' position for either a positive or negatively charged amino acid (such as Arg, Lys, Val, Gly, and Ala) (Dekker *et al.*, 2001). The active site is present on extracellular loops as a catalytic dyad: Asp 83 and Asp 85 are on one side and His 212 and Asp 210 on the other side of the loop, respectively. The Asp/His catalytic dyad uses water as a nucleophile to cleave its substrate by a novel catalytic mechanism that is different from other aspartic proteases (Vandeputte-Rutten *et al.*, 2001).

OmpT expression is higher in a lower concentration of glucose (0.02%) and lower in a higher concentration of glucose (2%) in M9 Minimal media (Yang *et al.*, 2011). Approximately 83% of *E. coli* isolates from urinary tract infections (UTIs) express the OmpT gene while 67% of faecal isolates express the protease (Lundrigan *et al.*, 1992). OmpT inactivates antimicrobial peptides secreted by epithelial cells as part of subverting the host innate immune response (Stumpe *et al.*, 1998) suggesting that OmpT is a virulence factor that supports tissue destruction within the host. OmpT permits bacterial adhesion to host cells during infections (Hritonenko *et al.*, 2007) and has been shown to cleave and inactivate tissue factor pathway inhibitor (TFPI) which would enhance the coagulation process and contribute to hemostatic imbalances that accompany bacterial infections (Yun *et al.*, 2008). In addition, OmpT can also cleave and activate plasminogen to plasmin, albeit inefficiently compared to Pla from *Yersinia pestis* (Grodberg *et al.*, 1988) and thereby facilitate the uropathogenic *E. coli* cell (UPEC) pathogenesis upon infection of the

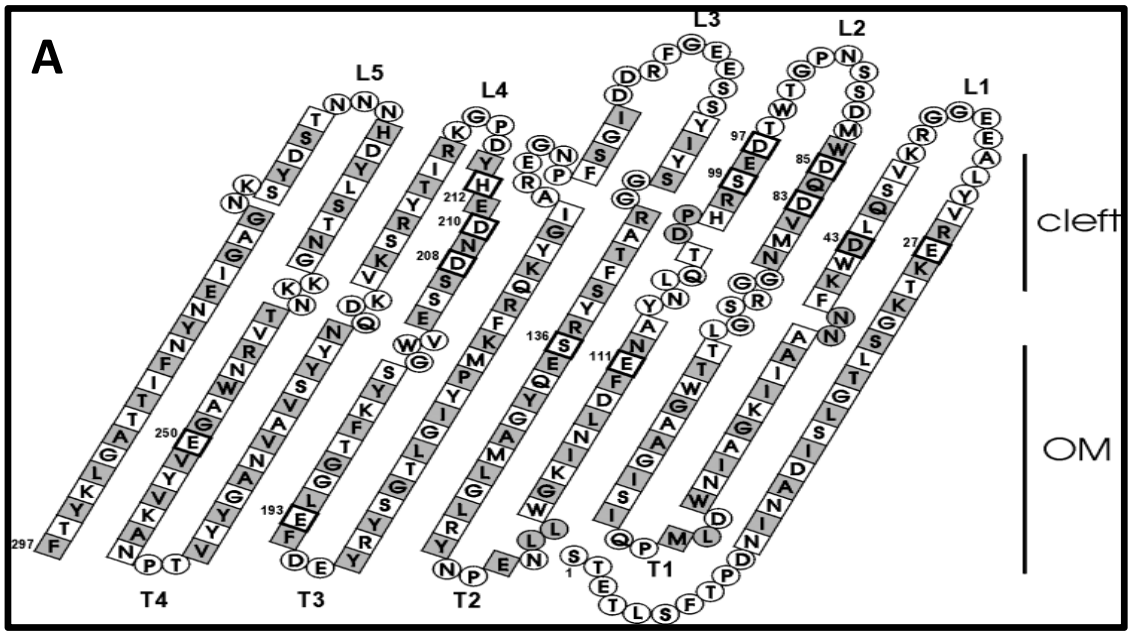
urinary tract. However, shortly after, plasmin would be inhibited by α_2-antiplasmin to levels that are insufficient for persistence of the infection (Lundrigan *et al.*, 1992). One study has shown that OmpT is a poor activator of plasminogen and was not able to cause fibrinolysis (Haiko *et al.*, 2009). Similary, Streptokinase/SK (resistant to α-antiplasmin) from *Streptococcus pyrogenes* (Li *et al.*, 1999), Staphylokinase/SAK (sensitive to α-antiplasmin) from *Staphylococcus aureus* (Esmon *et al.* 1998) aspartase from *Haemophilus influenza* (Sjostrom *et al.*, 1997) all engage the host plasminogen system to promote lysis of fibrin to facilitate pathogen dissemination and survival within the host environment. This study also showed that pro-matrix metalloproteinase-9 (MMP-9) was converted to its active form and responsible for cleaving the collagen tissue barrier that would immobilized cells. Collagen was cleaved by Pla protease from *Yersinia pestis* and PgtE of *Salmonellae enterica*, but not by OmpT of *E. coli* (Haiko *et al.*, 2009). In the case of plasminogen activation, two different studies showed contradictory results; stating that OmpT does (McCarter *et al.*,2004) or does not (Haiko *et al.*, 2009) activate plasminogen. This thesis will shed further light on OmpT in activation of the host plasminogen system.

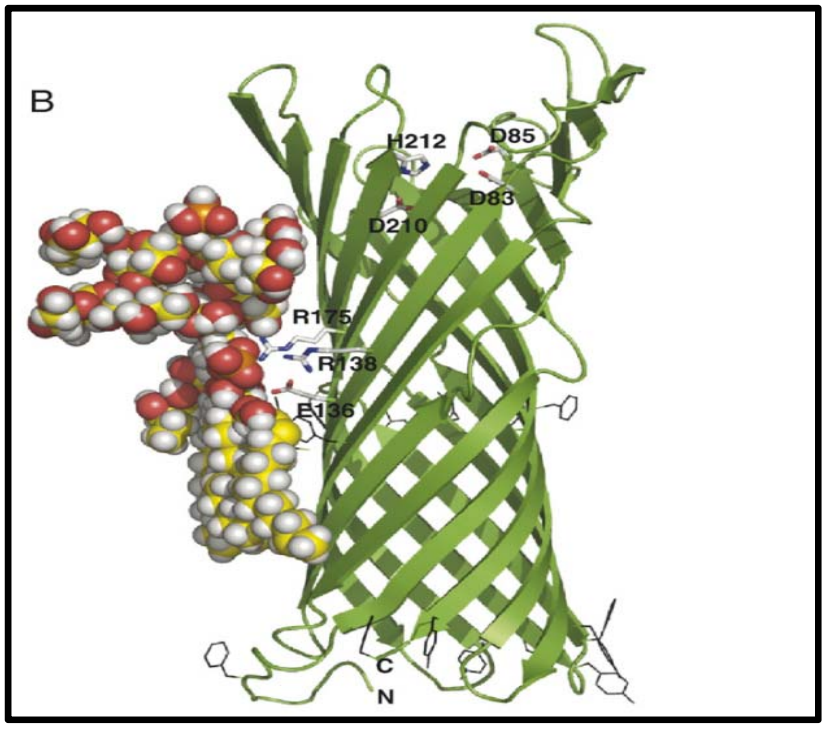
The importance of FV in the immune defence towards microbial pathogens is underscored by the observation that compared to wild type mice, mice deficient in either plasma or platelet FV displayed increased mortality upon infection with *Streptococcus pyrogenes* (Sun *et al.*, 2009). This result is consistent with dose-dependent inactivation of FV in both the baboon sepsis model (Samis *et al.* 2007) and recent studies by Tilley (MSc Thesis, 2011) describing OmpT inactivation of FV.

OmpT from the pathogenic strain of *E. coli O86a:K61* has been reported to cleave and inactivate FV and FVa (Tilley D, MSc Thesis, 2011) used in a baboon model of *E. coli* infections (Samis *et*

Figure 1.4 Structure of OmpT protease. OmpT has 10 antiparallel stranded β-barrel structures (Panel A) and a vase-shaped structure (Panel B) embedded in the outer membrane of *E. coli*. The amino acids are shown in the grey and white boxes. OmpT has five extracellular surface loops at the top, designated by L1, L2, L3, L4, and L5 and four periplasmic turns at the bottom, designated as T1, T2, T3, and T4 respectively (Panel A). The surface exposed loops have catalytic sites represented by H212 and D210 on one side and D83 and D85 on the other side (Panel B). The LPS binding sites (Red, white and yellow beaded structure) are represented on the left side as R175, R138 and R136 (Panel B). Adapted from Vandeputte-Rutten, L., Kramer, R. A., Kroon, J., Dekker, N., Egmond, M.R., & Gros, P., (2001). Crystal structure of the outer membrane protease OmpT from *Escherichia coli* suggests a novel catalytic site. *The EMBO Journal*, 20(18), 5033-5039.

Figure 1.4


A
L5
L4
L3
L2
L1
T4
T3
T2
T1
cleft
OM


B
H212
D85
D83
D210
R175
R138
E136
C
N

al., 2007). Hence, the aim of this research was to further characterize the effect of OmpT on FV and coagulation and examine its potential role in the pathogenesis of EHEC infections.

1.8 Study Rationale

Sepsis is responsible for increased morbidity and mortality as it progresses from a systemic inflammatory response syndrome (SIRS), to acute renal failure, adult respiratory distress syndrome, and eventually septic shock and DIC (Rangel *et al.*, 1998). Sepsis is caused by pathogenic compounds such as endotoxin (Lipopolysaccharide, LPS) which triggers the immune response and leads to microvascular thrombosis, multiple organ failure and DIC (LaPierre, 2010). The death rate from severe sepsis is 25-30% (Bernard *et al.*, 2001) and from septic shock is 40-70% (Annanne *et al.*, 2005). Every 300 in 100,000 people in North America suffer from sepsis related death and chronic diseases. Sepsis is the 13th most common cause of death in the United States with 75,000 new cases per year (LaPierre, 2010).

E. coli secrete OMVs as a part of a virulence mechanism that modulates inflammatory and clotting systems, exaggerates the host immune response and results in systemic inflammation and multiple organ failure (Park *et al.*, 2010). Bacterial proteases enhance the synthesis of host proinflammatory mediators such as TNF-α and IL-6 and cause the activation of platelets and resultant DIC in humans (Riedemann *et al.*, 2003). Pathogenic *E. coli* infections are a common cause of chronic diarrhoea, haemolytic uremic syndrome, haemorrhagic colitis and urinary tract infections (Joseph, 2011). These disorders in humans often occur in the presence of high molecular weight Serine protease autotransporters of *Enterobacteriaceae* (SPATEs) (Dautin, 2010). EspP has been shown to cleave FV and pepsin and may be responsible for haemorrhagic colitis (Brunder *et al.*, 1997). Although SPATEs such as EspC, EspP, Pet, Pic and Sat but not

Tsh and SepA, have been shown to cleave FV (Dutta *et al.*, 2002), proteolytic inactivation of FV was not demonstrated because FV activity assays were not performed.

E. coli O86a:K61 is the major contributor to infantile diarrhoea in humans and diarrhoeal diseases in wild birds (Ragione *et al.*, 2002). It is also present in cattle (Blanco *et al.*, 1993), pigs (Alex *et al.*, 1995) and horses (Holland *et al.*, 1996) where it causes significant diarrhoeal diseases and financial losses. *E. coli* infection has been associated with death and diarrhoea in children in developing countries (Egile *et al.*, 1997). ETEC is the major cause of diarrhoea in young children and is responsible for 300,000-500,000 deaths annually in developing countries (WHO, 2006).

EHEC was identified in 1982 (O'Brien *et al.*) and causes watery diarrhoea, hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). It usually affects people of all ages but young children and elderly patients develop severe symptoms and in most cases pregnant women and immune-compromised patients are at high risk. The symptoms include: bloody diarrhoea, severe stomach cramps, vomiting, nausea, renal damage, anemia, dehydration, disruption of red blood cells, depletion of platelets (thrombocytopenia), impaired kidney function, fever, and neurologic impairment (Brandt *et al.*, 1990; Karmali *et al.*, 1985; O`Brien & Mohawk, 2010).

EHEC was shown to secrete OMVs harbouring the Shigatoxin-1 (Stx-1) and Shigatoxin-2 also known as Shiga-toxin producing *E. coli* (STEC) and deliver toxins to host cells and contribute to pathogenesis causing life threatening diseases (O`Brien & Mohawk, 2010). According to the Food and Drug Administration (FDA), the mortality rate of *E. coli* infectons with TTP is as high as 50% in elderly patients. About 15% of hemorrhaghic colitis victims may develop HUS which

leads to permanent loss of kidney function. Recently, the Centre for Diseases Control and Prevention (CDC) estimated that nearly 265,000 cases of EHEC/STEC infections occur every year with 2000 hospitalizations in the United States. EHEC is a major contributor to infections from contaminated food in North America and is currently a major world-wide health problem.

1.9 Preliminary Studies

Using an experimental *E. coli* 086a:K61 infection model in baboons, there was a dose-dependent cleavage and inactivation of FV that was proportional to the amount of bacteria infused (Samis *et al.*, 2007). Plasmin, NE, APC, and protease(s) from *E. coli* JM109 lysates or culture supernatants were tested for their ability to cleave and inactivate FV upon addition to normal baboon plasma.The results are shown in Figure 1.5. The cleavage and inactivation of FV in normal baboon plasma was most consistent with the action of plasmin or protease(s) from *E. coli JM109* lysates or culture supernatants as assessed by immunoblotting (Samis, unpublished observations).

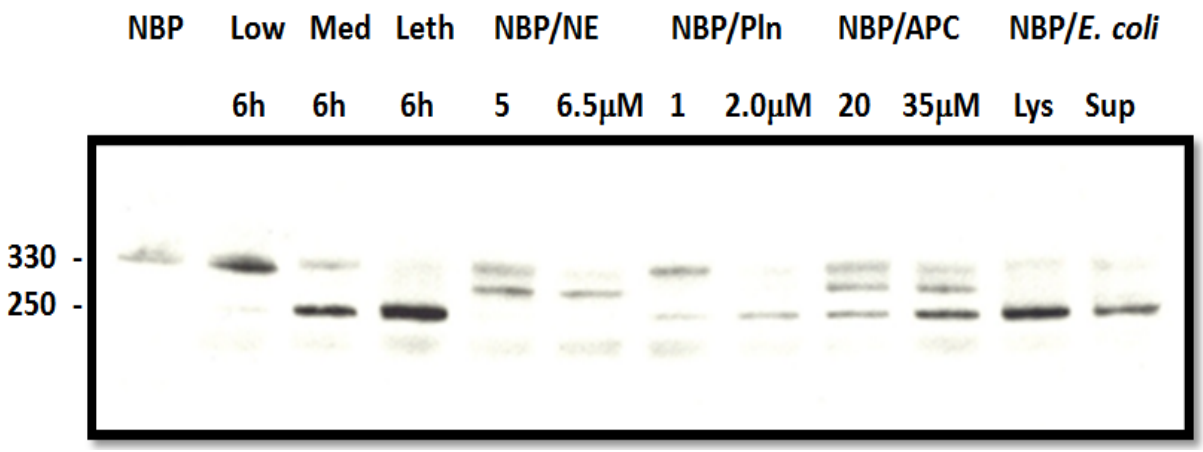
Neutrophil elastase (NE) has been reported to initially activate FV and inactivate the activated product (Samis *et al.*, 1997). During systemic inflammation, microbial infection triggers the release of proinflammatory mediators and NE. NE and proinflammatory mediators damage endothelial cells which induces the expression of tissue factor. Tissue factor activates the extrinsic pathway and further enhances coagulation and supresses fibrinolysis resulting in DIC (Russell *et al.*, 2006).

During *Escherichia coli* O86a:K61 induced sepsis in baboons, there was a dose-dependent relationship between the extent of FV inactivation and the amount of pathogenic *E. coli* infused (Samis *et al.*, 2003). Although the host neutrophil elastase (NE) and/or plasmin were candidate

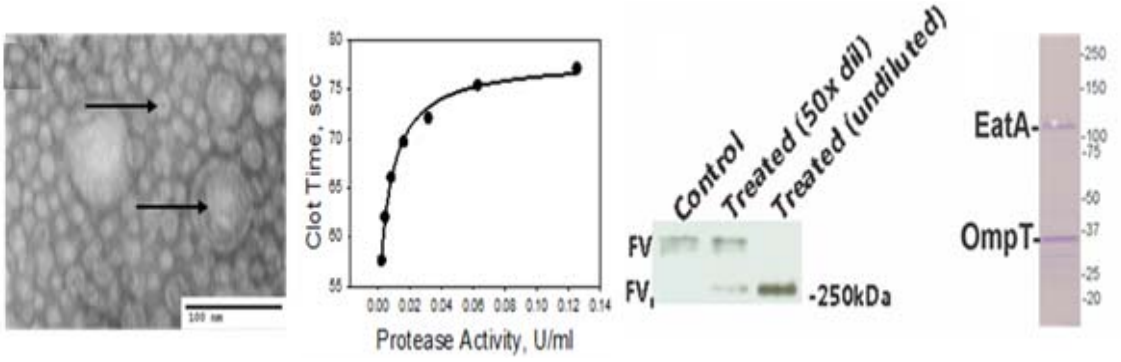
Figure 1.5 Cleavage and inactivation of FV during experimental sepsis and upon addition of proteases to normal baboon plasma. Normal baboon plasma (NBP) was untreated (Control) or taken from baboons or infused with Low, Medium (Med), and Lethal (Leth) doses of *E. coli* 086a:K61 after 6h. Also shown is NBP to which was added different concentrations (μM, final concentration) of neutrophil elastase (NE), Plasmin (Pln), activated protein C (APC) or *E. coli* JM109 lysate (Lys) or supernatant (Sup) fractions and incubated for 20min at room temperature. The samples were electrophoresed by SDS-PAGE and immunoblotted for FV. The molecular weight of intact FV (330kDa) and cleaved/inactivated FV (250kDa) are shown to the left of the panel. (Samis, unpublished observations). Panel B describes the two protease associated with OMVs OmpT and EatA (shown on right) from *E. coli* O86a:K61 which was shown to cleave and inactivate FV from 330kDa to 250kDa, as determined by Tilley *et al.* (2011).

Figure 1.5

A


NBP
Low 6h
Med 6h
Leth 6h
NBP/NE 5
6.5µM
NBP/Pln 1
2.0µM
NBP/APC 20
35µM
NBP/E. coli Lys
Sup
330 -
250 -

B


Clot Time, sec
Protease Activity, U/ml
Control
Treated (50x dil)
Treated (undiluted)
FV
FV*
-250kDa
EatA-
OmpT-

proteases for mediating FV inactivation during experimental sepsis in the baboon model, a secreted bacterial protease may have been responsible for the effects observed. Recently, Tilley (MSc. Thesis, 2011) demonstrated that *E. coli* O86a:K61 released OMVs which possessed an associated protease activity that inactivated both purified FV and FVa in normal human plasma. The proteolytic activity vs. FV/FVa was purified by approximately 1000-fold from *E. coli* OMVs by tangential flow filtration (TFF), sucrose density ultracentrifugation and Sephadex G-100 size exclusion chromatography. LC mass spectrometry identified 2 major proteins in the purified material as the proteases: OmpT and EatA as described in Figure 1.5B (Hospital for Sick Children, Toronto, ON) (Tilley, MSc Thesis, 2011). The FV microplate coagulation assay was used to show that this proteolytic activity associated with OMVs released by *E. coli* inactivated FV by up to 80% in normal human plasma. OMV associated proteolytic activity also prolonged the clot time of whole human blood as well as the prothrombin time (PT) and activated partial prothrombin time (aPTT) of normal human plasma (Tilley, MSc. Thesis, 2011).

The following are the biochemical characteristics of *E. coli* O86a:K61 OMV associated OmpT and EatA as determined by Tilley (MSc Thesis, 2011):

1) OmpT and EatA inactivate and hydrolyze FV/FVa as determined by microplate activity assay and immunoblotting, respectively.
2) OmpT and EatA prolong the clot time of whole blood initiated with tissue factor.
3) OmpT and EatA prolong the PT and aPTT clot times of human plasma.
4) OmpT and EatA activity in OMVs is heat stable at 60-70^{o}C vs FV/FVa in human plasma.

OmpT and EatA inactivation of FV/FVa in human plasma is inhibited by 90% by the addition of purified alpha-1 protease inhibitor (A1PI); but only by 10-20% by serine protease inhibitors

(Phenylmethylsulfonylflouride, PMSF). The above information provides the rationale for the following hypotheses:

1.10 Hypotheses

1) OmpT does not affect the growth of *E. coli* in liquid culture.
2) The EHEC OmpT may be involved in determining the production and/or composition of OMVs.
3) EHEC cellular and OMV associated OmpT inactivates FV to attenuate blood coagulation.

In view of the above, this research will focus on the characterization of EHEC OmpT to determine its role in the coagulation process and in the pathogenesis of *E. coli* infections. The following are the research objectives of this thesis:

1.11 Research Study Aims

1) Characterization and cloning of OmpT from *E. coli* O86a:K61 to determine its effect on FV/FVa.
2) To determine the effect of OmpT on the growth of EHEC in nutrient rich (LB) vs nutrient limited media (Minimal A media).
3) To determine the effect of OmpT on the cell size of EHEC by Digital microscopy.
4) To charaterize the EHEC genetic locus of OmpT by polymerase chain reaction (PCR) and OmpT protein expression in cells and OMVs by Immunoblotting.
5) To determine the effect of OmpT from EHEC and OMVs on FV in normal human plasma versus plasminogen-deficient plasma.
6) To determine the effect of EHEC OmpT on FV in normal human plasma by immunoblotting.
7) To determine the effect of OmpT from EHEC cells and OMVs on the activated partial prothrombin time (aPTT) and Prothrombin time (PT) in normal human plasma.
8) To determine the effect of OmpT from EHEC cells and OMVs on the activity of other coagulation factors in normal human plasma.

Chapter 2

Materials and Methods

2.1 Bacterial Strains and Growth Conditions

The three bacterial strains of EHEC (Wild type; ompT deletion mutant (ΔompT); and ompT over-expressing strain from a multicopy plasmid (pEHompT)) used in this work have been characterized in a previous study (Thomassin *et al.*, 2012). EHEC were grown at 37^{o}C with aeration at 200rpm in an orbital shaker (Eppendorf, Happauge, NY) in either Luria Bertani (LB) broth or Minimal A Media (60mM K_2HPO_4, 33mM KH_2PO_4, 7.5mM ammonium sulphate, 1.7mM trisodium citrate, pH7.0 with 0.2% (w/v) glucose, 0.006 % (w/v) tryptone, 1mM $MgSO_4$). Liquid growth media was supplemented with chloramphenicol (30µg/ml) for EHEC (pEHompT). *E. coli* O157:H7ΔompT was created by deleting the OmpT gene based on sacB gene based allelic exchange. *E. coli* O157:H7ΔompT(pEHompT) was created by transforming the pACYC184 multicopy plasmid (NEB England Biolabs Ltd., Pickering, ON, Canada) with the cloned OmpT gene under the control of its own promoter (Thomassin *et al.*, 2012).

2.2 Freezer stock of bacterial strains

The bacterial strains were generously provided by Dr. Herve Le Moual from McGill University (Montreal, QC). The strains were streaked on a LB agar plate with/without chloramphenicol (30µg/ml) and subcultured into LB broth. The frozen stocks were prepared by mixing a 1 ml overnight culture into glycerol to achieve a final concentration of 15%. The samples were pre-frozen on dry ice and stored at -80^{o}C. The frozen stock of OmpT construct/pUC18 in DH5α and BL21(DE3) cells were prepared in a similar manner and stored at -80^{o}C.

2.3 List of bacterial strains used

The following EHEC strains, listed in Table 2.1, were provided by Dr. Herve Le Moual (McGill University, Montreal, Canada). The wild type refers to *E. coli* O157:H7 strain; ΔOmpT refers to

an OmpT deletion mutant of EHEC and pEHOmpT refers to an OmpT deletion mutant reconstituted with OmpT overexpression from a plasmid (pACYC 184) driven by the enterohemorrhagic promoter. The abbreviation wild type, ΔOmpT, and pEHOmpT for *E. coli* O157:H7 strains will be used in all sections.

Table 2.1 Bacterial strains used in this study

	E. coli* strains	**Genotype/Description**	**References**
1	*E. coli* O157:H7	Wild type EHEC O157:H7	Riley *et al.*, 1983
2	EHEC ΔOmpT	EDL933 ΔOmpT	Thomassin *et al.*, 2012
3	EHEC ΔOmpT (pEHOmpT)	EDL933 ΔOmpT mutant expressing OmpT from pEHOmpT	Thomassin *et al.*, 2012

*EHEC strains were provided by Dr. Herve Le Moual.

2.4 Cloning of OmpT Gene from *E. coli* O86a:K61

2.4a Media and Growth Condition

DH5α/BL21(DE3) cells were grown in LB medium and incubated overnight at 37^{o}C with 200rpm agitation in an orbital shaker (Eppendorf, Happauge, NY). The concentration of ampicillin in LB media was 100μg/ml in order to maintain the plasmid within the cells.

2.4b Extraction of genomic DNA from *E. coli* O86a:K61 and Plasmid DNA from DH5αcells

The genomic DNA was isolated from *E. coli* O86a:K61 by an alkaline lysis method (Sambrook and Russel, 2001). The plasmid DNA (pUC18) was extracted using an EZ-spin column Plasmid DNA MiniPrep Kit from Biobasic (Biobasic Inc., Markam, ON, Canada) according to the manufacturer's instructions.

2.4c Polymerase Chain Reaction for amplification of the OmpT gene

The genomic DNA of *E. coli* O86a:K61 has not been previously sequenced. Hence, the forward and reverse primer for the OmpT gene was designed from the coding region based on the sequences from five different *E. coli* strains. The primers were flanked by the restriction sites to facilitate directional gene cloning after PCR amplification. The forward and reverse primers were designed according to % GC content and length were: 5'-GGATCCATGCGGGCGAAACTTCTGGGAATAGTCCTGACAACCCCTATTGCG-3' and 5'-AAGCTTTTAAAAGGTGTACTTAAGACCAGCAGTAGTGATGAAGTTATAGTT-3', respectively. The OmpT gene was amplified using genomic DNA of *E. coli* O86a:K61 as a template. The final concentration of genomic DNA was 80ng in a 25µl PCR reaction and the annealing temperature (Ta) was calculated based on the equation: 4 (G+C) + 2(A +T) -5 (^{o}C). Primers (0.4 µM), $MgCl_2$ (2.5mM), buffer (1x), deoxyribonucleotide triphosphates (dNTPs) (0.2mM), and Taq DNA polymerase (0.025U/ml) were used according to the manufacturer's instructions (Promega, Madison, WI, USA) in a total reaction volume of 25µl. The following conditions were used for OmpT gene amplification: denaturation at 94^{o}C for 2 minutes; denaturation at 94^{o}C for 30 seconds; and annealing Ta at 62^{o}C for a total 30 cycles; with 72^{o}C elongation for 1 minute; 72^{o}C final elongation for 10 minutes and 8^{o}C for final hold until the tubes were removed from the thermal cycler (MJ Research, Ramsey, MN). The PCR product was run on a 1.0% (w/v) agarose gel to confirm the amplification and size of the DNA with respect to the no template DNA control. Similarly, the above PCR method was followed to amplify and charaterize the OmpT gene locus in the three EHEC strains.

2.4d Gel Extraction procedure of PCR product from *E. coli* O86a:K61

The PCR product was gel purified using the EZ- Spin Column Gel Extraction Kit (Biobasic Inc., Markham, ON) as described in the manufacturer's protocol. The gel containing the DNA was incubated at 56^{o}C for 10 minutes and then transferred to a silica membrane containing column. The sample was centrifuged and washed twice with 80% (v/v) ethanol. The DNA was isolated with elution buffer (2.0mM Tris-HCl, pH 8.0) and stored at -20^{o}C.

2.4e Polishing of the PCR Product

The PCR product was polished with a T_4 DNA polymerase (3U/µl) (NEB England Biolabs Ltd., Pickering, ON, Canada) at 12^{o}C for 20 minutes and the enzyme was heat inactivated at 75^{o}C for 15 minutes. The enzyme has 3'-5' exonuclease activity, in order to remove 3' overhangs and to fill-in 5' overhangs to finally create a blunt-ended product for ligation.

2.4f Restriction digestion and de-phosphorylation of vector and ligation of OmpT gene

pUC18 plasmid was digested with a Sma-I according to the manufacturer's instructions (NEB England Biolabs Ltd., Pickering, ON). Briefly, the restriction digestion of pUC18 with Sma-I (0.5U/µl) was carried out at 25^{o}C in a water bath for 4 hours. Thereafter, pUC18 was dephosphorylated with antarctic phosphatase (5 units/µl) (New England Biolabs Ltd., Pickering, ON) at 37^{o}C for 15 minutes and finally the enzyme was heat inactivated at 65^{o}C for 5 minutes. The DNA concentration (OD_{260}) of plasmid (pUC18) and insert (OmpT) was determined using a microplate reader (Molecular Devices, Sunnyvale, CA). The insert (ng) was calculated with respect to vector size (kb) and concentration (ng), based on the equation: Insert (ng) = [Vector (ng) x size of the insert (kb)/size of the vector (kb)]. The ligation was carried out with varying amounts of vector (Sma-I digested pUC18) to insert (PCR product) ratios (1:3, 1:5, 1:10, 1:15)

in a 5x polyethylene glycol ligation buffer (250mM Tris HCl pH 7.6, 50mM MgCl2, 5mM Dithiothreitol (DTT), 25% (w/v) Polyethylene glycol PEG 4000) and T4 DNA ligase incubated for 16 hours at 16^{o}C according to the manufacturer's instructions (NEB England Biolabs Ltd., Pickering, ON). Restriction digestion with HindIII and BamHI was conducted to verify the insertion of the OmpT gene into pUC18 vector.

2.4g Preparation of Competent Cells and Transformations

The competent cells were prepared by the $CaCl_2$ treatment method (Sambrook and Russel, 2001). Briefly, an overnight culture (1%) was subcultured into 50ml LB media with aeration at 37^{o}C in an orbital shaker at 200 rpm (Eppendorf, Happauge, NY) and was grown to an optical density 600nm (OD600nm) of 0.5. The culture was then chilled on ice for 15 min. The cells were pelleted for 10 min at 4660 x g at 4^{o}C. The cells were re-suspended with 50 ml of cold 0.1M $CaCl_2$ and then incubated on ice for 30 min. The cells were centrifuged (Fisher Thermo Scientific, Wilmington, DE) at 4000 rpm for 10 min at 4^{o}C and resuspended into 5ml of 0.1 M $CaCl_2$ in 15% glycerol and stored at -70^{o}C. DNA (0.1-0.01ng) was transformed into 100µl of cells by heat shock to determine the transformation efficiency. Briefly, competent cells stored at -70^{o}C were thawed on ice for 5 min. The cells were heat shocked at 42^{o}C for 45 seconds and re-incubated on ice for 1 min and recovered in 250 µl LB broth. The cells were then incubated with DNA in an orbital shaker at 200 rpm (Eppendorf, Happauge, NY) at 37^{o}C for 1 hr. The cells were plated on LB agar/ampicillin plates and incubated (Fisher Scientific, Ottawa, ON) for 16 hours at 37^{o}C. Transformants were screened based on a blue-white selection method using X-gal and IPTG (Sambrook and Russel, 2001). LacZ gene in pUC18 produces β-galactosidase which converts X-gal (substrate) in the presence of IPTG (activator) to form blue colonies. If the

β-galactosidase gene is interrupted by the insertion of another gene (i.e. OmpT), this would lead to the formation of white colonies.

2.4h Directional Cloning and Sequencing of Insert in Cloning Vector

The DNA in the cloned vector was digested with HindIII and BamHI and directionally cloned into a BamHI/HindIII digested pUC18 vector. The presence of the expected 1kbp insert was confirmed by restriction digestion and DNA sequencing by the dideoxy chain termination method at McGill University (Genome Québec Innovation Centre, Montreal, QC) (Figures 3.1E and 3.2A). The sequence of the OmpT gene is shown in Figure 3.2A. The cloned vector (OmpT construct) and pUC18 vector was transformed into BL21 (DE3) competent cells (OmpT deficient strain). The cloning procedures are shown schematically in Figure 2.1.

2.4i *E. coli* O86a:K61 OmpT Sequence Analysis

The OmpT DNA sequence analysis was performed by the Basic Local Alignment Search Tool/BLAST (National Centre of Biotechnology Information, Bethesda, MD), multiple sequence analysis (EMBL-European Bioinformatics Institute, Cambridge, UK), DNA translate tool (Expasy) and the reverse complement tool (Gene Infinity, San Diego, CA).

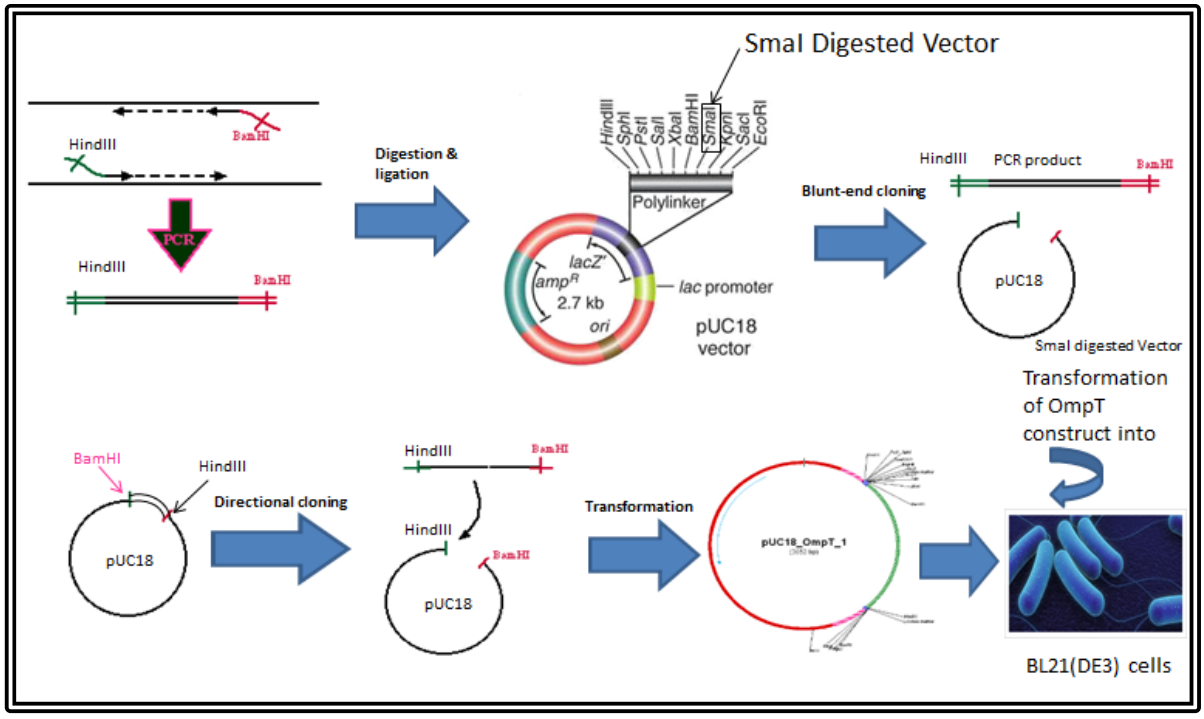
2.5 Preparation of Reagents and Materials

2.5a Preparation of Dialysis Tubing

The cellulose ester dialysis tube (Spectra/Por®, VWR Scientific, PA) was boiled in 1.9mM disodium EDTA and 6.6mM sodium bicarbonate for 5 minutes and then washed with distilled water. Subsequently, the tubing was boiled for 3 x 5 minutes and washed with distilled water. The dialysis tubing was stored in 15% (v/v) ethanol at 4^{o}C.

Figure 2.1 Cloning of OmpT Gene in *E. coli* O86a:61. This figure represents cloning of the OmpT gene from *E. coli* O86a:K61 upon its amplification by PCR, restriction digestion of pUC18 with SmaI, blunt end cloning, directional cloning and finally transformation of the genetic construct into *E. coli* BL21(DE3) cells (OmpT deficient strain).

Figure 2.1


SmaI Digested Vector
HindIII
BamHI
PCR
HindIII
BamHI
Digestion & ligation
HindIII SphI PstI SalI XbaI BamHI SmaI KpnI SacI EcoRI
Polylinker
lacZ'
ampR
2.7 kb
ori
lac promoter
pUC18 vector
Blunt-end cloning
HindIII
PCR product
BamHI
pUC18
SmaI digested Vector
Transformation of OmpT construct into
BamHI
HindIII
pUC18
Directional cloning
HindIII
BamHI
HindIII
BamHI
pUC18
Transformation
pUC18_OmpT_1
BL21(DE3) cells

2.5b Preparation of Prothrombin Time reagent

The thromboplastin/prothrombin time (PT) reagent (Trinity Biotech, Wicklow, Ireland) was reconstituted with 7 ml of HBS (20mM HEPES, 150mM NaCl, pH7.4) buffer per vial. The reagent was dialysed with 12-14 kDa molecular weight cut off (MWCO) dialysis tubing (VWR Scientific, PA) for 12-14 hours with stirring at 4^{o}C. The PT reagent was stored in aliquots at -80^{o}C.

2.5c Preparation of FV- Deficient Plasma

The method for the preparation of FV-deficient human plasma was modified from Bloom *et al.* (Bloom *et al.*, 1979). The blood was donated by healthy volunteer. The blood was collected in syringe containing a 1:9 volume ratio of 3.2% (w/v) sodium citrate to whole blood. The tubes containing the blood were mixed gently and then centrifuged at 4660 x g for 15 min at room temperature. The plasma was isolated and mixed with solid sodium EDTA to achieve a 5mM final concentration and pH of 7.4. The plasma was then incubated at 37^{o}C for 8 hours. The PT coagulation assay was measured to confirm the clot time increased from 12-13 seconds before EDTA addition to 100-130 seconds after EDTA treatment. The FV deficient plasma was dialysed (75mm, 12-14 MWCO; VWR Scientific, PA) against 4L of 20mM HEPES, 150mM NaCl, pH7.5 (HBS) for 12-14 hours at 4^{o}C. The plasma was aliquoted and stored at -80^{o}C until use.

2.6 Preparation of Outer Membrane Vesicles from *E. coli* O157:H7

Wild type EHEC, EHEC Δ*ompT*, and EHEC Δ*ompT*(pEHompT) were grown in 10 ml Minimal media (60mM K_2HPO_4, 33mM KH_2PO_4, 7.5mM ammonium sulphate, 1.7mM trisodium citrate, pH7.0) with 0.2% (w/v) glucose, 0.006 % tryptone, 1mM $MgSO_4$, with/without chloramphenicol

(30µg/ml) with aeration at 37°C in an orbital shaker at 200rpm (Eppendorf, Happauge, NY). The cultures were then subcultured into 1L of fresh minimal media and incubated at 37°C without agitation in an incubator (Fisher Scientific, Ottawa, ON) for 16-24 hours to reach an OD600nm of 0.5. The cultures were centrifuged at 10,000 x g at 4°C. The supernatants were filtered through 0.22 µm vacuum filters (Starstedt, Montreal, QC) to eliminate any remaining bacteria. The pH of the culture supernatants was adjusted to 7.4 and concentrated by approx. 33-fold (1L to 30ml) with a 100kDa molecular weight cutoff (MWCO) membrane (Pall Life Sciences, Ann Arbor, MI) using a tangential flow filtration (TFF) Capsule (Minimate, Pall Life Sciences, Ann Arbor, MI) at room temperature. The supernatants were further concentrated by 3-fold (30ml to 10ml) using 100kDa centrifugal filters (Millipore, Mississauga, ON) at 4660 x g at 4°C for 10mins. Outer membrane vesicles (OMVs) were pelleted by ultra centrifugation at 150,000 x g (Hitachi Koki Co, Hitachinaka, Japan) at 4°C for 1.5h and the pellets were resuspended in 250µl of HBS, pH 7.4. The OMVs were stored in aliquots in tightly sealed cryovials at -80°C and thawed/refrozen samples were not used for study.

2.7 Preparation of Cell Lysates

E. coli cells were grown in 50ml of Minimal media to an OD_{600}nm of 0.50 and centrifuged (Fisher Thermo Scientific, Wilmington, DE) at 10,000 x g for 30 minutes at 4°C. The pellets were resuspended in 5ml of HBS, pH 7.4 and sonicated on ice at setting #7 for 10 mins using a Dismembranator (Fisher Thermo Scientific, Wilmington, DE). Aliquots of the cell lysates were stored in tightly capped cryovials -70°C and thawed/refrozen samples were not used for study.

2.8 Bicinchoninic Acid Protein Assay

The protein concentration of EHEC OMVs and cell lysates were determined using the bicinchoninic acid (BCA) protein assay using bovine serum albumin (BSA, Fisher Thermo Scientific, Nepean, ON) as the standard according to the manufacturer's instructions. The absorbance of standards and samples were read at 562nm using a microplate reader (SpectroMax Plus, Molecular Devices, Sunnyvale, CA) corrected with HBS, pH 7.4 as a control. Standard curves of Absorbance at 562nm vs BSA protein concentration were used to interpolate the protein concentrations of the OMV and cell lysate preparations using Sigma Plot 12.0 (San Jose, CA).

2.9 Effect of OmpT Expression on Bacterial Growth in Liquid Culture

Wild type EHEC, EHEC Δ*ompT*, and EHEC Δ*ompT* (pEHompT) were grown in 5ml of either LB broth or Minimal A Media with/without chloramphenicol (30μg/ml) overnight at 37^{o}C with aeration at 200 rpm in an orbital shaker (Eppendorf, Happauge, NY). The cultures were subcultured with 1% (v/v) of overnight culture inoculated into 50ml of fresh LB broth or minimal media and further incubated at 37^{o}C with aeration at 200rpm. The OD600nm of culture aliquots were measured using a UV/Vis spectrophotometer (Thermoscientific, Wilmington, DE) every hour for 12-15 hours. The OD600nm versus incubation time (in hours) of the *E. coli* strains in different media was plotted using Sigma plot 12.0 (San Jose, CA). Statistical analysis of the growth curves was performed using ANOVA (Sigma Plot 12.0, San Jose, CA) with significance set at $p \leq 0.05$. The same procedure was repeated for BL21(DE3)OmpT, BL21(DE3)pUC18, and Bl21(DE3) cells as a control in both LB and Minimal A media.

2.10 Transmission Electron Microscopy

OMVs were prepared as described above. Copper grids (Glider grids, 200 mesh copper Electron Microscopy Sciences, Hatfield, PA) were loaded with 10µl of the OMV preparations and incubated for 1 minute to allow the grid to adsorb the specimen. Then, excess volume was removed by blotting on filter paper. The copper grids were then floated on a drop of 2% (w/v) uranyl acetate on parafilm for 30 seconds; blotted with filter paper and then air dried for 1 min at room temperature. Specimens were examined with a transmission electron microscope (TEM; FEI Company, model Technai 20, Hillsboro, Oregon; HV = 200.0kV; Mount Sinai Hospital, Toronto, ON) at 100,000 x magnification. The numbers and dimensions of OMV preparations from the three EHEC strains were measured using Magnification software (Orbicule Inc, Belgium). Number of OMVs/TEM field and OMV size distributions of the three EHEC strains were plotted and analyzed for significant differences using Sigma Plot 12.0 (San Jose, CA).

2.11 Digital Microscopy

The three different EHEC strains were stained with the Gram-staining procedure (Mahon *et al.*, 2011). Briefly, cells were smeared on dry and cleaned slides and stained with crystal violet 1% (w/v) for 1minute and gently rinsed with distilled water (0.22µm filtered). The cells were stained with Gram's iodine 1% (w/v) for 1min and excess stain rinsed with distilled water. Specimens were then decolorized with 95% (v/v) ethanol and rinsed with distilled water. The specimens were counter stained with safranin 1% (w/v) for 1-2 min and excess stain removed with distilled water. The cells were observed by Digital Microscopy (DM; Keyence Corporation, Osaka, Japan) at 4000 x magnification and photographed using an attached camera unit (VHX-1100). The cell size (Length and Width) was measured using digital microscope software (VH-M100

XY measurement and VHX-H1M1 measurement) for at least 500 different cells from each of the three EHEC strains (wild type EHEC, ΔompT EHEC, ΔompT pEHompT EHEC). The cell sizes were compared and analysed using Student's t-tests in Sigma Plot 12.0 with significance set at $p \leq 0.05$.

2.12 Determination of Viable *E. coli* Cell Number by the Spread Plate Technique

The three different EHEC strains were grown in 10ml of Minimal A media (60mM K_2HPO_4, 33mM KH_2PO_4, 7.5mM ammonium sulphate, 1.7mM trisodium citrate, pH7.0 with 0.2% (w/v) glucose, 0.006 % (w/v) tryptone, 1mM $MgSO_4$) with/without chloramphenicol (30µg/ml). The cultures were subcultured into 50ml of fresh Minimal A media with 1 % overnight culture volume and OD600nm was measured at different times by spectrophotometer (Thermo Scientific, Wilmington, DE) for specific ODs (0.1, 0.2, 0.3, etc.). The culture at specific OD600nm was diluted 10-fold serially in Minimal A media and the cells were plated on LB/agar plates with/without chloramphenicol in duplicate and incubated at overnight 37^{o}C. The cfu/ml was calculated based on the equation: cfu/ml = # of colonies/(dilution factor x volume of sample plated). The plates having 20-200 cells were counted and cfu/ml calculated for each the strain at specific OD600 values and the procedure is described schematically in Figure 2.2. The growth rate constant and generation time was calculated for three different EHEC strains according to Todar K, 2008.

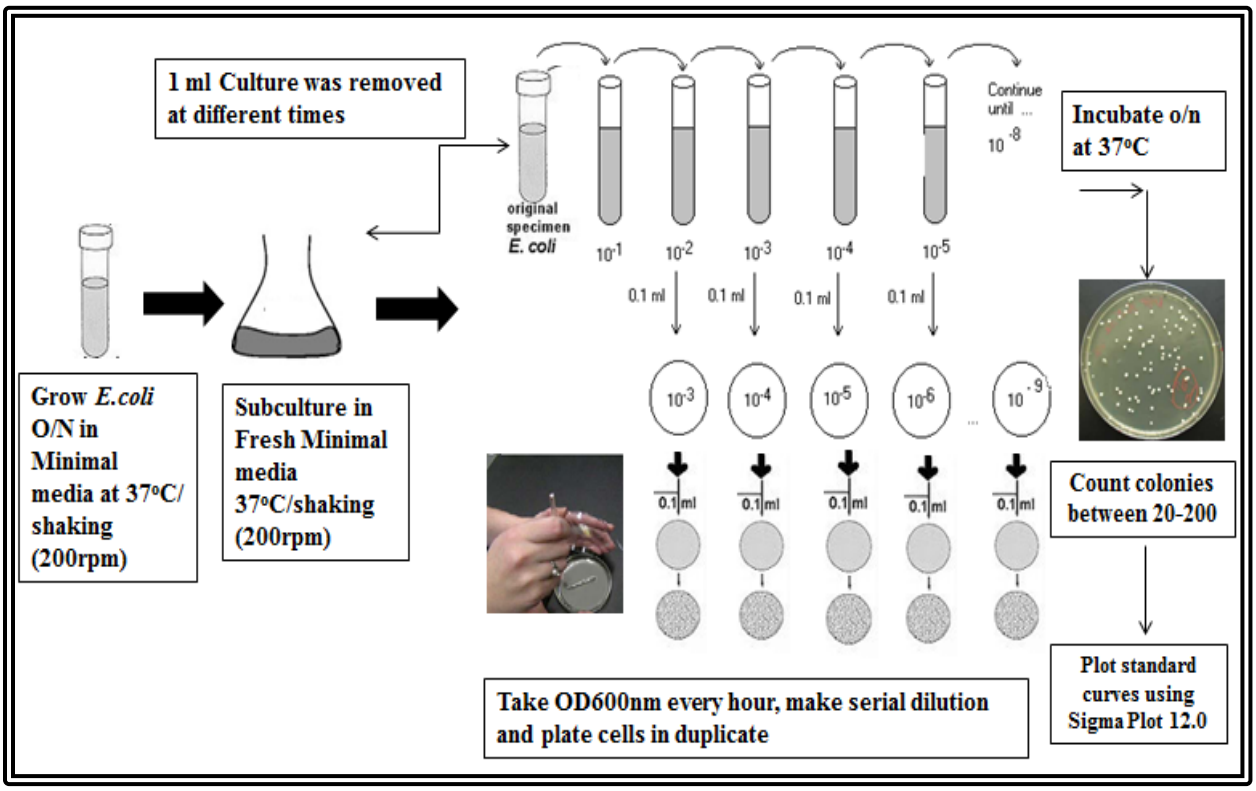
2.13 Activated Partial Thromboplastin Time Coagulation Assay

Citrated normal human plasma (NHP; 30µl; Precision Biologicals, Halifax, NS) (30µl) was incubated with 30µl of OMVs (0.25µg, 0.5µg, 1.0µg and 2.0 µg protein) or with 30µl whole *E. coli* cells (10^7, 10^8, 10^9, and 10^{10} cells) or HBS, pH 7.4 as a control at room temperature for 30

Figure 2.2 Spread plate technique to determine the cell concentration in cfu/ml of EHEC.

The spread plate technique was performed to determine the cfu/ml of the three EHEC strains. The cells were cultuered in Minimal A media 12-14 hours at 37^{o}C with aeration at 200 rpm. The culture was then subcultured into the fresh Minimal A media and absorbance at 405 (nm) was recorded at regular intervals (0.1, 0.2, 0.3, etc.). The culture was serially diluted by 10-fold and aliquots were duplicated and incubated 12-14 hours at 37^{o}C. The plates having 20 to 200 colonies were counted and the cfu/ml was determined as # of colonies/(dilution factor x volume of sample plated). The standard curves of cfu/ml vs. OD600nm for the different strains were plotted using Sigma Plot 12.0.

Figure 2.2


1 ml Culture was removed at different times
Grow E.coli O/N in Minimal media at 37°C/ shaking (200rpm)
Subculture in Fresh Minimal media 37°C/shaking (200rpm)
original specimen E. coli
Continue until ... 10 -8
0.1 ml
Incubate o/n at 37°C
Count colonies between 20-200
Plot standard curves using Sigma Plot 12.0
Take OD600nm every hour, make serial dilution and plate cells in duplicate

minutes or 1hour. The activated partial thromboplastin time (aPTT) assay was performed in microplate reader (SpectraMax Plus, Molecular Device, Sunnyvale, CA). Fibrin clot formation was initiated with 30 µl aPTT reagent (bioMerieux, Durham, NC). The samples were mixed for 5 seconds and incubated in the plate reader for 5 minutes and 30µl of 25mM $CaCl_2$ was added to initiate fibrin formation. The samples were shaken for 5 seconds and the absorbance at 405nm was measured every 5 seconds for 6 minutes at absorbance 405nm in a microplate reader (SpectraMax Plus, Molecular Devices, Sunnyvale, CA). The clot time was determined as the time after $CaCl_2$ addition to reach the half maximal increase in absorbance at 405nm achieved during the fibrin clot formation event.

2.14 Prothrombin Time Assay

Citrated normal human plasma (NHP; Precision Biologicals, Halifax, NS) (30µl) was incubated with 30µl OMVs (0.25µg, 0.5µg, 1.0µg and 2.0 µg protein) or with 30µl whole *E. coli* cells (10^7,10^8,10^9, and 10^{10} cells) or HBS, pH 7.4 as a control at room temperature for 30 minutes or 1 hour. The prothrombin time (PT) assay was performed in a microplate reader (SpectraMax Plus, Molecular Devices, Sunnyvale, CA). Fibrin clot formation was initiated with 60µl each of thromboplastin reagent (Trinity Biotech, Wicklow, Ireland) and 25mM $CaCl_2$. The samples were shaken for 2 seconds and the absorbance at 405nm was measured every 5 seconds for 6 minutes with a microplate reader (SpectraMax Plus, Molecular Devices, Sunnyvale, CA). The clot time was determined as the time after $CaCl_2$ addition to reach the half maximal increase in absorbance at 405nm achieved during the fibrin clot formation event.

2.15 FV Coagulation Assay with OMVs and Lysates

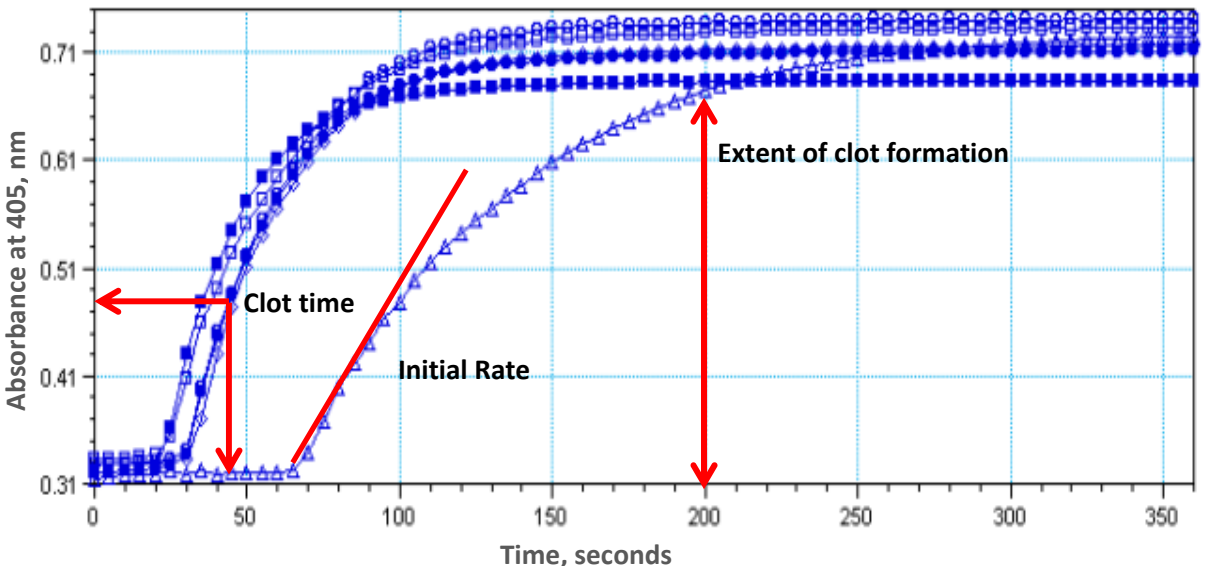
Citrated normal human plasma (NHP, Precision Biologicals, Halifax, NS) was diluted 5x-fold in HBS, pH 7.4 and incubated with OMVs (1μg) or HBS, pH 7.4 as a control at room temperature for 30 minutes. For cell lysates, the cells were grown to an OD_{600} of 0.5. NHP was finally diluted 20-fold and analysed with the FV assay. The FV assay was performed in a microplate reader (SpectraMax Plus, Molecular Devices, Sunnyvale, CA) as described by Tilley *et al.* (2012). Briefly, FV deficient plasma (50μl) was mixed with treated NHP samples and clot formation was initiated with thromboplastin/prothrombin time (PT) reagent (50μl). The samples were mixed for 10 seconds and 50μl of 25 mM $CaCl_2$ was added to initiate fibrin formation. The absorbance was read at 405nm every 6 seconds for 6 minutes in a microplate reader (SpectraMax Plus, Molecular Devices, Sunnyvale, CA). The clot time was defined as the time after $CaCl_2$ addition to reach the half maximal increase in absorbance at 405nm. The three important parameters can be determined from the graph and compared between the samples as shown in Figure 2.3. The clot time was defined as the time to reach the half maximal absorbance increases after $CaCl_2$ addition. The initial rate of fibrin clot formation (mUnits/minute) was defined as the rate of increase of absorbance at 405nm during the initial 5-6 time points of fibrin clot formation. The extent of clot formation was defined as the difference between the maximal and minimal absorbance at 405nm during fibrin clot formation.

2.16 FV Coagulation Assay with whole EHEC cells

Wild type EHEC, EHEC ΔompT, and EHEC ΔompT (pEHompT) cells were grown in 5ml of Minimal A media overnight at 37^{o}C with aeration at 200rpm. The cells were subcultured into 50 ml fresh Minimal media and grown to an OD 600nm of 0.5. The cells were centrifuged at

Figure 2.3 Fibrin Clot formation in normal human plasma using the kinetic microplate FV activity assay. The FV activity assay was performed by measuring the change of absorbance at 405 nm over time in the kinetic FV microplate assay (Tilley *et al.*, 2011). The graph represents the clot formation event using 20-fold diluted NHP. The x-axis represents the time for clot formation (in minutes) and the y-axis represents the change in absorbance at 405 nm. The first two red arrows represent the half maximum increase in absorbance (24.26sec) at 405nm. The second line in red represents the initial rate of clot formation for the first five time points (535.4 milliUnits/minute with r^2 of 0.969). The third line with two sided arrows in red represents the extent of clot formation or the difference between the maximal and minimal absorbance at 405nm during fibrin clot formation.

Figure 2.3


Absorbance at 405, nm
0.71
0.61
0.51
0.41
0.31
0
50
100
150
200
250
300
350
Time, seconds
Clot time
Initial Rate
Extent of clot formation

10,000 x g at 4°C for 30 minutes. The cell pellets were resuspended in HBS, pH 7.4 in different volumes to give 10^{10} cells/ml which was further diluted 10-fold to 10^9 cells/ml. Normal Human Plasma (NHP, 30 µl of 5-fold diluted; Precision Biological, Halifax, NS) in HBS, pH 7.4, was treated with 10^9cells/ml at room temperature for 1 hour. The samples were diluted in HBS, pH 7.4 20-fold with respect to plasma and cells were pelleted in a microcentrifuge (Thermo Electron Corporation, Waltham, MA) at 17,000 x g for 5 minutes at 4°C. The supernatants were then assayed in the FV coagulation microplate assay as described above.

2.17 Effect of cellular and OMVs associated OmpT on other coagulation and fibrinolytic factor activities in normal human plasma

The standard curves of FI, FII, and FX were generated in the same manner as the FV standard curves as mentioned earlier. Different dilutions of NHP (0-fold to 1024-fold) in HBS, pH7.4 were assayed with factor deficient FI (Affinity Biologicals, Ancaster, ON), or FII or FX deficient (Geroge King Biomedical, Overland Park, KS, USA) human plasma by monitoring the change in absorbance at 405nm for 6 minutes for every 5 seconds. In order to perform the coagulation factor activity assays, 30 µl of 5-fold diluted Normal Human Plasma (NHP, Precision Biological, Halifax, NS) in HBS, pH 7.4, was treated with 10^9cells/ml for 1 hour or 1µg protein from the OMVs from the three EHEC strains for 30 minutes at room temperature. The samples were then diluted 20-fold with respect to plasma except for the Fibrinogen (FI) assay which was diluted 2-fold with repect to plasma and the cells were pelleted before performing activity assays as described above. Factor deficient (fibrinogen, FII, and FX) plasma (50µl) was mixed with the samples and then clot formation was initiated with thromboplastin/prothrombin time (PT) reagent (50µl). The samples were mixed for 10 seconds and 50µl of 25 mM $CaCl_2$ was added to initiate fibrin formation. The absorbance was read at 405nm every 6 seconds for 6 minutes in a

microplate reader (SpectraMax Plus, Molecular Device, Sunnyvale, CA). The clot time was defined as the time after $CaCl_2$ addition to reach the half maximal increase in absorbance at 405nm. In the case of the FV standard curve and activity assay, plasminogen (PLG)-deficient plasma (Affinity Biological, Ancaster, ON) was used instead of NHP and assayed using FV-deficient plasma in the FV activity assay as described above.

2.18 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

EHEC OMVs (75-80 ng) were added to an equal volume of 2x Loading Dye (2xLD: 0.025% bromophenol blue, 12.5% glycerol, 2% SDS, 5% mercaptoethanol, 10mM HEPES, pH 7.4, 75mM NaCl). Cells were normalised to an OD_{600} of 0.5 in Minimal A media and the cell pellets from 1 ml culture aliquots were dissolved in 100µl of 2xLD. All samples were heated at 95^{o}C for 5 minutes and loaded in 4-20% polyacrylamide Criterion gradient gels (BioRad, Mississauga, ON) along with 5-20 µl of prestained molecular weight markers. The gels were electrophoresed in 25mM Tris, 192mM glycine, 0.1% SDS, pH-8.3 at constant voltage (150V) for 1.5 hours at room temperature. The gels were either stained with Coomassie Brilliant Blue or silver.

2.19 Coomassie Brilliant Blue Staining

After electrophoresis, the gels were submerged into Coomassie Brilliant Blue stain (0.0016% (w/v) Coomassie brilliant blue, 5% (v/v) glacial Acetic acid, 7.5% (v/v) ethanol) for approximately 16 hours at room temperature with agitation. Thereafter, the gels were destained in 15% (v/v) methanol, and 3% (v/v) glycerol for at least 1 hour at room temperature with agitation and then stored at 4^{o}C until photography.

2.20 Silver Staining

All the solutions were made prior to carrying out the staining procedure. The procedure was a modification of the method of Merril *et al.*, 1981. The gel was gently removed and submerged into 200 ml prefix solution (50% methanol, 10% ethanol, 40% water). The gel was then microwaved for 1.5 minutes at 50% power. While heating, the gel was shaken for 30 seconds for every 1.5 minutes. The prefix solution was decanted and replaced with 200 ml of water, then heated in a microwave for 2 minutes at 50% power with frequent shaking. The gel was then incubated in water with agitation for 2 minutes at room temperature. The water was decanted, replaced with 200 ml of 100µM DTT, and heated for 2 minutes at 50% power with frequent shaking. The gel was incubated with the DTT solution with agitation for 2 minutes at room temperature. The DTT solution was discarded and replaced with 0.1% (w/v) silver nitrate in water and heated for 1.5 minutes at 50% power with frequent shaking. The gel was washed twice with 200 ml distilled water. The water was decanted and replaced with a 200 ml developer solution (3% (w/v) sodium carbonate, 0.05% (v/v) formaldehyde) and agitated until all the protein bands were clearly visible. The reaction was stopped with 2.3M citric acid for 1 minute. The gel was again washed with distilled water and stored in 0.03% (w/v) carbonate at 4^{o}C until photography.

2.21 Western Blotting

2.21a) Western Blotting for FV in Human Plasma

For this assay NHP (30µl; Precision Biologicals, Halifax, NS) was treated with 1 µg of OMV protein or 10^9 cells/ml from wild type EHEC, EHEC ΔompT, and EHEC ΔompT (pEHompT) and incubated for various times (0 min, 20 min, 1 hour, 1.5 hours, 2 hours) at room temperature.

At different times, 60 µl of sample was removed and diluted 2x-fold further with HBS, pH 7.4. For whole cells, the cells were centrifuged at 17,000 x g for 5 minutes at 4^{o}C and the supernatant fractions recovered. Samples of NHP treated with OMVs or cells (120µl) were concurrently analyzed with the FV microplate coagulation assay (50µl; See above) and Western blotting (50µl). For Western blotting, the samples (50µl) were diluted 6x-fold further in 2x Loading dye (2xLD: 0.025% bromophenol blue, 12.5% glycerol, 2% SDS, 5% mercaptoethanol, 10mM HEPES, 75mMNaCl) and heated at 95^{o}C for 5 minutes. For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 40µl of samples (0.33µl of NHP) were electrophoresed in 4-20% polyacrylamide gradient Criterion gels (BioRad, Mississauga, ON) along with 10-20µl prestained molecular weight markers (BioRad, Mississauga, ON) in 25mM Tris, 192mM glycine, 0.1% SDS, pH-8.3 at constant voltage (150V) for 1.5 hours at room temperature. The gel was gently removed and electroblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Etobicoke, ON) at 35 V (Constant voltage) in 25mM Tris, 192mM glycine, 0.05% SDS, 10% (v/v)methanol, pH 8.3 with gentle stirring overnight for 16 hours at 4^{o}C. The membrane was blocked with phosphate buffered saline (PBS, 14mM NaCl, 1.5mM KH_2PO_4, 10mM $NaHPO_4$, 2.5mM KCl, pH 7.4) containing 2% (w/v) BSA for 1 hour with agitation at room temperature. The membrane was washed 4x in PBS-Tween (14mM NaCl, 1.5mM KH_2PO_4, 10mM $NaHPO_4$, 2.5mM KCl, 0.1% (v/v) Tween-20, pH 7.4) for 5 minutes each with agitation at room temperature. The membrane was incubated with sheep anti-human FactorV-IgG (30,000-fold dilution; Haematologic Technologies, Burlington, VT) in 5% (w/v) non-fat milk powder in PBS-Tween for 1 hour with shaking at room temperature. The membrane was washed 4x in PBS-Tween at room temperature for 5 minutes each with gentle shaking. The membrane was then incubated with donkey anti-sheep-IgG conjugated with horseradish

peroxidase (21,000-fold diluted; Jackson Immunochemicals, West Grove, PA) in 5% (w/v) non-fat milk powder in PBS-Tween for 1 hour with agitation at room temperature. The membrane was washed 4x in PBS-Tween for 5 minutes each at room temperature and once with distilled water for 5 minutes at room temperature. The membrane was then exposed to 10ml each of enhanced chemiluminescence (ECL) solutions 1 and 2 (Perkin Elmer, Waltham, MA) reagents for 1 minute at room temperature. The membrane was wrapped in Saran wrap and exposed to XOMAT film (Kodak, Toronto, ON) with an intensifying screen for various times (30seconds, 1 minute, 2 minutes or 5 minutes) at room temperature. The film was developed in an automated developer (Kodak X-OMAT 1000A, Toronto, ON) and the image scanned with a Hewlett-Packard Scanjet 4750c scanner (Mississauga, ON).

2.21b) Western Blotting with Cro-P Antibody

The Western blotting procedure for cell lysates or OMVs from wild type EHEC, EHEC ΔompT, and EHEC ΔompT (pEHompT) using the rabbit anti-Cro-P antibody (74% identical with OmpT at amino acid level) was performed as described by Thomassin *et al.* (2012). For preparing whole cell lysates, the cell numbers were normalised to OD_{600}- 0.5. Briefly, cell cultures (1ml in Minimal A media with an OD600 of 0.50) were centrifuged for 5 mins at 13,000 x g at room temperature and the cell pellets resuspended in 100µl of 2x Loading dye (2xLD; 62.5mM Tris pH6.8, 12.5% glycerol, 2% SDS, 0.025 % bromophenol blue, 2% mercaptoethanol). OMVs were resuspended into equal volumes of 2x LD. Both sets of samples were heated at 95^{o}C for 5 minutes and electrophoresed (0.0036-0.18ug of OMV protein in 20µl sample or 1.0µg protein in 7.5µl for the cell lysates) in 10% polyacrylamide gels under denaturing conditions (Sambrook and Russell, 2001) along with 10-20µl of prestained molecular weight standards (BioRad,

Mississauga, ON) in 25mM Tris, 192mM glycine, 0.1% SDS, pH-8.3 for 40 minutes at 80V and 90 minutes at 100V. The gel was gently removed and electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Etobicoke, ON) at 35 V (Constant voltage) in 25mM Tris, 192mM glycine, 0.05% SDS, 10% methanol, pH 8.3 with gentle stirring for 16 hours at 4^{o}C. The membrane was blocked with Tris-Buffered Saline-Tween (TBS-Tween; 20mM Tris, 500mM NaCl, 0.025% (v/v) Tween-20, pH 7.4) containing 5% non-fat dry milk powder for 1 hour at room temperature with shaking. The blot was washed 3x in TBS-Tween with gentle shaking for 5 minutes each at room temperature. The blot was incubated overnight at 4^{o}C with a rabbit polyclonal anti-Cro-P antibody (1:10,000 dilution, McGill University, Montreal, QC) in TBS-Tween containing 5% (w/v) non-fat dry milk. The blot was washed with 3x in TBS-Tween for 5 minutes each at room temperature and transferred to goat anti-rabbit IgG conjugated with horseradish peroxidase (1:5,000 dilution, Sigma-Aldrich, St Louis, MO) in TBS-Tween containing 5% non-fat dry milk and incubated for 1 hour at room temperature with gentle shaking. The blot was washed 3x in TBS-Tween, 1x with distilled water for 5 minutes each at room temperature and then exposed to 10ml each of ECL reagents 1 and 2 (Perkin Elmer, Waltham, MA) for 1 minute at room temperature. The membrane was wrapped in Saran wrap and exposed to a XOMAT film (Kodak, Toronto, ON) with an intensifying screen for 30 seconds at room temperature. The film was developed in an automated developer (Kodak X-OMAT 1000A, Toronto, ON) and an image scanned into a Hewlett-Packard Scanjet 4750c scanner (Mississauga, ON). The density of the individual protein species was determined by densitometry using Corel Photo Paint Software (Ottawa, ON).

2.22 Statistical Analysis

All the statistical analysis was performed by SigmaPlot Version 12.0 (San Jose, CA). The analysis was performed by one-way analysis of variance (ANOVA; Kruskal-Wallis test; Holm-Sidak test; Tukey's test) and the Student's t-test. Statistical significance was set as $p \leq 0.05$.

Chapter 3

Results Section (A&B)

Results Section A

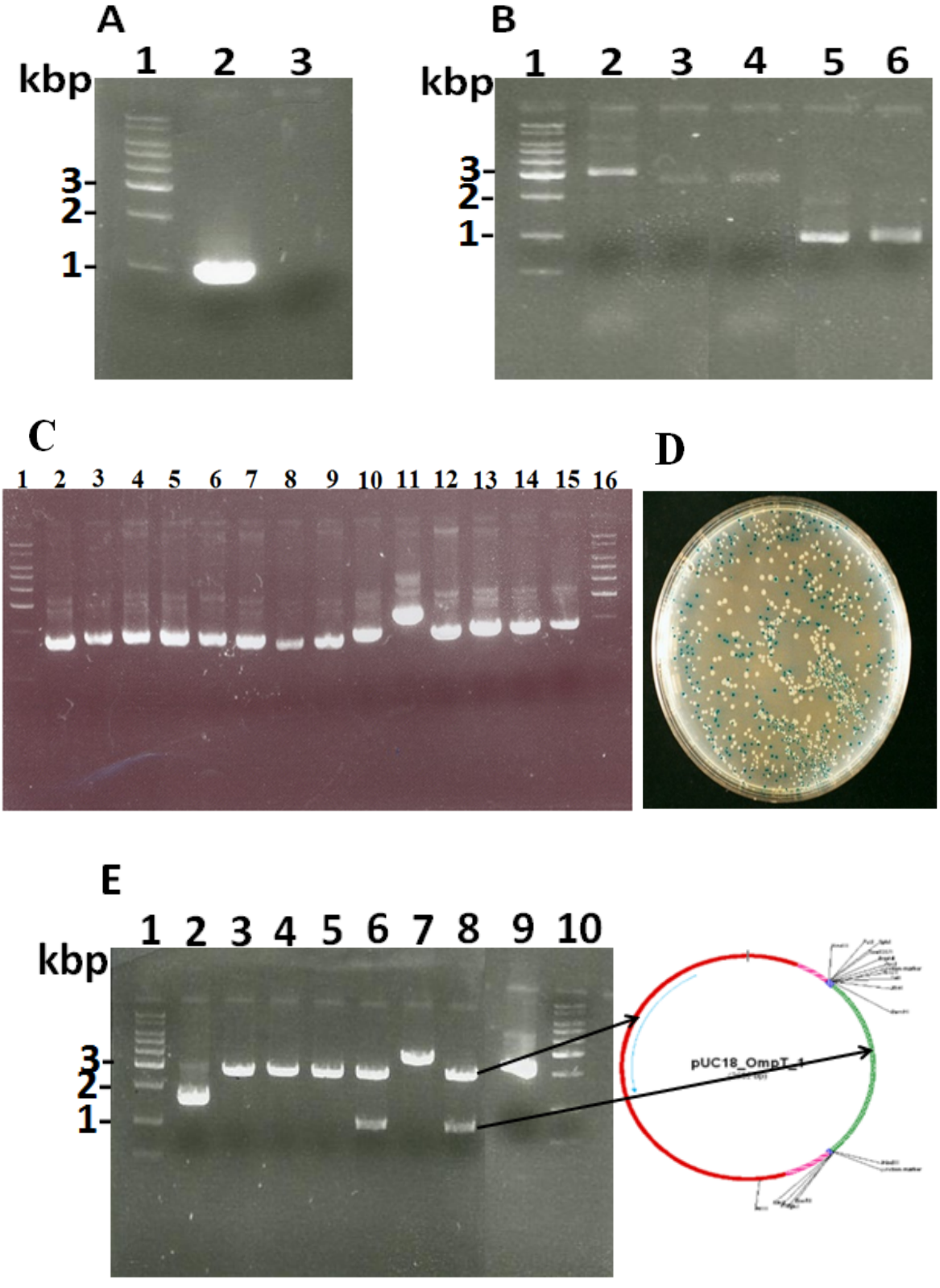
3.1 Cloning and characterization of OmpT gene from *E. coli* O86a:K61

The OmpT protein from *E. coli* O86a:K61 was shown to cleave and inactivate FV in human plasma to attenuate blood coagulation as a part of innate immune response (Tilley D, M.Sc. thesis, 2011). In order to further characterize OmpT from this strain of *E. coli*, the OmpT gene was cloned.

The nucleotide sequence of *E. coli* O86a:K61 is not presently known. Hence, the forward and reverse primers were designed based on a consensus sequence of five different *E. coli* strains (*E. coli* O157:H7, *E. coli* O55:H7, *E. coli* O103:H2, *E. coli* K-12, and *E. coli* O127:H6). The OmpT gene was amplified using genomic DNA from *E. coli* O86a:K61 as a template and no DNA template was used as a control by the polymerase chain reaction (PCR) as described in Figure 3.1A. For the cloning process, pUC18 was isolated and digested with SmaI (Figure 3.1 B) for blunt end cloning. The OmpT gene was polished with T_4 DNA polymerase (Figure 3.1B) to remove or fill up overhangs and pUC18 was dephosphorylated for efficient blunt cloning (Figure 3.1B). The OmpT gene was ligated into the pUC18 vector with various vector:insert ratios as shown in Figure 3.1C. The OmpT gene was then cloned into the pUC18 vector and transformed into *E. coli* DH5α cells. The cloned vector, having the OmpT gene, was selected based on blue white screening as shown in Figure 3.1D (Sambrook & Russel, 2001). The recombinant vector was digested with HindIII and BamHI to confirm the insertion of the OmpT gene into the pUC18 vector (Figure 3.1E). Further, the recombinant vector was digested with HindIII and BamHI for directional cloning into the HindIII and the BamHI digested pUC18 vector and finally transformed into *E. coli* BL21 (DE3).

Figure 3.1 Cloning of OmpT gene from *E. coli* O86a:K61. The OmpT gene was amplified using genomic DNA from *E. coli* O86a:K61 as a template using PCR. Lane 1 was the DNA marker (0.5µg/µl, New England Biolabs Ltd., Pickering, ON); lane 2 had the OmpT gene amplified from genomic DNA and lane 3 contained the no template control (Panel A). pUC18 (undigested in lane 2) was digested with Sma-I (lane 3), and dephosphorylated with Antarctic phosphatase (lane 4) as shown in Panel B. The OmpT gene product was polished with T4 DNA polymerase (Panel B) is shown in lane 6, and unpolished product shown in lane 5 (Panel B). The vector (pUC18) and insert (OmpT gene) was ligated using different ratios: 1:3 (lane 2, 3, 4, 5, 6, 7), 1:5 (lanes 8, 9), 1:10 (lanes 10, 11), 1:20 (lanes 12, 13) and 1:50 (lanes 14, 15) with a DNA marker lane in the outer wells (lanes 1 and 16) as shown in Panel C. The OmpT gene was cloned into the pUC18 vector and selected based on the blue white screening (Panel D).The OmpT construct was isolated from the white colonies and pUC18 from the blue colonies and then sequentially digested with BamHI and HindIII to confirm the insertion of the OmpT gene (Panel E). pUC18 (lane 2) was digested with HindIII (lane3), BamHI (lane 4), and HindIII & BamHI (lane 5). The OmpT construct (lane 9) was digested with HindIII (lane 6), BamHI (lane 7), and HindIII & BamHI (lane 8) with DNA marker added to the outer two wells (lanes 1, and 10).

Figure 3.1


A
kbp
1 2 3
3
2
1
B
kbp
1 2 3 4 5 6
3
2
1
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
D
E
1 2 3 4 5 6 7 8 9 10
kbp
3
2
1
pUC18_OmpT_1

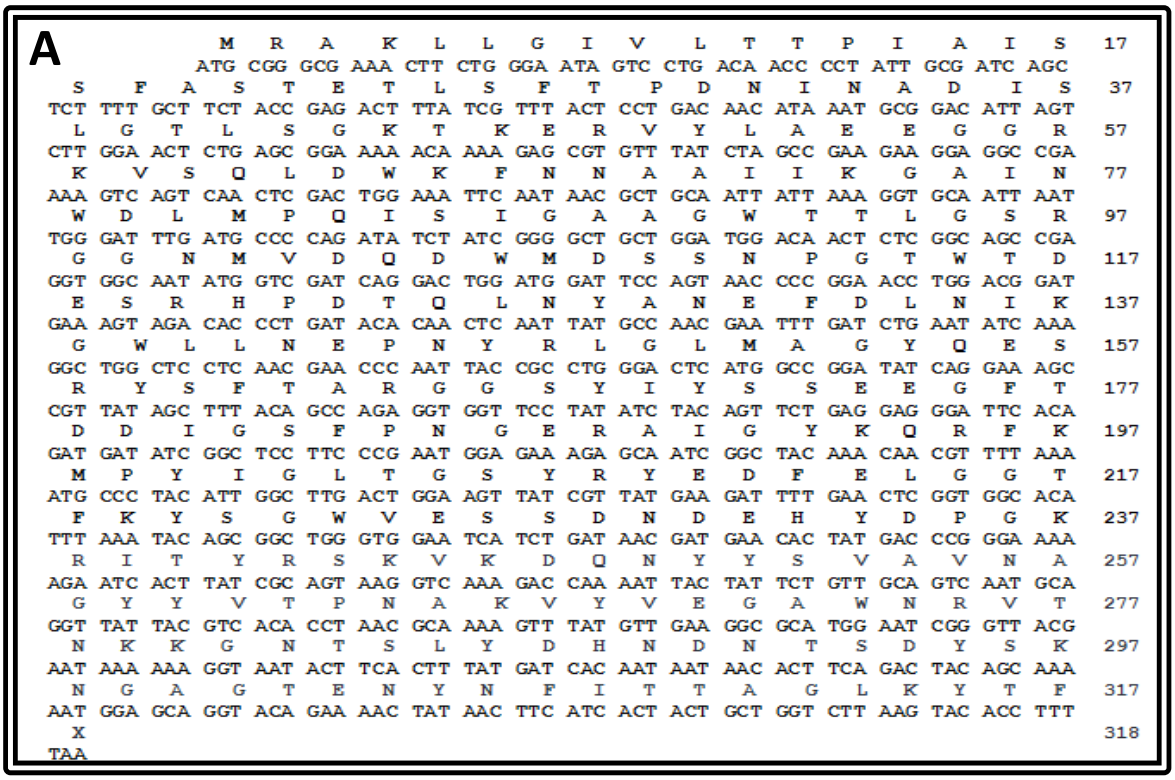
The PCR amplification and insertion of the OmpT gene into the pUC18 vector was confirmed by two methods: restriction digestion and sequencing by the dideoxy chain termination, as shown in Figure 3.1E and 3.2A, respectively. There are also LPS binding sites at Arg 175, Arg 138, Lys 226, Glu 136 and Tyr 134 (Kramer *et al.*, 2001) as shown in Figure 3.2B. The gene encoding the active sites and LPS binding sites were compared to genes from different *E. coli* strains which displayed no significant differences as verified by multiple comparison analyses, at both the nucleotide and amino acids levels. Hence, the active and LPS binding sites of the OmpT gene from *E. coli* O86a:K61 are similar to the OmpT gene from different *E. coli* strains. OmpT cleaves its substrate at a highly specific P1 position at arginine/lysine and less stringently at P1' positions having arginine/ lysine/ valine/ glycine/alanine (McCarter *et al.* 2004). OmpT is a vase-shaped β-barrel structure, 70Å long as shown in Figure 1.4B. OmpT is embedded in the membrane along with lipopolysaccharide (LPS) which permits the proper orientation of the proteolytic active sites (Asp 83 & Asp 85, His 212 &Asp 210) allowing them to extend facing the extracellular environment as shown in Figure 3.2B. Based on cleavage specificity between dibasic residues, there are 12 different potential OmpT cleavage sites in human FV.

3.2 OmpT Sequence Analysis from *E. coli* O86a:K61

The sequence analysis was performed using the Basic Local Alignment Search Tool (BLAST; National Centre of Biotechnology Information, Bethesda, MD). The OmpT gene of *E. coli* O86a:K61 was compared against gene sequence from different *E. coli* strains at the nucleotide and protein levels, and the result of this comparison is described below in Table 3.1. The OmpT gene from *E. coli* O86a:K61 was compared to the same locus in different strains for conservative and non-conservative changes. There were no conservative and non-conservative changes found

Figure 3.2 OmpT nucleotide and amino acid sequence in *E. coli* O86a:K61.The OmpT gene was sequenced by the dideoxy chain termination method at McGill University, QC (Panel A). The gene has an initiation codon ATG and a termination codon TAA. As shown in Panel B, the first 10 amino acids form the signal sequence (not underlined) and the remaining is mature amino acid sequence (underlined) that result in a total of 318 amino acids (Panel B). The active sites are a catalytic dyad, Asp83-Asp85 and His 212-Asp210, highlighted in turquoise blue, (Panel B). The five amino acids that form the LPS binding sites are shown in grey boxes as LPS. The L1-L5 extracellular folds are highlighted in pink and the T1-T4 periplasmic folds are highlighted in yellow. The active sites, LPS binding sites, and extracellular and periplasmic folds (Panel B) were adapted from Kramer, Vandeputte-Rutten, Roon, Dekker, Egmond, & Gros, (2001). Identification of essential acidic residues of outer membrane protease OmpT supports a novel active site. *FEBS Letters*. 505(3), 426-430.

Figure 3.2


A
M R A K L L G I V L T T P I A I S 17
ATG CGG GCG AAA CTT CTG GGA ATA GTC CTG ACA ACC CCT ATT GCG ATC AGC
S F A S T E T L S F T P D N I N A D I S 37
TCT TTT GCT TCT ACC GAG ACT TTA TCG TTT ACT CCT GAC AAC ATA AAT GCG GAC ATT AGT
L G T L S G K T K E R V Y L A E E G G R 57
CTT GGA ACT CTG AGC GGA AAA ACA AAA GAG CGT GTT TAT CTA GCC GAA GAA GGA GGC CGA
K V S Q L D W K F N N A A I I K G A I N 77
AAA GTC AGT CAA CTC GAC TGG AAA TTC AAT AAC GCT GCA ATT ATT AAA GGT GCA ATT AAT
W D L M P Q I S I G A A G W T T L G S R 97
TGG GAT TTG ATG CCC CAG ATA TCT ATC GGG GCT GCT GGA TGG ACA ACT CTC GGC AGC CGA
G G N M V D Q D W M D S S N P G T W T D 117
GGT GGC AAT ATG GTC GAT CAG GAC TGG ATG GAT TCC AGT AAC CCC GGA ACC TGG ACG GAT
E S R H P D T Q L N Y A N E F D L N I K 137
GAA AGT AGA CAC CCT GAT ACA CAA CTC AAT TAT GCC AAC GAA TTT GAT CTG AAT ATC AAA
G W L L N E P N Y R L G L M A G Y Q E S 157
GGC TGG CTC CTC AAC GAA CCC AAT TAC CGC CTG GGA CTC ATG GCC GGA TAT CAG GAA AGC
R Y S F T A R G G S Y I Y S S E E G F T 177
CGT TAT AGC TTT ACA GCC AGA GGT GGT TCC TAT ATC TAC AGT TCT GAG GAG GGA TTC ACA
D D I G S F P N G E R A I G Y K Q R F K 197
GAT GAT ATC GGC TCC TTC CCG AAT GGA GAA AGA GCA ATC GGC TAC AAA CAA CGT TTT AAA
M P Y I G L T G S Y R Y E D F E L G G T 217
ATG CCC TAC ATT GGC TTG ACT GGA AGT TAT CGT TAT GAA GAT TTT GAA CTC GGT GGC ACA
F K Y S G W V E S S D N D E H Y D P G K 237
TTT AAA TAC AGC GGC TGG GTG GAA TCA TCT GAT AAC GAT GAA CAC TAT GAC CCG GGA AAA
R I T Y R S K V K D Q N Y Y S V A V N A 257
AGA ATC ACT TAT CGC AGT AAG GTC AAA GAC CAA AAT TAC TAT TCT GTT GCA GTC AAT GCA
G Y Y V T P N A K V Y V E G A W N R V T 277
GGT TAT TAC GTC ACA CCT AAC GCA AAA GTT TAT GTT GAA GGC GCA TGG AAT CGG GTT ACG
N K K G N T S L Y D H N D N T S D Y S K 297
AAT AAA AAA GGT AAT ACT TCA CTT TAT GAT CAC AAT AAT AAC ACT TCA GAC TAC AGC AAA
N G A G T E N Y N F I T T A G L K Y T F 317
AAT GGA GCA GGT ACA GAA AAC TAT AAC TTC ATC ACT ACT GCT GGT CTT AAG TAC ACC TTT
X 318
TAA
B
Catalytic Site,10 β-strands and L1-L5 folds:
L1
L2
L3
L4
L5
LPS
LPS
LPS
LPS
Asp83-Asp85
His212-Asp210

in the OmpT DNA sequence of *E. coli* O86a:K61compared to the other *E. coli* strains in both LPS binding sites and the active sites.

Table: 3.1 Sequence analysis of OmpT from *E. coli* O86a:K61 with other bacterial strains

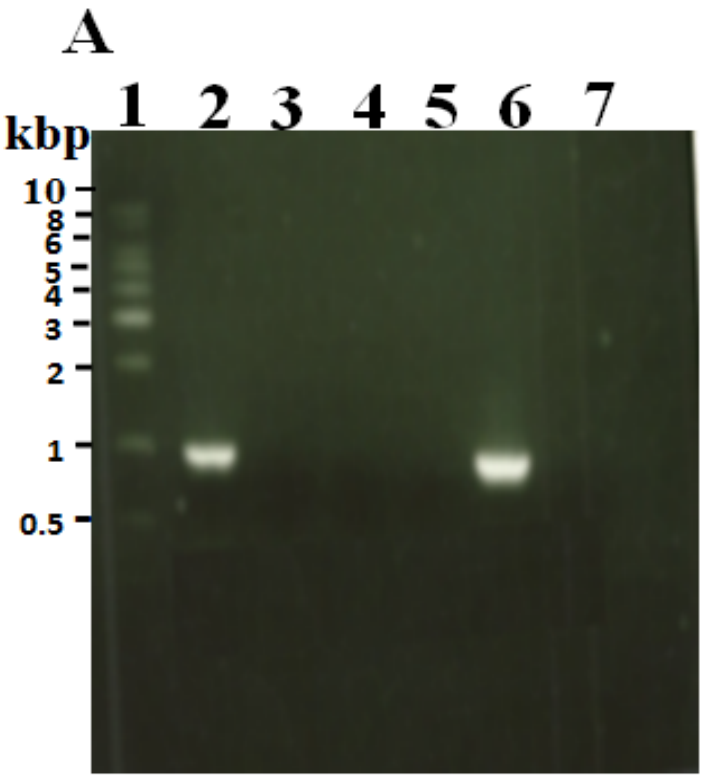
Bacterial strains and its protease	Nucleotide %	Amino acid %
E. coli K-12 (OmpT)	99%	99%
E. coli O157:H7(OmpT)	98%	98%
E. coli O55:H7 (OmpT)	99%	99%
E. coli O127:H6 (OmpT)	98%	98%
E. coli O103:H2 (OmpT)	99%	99%
E. coli S88 (OmpT)	98%	95%
E. coli DEC 15A(OmpP)	98%	95%
E. coli O83:H1 (Outer membrane protease)	98%	98%
E. coli W26/ E. coli TA271 (OmpP)	70%	70%
E. coli STEC 031(Coagulase or fibrinolysis)	99%	99%
E. coli APEC 01 (Outer membrane protease)	90%	90%
E. coli UMNO6 (Protease 7)/E. coli TA280 (Protease A)	99%	99%
Salmonella enterica (PgtE)	75%	75%
Shigella flexneri (SopA protein)	57%	56%
Yersinia pestis (Plasminogen Activator-Pla)	48%	48%

3.3 Characterization of OmpT genetic locus in EHEC strains

The OmpT gene was amplified using genomic DNA from wild type and ΔOmpT, and plasmid DNA from pEH OmpT using PCR. The 1 kbp product of the OmpT gene was present in the wild type EHEC and pEHOmpT, but not in the ΔOmpT strain as shown in the gel image of Figure 3.3.

Figure 3.3 PCR amplification of OmpT gene locus in EHEC. The presence of the OmpT gene locus in the wild type, ΔOmpT, and pEHOmpT was determined by PCR. The OmpT gene was present as a 1 kbp product as shown in Figure 3.4A (lane 2 and lane 6) in comparison to the DNA marker lane (lane 1). The OmpT gene was present in the wild type (lane 2) and pEHOmpT (lane 6) but not in ΔOmpT (lane 4) with respect to the no template control for all strains (lanes 3, 5, 7).

Figure 3.3



3.4 Effect of OmpT on the growth of EHEC and *E. coli* BL21(DE3) in nutrient-rich and nutrient-limited media

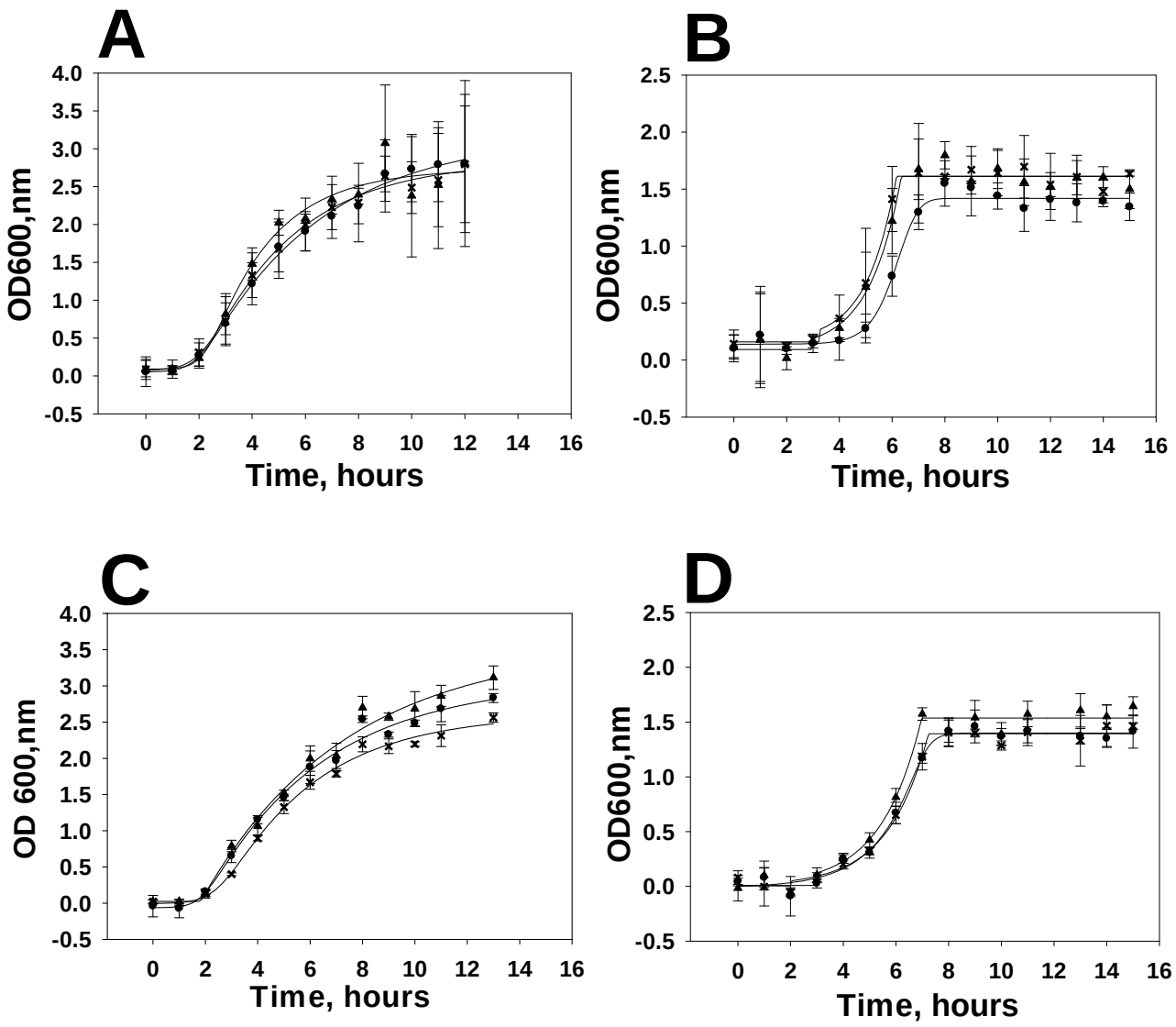
The effect of OmpT was studied on the growth of EHEC and *E. coli* BL21(DE3) in nutrient rich (LB media) and nutrient-limited medium (Minimal A media), as shown in Figure 3.4. The growth of the different strains was compared using ANOVA (Analysis of variance - Holm-Sidak method). The growth curve of EHEC in LB media indicated that there was no significant difference between the growth of wild type, ΔOmpT, and pEHOmpT ($p=0.142$, $p>0.05$) in nutrient rich liquid media. The growth curve of EHEC in Minimal A Media indicated that pEHOmpT displayed significantly reduced growth compared to the wild type and ΔOmpT ($p<0.001$). Conversely, the OmpT-containing BL21 (DE3) strain in LB media, grew significantly less than BL21 (DE3) and BL21(DE3) with pUC18 ($p<0.001$). BL21(DE3) growth was enhanced compared to the pUC18 strain ($p<0.001$). Also, BL21 (DE3) pUC18 and Bl21(DE3) with the OmpT construct from *E. coli* O86a:K61 grew significantly slower compared to BL21(DE3) cells in Minimal A media ($p<0.001$). The results indicated that over expression of OmpT retards growth significanlty in both EHEC under nutrient limiting condition and BL21(DE3) under both nutrient rich and limiting conditions.

3.5 Growth rate constant and Generation time of EHEC

The generation time or doubling time is the average time required for the cells to increase in number by two-fold. The rate of increase in cell number is inversely proportional to the number of cells present at any time. The generation time and growth rate constant was calculated from the equations: A) Growth Rate constant = $(\log_{10}Nt - \log_{10}No) / \log_{10}2$; and B) Generation time = time (min) / growth rate index (μ). The growth rate constant and the generation time were calculated from the graph of log of viable cell number (cfu/ml) versus time (minutes) required

Figure 3.4 Growth curves of EHEC and *E. coli* BL21 (DE3) in LB media and Minimal media. The growth curves in LB media and Minimal A media for EHEC are shown in Figures 3.4A and 3.4B, respectively. The growth curves of *E. coli* BL21(DE3) in LB and Minimal media are shown in Figure 3.4C and 3.4D, respectively. The graphs illustrate the optical density at 600nm (OD_{600} nm) versus growth time (in hours) and were plotted using the non linear regression model (Sigma Plot, Version 12) which indicated the strong relationship between these variables for all three strains studied ($r^2 \sim 0.99$). In Panel A and B, the closed triangle refers to wild type EHEC; crossed mark refers to ΔOmpT; and closed circle refers to pEHOmpT. In Panel C and D, the closed triangle refers to BL21(DE3); the closed circle refers to BLl21(DE3) with pUC18; and crossed mark refers to BL21(DE3) with the OmpT construct from *E. coli* O86a:K61.

Figure 3.4


A
OD600,nm
Time, hours
B
OD600,nm
Time, hours
C
OD 600,nm
Time, hours
D
OD600,nm
Time, hours

for cell growth. The growth rate constant and generation time for the EHEC strains is shown in Table 3.3. The pEHOmpT had a decreased growth rate and increased generation time compared to the wild type and ΔOmpT. There was no significant difference in the growth rate and generation time between the wild type and ΔOmpT strains. This result is consistent with the growth curve of the wild type, ΔOmpT and pEHOmpT in Minimal A media, where pEHOmpT grew significantly slower than the wild type and ΔOmpT strains ($p<0.001$).

Table 3.2 Growth rate and generation time of EHEC.

EHEC strains	Growth Rate constant, minutes	Generation Time, minutes
Wild type	57.3	43.6
ΔOmpT	45.6	54.6
pEHOmpT	35.4	70.3

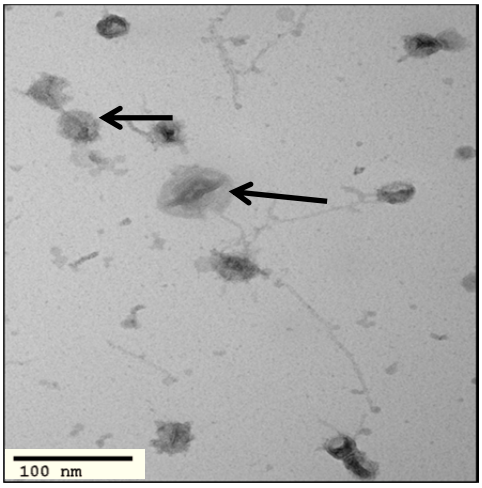
3.6 OmpT effect on EHEC Outer Membrane Vesicle production.

The OMVs from EHEC were isolated using a standard protocol as described in detail in the Methods section. The OMVs from different strains of EHEC *E. coli* strains were observed with transmission electron microscopy (TEM) at 100,000 x magnification and at a 200 kV accelerating voltage. Wild type EHEC produced a significantly larger number of OMVs than ΔOmpT, shown by the arrow in Figure 3.5A ($p<0.001$). ΔOmpT produced significantly less OMV as shown in Figure 3.5B. However, the pEHOmpT strain produced significantly more OMVs compared to the wild type ($p<0.001$, Figure 3.5C). pEHOmpT produced

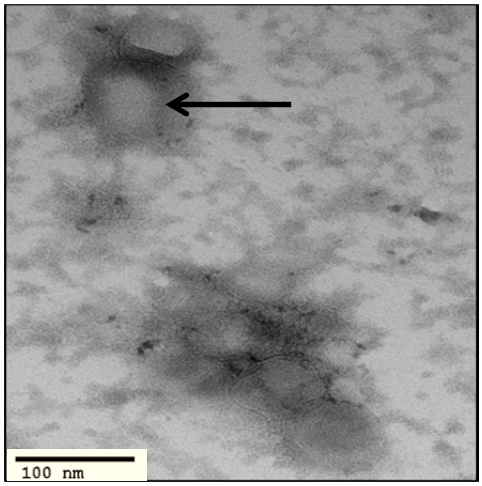
Figure 3.5 Transmission Electron Microscopic analysis of EHEC outer membrane vesicles. The OMVs were observed at 100,000 x magnification at 200kV accelerating voltage. The wild type produced approximately 60-80 OMVs per field area (Panel A); ΔOmpT produced few OMVs (Panel B) and pEHOmpT produced a large number of OMVs, approximately 2000-3000 per field area (Panel C). The OMVs are shown by black arrows in all Panels.

Figure 3.5

A



B



C

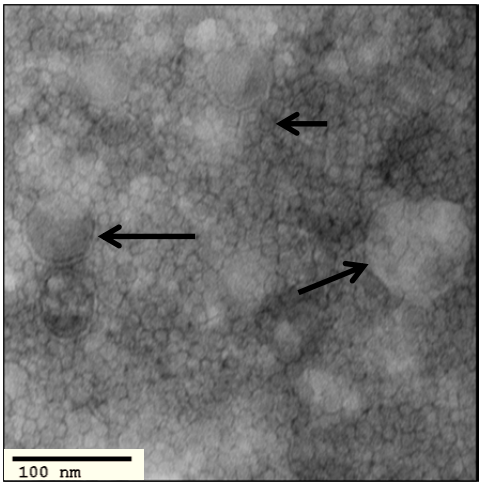


Figure 3.6 Outer membrane vesicles production from EHEC strains. The OMVs were measured by Macnification software (Orbicule Inc, Belgium). The graph illustrates the number of OMVs secreted (No. of OMVs) for each of the three EHEC strains. The black bar refers to the wild type, white bar to ΔOmpT and grey filled bar to pEHOmpT EHEC strain, respectively.

* $p<0.001$

Figure 3.6

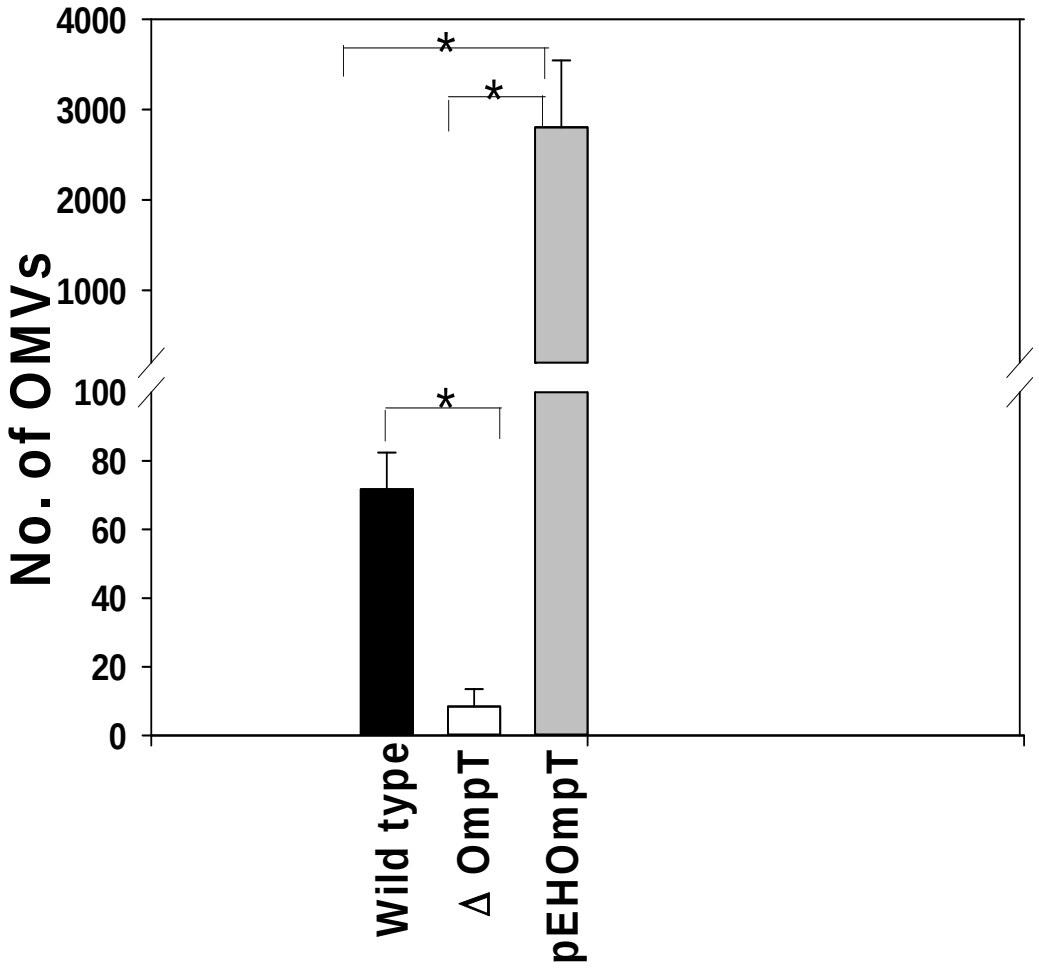
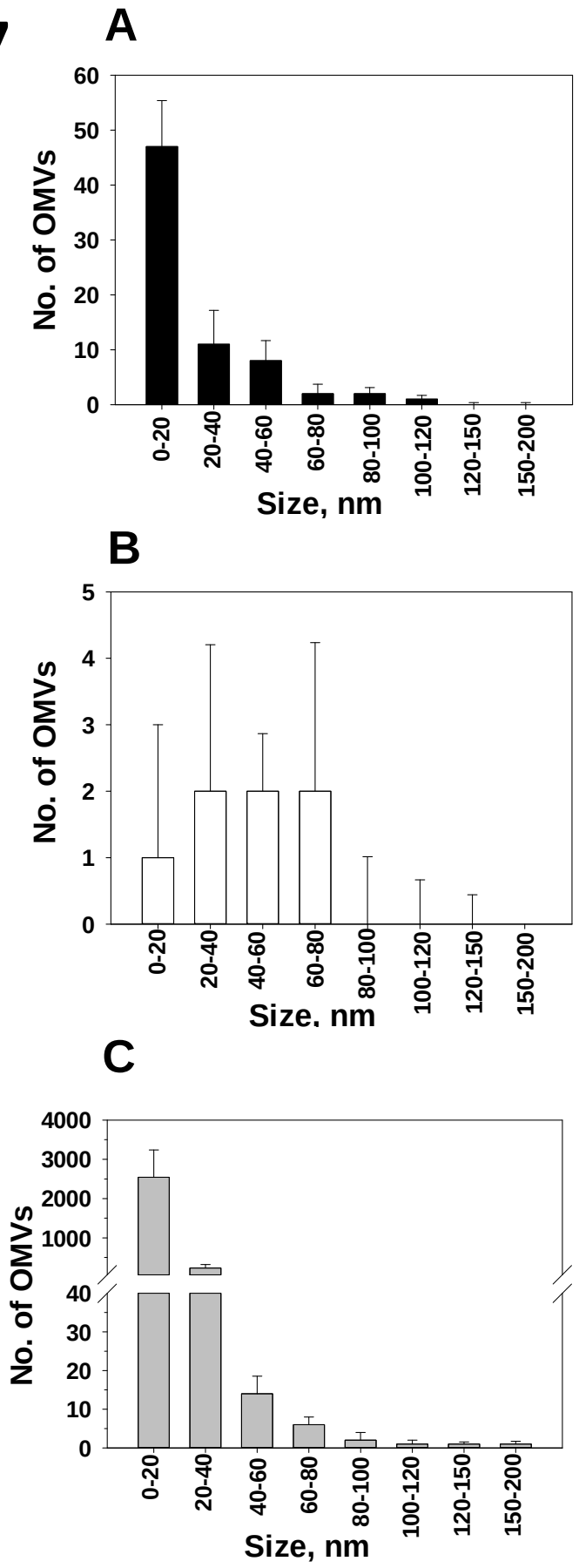

4000
3000
2000
1000
100
80
60
40
20
0
No. of OMVs
*
*
*
Wild type
Δ OmpT
pEHOmpT

Figure 3.7 Size distributions of outer membrane vesicles from EHEC. The OMVs were measured by Macnification software (Orbicule Inc, Belgium). The graph illustrates the different size range of OMVs (nm) and number of OMVs (No. of OMVs) for the wild type (Panel A), ΔOmpT (Panel B) and pEHOmpT (Panel C) EHEC strains, respectively. The black bar refers to the wild type, white bar to ΔOmpT and grey filled bar to pEHOmpT, respectively.

Figure 3.7


A
No. of OMVs
60
50
40
30
20
10
0
0-20
20-40
40-60
60-80
80-100
100-120
120-150
150-200
Size, nm
B
No. of OMVs
5
4
3
2
1
0
0-20
20-40
40-60
60-80
80-100
100-120
120-150
150-200
Size. nm
C
No. of OMVs
4000
3000
2000
1000
40
30
20
10
0
0-20
20-40
40-60
60-80
80-100
100-120
120-150
150-200
Size, nm

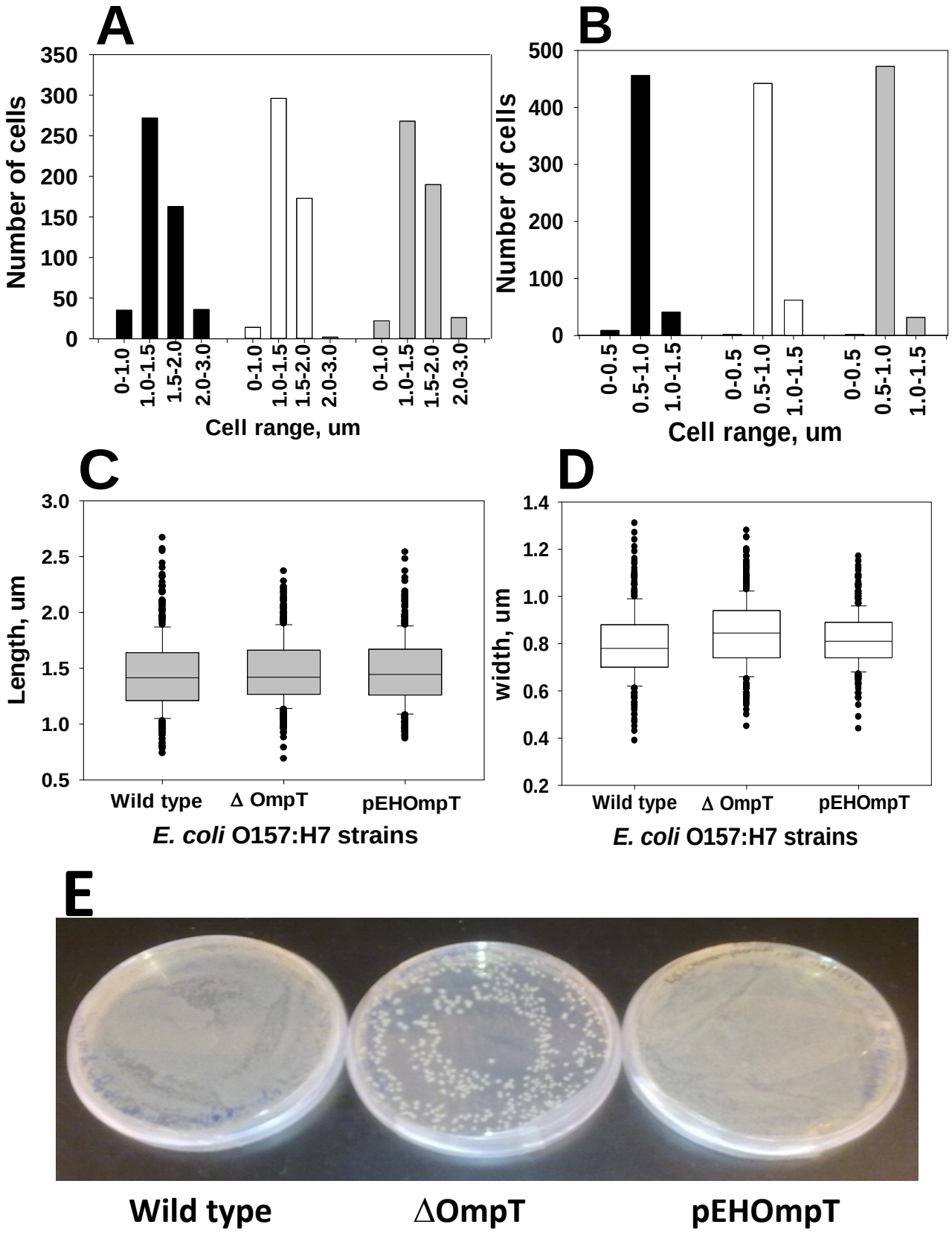
approximately 40-fold more OMVs than the wild type, and approximately 300-fold more OMVs than ΔOmpT and these differences were highly significant ($p<0.001$, Figure 3.6). Compared to the wild type cells, the pEHOmpT produced a 50-fold greater number of smaller sized OMVs (20-40nm) and a significantly larger number of 40-60nm, and 60-80nm of OMVs and these differences were also significant ($p<0.001$, Figure 3.7A and 3.5 C). Compared to ΔOmpT, pEHOmpT produced a 1250-fold greater number of smaller sized OMVs (20-40 nm) and a larger number of 40-60nm, and 60-80nm of OMVs and these differences were also significant ($p<0.001$, Figure 3.7 B and 3.7C). Compared to ΔOmpT cells, wild type cells produced a significantly larger number of different sized OMVs such as 0-20nm, 20-40nm, 40-60nm, 80-100nm, 100-120nm of OMVs ($p<0.05$, Figure 3.7A and 3.7B). TEM analyses indicated that change in OmpT gene expression has profound effect on the size and number of OMVs produced by EHEC.

3.7 Cell dimension analysis of EHEC

The EHEC strains were stained with a standard Gram-staining procedure (Mahon *et al.*, 2011) and observed with a Digital microscope at 4000 x magnification. The cells were photographed and measured with at least 500 different cells for each of the three strains using VH-M100 XY measurement and VHX-H1M1 measurement. The length range of OMVs in wild type cells was 0.74-2.67μm; in ΔOmpT was 0.69-2.37μm; and in pEHOmpT was 0.87-2.54μm. The average length of the wild type cells (1.44μm $\pm$ 0.33μm), ΔOmpT (1.47μm $\pm$ 0.28μm) and pEHOmpT (1.47μm $\pm$ 0.30μm) indicated that there was no significant difference between the three strains ($p=0.191$; p.0.05; Figure 3.8A and 3.8C). The width range of the wild type was 0.39-1.31μm; ΔOmpT was 0.45-1.28μm; and pEHOmpT was 0.44-1.17μm. The average width of the wild type

Figure 3.8 Digital Microscopy analyses of EHEC. The three EHEC strains were stained by Gram-staining (Mahon *et al.*, 2011). The cells were observed under a Digital Microscope (DM; Keyence Corporation, Osaka, Japan) at 4000 x magnification to determine the length and width. Figure 3.6 describes the length (Panel A), width (Panel B) and size comparison of the wild type, ΔOmpT, and pEHOmpT (Panel C and D) EHEC cells. Image of the LB agar plates with wild type (left), ΔOmpT (centre), and pEHOmpT (right) EHEC strains (Panel E). The black bar refers to the wild type, white bar to ΔOmpT and grey filled bar to pEHOmpT, respectively (Panels A and B).

Figure 3.8



E

Wild type **ΔOmpT** **pEHOmpT**

was 0.79µm ± 0.14µm; ΔOmpT was 0.84µm ± 0.14µm; and pEHOmpT was 0.81µm ± 0.11µm; indicating that the ΔOmpT cells had a significantly larger width compared to the wild type and pEHOmpT ($p<0.001$). Also pEHOmpT was wider than the wild type cells ($p<0.05$) as described in Figure 3.8 B and 3.8D. This indicates that the OmpT overexpression and deletion had a significant effect on the cell width but not the length of EHEC.

3.8 Analysis of OmpT protein expression in EHEC cell lysates and outer membrane vesicles.

Immunoblotting was conducted as per the procedure optimised by Dr. Herve Le Moual and co-workers (Thomassin *et al.*, 2012. The immunoblotting was performed with EHEC OMVs and cell lysates using a Cro-P antibody (74% identical to OmpT at the amino acid level) to determine the OmpT expression in these two environments. As shown in Figure 3.9, OmpT is expressed in the wild type and pEHOmpT whole cell lysates and OMVs, but not in cell lysates and OMVs from ΔOmpT. Table 3.4 illustrates the OmpT band density in cell lysates and OMVs from EHEC. The density was calculated based on the equation: (Background intensity – Mean Intensity) x Number of Pixels. The relative density (i.e fold expression) is the ratio of either ΔOmpT or pEHOmpT density relative to the wild type density. The densitometry analysis indicated that the OmpT is expressed approximately 5-fold higher in pEHOmpT cell lysate than the wild type and approximately 50-fold greater in pEHOmpT OMVs than wild type. Negligible OmpT protein expression was observed in both cell lysates and OMVs from the ΔOmpT strain.

3.9 Protein composition of *E. coli* O157:H7 cell lysates and outer membrane vesicles.

The method for silver staining was modified from the Merril *et al.*, 1981. Silver staining was performed with cell lysates and OMVs from the three EHEC strains (Figure 3.10A and B).

Figure 3.9 Immunoblotting for OmpT in EHEC cell lysates and outer membrane vesicles. The immunoblotting was performed with the wild type, ΔOmpT and pEHOmpT of EHEC whole cell lysates and OMVs. The whole cell lysates were prepared by growing EHEC to $0D_{600}$ of 0.5 (1ml culture, centrifuge at 17,000 x g for 5 minutes $4^{o}C$), resuspended and heated in 100µl of 2x LD at $95^{o}C$ for 5 minutes; loaded on 4-20% polyacrylamide and electrophoresed at 150V constant voltage. The protein was electroblotted onto a PVDF membrane and proteins were detected with primary antibody (anti-Cro-P antibody) and secondary antibody (Goat anti-rabbit IgG with conjugated HRP). The wild type, ΔOmpT, pEHOmpT cell lysates are shown in lane 1, lane 2, and lane 3, respectively; Wild type OMVs in lane 4 (0.09µg), and lane 5 (0.018µg), ΔOmpT OMVs in lane 6 (0.09µg) and lane 7 (0.018µg); and pEHOmpT OMVs in lane 8 (0.0036µg), and lane 9 (0.009µg). The OmpT protein expression in the wild type and pEHOmpT is indicated by a filled arrow at approximately 35kDa and cross-reactive bands by asterisks.

Figure 3.9

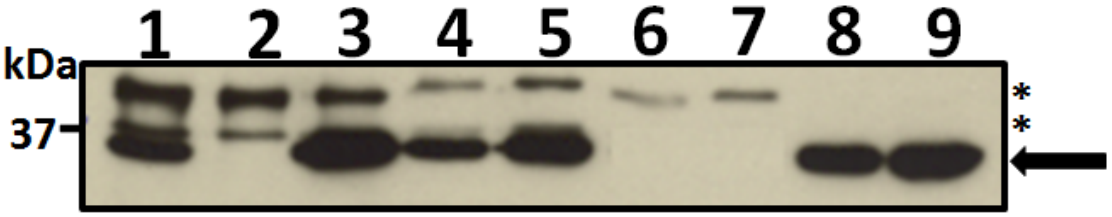


Table 3.3 Densitometric analysis of EHEC cell lysates and outer membrane vesicles OmpT. This Table describes the OmpT density of the bands in the immunoblotting image (Figure 3.7). The density was obtained using Corel Photo Paint (Ottawa, ON). The comparison between the densities of OmpT (in the wild type and pEHOmpT) is described as the fold-expression per μg of protein. The relative density refers to the ratio of OmpT density of the pEHOmpT strain with respect to the wild type strain.

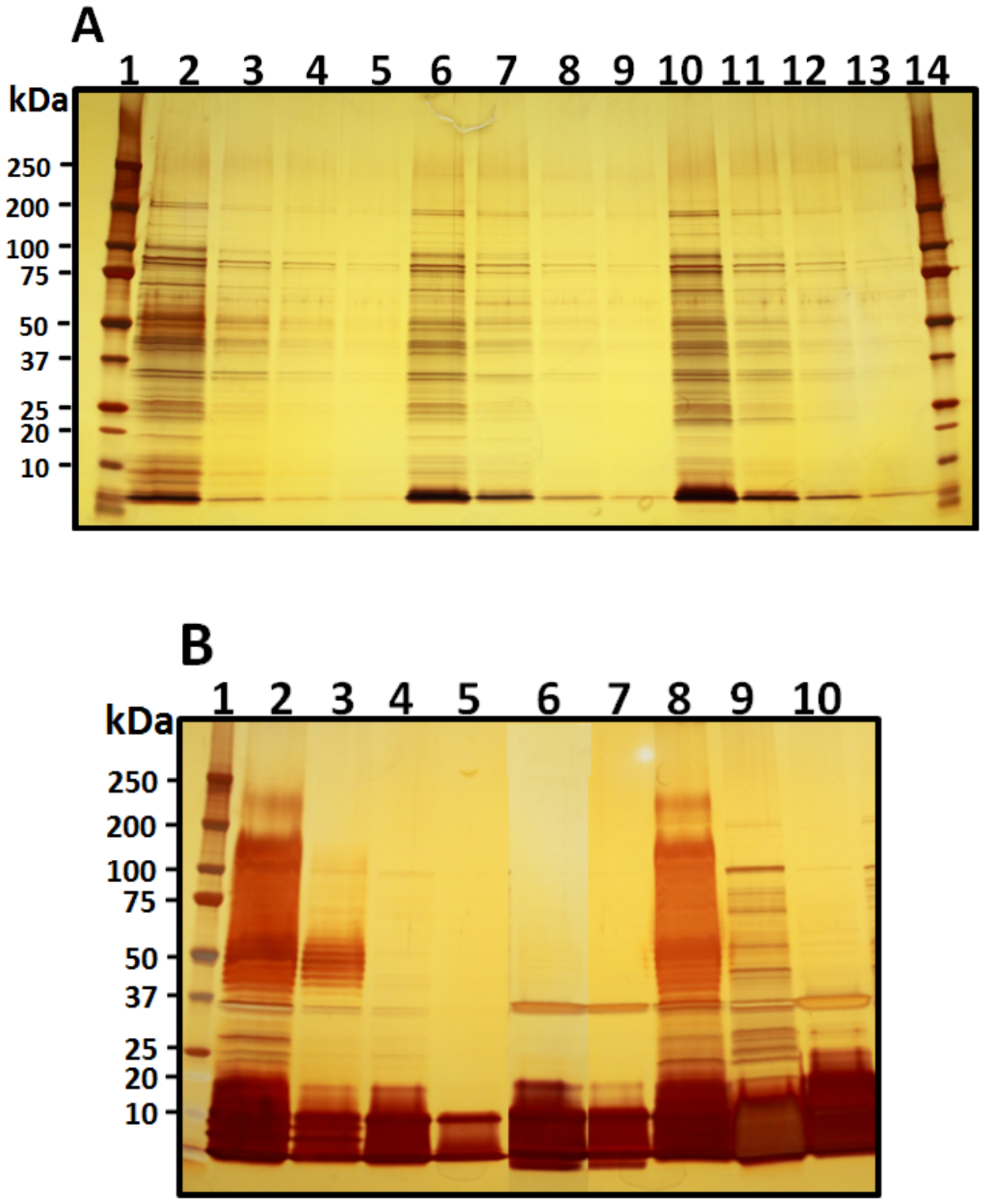
Table 3.3 Densitometric Analysis of OmpT Expression in EHEC

	EHEC strains	Relative density
Lysates	wild type (1µg)	1.00
	Δ OmpT (1µg)	0.00
	pEHOmpT (1µg)	3.08
OMVs	wild type (0.18ug)	1.00
	wild type (0.09ug)	1.00
	Δ OmpT(0.18ug)	0.00
	Δ OmpT(0.09ug)	0.00
	pEHOmpT(0.0036ug)	44.90
	pEHOmpT (0.009ug)	20.45

There were no large differences in protein composition of the cell lysates from the three different EHEC strains (Figure 3.10A). However, the silver staining of the gel of the OMVs from EHEC strains showed that the wild type produced a greater number of proteins (Approximately 60-80) with stainable lipid compared to ΔOmpT and pEHOmpT (Figure 3.10B). On the other hand, there are fewer proteins (approximately 30-40) present with low stainable lipid in OMVs from ΔOmpT. There was one major protein present at 37kDa (OmpT) with approximately 4-5 other proteins in pEHOmpT with low stainable lipid levels. Silver staining analysis revealed that lane 1 and lane 8 has stainable lipid in the region between 10-20 kDa and 40-200 kDa indicating the presence of Lipid A and O antigen polysaccharide of rough LPS as described by others (Haurat *et al.*, 2011). Staining in the region 40-200 kDa was absent in ΔOmpT and pEHOmpT, but the region between10-20 kDa was staining prominently in pEHOmpT but was barely detectable in ΔOmpT. This indicates that alterations in OmpT gene expression in EHEC not only profoundly altered OMV biogenesis, but resulted in changes in the lipid and protein content of OMVs as well. There are two different possibilities; either the OmpT gene is directly involved lipid and protein sorting in OMVs or is indirectly controling the expression of another gene which is involved in determing the OMV composition. The mechanism of sorting protein and lipid into OMVs remains to be elucidated.

Figure 3.10 Protein profile of EHEC cell lysates and outer membrane vesicles. Lanes 1 and 14 in Panel A refer to the molecular size protein ladder (BioRad, Mississauga, ON). The wild type lysate was loaded in lane 2 (6.9µg), lane 3 (1.34µg), lane 4 (0.69µg), and lane 5 (0.34µg); ΔOmpT lysate in lane 6 (6.9µg), lane 7 (1.34µg), lane 8 (0.69µg) and lane 9 (0.34µg); pEHOmpT lysate in lane 10 (6.9µg), lane 11 (1.34µg), lane 12 (0.69µg) and lane 13 (0.34µg). The cell lysates from EHEC strain cell lysates (50ml of culture grown to OD_{600}-0.50, centrifuged at 10,000 x g, 30 minutes, 4^{o}C and resuspended in 5ml of HBS, pH7.4) was sonicated at setting #7 for 10 minutes on ice and mixed with 2x loading dye, and boiled for 5 minutes. The samples were loaded on a 4-20% polyacrylamide gel and electrophoresed at 150V, constant voltage (Panel A). The OMVs from EHEC strains were prepared from the culture supernatant concentrating by 33-fold using tangential flow filtration and further 3-fold by centrifugal filtration, and then pelleted by ultracentrifugation. In Panel B, the protein ladder was loaded in lane 1; the wild type OMVs in lane 2 (370 ng), and lane 3 (75 ng); ΔOmpT OMVs in lane 4 (75 ng), and lane 5 (15 ng); pEHOmpT OMVs in lane 6 (80ng) and lane 7 (40ng). OMVs in the supernatant fractions from wild type (lane 8), ΔOmpT (lane 9) and pEHOmpT (lane 10) are also shown in Panel B. Each sample was loaded on the 4-20% polyacrylamide gel and electrophoresed at 150V, constant voltage (Panel B). The gels were stained with silver staining according to Merril *et al.*, 1981.

Figure 3.10


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
kDa
250
200
100
75
50
37
25
20
10
B
1 2 3 4 5 6 7 8 9 10
kDa
250
200
100
75
50
37
25
20
10

Results Section B

3.10 Determination of the EHEC cell number by spread plate technique

EHEC cell numbers were determined by a spread plate technique. It is important to determine the specific cell number at OD_{600} of 0.5 to ensure that the same numbers of cells were used for the FV assay, prothrombin time (PT) assay, activated partial thromboplastin time (aPTT) assay and Western blotting. The standard curves for each of the three EHEC strains are shown in Figure 3.11.

3.11 Determination of protein concentration by BCA assay

The protein content of the OMVs and lysates from the three EHEC strains were determined by BCA assay. BSA was used as a standard and was diluted serially by 2-fold to construct standard curves. The standard curves were plotted using Sigma Plot 12.0 as shown in Figure 3.12. The standard curve was used to determine the protein concentration in EHEC OMVs and cell lysates as shown in Table 3.5. This result indicated that the amount of protein produced by the wild type OMVs was 2-fold higher than the level of protein produced in the OMVs from the ΔOmpT strain. The pEHOmpT had a protein content in OMVs that was 7-fold higher than ΔOmpT and 4-fold higher than the wild type. The protein concentration is important since it ensures that the same amount of protein is used for performing different assays as mentioned above.

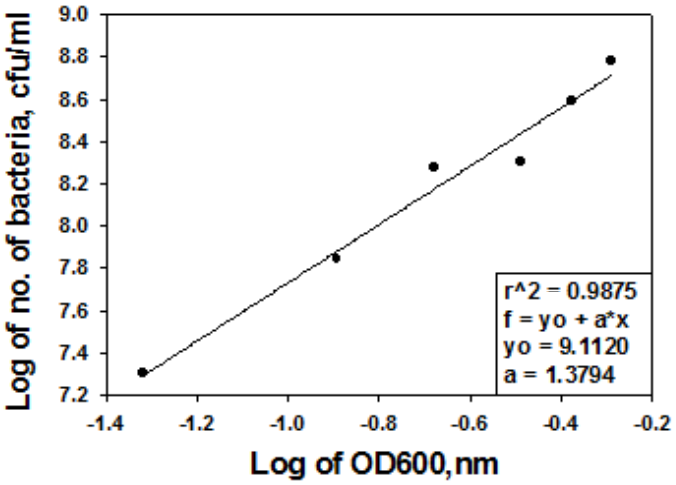
3.12 Prothrombin Time and Activated Partial Thromboplatin Time assay with whole cells and outer membrane vesicles

The PT and aPTT assays measure the activity of different factors involved in extrinsic and

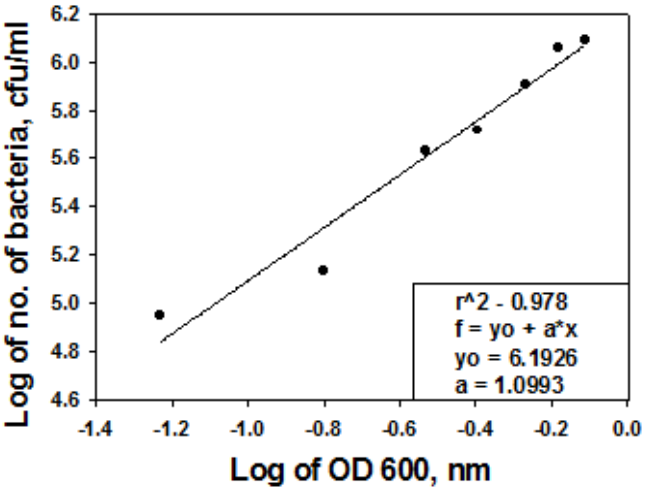
Figure 3.11 Standard curves of EHEC strains cell numbers. The spread plate technique was performed to generate the standard curves for wild type, ΔOmpT and pEHOmpT. The graphs show the log of bacteria (cfu/ml) and log of optical density at 600nm (OD_{600} nm). The graph displayed the linear relationship between these two variables ($r^2 \sim 0.99$). Panel A shows the graph for the wild type; Panel B for ΔOmpT; and Panel C for pEHOmpT, respectively.

Figure 3.11

A



B



C

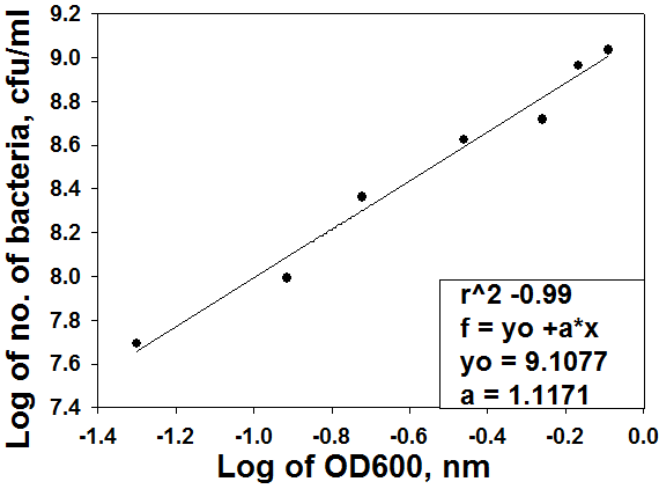


Figure 3.12 BCA assay to determine the protein concentration of EHEC cell lysates and outer membrane vesicles. Serial dilutions of BSA were used as a standard protein for preparing the standard curve. The standard curve was used to determine the unknown protein concentration of OMVs and cell lysates from the three EHEC strains. The graph shows the optical density at 562 nm (OD_{562} nm) and the BSA concentration (µg/ml). Table 3.4 describes the protein concentration (µg/ml) for OMVs (ultracentrifuged pellet and supernatant) and cell lysates from the wild type, ΔOmpT, and pEHOmpT EHEC strains.

Figure 3.12

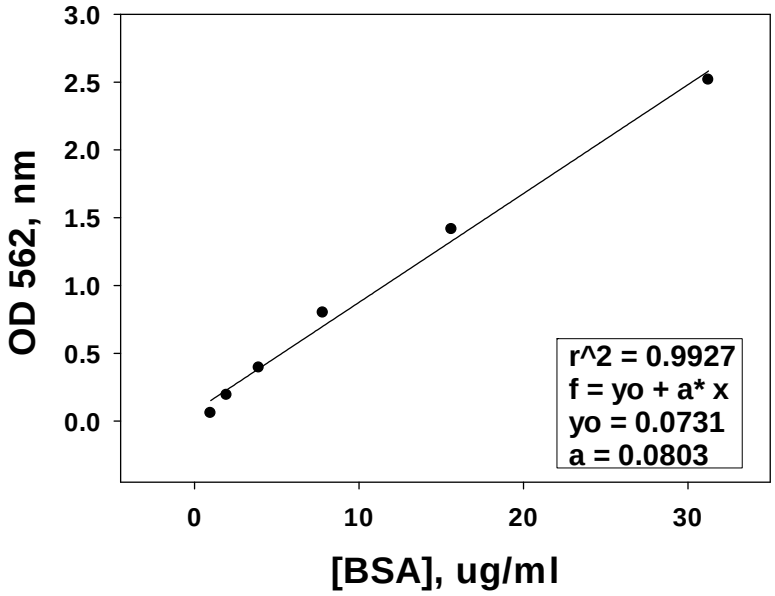


Table 3.4 **Protein concentration of outer membrane vesicle and cell lysates from EHEC strains**

EHEC strains	Ultra Centrifuged Pellet (µg/ml)	Ultra Centrifuged Supernatant (µg/ml)	Cell Lysates (µg/ml)
Wild type	75.46 ± 20.21	16.30 ± 6.83	134.6 ± 10.00
ΔOmpT	35.14 ± 24.37	16.00 ± 3.15	135.57 ± 29.00
pEHOmpT	271.09 ± 163.99	42.85 ± 11.96	133.7 ± 19.44

intrinsic coagulation pathways, respectively. Normal human plasma (NHP, diluted 5-fold in HBS, pH 7.4) was treated with varying numbers of cells (10^7,10^8, 10^9, & 10^{10}) or OMVs (0.25µg, 0.5µg, 1.0µg and 2.0µg of protein) to determine the effect on the PT and aPTT clot times. As shown in Figure 3.13A, 3.13B, 3.14A, and 3.14B, there was dose-dependent prolongation of PT and APTT clot time with increasing amounts of OMVs and whole cells. The effect of OMVs on the PT clot time is comparatively more prominent with the pEHOmpT than the wild type and ΔOmpT ($p<0.05$), but the difference between the wild type and ΔOmpT was not significant ($p>0.05$). Moreover, the pEHOmpT strain has significantly prolonged PT clot time compared to the wild type, ΔOmpT and HBS control ($p<0.05$), but the difference between the wild type and ΔOmpT was not significant ($p>0.05$). The effect of whole cells on the PT clot time was considerably more prolonged with pEHOmpT compared to the wild type and ΔOmpT ($p<0.05$), but there was no significant difference between the wild type and ΔOmpT ($p>0.05$). On the other hand, the wild type and ΔOmpT have a similar effect on aPTT clot times. The aPTT clot times effect is prolonged with the pEHOmpT compared to the HBS control ($p<0.05$) but the difference between the three strains was not significant ($p>0.05$). Strikingly, a maximum clot time prolongation effect with pEHOmpT was demonstrated with 2.0µg of OMVs and 10^{10} cells, when there was no clotting observed with normal human plasma in the aPTT assay. These results showed that OmpT associated with whole EHEC cells and OMVs prolonged both PT and APTT clot times. The specific coagulation factors that are inactivated in the intrinsic and extrinsic pathways will need to be determined in future studies to demonstrate OmpT cleavage specificity for individual coagulation factors.

Figure 3.13 Prothrombin Time assay with OmpT associated with EHEC whole cells and OMVs. The PT measures the activity of factors involved in the extrinsic pathway. The PT assay was performed with various concentrations of OMVs (0.25µg, 0.5µg, 1.0µg, and 2.0µg protein) and cells (10^7,10^8, 10^9, and 10^{10} cells/ml) from the three EHEC strains. The graphs illustrate time for clot formation and EHEC strain (Wild type, ΔOmpT and pEHOmpT) used. The white bar represents 0.25µg, the light grey bar is for 0.5µg, the grey bar for 1.0µg, the black bar for 2.0 µg, while the stippled bar represents HBS control in the Panels A. The white bar represents 10^7 cells, the light grey bar is for 10^8 cells, the grey bar for 10^9 cells, the black bar for 10^{10} cells, while the stippled bar represents HBS control in the Panels B.

Figure 3.13

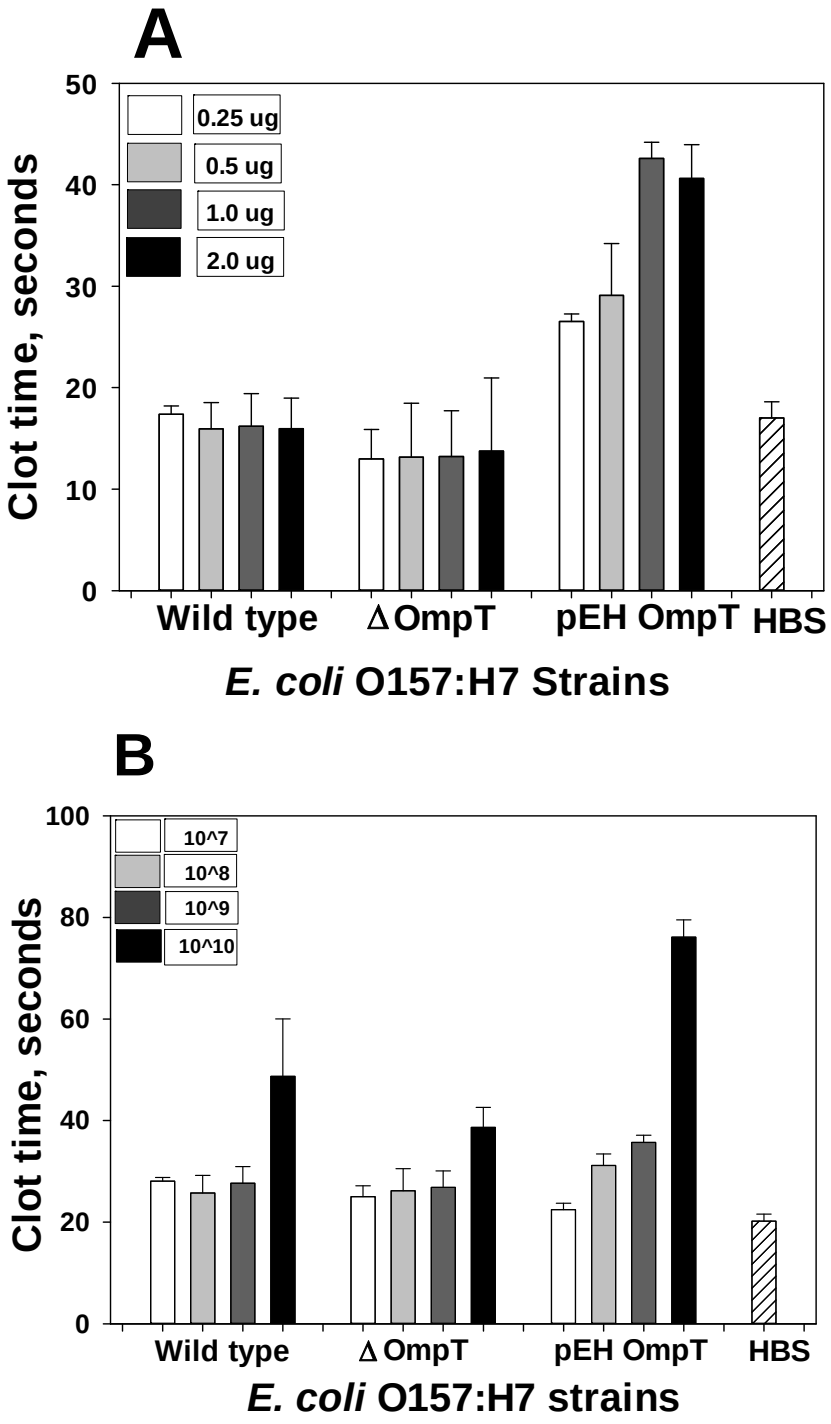
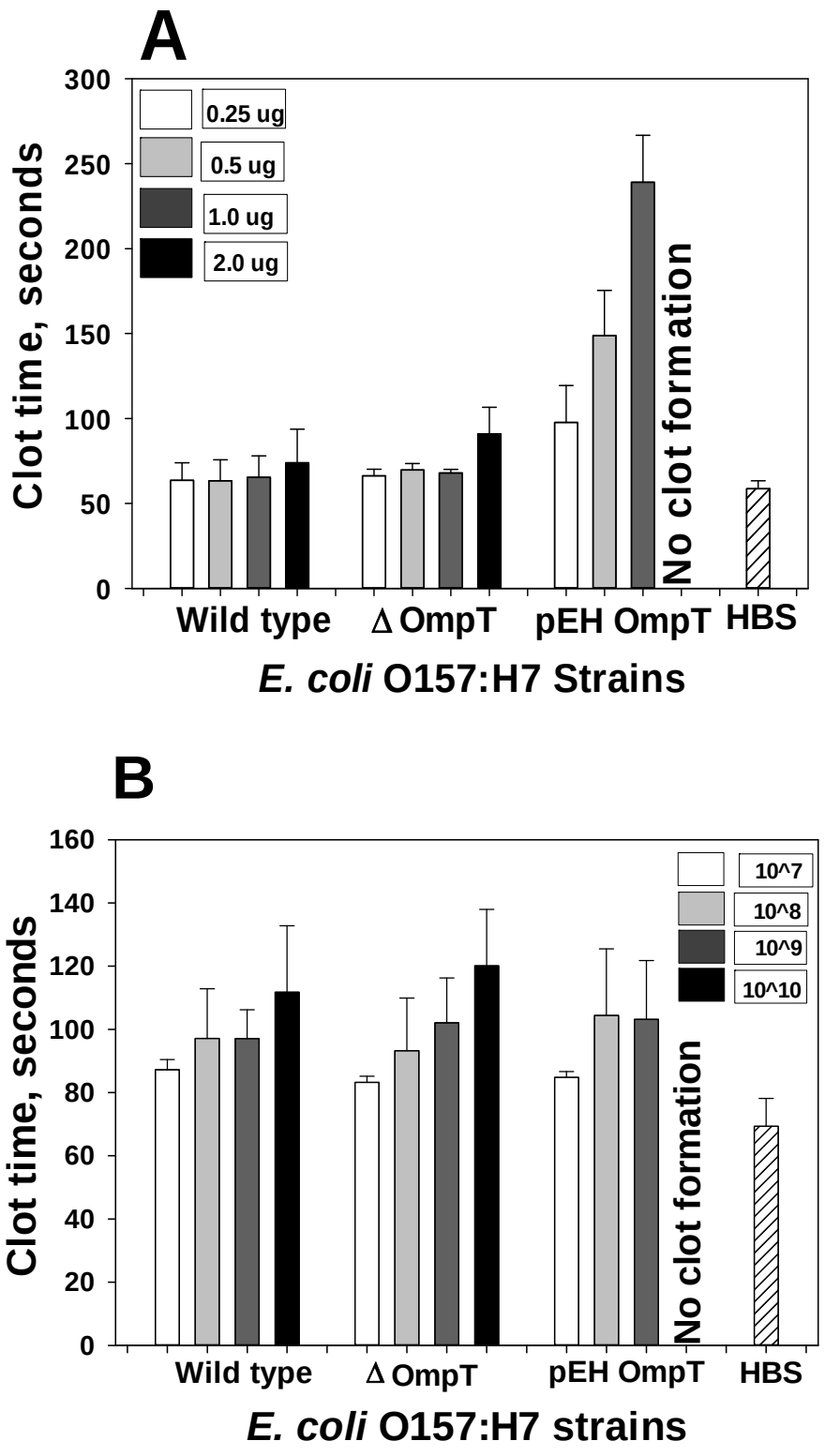

A
0.25 ug
0.5 ug
1.0 ug
2.0 ug
Clot time, seconds
0
10
20
30
40
50
Wild type
ΔOmpT
pEH OmpT
HBS
E. coli O157:H7 Strains
B
10^7
10^8
10^9
10^10
Clot time, seconds
0
20
40
60
80
100
Wild type
ΔOmpT
pEH OmpT
HBS
E. coli O157:H7 strains

Figure 3.14 Activated partial thromboplastin time assay with OmpT associated with EHEC whole cells and outer membrane vesicles. The aPTT measures the activity of factors involved in the intrinsic pathway. The aPTT assay was performed with various concentrations of OMVs (0.25µg, 0.5µg, 1.0µg, & 2.0µg of protein) and cells (10^7,10^8, 10^9, &10^{10}). The graphs illustrate time for clot formation and EHEC strain (Wild type, ΔOmpT and pEHOmpT) used. The white bar represents 0.25µg, the light grey bar is for 0.5µg, the grey bar for 1.0µg, the black bar for 2.0 µg, while the stippled bar represents HBS control in the Panels A. The white bar represents 10^7 cells, the light grey bar is for 10^8 cells, the grey bar for 10^9 cells, the black bar for 10^{10} cells, while the stippled bar represents HBS control in the Panels B.

Figure 3.14


A
300
250
200
150
100
50
0
Clot time, seconds
0.25 ug
0.5 ug
1.0 ug
2.0 ug
No clot formation
Wild type
Δ OmpT
pEH OmpT
HBS
E. coli O157:H7 Strains
B
160
140
120
100
80
60
40
20
0
Clot time, seconds
10^7
10^8
10^9
10^10
No clot formation
Wild type
Δ OmpT
pEH OmpT
HBS
E. coli O157:H7 strains

3.13 Factor V Coagulation Activity Assay

Based on the results from previous experiments, it was shown that there was dose-dependent cleavage and inactivation of FV/FVa in baboon sepsis model when the baboons were infused with increasing doses of *E. coli* O86a:K61 (Samis *et al.*, 2007). It was later proved that the OmpT protease from *E. coli* O86a:K61 was responsible for the FV/FVa inactivation observed (Tilley D, M.Sc. thesis, 2011). An attempt to further characterize the OmpT inactivation effect on FV was conducted by employing a genetic approach using whole cells and OMVs from wild type, an OmpT gene deletion and an OmpT overexpressing strain. The FV activity standard curve of log clot time (seconds) versus log activity (U/ml) was prepared by serially diluting NHP in HBS, pH 7.4. The standard curve was utilised to determine the FV activity (U/ml) of NHP treated samples and controls. The FV activity in NHP is defined as 1 Unit of activity in 1 ml of NHP. The concentration of FV in human plasma is approximately 20-40nM (Mann *et al.*, 1981). The FV activity standard curve is illustrated in Figure 3.15. Table 3.6 refers to the FV inactivitation percent/µg of protein when NHP was treated with 1 µg of OMVs. This table indicates that the OMVs from the wild type strain inactivated FV by approximately 40%, ΔOmpT by 5-10% and pEHOmpT by 95%. The pEHOmpT inactivates FV at a significantly higher rate than the wild type and ΔOmpT ($p<0.001$) and inactivation by the wild type was significantly higher than ΔOmpT ($p<0.05$). Since the OMVs were not completely pelleted by ultracentrifugation, 1 µg of OMV protein in the supernatant fractions was also analysed for their effect on FV. The same effect with OMVs in supernatant was demonstrated: the wild type inactivated FV by 30% and pEHOmpT by 95%, but the effect was completely abolished by ΔOmpT when compared to the HBS control. The significance levels observed for the FV inactivation ($p<0.05$) in the pelleted OMVs were the same as those for the OMVs in the

Figure 3.15 Factor V standard curve using FV-1 stage microplate assay and effect of EHEC OMVs and cells on FV. The FV standard curve was created based on the method used by Tilley *et al.*, 2011. The NHP was diluted (0-fold to 512-fold) and assayed using a FV-1 stage microplate assay. As shown in Figure 3.15A, the standard curve plots log FV activity (U/ml) vs log clot time (seconds). The graph was plotted using Sigma Plot 12.0 and indicated a strong linear relationship between these two variables (r^2- 0.98). The graph in Figure 3.15B illustrates the effect of OMVs on NHP treated with OMVs from wild type, ΔOmpT, pEHOmpT. The black bar refers to the OMVs from the wild type, the white bar to OMVs from ΔOmpT, the grey filled bar to OMVs from pEHOmpT and the stippled filled bar to HBS. The difference is significant between pEHOmpT OMVs and the wild type OMVs ($p<0.001$), pEHOmpT OMVs and ΔOmpT OMVs ($p<0.001$), and the wild type OMVs and ΔOmpT OMVs ($p<0.05$). The difference is significant between pEHOmpT cells and the wild type cells ($p<0.05$), pEHOmpT cells and ΔOmpT cells ($p<0.001$), and the wild type cells and ΔOmpT cells ($p<0.001$). * $p<0.001$, ** $p<0.05$.

Figure 3.15

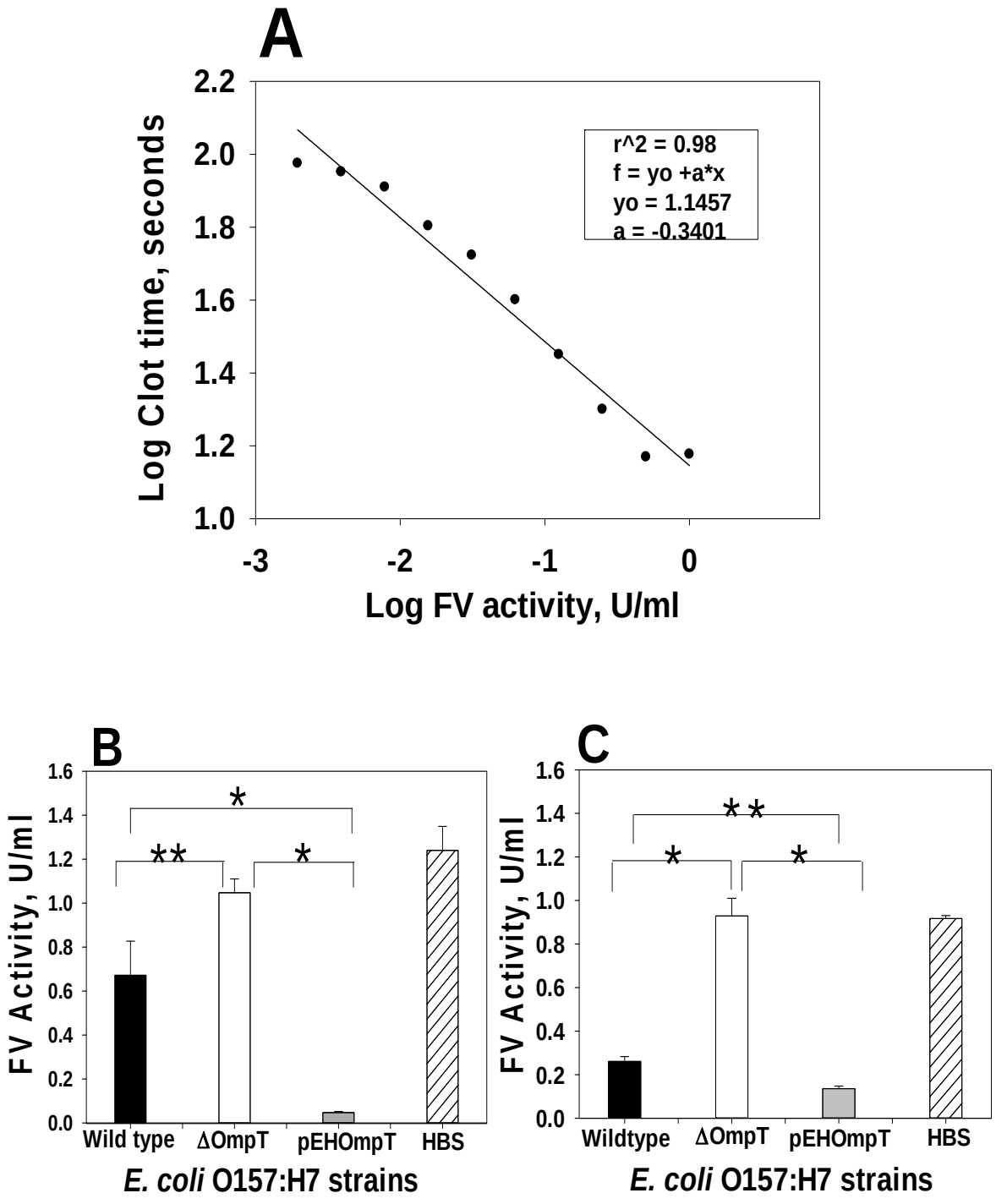

A
Log Clot time, seconds
Log FV activity, U/ml
r^2 = 0.98
f = yo +a*x
yo = 1.1457
a = -0.3401
2.2
2.0
1.8
1.6
1.4
1.2
1.0
-3
-2
-1
0
B
FV Activity, U/ml
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
**
*
Wild type
ΔOmpT
pEHOmpT
HBS
E. coli O157:H7 strains
C
FV Activity, U/ml
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
*
*
Wildtype
ΔOmpT
pEHOmpT
HBS
E. coli O157:H7 strains

Table 3.5 FV inactivation by EHEC outer membrane vesicles. The Table indicates the FV activity % remaining and FV inactivation %/μg of protein using the OMVs from EHEC wild type, ΔOmpT and pEHOmpT. OMVs, present in pellet and supernatant, were used for assaying FV from NHP in the FV activity assay. The NHP was treated with 1μg of OMVs for 30 minutes or 10^9 cells/ml for 1 hour at room temperature and the FV activity (U/ml) remaining was determined from the standard curve in Figure 3.15A.

Table 3.5

EHEC strain	FV activity (Percent activity remaining)	FV inactivation (Percent inactivation /µg of protein)
Wild type (OMVs)	62.67 ± 9.62	37.33 ± 9.62
ΔOmpT (OMVs)	88.19 ± 7.36	11.81 ± 7.36
pEHOmpT (OMVs)	4.63 ± 1.35	95.37 ± 1.35
Wild type (Supernatant/OMVs)	73.39 ± 7.27	26.61 ± 7.27
ΔOmpT(Supernatant/OMVs)	~100%	No inactivation
pEHOmpT(Supernatant/OMVs)	6.70 ± 1.00	93.30 ± 1.00
Wild type (cells)	24.17 ± 3.54	75.83 ± 3.54
ΔOmpT (cells)	87.05 ± 12.66	12.94 ± 12.66
pEHOmpT (cells)	4.56 ± 0.16	95.44 ± 0.16

supernatant fractions.

3.14 Time-dependent inactivation of FV by FV activity assay and immunoblotting

The time-dependent OmpT inactivation of FV was assessed by the FV activity assay and concurrently with the same sample by immunoblotting. NHP was treated with 1 μg of EHEC OMVs protein or 10^9 cells for various times (20 minutes, 1 hour, 1.5 hours, and 2.0 hours). As shown in Figures 3.16 and 3.17, the FV was cleaved by pEHOmpT cells and OMVs from 330 kDa to 250 kDa in NHP at room temperature and inactivated by 80% in 20 minutes and 95% in 2 hours; significantly higher activity than the EHEC wild type and ΔOmpT cells and OMVs ($p<0.05$). The wild type cells and OMVs inactivated FV by 60% in 2 hours under the same conditions and the FV inactivation effect was abolished with EHEC ΔOmpT cells and OMVs ($p<0.05$). The inactivation of FV by OmpT from OMVs and cells from the wild type and pEHOmpT EHEC strains correlated well with its cleavage by immunoblotting.

3.15 OmpT inactivation of FV in normal and plasminogen deficient human plasma

There are at least two possible explanations for the OmpT dependent inactivation of FV in NHP: direct or indirect. Experiments were carried out to determine whether the effect of OmpT from EHEC cells and OMVs was by OmpT directly or indirectly by activating plasminogen to plasmin. OmpT may render FV inactive either directly by itself or indirectly by activating plasminogen to plasmin. Different studies conducted with OmpT showed that OmpT does activate plasminogen to plasmin (McCarter *et al.*, 2004; Lundrigan & Webb, 1992) while another study showed OmpT was a poor plasminogen activator (Haiko *et al.*, 2009). These possibilities were tested by measuring FV inactivation with the cells and OMVs from the different EHEC

Figure 3.16 Effect of EHEC outer membrane vesicles OmpT on FV in normal human plasma. NHP was treated with 1µg of OMVs for 30 minutes at room temperature and a 60 µl aliquot of NHP was withdrawn at different times and 30µl of treated NHP was utilised for FV activity assay. The other aliquot of NHP was diluted further and 0.33µl was added to 2x loading dye and heated at 95^{o}C for 5 minutes and samples loaded on 4-20% polyacrylamide SDS-PAGE gel (Bio-Rad Criterion Gel, Mississauga, ON) and electrophoresed for 1.5 h at 150V. The FV inactivation in the FV activity assay correlated with its cleavage by immunoblotting for different times (20 minutes, 1 hour, 1.5 hours and 2 hours). Panel A depicts the NHP treated with OMVs from wild type cells which inactivated FV by 60% and FV was cleaved from 330kDa to 250kDa on the blot. Panel B depicts the NHP treated with OMVs from ΔOmpT cells which did not inactivate FV and was similar to the HBS control. Panel C depicts the NHP treated with OMVs from pEHOmpT cells which inactivated FV by 80% in 20 minutes and 90% in 2 hours which correlated well with FV cleavage from 330kDa to 250kDa and 150kDa on the blot. The black bar refers to the OMVs from wild type, the white bar to the ΔOmpT, and the grey filled bar to pEHOmpT EHEC strains.

Figure 3.16

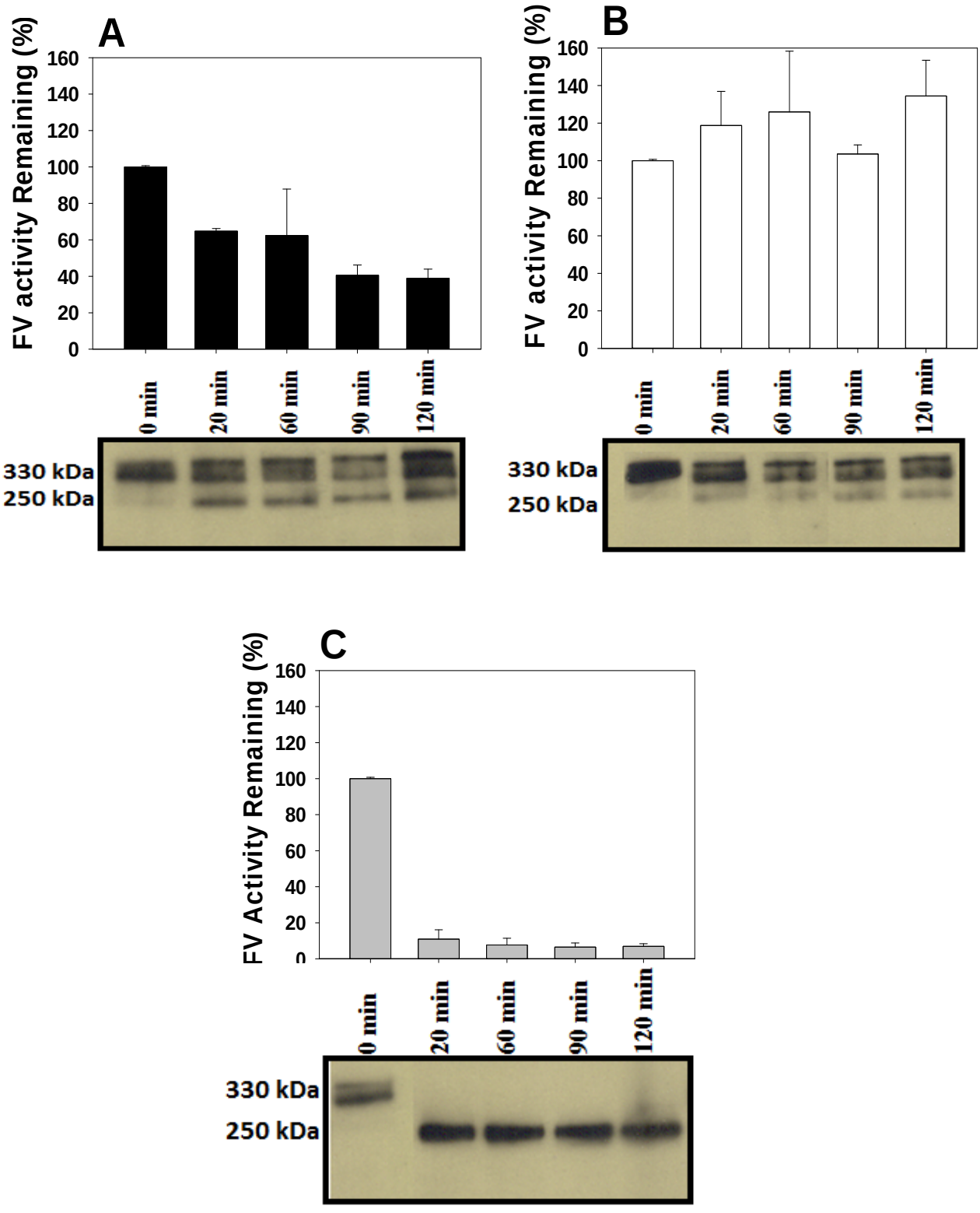

A
FV activity Remaining (%)
160
140
120
100
80
60
40
20
0
0 min
20 min
60 min
90 min
120 min
330 kDa
250 kDa
B
FV activity Remaining (%)
160
140
120
100
80
60
40
20
0
0 min
20 min
60 min
90 min
120 min
330 kDa
250 kDa
C
FV Activity Remaining (%)
160
140
120
100
80
60
40
20
0
0 min
20 min
60 min
90 min
120 min
330 kDa
250 kDa

Figure 3.17 Effect of EHEC cell OmpT on FV in normal human plasma. NHP was treated with 10^9 cells, 60 µl of NHP was withdrawn at different times (20 minutes, 1hour, 1.5 hours and 2 hours) and then diluted, and centrifuged at 17,000 x g, 4^{o}C. From the centrifuged sample, 30µl NHP was utilised for the FV activity assay and another aliquot of the treated NHP was diluted further (0.33µl) added to 2x Loading dye, heated at 95^{o}C for 5 minutes, samples loaded on 4-20% polyacrylamide SDS-PAGE gel (Bio-Rad Criterion Gel, Mississauga, ON) and electrophoresed for 1.5 h at 150V. FV inactivation in the FV activity assay correlated well with its cleavage by immunoblotting for different times (20 minutes, 1 hour, 1.5 hours and 2 hours). Panel A depicts the NHP treated with the wild type cells which inactivated FV by 60% and resulted in FV cleavage from 330kDa to 250kDa. Panel B depicts the NHP treated with ΔOmpT cells which did not inactivate FV and was equivalent to the HBS control. Panel C depicts the NHP treated with pEHOmpT cells which inactivated FV by 80% in 20 minutes and 90% in 2 hours which was correlated with FV cleavage from 330kDa to 250kDa. The black bar refers to the wild type, the white bar to the ΔOmpT, and the grey filled bar to pEHOmpT EHEC strains.

Figure 3.17

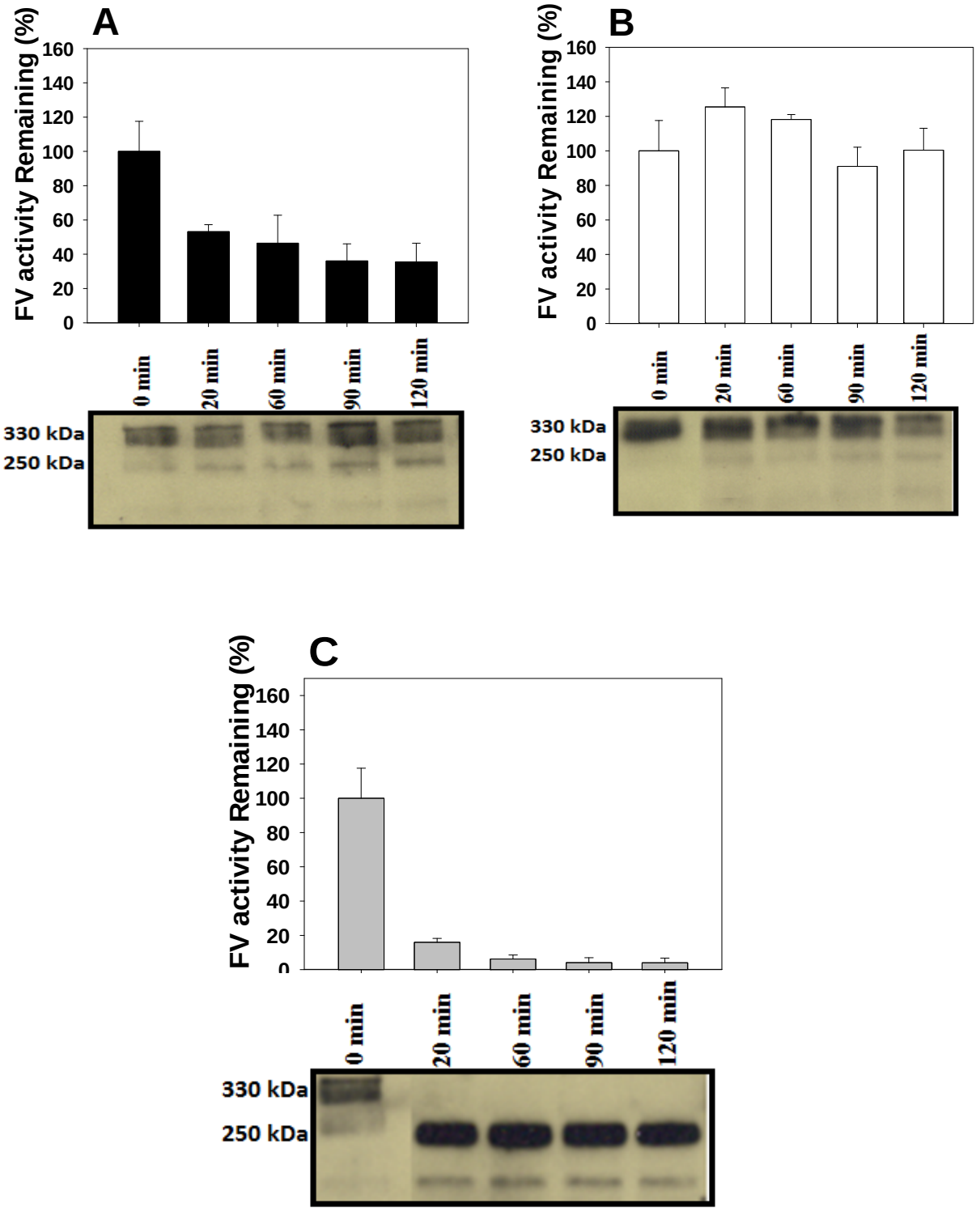
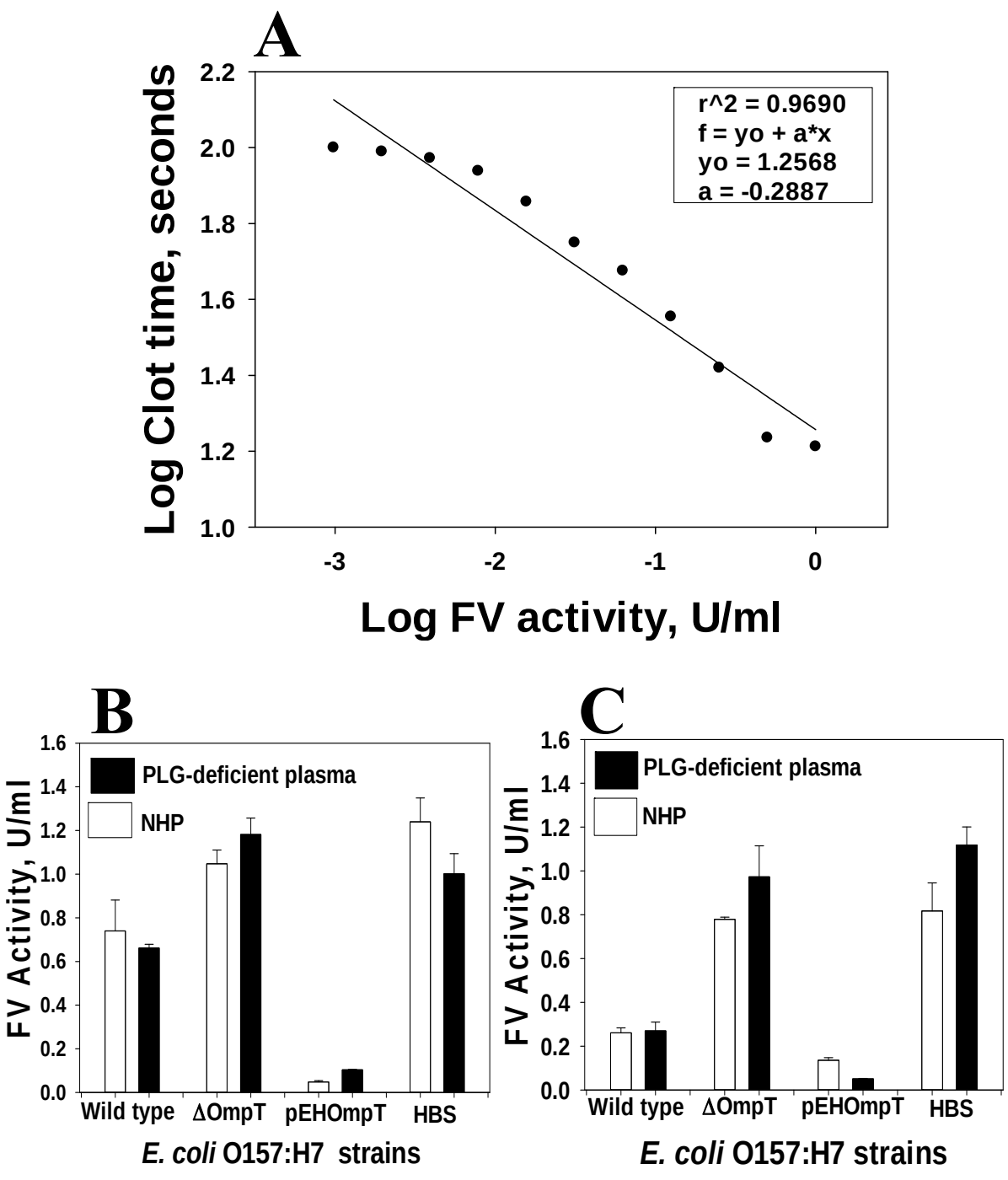

A
FV activity Remaining (%)
160
140
120
100
80
60
40
20
0
0 min
20 min
60 min
90 min
120 min
330 kDa
250 kDa
B
FV activity Remaining (%)
160
140
120
100
80
60
40
20
0
0 min
20 min
60 min
90 min
120 min
330 kDa
250 kDa
C
FV activity Remaining (%)
160
140
120
100
80
60
40
20
0
0 min
20 min
60 min
90 min
120 min
330 kDa
250 kDa

Figure 3.18 OmpT inactivation of FV in plasminogen-deficient human plasma versus normal human plasma. The FV standard curve was constructed with various concentrations of FV in Plasminogen (PLG) deficient human plasma (0-fold to 1024-fold in HBS) in the FV activity assay (Tilley *et al*., 2011). In Panel A, the graph illustrates log FV activity (U/ml) versus log of time for clot formation (seconds). The data was plotted using the linear regression model which indicated a strong relationship between these two variables ($r^2 = 0.969$). The NHP and PLG-deficient treated plasma were treated with OmpT from EHEC OMVs (Panel B) or cellular associated OmpT (Panel C) from the three EHEC strains used. The graph illustrates the FV activity remaining and EHEC strains (wild type, ΔOmpT and pEHOmpT) and the HBS control. The black bar refers to the result for PLG-deficient treated plasma and the white bar refers to NHP treated plasma with OMVs and cellular associated OmpT from the three EHEC strains used.

Figure 3.18


A
Log Clot time, seconds
2.2
2.0
1.8
1.6
1.4
1.2
1.0
r^2 = 0.9690
f = yo + a*x
yo = 1.2568
a = -0.2887
-3
-2
-1
0
Log FV activity, U/ml
B
FV Activity, U/ml
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PLG-deficient plasma
NHP
Wild type
ΔOmpT
pEHOmpT
HBS
E. coli O157:H7 strains
C
FV Activity, U/ml
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PLG-deficient plasma
NHP
Wild type
ΔOmpT
pEHOmpT
HBS
E. coli O157:H7 strains

strains using normal human plasma or plasminogen (PLG) deficient human plasma with the FV activity assay. The PLG-deficient plasma has all the clotting factors except plasminogen, which after activation to plasmin is involved in the fibrinolysis pathway. Further, plasmin has been shown to cleave and inactivate FV (Omar & Mann, 1979; Hoover-Plow, 2010). The FV activity standard curve for PLG-deficient plasma was plotted in the same manner as NHP as described in Figure 3.18 A. Finally, the plasma was treated with 1µg of EHEC OMVs or 10^9 EHEC cells and FV activity assay was performed. The data was plotted using Sigma Plot 12.0, as described in Figure 3.18B and 3.18C. Cellular and OMV OmpT from the wild type cells inactivated FV in PLG-deficient human plasma to levels similar to those in NHP. pEHOmpT cellular and OMV OmpT inactivated FV in PLG deficient plasma (80-90%) approximately to the same level as the FV in NHP (approx. 80-90%). Strikingly, FV inactivation was completely abolished with ΔOmpT cellular and OMV OmpT in both PLG-deficient plasma and NHP. Hence, cells and OMVs OmpT can inactivate FV directly in NHP without the involvement of the plasminogen/plasmin system.

3.16 Effect of OmpT from EHEC on other coagulation factors

In order to determine the effect of OmpT on other coagulation factors involved in extrinsic and common pathways, different coagulation factor deficient plasma were utilised to establish the standard curves and activity assays. The activity of different factors was defined similarly as for FV (1 ml of NHP has 1 Unit of coagulation factor activity). The calibration curves of the different coagulation factors were performed in the same way as for the FV activity standard curve. The activity assays for different coagulation factors were as follows.

i) Fibrinogen coagulation factor assay The PT-based fibrinogen assay was modified based on the method used by Mackie *et al.* 2003. The standard curve of fibrinogen (FI) is shown in Figure 3.19. The concentration of fibrinogen in normal human plasma is 2-4mg/ml (6-12µM) (Grannis, 1970). It was observed that there was no clot formation after reaching the threshold value with 8-fold diluted NHP. Therefore, the standard curve was plotted with four points (0-, 2-, 4-, and 8-fold diluted NHP). The fibrinogen activity assay was performed with 2-fold diluted NHP (30µl) treated with 1 µg OMVs/10^9 cells (30µl). As shown in Figure 3.19B and 3.19C, there was no significant inactivation with the wild type and ΔOmpT cellular and OMV associated OmpT, but the pEHOmpT cells and OMVs inactivated fibrinogen by approximately 40-50%. The fibrinogen inactivation difference is significant between the wild type, ΔOmpT, and pEHOmpT whole cells as well as HBS control ($p< 0.05$). There was a significant difference in fibrinogen inactivation by pEHOmpT OMVs compared to the wild type, ΔOmpT OMVs and HBS control ($p<0.05$). This suggests that over expression of OmpT in EHEC cells and OMVs can inactivate fibrinogen significantly.

ii) Prothrombin coagulation factor assay: The prothrombin concentration in normal human plasma is approximately100µg/ml (1400nM) (Butenas & Mann, 2002). A standard curve was plotted using 2-fold serially diluted NHP and assayed using prothrombin (FII) deficient plasma as for the FV and fibrinogen microplate assays. The graph for the FII standard curve is as shown in Figure 3.20. The FII was not inactivated by OMVs from wild type and ΔOmpT, but FII was inactivated by 40% by pEHOmpT OMVs. The wild type and pEHOmpT OMVs inactivated FII by 20% andOMVs from ΔOmpT inacitvated FII in NHP by 10%.There was no significant difference in the FII inactivation with EHEC cells ($p=0.216$) and OMVs ($p=0.175$) compared to the HBS control.

Figure 3.19 Fibrinogen calibration curve and activity assay with EHEC whole cells and OMVs. Due to the sensitivity of the assay, the fibrinogen standard curve was assayed with four different concentrations of NHP (0-fold to 8-fold in HBS) using fibrinogen deficient plasma based on the PT-based coagulation assay. In Panel A, The graph illustrates log fibrinogen activity (U/ml) versus log of time for clot formation (seconds). The data was plotted using the linear regression model which indicated a strong relationship between these two variables ($r^2 = 0.84$). The NHP was treated with OmpT from EHEC OMVs (Panel B) and cells (Panel C). The graph shows the FI activity remaining and EHEC strains (Wild type, ΔOmpT and pEHOmpT) and HBS control. The black bar denotes the wild type, the white bar the ΔOmpT strain, the grey filled bar pEHOmpT strains, while the stippled filled bar represents the HBS control.

Figure 3.19

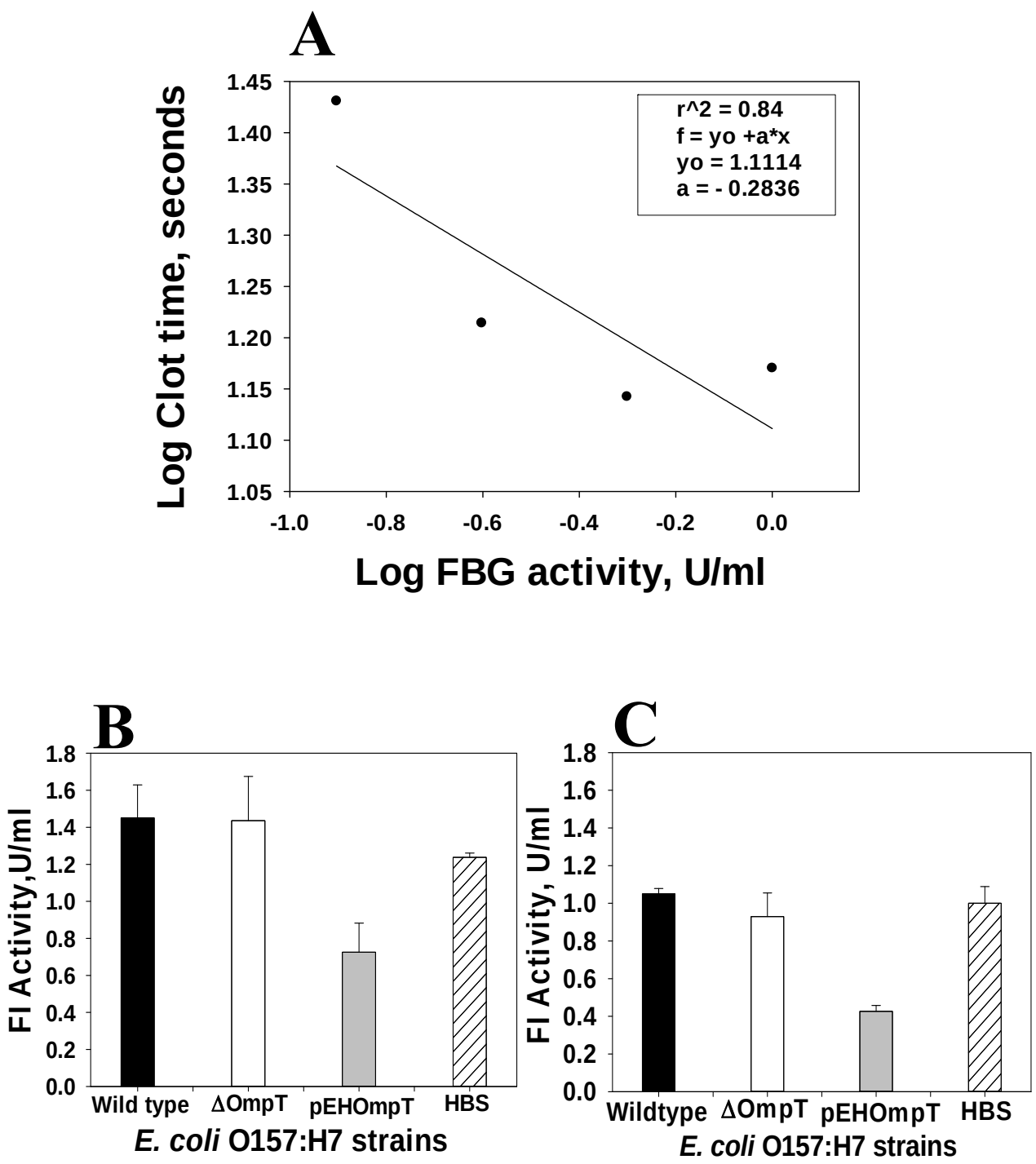
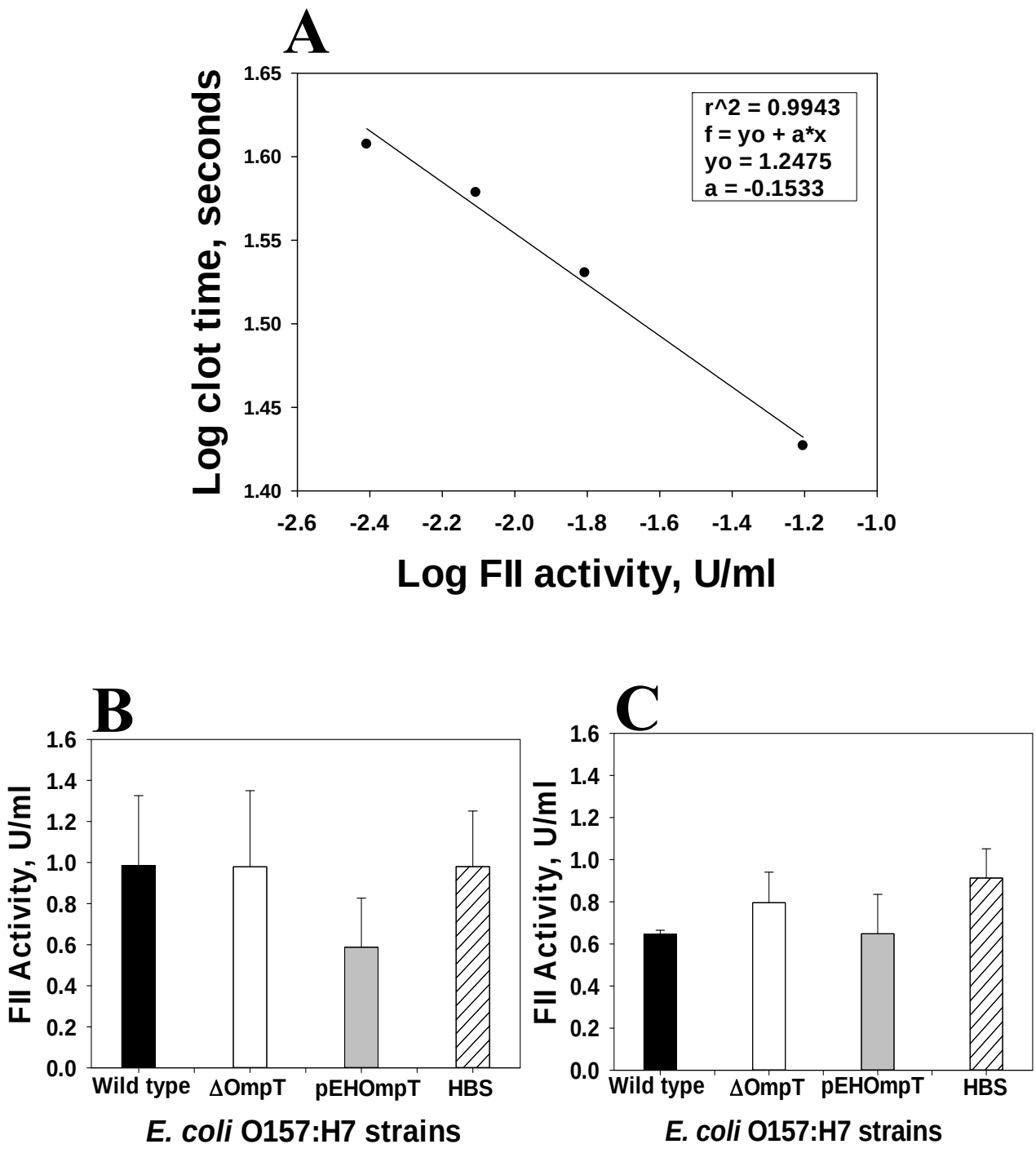

A
Log Clot time, seconds
1.45
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
r^2 = 0.84
f = yo +a*x
yo = 1.1114
a = - 0.2836
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
Log FBG activity, U/ml
B
FI Activity,U/ml
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Wild type
ΔOmpT
pEHOmpT
HBS
E. coli O157:H7 strains
C
FI Activity, U/ml
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Wildtype
ΔOmpT
pEHOmpT
HBS
E. coli O157:H7 strains

Figure 3.20 Prothrombin calibration curve and activity assay with EHEC whole cells and OMVs. The prothrombin standard curve was assayed with varying concentrations of NHP (0-fold to 1024-fold in HBS) using prothrombin deficient plasma based on the PT-based coagulation assay. In Panel A, the graph illustrates log FII activity (U/ml) versus log of time for clot formation (seconds). The data was plotted using the linear regression model which indicated a strong relationship between these two variables (r^2= 0.9943). The NHP was treated with the OmpT from OMVs (Panel B) and the cellular associated OmpT (Panel C) from the three EHEC strains used. The graph describes the FII activity remaining and EHEC strains (Wild type, ΔOmpT and pEHOmpT) and HBS control. The black bar refers to the wild type, the white bar to the ΔOmpT, the grey filled bar to the pEHOmpT strain while the stippled filled bar represents the HBS control.

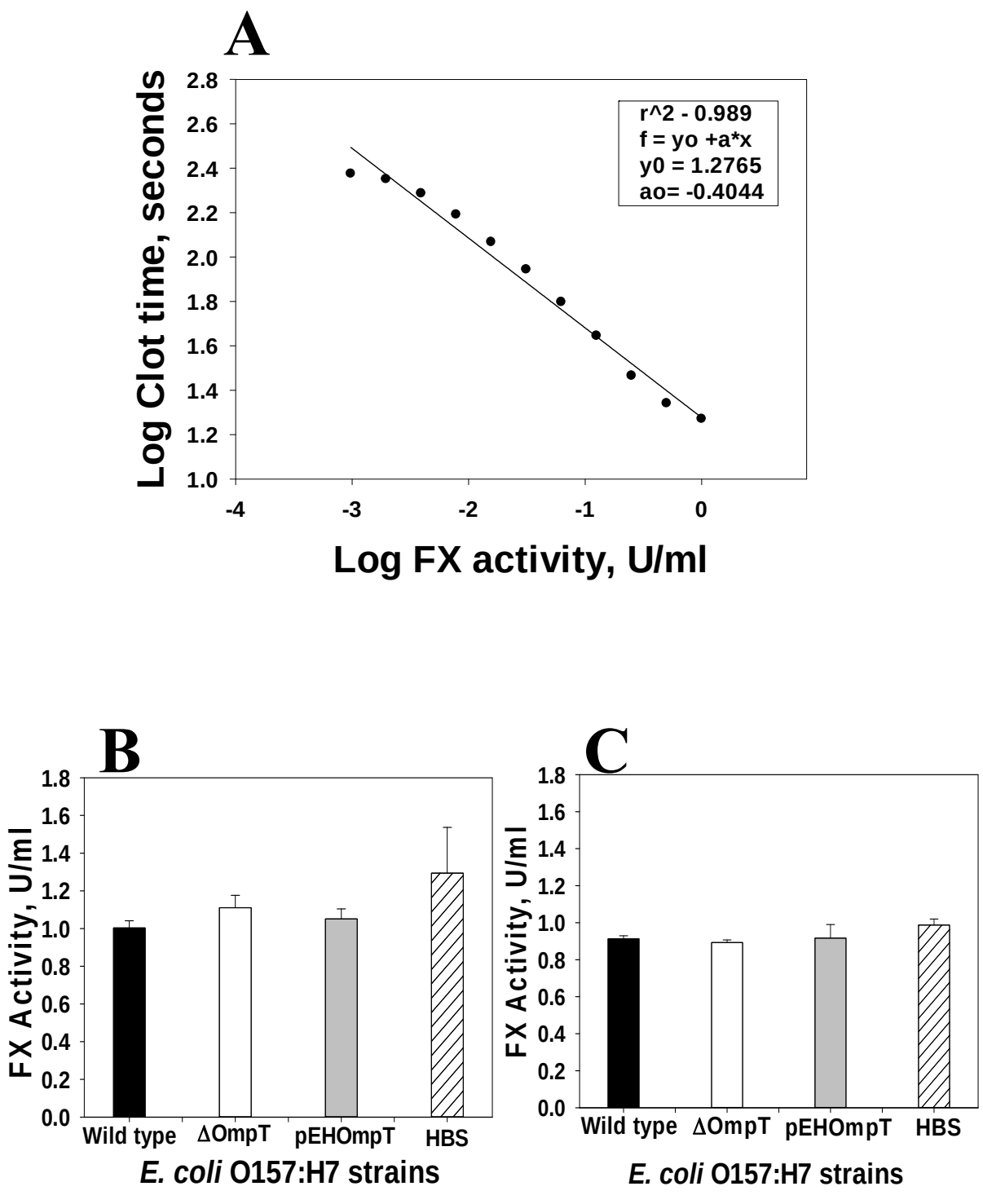
Figure 3.20


A
Log clot time, seconds
1.65
1.60
1.55
1.50
1.45
1.40
r^2 = 0.9943
f = yo + a*x
yo = 1.2475
a = -0.1533
-2.6
-2.4
-2.2
-2.0
-1.8
-1.6
-1.4
-1.2
-1.0
Log FII activity, U/ml
B
FII Activity, U/ml
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Wild type
ΔOmpT
pEHOmpT
HBS
E. coli O157:H7 strains
C
FII Activity, U/ml
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Wild type
ΔOmpT
pEHOmpT
HBS
E. coli O157:H7 strains

iii) Factor X coagulation factor assay: The Factor X (FX) microplate assay was performed as described previously for the other coagulation factors. The standard curve for FX is as shown in the Figure 3.21A. The normal concentration of FX is approximately10µg/ml (170nM) (Butenas & Mann, 2001). NHP was treated with 1 µg of EHEC OMVs or10^9 EHEC cells and remaining FX activity was determined with of the PT-based fibrin clot formation assay. As shown in Figures 3.21B and 3.21C, there was no significant difference in FX activity between the wild type and pEHOmpT cellular ($p= 0.375$, $p>0.05$) and OMVs associated OmpT ($p= 0.375$, $p>0.05$) compared to the effect of cells and OMVs from ΔOmpT and the HBS control. The EHEC cell and OMV OmpT did not significantly target FX for inactivation with any of the three strains used.

Figure 3.21 Factor X calibration curve and activity assay with EHEC cells and outer membrane vesicles. The Factor X (FX) standard curve was assayed with varying concentrations of NHP (0-fold to 1024-fold diluted in HBS, pH 7.4) using FX deficient plasma based on the PT-based coagulation assay. In Panel A, the graph shows log of FX activity (U/ml) versus log of time for clot formation (seconds). The data was plotted using the linear regression model which displayed a strong relationship between these two variables (r^2=0.989). NHP was treated with OmpT from EHEC OMVs (Panel B) and EHEC cells (Panel C). The graph illustrates the FX activity remaining and EHEC strains (wild type, ΔOmpT and pEHOmpT) and the HBS control. The black bar refers to wild type, the white bar to the ΔOmpT, the grey filled bar to pEHOmpT strains while the stippled filled bar refers to the HBS control.

Figure 3.21


A
Log Clot time, seconds
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
-4
-3
-2
-1
0
r^2 - 0.989
f = yo +a*x
y0 = 1.2765
ao= -0.4044
Log FX activity, U/ml
B
FX Activity, U/ml
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Wild type
ΔOmpT
pEHOmpT
HBS
E. coli O157:H7 strains
C
FX Activity, U/ml
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Wild type
ΔOmpT
pEHOmpT
HBS
E. coli O157:H7 strains

Chapter 4

Discussion and Conclusions

Discussion

Previous research has shown in a baboon sepsis model with *E. coli* O86a:K61 that there was a dose-dependent cleavage and inactivation of plasma FV (Samis *et al.*, 2007). Immunoblotting indicated that the FV was cleaved and inactivated from 330kDa to 250kDa in the baboon sepsis model which was consistent with proteolytic action of plasmin, neutrophil elastase, or a secreted protease from *E. coli* O86a:K61. It was later shown that a secreted protease from *E. coli* O86a:K61 was capable of cleaving and inactivating FV in human and baboon plasma. The protease was purified from culture supernatant of *E. coli* O86a:K61 and identified as OmpT by Tilley D (Masters Thesis, 2011).

In order to further analyse the effect of *E. coli* O86a:K61 OmpT on the blood coagulation process, the OmpT gene was blunt-end cloned into pUC18 vector, directionally cloned using BamH1 and HindIII into the same vector and then the construct was transformed into *E. coli* BL21 (DE3) cells. The OmpT gene insert was characterized by restriction digestion and sequenced by the dideoxy chain termination method. The sequence of the OmpT gene of *E. coli* O86a:K61 was compared with the omptin protease from other bacterial strains indicating that the OmpT gene from *E. coli* O86a:K61 was 90-99% identical at both the nucleic acid and amino acid levels to the OmpT gene of other *E. coli* strains. The OmpT gene from *E. coli* O86a:K61 was 47% similar to Pla protease gene from *Yersinia pestis* and 57% similar to SopA gene of *Shigella flexneri*. There were no significant changes at the amino acid level found in the conservative and non-conservative replacement/alterations that can alter the LPS binding and the active sites which can alter the activity of OmpT toward its protein substrates.

A research collaboration with Dr. Herve Le Moual (Associate Professor, Department of Microbiology, McGill University, Montreal, PQ), was established to study the effect of OmpT

on the coagulation process using a genetic approach with defined EHEC strains. This involved the use and comparison of the effect of EHEC wild type, an OmpT deletion mutant and an OmpT overexpression strains on the human blood coagulation system. These EHEC strains were also used to determine the effect of OmpT on the EHEC growth in liquid culture and OMV composition and biogenesis. In addition, these same EHEC strains were used for experiments to characterize the effect of cellular and OMV associated OmpT on FV and other coagulation factors.

The presence of the OmpT gene locus in EHEC strains was confirmed by PCR from wild type and pEHOmpT, but not from the ΔOmpT strain. In addition, the presence of the OmpT protein was confirmed using a Cro-P antibody by the immunoblotting in OMVs and cell lysates from the wild type and pEHOmpT and its absence from cell lysate OMVs from ΔOmpT. The results from these experiments were consistent with the EHEC strains constructed and characterized by Thomassin *et al.* 2012. These studies were also done to validate the approach employed for future experiments.

Gram-negative bacterial infections are a leading cause of severe sepsis and death worldwide, particularly in developing countries (Galdiero *et al.*, 2012). Gram-negative bacteria, such as *E. coli,* have been treated with antibiotics, but this micro-organism has evolved to become antibiotic resistant (Johnson *et al.*, 2005) and multi-drug resistant as well (Tadesse *et al.*, 2012). It was recently shown that children have developed a significantly increased rate of HUS from *E. coli O157:H7* infection when treated with antibiotics (Wong *et al.*, 2012) which can also induce the expression of Shiga toxins (Zhang *et al.*, 2000). *E. coli* is commonly found in humans, animals, water, and food and is responsible for causing extra intestinal infections, gastroenteritis, urinary tract infections, meningitis, peritonitis, and septicemia (Baum & Marre, 2005). *E. coli* uses

several virulence factors to evade the host immune system such as LPS and associated proteases. Virulence factors such as LPS and proteases can potentially target the blood coagulation system which is a part of host innate immune response (Galdiero *et al.*, 2012). LPS alone is toxic, damages tissue and is responsible for septic shock resulting in septicemia during the host innate immune response. LPS can also induce a host inflammatory response which prevents the bacteria from being phagocytosed by neutrophils (Todar, 2008). There have been discoveries made with LPS and its involvement in sepsis, but the proteases associated with it have not yet been explored (Galdiero *et al.*, 2012). In the present study, the OmpT protease, whose biological function remains unknown, was studied in detail and found to have on the OMVs biogenesis and composition and coagulation system as well. The effect of OmpT on the blood coagulation system needs to be further investigated and explored in future work to have a better understanding of how this secreted protease may alter this host system which is an effector arm of the innate immune response.

E. coli is ubiquitously found in the environment. EHEC is resistant to harsh environments and normally grows on any living or non-living surface (Davis and Kendall, 2012). The doubling time of *E. coli* was 20-30 minutes in LB media and 45-60 minutes to several hours in Minimal A media depending upon the concentration of glucose supplied in the media (Stanley *et al.*, 1998). The effect of OmpT on the growth of *E. coli* was determined in this thesis by using EHEC and BL21(DE3) in LB and Minimal A media. Cells were grown to OD_{600} of 0.15 in Minimal A media and up to OD_{600} of 3.0 in LB media, respectively. The reason for less growth in Minimal A media is that this media has a limited amount of nutrients such as glucose necessary for cell growth. Result in this thesis have shown that that OmpT over expression significantly impaired the growth of EHEC in Minimal media and BL21(DE3) in both LB and Minimal media

($p<0.001$). There are two possible reasons for OmpT over expression retarding cell growth. The first may be that given OmpT is an outer membrane protein, its higher than normal level expression may be toxic to cells. This observation is consistent with other studies which indicate that the overexpression of membrane proteins leads to the accumulation of protein aggregates such as chaperones, proteases, periplasmic proteins, and cytoplasmic proteins (Choi & Lee, 2004). Such event would hinder the AcO-pta pathway for ATP production and the TCA cycle to reduce the energy metabolism for ATP production (Wagner *et al.*, 2008). Secondly, OmpT over-expression into mRNA may compete with the other cellular protein's mRNA for translation which is essential for cell growth. This possibility is consistent with a study conducted that measured the growth of *E. coli* and indicated that 30% of proteins are replaced by overexpressed protein that down regulated translation (Dong *et al.*, 1995). Eventually, the ribosomes may be destroyed, which ultimately would lead to the cessation of cell growth. At the same time, the rRNA level would also decrease and mRNAs would compete for the ribosomes. This would in turn affect the growth (Dong *et al.*, 1995). This possibility was consistent with the generation time and growth rate constant as described here: EHEC pEHOmpT had a growth rate that decreased by 1.6-fold and generation time that increased by 1.6-fold compared to the wild type. EHEC pEHOmpT decreased the growth rate by 1.3-fold and increased the generation time by 1.3-fold compared with ΔOmpT. There was no significant difference in the growth rate and generation time between the EHEC wild type and ΔOmpT deletion mutant strains in nutrient poor media.

Plating of the EHEC strains on LB agar demonstrated differences in the resultant colony size. The ΔOmpT strain formed a larger colony compared to the wild type and pEHOmpT. The size of *E. coli* was further studied using Digital microscopy and indicated that there was no significant

difference between the length of the three strains ($p>0.05$). However, the EHEC ΔOmpT strain had a larger width than the wild type and pEHOmpT ($p<0.001$) and the pEHOmpT EHEC strain had a significantly larger width than the wild type ($p<0.05$).

It has been shown previously that *E. coli* 086a:K61 secretes outer membrane vesicles (OMVs) that attenuate the blood coagulation process (Tilley *et al.*, Masters thesis, 2011). It was shown in the present study that EHEC strains can secrete OMVs in the extracellular milieu of Minimal A media. The OMVs from the three EHEC strains were subjected to SDS-PAGE and silver staining to assess their protein and lipid composition. The results indicated that the OMVs from the EHEC wild type strain had more proteins (approximately 80) and stainable lipid than OMVs from the ΔOmpT and pEHOmpT strains. The lipid is required as LPS for OmpT as a co-factor and for proteolytic activity (Vandeputte-Rutten *et al.*, 2001). OMVs from EHEC ΔOmpT had a lower number of proteins (approximately 40) and low stainable lipid compared to the wild type. However, OMVs from EHEC pEHOmpT had an even lower number of proteins (approximately 5-10) and low stainable lipid content. This result indicated that alteration in OmpT expression alters both the protein and lipid composition of OMVs. Other studies have shown that the overexpression of protein inhibits the production of other cellular proteins due to the competition of mRNAs for translation (Dong *et al.*, 1995). This may explain, in part, how OmpT was the main protein expressed in OMVs from pEHOmpT. A 37kDa protein was present in OMVs of the wild type and pEHOmpT strain, but not in OMVs from ΔOmpT.

OMVs production from *E. coli* is induced by stressful growth conditions, T4 infection, the stress response to antibiotics treatment, internal stress such as production of misfolded proteins, and overexpression of periplasmic proteins (Loeb & Kilner, 1979; McBroom and Kuehn, 2007; Kulp & Kuehn, 2010). In the present study, *E. coli* was grown in Minimal A liquid media with a

limited supply of nutrients, thereby inducing the production of OMVs in the EHEC wild type and pEHOmpT. It was shown here by TEM that the wild type EHEC produced 60-80 OMVs per field; and OMV production was enhanced with the EHEC pEHOmpT strain, which produced 2000-3000 OMVs per field. OMV production was significantly reduced with the EHEC ΔOmpT strain which produced approximately 5-10 OMVs per field. The wild type EHEC strain produced a significantly larger number of different sized ranges of OMVs than ΔOmpT ($p<0.05$). However, the EHEC pEHOmpT strain produced an even larger number of different sizes of OMVs compared to the wild type ($p<0.001$). The experiments indicated that change in OmpT expression had a profound effect on OMV production and composition in EHEC strains used here. OmpT gene deletion not only decreased OMV production but decreased protein and lipid composition as well. OmpT gene over expression profoundly increased OMV production but decreased the protein composition and stainable lipid too.

The OMV production in EHEC strains was consistent with another study which showed that the vesiculation process was activated by an envelope stress pathway, by disrupting the peptidoglycan layer or by the outer membrane protein linking to the peptidoglycan layer (Kulp & Kuehn, 2010). In addition, immunoblotting showed that the OmpT was indeed present in the cell lysates and OMVs of the EHEC wild type and pEHOmpT strains but not in the EHEC ΔOmpT strain. It has been also shown that EHEC produced OMVs that were found in association with Shiga toxin I and II (Yokohama *et al.*, 2000). OMV associated with Shiga toxin I and II was potent and cytotoxic that resulted in cell death (Kim *et al.*, 2010). Besides being toxic to cells, OMVs may also serve as a protective shield against antimicrobial peptides and phage infections (Manning & Kuehn, 2011). A recent study has shown that OMVs without outer membrane proteins can be utilised as a vaccine for bacterial sepsis or *E. coli* infections, but this was

disadvantageous in that it posed a high risk of systemic inflammatory response syndrome (SIRS) when injected in higher doses and became toxic due to the presence of pattern recognition molecules. It is challenging to make a vaccine with this approach because *E. coli* expresses different types of outer membrane proteins (Kim *et al.*, 2013; Rompikuntal. P K, 2012).

Four to five different sized outer membrane proteins ranging from 10 to 35kDa are found associated with the LPS in gram negative bacteria (Galdiero *et al.*, 2012). It was also shown in another study that unidentified outer membrane proteins of apparent molecular weight of 5-9, 18 and 35kDa associated with LPS and are shed into human serum (Binkley, 1945). However, other studies identified more than 200 proteins associated with OMVs from Gram-negative bacteria such as porins (PorA, PorB, OprF), murein hydrolases (Met, SLT), multidrug efflux pumps (Mtr, Mex, TolC), ABC transporters (LamB, FadL), protease/chaperone proteins (DegQ, SurA), and motility proteins (FliC, PilQ) (Chatterjee & Chaudhuri, 2012). The research outlined here has discovered that OmpT, a protease with apparent molecular weight of 33.5kDa was found in association with OMVs released from EHEC. However, different studies showed that OMVs when detected by mass spectrometry do contain the cytoplasmic proteins when OMVs were collected from cells grown to stationary phase (Lee *et al.*, 2007). Conversely, OMVs did not contain the cytoplasmic and inner membrane protein from a Tol/Pal mutant of *E. coli* (Berlanda Scorza *et al.*, 2008). However, the present study has shown that the OmpT was present in whole cells as well and OMVs of the EHEC wild type and pEHOmpT but not ΔOmpT when cells were collected from the mid-log phase of growth in liquid media.

OmpT is an outer membrane protein, with active sites are facing the outer leaflet of the outer membrane. It requires LPS as a co-factor for proteolytic activity which determines the orientation of active sites and results in substrate cleavage between the dibasic amino acid

residues (Vandeputte-Rutten *et al.*, 2001). LPS along with outer membrane proteins are comparatively more toxic and potent compared to the effect of LPS alone as shown in animal sepsis models (Galdiero *et al.*, 2012). One study has shown that *E. coli* BL21(DE3) with pET28a+OmpT when incubated with antimicrobial peptide (LL-37) was not affected compared to the BL21(DE3) with pET28a alone indicating that OmpT showed increased resistance in *E. coli* Bl21(DE3) growth in the presence of antimicrobial peptide (Shi-lei *et al.*, 2010). In addition, outer membrane proteins affect the hemodynamics, blood coagulation, body temperature, cellular and humoral immunities, proliferation of B lymphocytes and macrophages, and release of endogenous mediators which leads to tissue pathology, severe sepsis, DIC and multiple organ failure (Levi *et al.*, 2004; Galdiero *et al.*, 2012). However, the precise function of OmpT associated with OMVs and bacterial cells remains to be elucidated.

The reason for using whole cells and OMVs for coagulation assays in this research is that the OmpT is present in the outer membrane of both structures would be expected to have access to coagulation factors in blood borne systemic infections. Thus, the OMVs and cells from EHEC were utilised to determine the effect of OmpT on blood coagulation. In the present study, the concentration of 10^7-10^9 cells/ml and 0.25-2 µg of OMVs were utilised to assess the effect on FV and also on other coagulation factors. The same concentrations of bacteria are attainable in the severe sepsis patient (Yun *et al.*, 2009) and 1-5µg of OMVs would be constitutively secreted by *E. coli* during sublethal septicimia (Park et al., 2010).

It was shown that the EHEC pEHOmpT cellular and OMV associated OmpT showed a significant prolongation of the PT and aPTT clot times compared to the cells and OMVs from the wild type and ΔOmpT strains. The EHEC pEHOmpT cellular (10^{10}cells) and OMV (2µg of protein) associated OmpT completely abolished the clot formation in the aPTT assay which has

not been reported previously. Similarly, another study has shown prolonged PT and aPTT in neonatal diarrhoeic calves with DIC infected from *E. coli* infection (Gokce *et al.*, 2006) and in septic condition in humans (Chandrashekar, 2011). Another study has shown that LPS can also prolong the PT and APTT in chickens (Pliszczak-Krol *et al.*, 2012). On the other hand, *Streptococcus pyogenes* can prolong the APTT clot time but does not affect the PT when injected into mice (Loof *et al.*, 2011). Therefore, it is concluded that *E. coli* and other micro-organisms could target specific coagulation factors for inactivation. Initially, coagulation factors, such as fibrinogen, FII, FV, and FX, involved in the common pathway were tested for cleavage and inactivation using microplate based clotting assays. These factors were examined initially because they belong to both the intrinsic and extrinsic pathways. The effect of *E. coli* cell and OMV associated OmpT on coagulation factors involved in the intrinsic and extrinsic pathways will need to be tested in future studies.

The kinetic microplate-based FV activity assay is a novel, fast, economical, simple, high quality, has high throughput, and requires small sample volumes (Tilley *et al.*, 2011). In addition, the same microplate PT-based coagulation assay was implemented for analysing the effect of EHEC cell and OMV OmpT on coagulation factors of the common pathway such as FI, FII, and FX. Finally, FV inactivation by EHEC cell and OMV associated OmpT was compared between NHP and PLG-deficient human plasma.

The FV microplate assay was performed with OmpT associated with OMVs and cells from EHEC strains. The normal concentration of FV in human plasma is 20-40nM (Mann & Kalafatis, 2011), and one unit/ml of human plasma corresponds to approximately 20nM (Tracy *et al.*, 1982). The threshold value of FV required for clot formation is 1nM (Mann, 2000). In order to perform the FV assay, normal human plasma was diluted 20-fold to a final concentration of

1-2nM FV which is sufficient for a clot formation event. The FV was inactivated by 40%/μg of protein with OMVs and cells from the EHEC wild type (0.4-0.8nM of FV remaining) and 90-95%/μg of protein with OMVs and cells from the EHEC pEHOmpT strain (0.05-0.1nM of FV remaining), which was significantly higher than OMVs and cells from EHEC ΔOmpT strain (5-10%/μg of protein) ($p<0.05$). This finding is consistent with FV inactivation below 1-5% (0.2-1nM of FV), which would significantly delay the thrombin generation in NHP (Mann, 2000). As the OMVs were not completely pelleted from the culture supernatants by ultracentrifugation, the supernatant fraction were also studied for their effect on FV in human plasma using the FV microplate assay. Similarly, the FV in human plasma was significantly inactivated by the supernatant fractions after ultracentrifugation by approximately 40%/μg of EHEC wild type OMVs, 90%/μg of EHEC pEHOmpT OMVs and this effect was completely abolished with EHC ΔOmpT OMVs ($p<0.05$). Hence, a similar inactivation effect on FV in human plasma was observed with the OMVs in pelleted and supernatant fractions. OMVs from the pelleted fractions had a higher concentration of protein than those in the ultracentrifuged supernatants and were employed for all the other experiments.

There was no significant difference in the extent of clot formation between the EHEC cells and OMVs from the wild type, ΔOmpT and pEHOmpT, although the EHEC pEHOmpT cells and OMVs also decreased the initial rate of fibrin formation compared to the EHEC cells and OMVs from the wild type and ΔOmpT. These findings are in complete agreement with FV inactivation as shown in the baboon sepsis *E. coli* model from Samis *et al.* (2007). It was shown that FV deficient mice demonstrated increased mortality with streptococcal infection compared to the FV Leiden mutation mice or the wild type control mice (Sun *et al.*, 2009). Moreover, the plasma concentration of FV in mice is 4.6-fold higher (92nM) than in humans with this condition (Mann

2000); therefore, mice deficient in FV demonstrate increased mortality compared to the humans which show a severe bleeding tendency and a hemorrhagic condition (Yang *et al.*, 2000). One study has shown that FV is also targeted for cleavage and inactivation by culture supernatant fractions of *Acinetobacter baumannii* clinical isolates (Tilley *et al.*, 2012) indicating that the FV in plasma is also targetted by different bacteria to evade this part of the host immune response. Also, FV is a critical regulator of thrombin generation and fibrin clot formation (Mann & Kalafatis, 2011); its over-activation leads to thrombosis and deficiency results in a bleeding disorder. It was also shown previously that the EspP from *E. coli* O157:H7 can cleave FV in human plasma (Brunder *et al.*, 1997), but FV activity assay were not performed to determine the effect on FV function. In the work reported here, there was no significant FV inactivation with EHEC OMVs and cells from the ΔOmpT strain, indicating other proteases from EHEC did not have a inactivation effect on FV in NHP.

FV cleavage by OmpT was also demonstrated by immunoblotting. NHP was treated with either EHEC OMVs or whole cells. EHEC cellular and OMV associated OmpT from the EHEC wild type inactivated FV by 60% in 2 hours and pEHOmpT inactivated FV by 95% in 2 hours. No FV was inactivation was observed by EHEC cells and OMVs from ΔOmpT. The FV was cleaved from 330kDa to 250kDa by EHEC cellular and OMV OmpT from the wild type and pEHOmpT strains and pEHOmpT cells cleaved the 250kDa species further to a 150kDa product. These results are consistent with the findings from the baboon sepsis *E. coli* model which showed a dose-dependent inactivation of FV (Samis *et al.*, 2007) and also with studies conducted for FV inactivation using culture supernatant of *E. coli* 086a:K61 by Tilley *et al.* (2011). Thus, these results show collectively that OmpT, either on the outer cell membrane or as a part of OMVs, specifically targets FV for inactivation to attenuate blood coagulation in order block fibrin

formation to enhance pathogen growth and spread. It was shown in the present study that EHEC secretes OMVs as a part of virulence mechanism to attenuate the blood coagulation process by OmpT dependent inactivation of FV in human plasma.

The coagulation pathway is also controlled by plasmin, a protease involved in the fibrinolysis pathway. The function of plasmin is to dissolve fibrin barrier to fibrin degradation production (FDPs) to restore normal blood flow after injury. However, FDPs interfere with thrombin generation (Weitz *et al.*, 1998) and plasmin interferes with clot formation by inactivating FV, FVIII, FIX, and FX as described by Hoover-Plow (2010). Thus, it was not clear whether EHEC OmpT was directly inactivating FV or indirectly inactivating FV by activating plasminogen to plasmin. OmpT has been shown by others to be a plasminogen activator (McCarter *et al.*, 2004) that contradicts another observation made by a different study which showed that OmpT was a poor plasminogen activator (Haiko *et al.*, 2009). Hence, the involvement of plasmin was examined by comparing the inactivation of FV by EHEC cells and OMVs using PLG deficient human plasma and NHP. FV was inactivated with EHEC cells and OMVs in the PLG-deficient plasma to similar levels observed in NHP. However, EHEC ΔOmpT cells and OMVs did not inactivate FV in both NHP and PLG deficient plasma and the FV inactivation effect was enhanced with pEHOmpT whole cells and OMVs over and above that observed with wild type EHEC cell and OMVs. There was no significant difference in the resultant FV activity by EHEC cells and OMVS associated OmpT with both PLG-deficient plasma and NHP. Therefore, this data indicates that OmpT does not utilise host plasmin for an indirect inactivation effect on FV. Inactivation by OmpT EHEC cell and OMV is direct and does not involve the host plasminogen system. This finding is consistent with the observation made by Haiko *et al.* (2009) which showed that OmpT is a poor plasminogen activator. The OmpT inactivation of FV reported here

is different from the actions of plasminogen activator (Pla) protease from *Yersinia pestis* (Sodeinde *et al.*, 1992), streptokinase (SK) from *Streptoccocus pyrogenes* (Sun *et al.*, 2004), and staphylokinase from *Staphylococcus aureus* (Esmon & Mather, 1998) which all down regulate fibrin formation by activating host plasminogen to plasmin.

OmpT protease activity versus other coagulation factors (Fibrinogen, FII, & FX) involved in the common pathway was also examined here. The OMVs and cellular associated OmpT from wild type EHEC did not inactivate fibrinogen, while OmpT in OMVs and cells from pEHOmpT inactivated fibrinogen by approximately 40-50%. This suggests that the pEHOmpT may also inactivate the fibrin barrier directly when it is highly expressed without using the host plasminogen system. This finding is consistent with the observation that *Bacteroides fragilis* can reduce the fibrin barrier using a putative fibrinogen binding protein (BF-FBP) as virulence factor for dissemination and during clinical infection (Houstan *et al.*, 2010). However, FII and FX were not significantly inactivated by the OmpT from OMVs and cells from the EHEC wild type, ΔOmpT and pEHOmpT ($p>0.05$). Thus under the condition used here, EHEC cell and OMV OmpT can specifically target and inactivate FV and to some extent fibrinogen when the OmpT gene is highly expressed.

Conclusion

In summary, EHEC is a Gram-negative bacterium that can cause sepsis in humans. OmpT is involved in OMVs biogenesis and production and its altered expression alters OMV lipid and protein composition as well. The EHEC pEHOmpT had a slower growth rate constant and longer generation time and grew significantly slower than the EHEC wild type and ΔOmpT strain in the Minimal A liquid media, but the difference in growth between strains was not significant in the LB nutrient rich media. The OmpT gene was present in the wild type and pEHOmpT but not in the ΔOmpT strain. In addition, OmpT protein expression was consistent with the presence or absence of the genetic locus: OmpT protein was expressed in the EHEC wild type and pEHOmpT in cell lysates and OMVs, but not in the EHEC cell and OMVs from the ΔOmpT strain. The fact that the OmpT can prolong the PT and aPTT clot time implies that it attenuates the blood coagulation system by targeting and inactivating clotting factors involved in the extrinsic and intrinsic pathways, respectively. The results indicate that OmpT is a potent virulence factor associated with the outer membrane of EHEC which can attenuate blood coagulation by inactivating FV and to some extent fibrinogen, but not the other factors such as FII or FX of the common pathway. The OMVs and cellular associated OmpT from the EHEC wild type inactivated FV by 60% in two hours and the effect was enhanced with OMVs and cells from the EHEC pEHOmpT strain which inactivated FV by 90% in two hours. Virtually no inactivation was observed with the cells and OMVs from EHEC ΔOmpT strain. OmpT renders FV inactive by cleaving it from 330kDa to 250kDa as revealed by immunoblotting. In addition, OmpT does not inactivate FV indirectly by engaging and activating the host plasminogen system. The functional effect of OmpT from the EHEC strains used here on the coagulation process needs to be investigated in future studies.

Impact and Significance of the Research

EHEC express OmpT proteolytic activity as a part of the OMVs or as a part of the outer membrane protein in whole cells to overcome the fibrin barrier function to evade the host innate immune response. Since FVa is a critical factor responsible for the *prothrombinase* complex mediated generation of fibrin, OmpT inactivation of FV would be expected to attenuate the coagulation process to maximize pathogen growth, survival, and transmission. This research provides in-depth knowledge of how EHEC evades the host innate immune response by specifically targeting coagulation factors such as FV and to some extent Fibrinogen (FI) for inactivation, but not other factors (FII, and FX). This research will lead to a greater understanding of *E. coli* virulence in humans and to the development of new vaccines, antimicrobial drug targets, diagnostic techniques, and treatments to combat EHEC infections of humans in the future.

Chapter 5

Future Directions

1) The effect of EHEC OmpT (Whole cells and OMVs) may be studied with the factors involved in intrinsic pathway of coagulation such as FVIII, FIX, FXI, FXII, prekallikrein and high molecular weight kininogen. Since FXIII is involved in the cross-linking of fibrin, it could be can also be analysed to determine whether it is also susceptible to inactivation by cellular and OMV associated EHEC OmpT.
2) The effect of OmpT (Whole cells and OMVs) may be studied in whole blood. The effect of OmpT on clot formation in whole blood could be assessed upon addition of defined amounts of protease to citrated human blood for various times at room temperature. Then, either thromboplastin and $CaCl_2$ (25mM) or the aPTTreagent and $CaCl_2$ would be added to assess the time for clot formation via the extrinsic and intrinsic pathways, respectively. These experiments would address whether EHEC cells and OMV associated OmpT significantly alters the time for clot formation in whole human blood. Such information would provide the basis for protease functional inactivation of the coagulation system in a relevant biological fluid containing blood cells.
3) The effect of EHEC OmpT (Whole cells and OMVs) on fibrin clot formation and clot lysis in human plasma could also be studied in future work. Thrombin and $CaCl_2$ or thrombin, $CaCl_2$ and a tissue plasminogen activator (tPA) could be added to determine the effect of EHEC OmpT on clot formation and clot degradation, respectively. The initial rate and extent of fibrin formation and degradation could also be evaluated as part of this work in the future.
4) The effect of EHEC OmpT on thrombin generation in human plasma using fluorescently tagged prothrombin with whole cells and OMVs from the three EHEC strains could also

be explored in future work. This approach could be also used as a rapid screening assay to measure thrombin generation with OMVs and cells different EHEC strains.

5) The proteins present in the OMVs from the three EHEC strains could also be determined by comparative proteomic analysis with 2 dimensional electrophoresis gel and mass spectrometry.
6) The lipid composition in the OMVs from the three different EHEC strains could also be determined by using gas chromatography in combination with mass spectrometry identification and quantification.
7) A long term goal for this research is to identify and charaterize different proteases from the other micro-organisms such as viruses and fungi and determine their effect on the blood coagulation system.

Chapter 6

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