The identification and characterization of bacterial and host epithelial factors involved in the pathogenesis of invasive *Francisella tularensis* and extracellular *enteropathogenic E. coli* (EPEC)

by

Hong Ting (HT) Law

B.Sc. (Hons), Simon Fraser University, 2008

Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

in the Department of Biological Sciences Faculty of Science

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Spring 2015

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Abstract

Enteropathogenic Escherichia coli (EPEC) and Francisella tularensis possess a toolkit of virulence factors that allow them to adapt to the host environment and prevail over the body’s natural defenses in different ways. For diarrheagenic EPEC, a type III secretion system is used to inject virulence factors directly into the host cell allowing the extracellular microbe to co-opt key host processes and generate motile ‘pedestal’-shaped structures at the site of intimate bacterial-host contact. Because many of the same principles that drive the biogenesis and movement of EPEC pedestals are paralleled at the leading edge of migrating cells, I used EPEC pedestals as a model of the leading edge and developed a strategy to identify pedestal proteins and tease out their biological functions. Using mass spectrometry-based proteomics of concentrated pedestal preparations, I identified 17 highly abundant novel proteins as well as 11 previously known pedestal proteins. One of the identified molecules, nexilin, was characterized in depth. Using EPEC and Listeria monocytogenes as bacterial models for actin-based dynamics, I revealed that nexilin is concentrated towards the rear of pedestals and Listeria comet tails when these actin-rich structures become motile. The use of siRNA-mediated knockdowns further suggested that depletion of nexilin results in unusually thin and short filamentous comet tails. Another pathogen that can colonize non-phagocytic cells is F. tularensis—the etiological agent of Tularemia. Here, I examined the internalization process and intracellular fate of Francisella in hepatocytes. To study the strategy that F. tularensis uses to invade epithelial cells I developed in vitro infection models and used those models to uncover clathrin, its associated endocytic components and cholesterol as key molecules needed for F. tularensis internalization. Finally, I elucidated the role of two Francisella virulence factors (IglC, PdpA) and showed that both PdpA and IglC are needed for the efficient invasion and intracellular growth of F. tularensis. Taken together, I have identified multiple novel targets co-opted by the extracellular EPEC and the intracellular pathogens L. monocytogenes and F. tularensis that play a central role in their pathogenesis such as the actin associated protein nexilin and clathrin endocytic components clathrin, epsin1, and Eps15.
**Keywords:**  EPEC; *Francisella tularensis*; Clathrin-dependent endocytosis; Actin cytoskeleton; Epithelial cells; Host-pathogen interactions
I’d like to dedicate this thesis to my fiancé Stephanie Cheung who has opted to live in Vancouver as I complete this significant chapter of my life. To my parents and grandmother and late grandfather, for your resolute support of my career goals.
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I want to express my gratitude to all the past and present Guttman lab members (Dr. Ann Lin, Tyson Ruetz, Karen Lo, Dr. Kuo Ming Yeh, Dr. Ka Yin Leung, Byron Tenkink, Michael Chua, Priyanka Aggarwal, and Aaron Dhanda). We’ve shared some epic events that will likely be told in the Guttman lab for many years to come. Also, I want to recognize all of the undergraduate students that I helped supervise (Aarati Sriram, Aaron Dhanda, Brian Quach, Charlotte Fevang, Jeremy Jackson). Having you guys in the lab was a lot of fun and I am very fortunate to have such kind and hard working students over these past several years.

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I am very grateful to my committee members Dr. Margo Moore and Dr. Wayne Vogl for being outstanding mentors and as role models that I deeply cherish.

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<th>Description</th>
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<tbody>
<tr>
<td>A/E</td>
<td>Attaching and effacing</td>
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<td>Focal adhesion kinase</td>
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FPI  *Francisella* pathogenicity island
GAP  GTPase-activating proteins
GEF  Guanine exchange factor
Grb  Growth factor receptor-bound protein
h  Hour(s)
Hcp  Hemolysin coregulated protein
I-BAR  Inverted Bin/Amphyphysin/Rvs
IBD  Intimin-binding domain
ID  Infectious dose
ID  Infectious dose
Igl  Intracellular growth locus
Inv  Invasin
IRSp53  Insulin receptor substrate protein of 53kDa
IRTKS  Insulin receptor tyrosine kinase substrate
IVOC  In vitro organ culture
kb  Kilobase pair
kDa  Kilodalton
*L. monocytogenes*  *Listeria monocytogenes*
LAMP1  Lysosomal-associated membrane protein 1
LEE  Locus of enterocyte effacement
LPS  Lipopolysaccharide
LVS  Live vaccine strain
Map  Mitochondria-associated protein
Mb  Megabase pair
min  Minute(s)
MOI  Multiplicity of Infection
N-  Amino-
N-WASP  Neural Wiskott-Aldrich syndrome protein
NADPH  Nicotinamide adenine dinucleotide phosphate-oxidase
Nck  Non-catalytic region of tyrosine kinase adaptor protein
NHE1  Sodium/Hydrogen exchanger 1
Nle  Non-LEE effector
ORF  Open reading frame
P. aeruginosa  $Pseudomonas$ aeruginosa
Pdp  Pathogenicity determinant protein
PI  Post-infection
PI3K  phosphatidylinositol 3-kinase
PI3K  phosphatidylinositol 3-kinase
PI3K  Phosphatidylinositol 3-kinase
ROS  Reactive oxygen species
S. enterica  $Salmonella$ enterica
S. flexneri  $Shigella$ flexneri
Salmonella Typhimurium  $Salmonella$ enterica serovar Typhimurium
Sep  Secretion of $E$. coli proteins
SFU  Simon Fraser University
SGLT  Sodium-glucose linked transport
SH  SRC homology
ssp.  Subspecies
T3SS  Type III secretion system
T6SS  Type VI secretion system
TccP  Tir cytoskeleton coupling protein
TER  Transepithelial resistance
Tfp  Type IV pilus
Tir  Translocated intimin receptor
TJ  Tight junctions
TLR  Toll-like receptor
TSA  Tryptic Soy Agar
TSB  Tryptic Soy Broth
V. cholera  $Vibrio$ cholerae
VCA  Verpolin, Cofilin, Acidic
Vgr  Valine-glycine repeat protein
Vir  Virulence gene
WICH  WIP-CR16-homologous protein
WIP  WASP-interacting protein
WIRE  WASP-interacting protein-related
WT  Wildtype
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Chapter 1.

Introduction

1.1. Enteropathogenic *Escherichia coli* (EPEC)

Enteropathogenic *Escherichia coli* (EPEC) is one of several different pathotypes of diarrheagenic *E. coli*. Phenotypic virulence traits characteristic of EPEC are defined by their ability to intimately adhere to enterocytes on the luminal surface, clear the surrounding microvilli and form characteristic ‘pedestal’-shaped lesions.

EPEC is typically transmitted from person to person through the ingestion of feces in contaminated food or water \(^1,2\). Once ingested, EPEC preferentially colonizes the small intestine where it typically remains extracellular. This can lead to profuse watery diarrhea usually within a few days \(^3\). EPEC-generated diarrhea can persist for more than 2 weeks \(^4\) leading to excessive fluid and electrolyte loss, which can result in death, particularly in children 5 years of age and younger \(^5\). In the first half of my introduction, I will provide general information on EPEC, its pathogenesis, and its virulence factors.

1.1.1. Early discoveries that defined EPEC as an ‘attaching and effacing’ pathogen

In the early 1900’s, *E. coli* were considered harmless bacteria. It was not until 1955 that the term EPEC was coined to refer to an aetiological agent \(^6\) responsible for a rash of neonatal diarrhea outbreaks in the 1940’s. The earliest EPEC outbreak can be traced back to 1943 during the period known as ‘Summer diarrhea’ because the highest frequency of incidences coincided during the hot summer periods. \(^7,8\). Erroneously, a number of early studies in the 60’s and 70’s perceived EPEC as a bacterium with invasive and toxigenic properties comparable to enterotoxigenic *E. coli* (ETEC), which at
the time was widely known to cause traveller’s diarrhea \(^9,10\). To quell this misconception, Levine and colleagues (1978) initially tested a set of EPEC strains that were isolated from infants with persistent diarrhea and found that none of the strains were invasive or toxin producing. Then the authors challenged healthy adult volunteers with these EPEC strains and, by day 2, nearly all of their subjects developed profuse diarrhea \(^11\). This was the first comprehensive study that satisfied Koch’s postulates in validating the causative relationship between non-toxigenic and non-invasive EPEC and diarrheal disease.

In the 1980’s, various ultrastructural studies on EPEC-infected intestinal biopsy samples substantiated the microbe’s extracellular pervasiveness \(^12-15\). Importantly, this technique also highlighted its remarkable ability to dramatically alter the cellular landscape along the apical region of enterocytes \(^13\). The term ‘attaching and effacing’ (A/E) lesions was conceived to reflect the characteristic sites where EPEC tightly ‘attached’ to the epithelium and ‘effaced’ the surrounding microvilli \(^13\). Later discoveries of other intestinal bacteria that possessed the same general virulence traits \(^16\) led to the classification of a family of pathogens, called ‘A/E bacteria’.

The EPEC pathotype was originally defined using a screening process known as serotyping. At least in the research setting, this method is not an accurate method to identify EPEC strains because there have been multiple instances where non-EPEC bacteria have tested positive even though they do not possess key virulence traits that are representative of EPEC. An example of this was demonstrated by Knutton and colleagues (1991). During EPEC infections the microbes concentrate actin filaments at the region where EPEC attach to the host cells. In that study Knutton and co-workers used a fluorescence actin staining test to identify those concentrated sites of actin accumulation beneath the site of EPEC attachment and found that only a fraction of ‘EPEC’ serotypes were able to recruit actin to those sites. Their results surmised that serotyping is an inaccurate method for distinguishing EPEC pathotypes \(^17\). More recently, it appears that some atypical EPEC strains are not able to accumulate actin at sites of EPEC attachment in cultured cells, but can do so \textit{in vivo} or \textit{ex vivo} \(^18,19\). Currently, genetic sequencing is commonly used to define EPEC based on the presence of virulence genes \textit{eae} (intimin), \textit{bfp} (bundle forming pili), and absence of \textit{stx} (shiga toxin).
1.1.2. Epidemiology and treatment

Diarrheal disease in children younger than 5 years of age account for ~11 million deaths worldwide per year. EPEC is endemic in some of the poorest regions in the world, especially those without adequate access to clean water and sanitation. Current figures estimate that EPEC is the second leading cause of diarrhea-related deaths (or 7-15%) in infants and young children under the age of five in many developing countries. The decline in the prevalence of EPEC, which was once considered the foremost cause of infantile diarrhea, may be attributed to increased awareness and compliance with basic hygiene measures.

In industrialized countries, EPEC outbreaks are very rare. Epicentres where these infections are more likely to occur are in places such as day cares, nurseries, and even high schools. Unfortunately, information regarding the frequency of EPEC infections in developed countries is scarce. The lack of a national surveillance program for tracking EPEC infections in industrialized countries and the fact that EPEC carriage can occur in apparently healthy adults are two major challenges that researchers face when trying to understand its epidemiology. The infectious dose (ID) of EPEC in an adult is understood to be $10^8$ bacteria or higher, but presumably this is much lower in infants. By contrast, other enteric pathogens for example Listeria monocytogenes and enterohemorrhagic E. coli (EHEC) require as few as ten microorganisms to cause disease.

The recommended treatment protocol for early onset of diarrhea entails rehydration of the patient to restore electrolyte and fluidic balance. Unless absolutely necessary, patient care providers typically withhold antibiotic treatment for acute gastroenteritis due to growing concerns regarding antibiotic resistance. Superfluous use of antibiotics over the past several decades have contributed to the global emergence and spread of drug resistant populations of EPEC and other pathogenic microbes. In fact, multidrug-resistance has been detected in up to 60-70% of cases of EPEC-induced diarrhea in infants and young children under the age of 5.
1.2. Mechanisms of EPEC virulence

A/E pathogens share a common genetic cluster called the locus of enterocyte effacement (LEE). The LEE is located within the bacterial chromosome and is central for the virulence of all A/E pathogens. As would be expected, the LEE has been identified in not only EPEC but other members of the A/E family including *Citrobacter rodentium* and EHEC. This pathogenicity island encodes all of the necessary components of the type III secretion system as well as many virulence factors, chaperones, and transcriptional regulators. A number of virulence genes have been found outside of the LEE. For example, virulence genes encoded in the large extrachromosomal DNA, referred to as the EPEC adherence factor (EAF) plasmid, enhance the colonization of EPEC in cultured epithelial cells and in the small intestine of colostrum-deprived piglets. Consistent with this, just 2 out of 9 healthy human volunteers infected with an EPEC mutant, which lacked the EAF plasmid, developed diarrhea compared to 9 out of 10 subjects that were infected with WT EPEC. This established that genes encoded within the EAF plasmid and linked to intestinal adherence are required for EPEC to be fully virulent in humans. Because most of our understanding of the underlying pathogenic mechanisms are derived from typical EPEC stains, which carry the EAF plasmid, I will refer to all typical EPEC strains simply as ‘EPEC’ for the purpose of this thesis.

1.2.1. Bundle forming pili (BFP)

The EAF plasmid contains a *bfp* locus that encodes core components of the type IV bundle forming pili (BFP). BFP are rope-like appendages that emerge from the bacterial surface and function to promote microbial autoaggregation and microcolony formation. Though EPEC appears to utilize a variety of ‘adhesin’ molecules to attach onto the host epithelium, the most well studied EPEC adhesin is BFP. BFP is one of two known fimbriae (aka. pili) encoded by this extracellular pathogen. Studies in human volunteers have shown that *bfpA*+ EPEC are more virulent and have a greater capacity of adhering to the intestinal epithelium, compared to BFP deficient mutants. Cell culture models have also been exploited in an attempt to understand the role of BFP in the pathogenesis of EPEC. During the early stages of EPEC attachment, BFP are able
to make contact with neighbouring bacteria as well as epithelial cells. Interactions between pilin, the major subunit of BFP, and a number of molecules on the host surface (ie. phosphatidylethanolamine and N-acetyllactosamine) have been identified. Moreover, these interactions were shown to enhance initial bacterial attachment to the host plasma membrane. Juxtaposed to the epithelial cell surface, BFP filaments autoaggregate to form a sizeable network that tethers neighbouring bacteria together. This observable phenotype is common among EPEC strains and is referred to as a ‘localized adherence’ pattern. By contrast, atypical EPEC isolates—that lack BFP—show different localization patterns depending on the adhesin factors encoded by those microorganisms.

Within the BFP are thousands of pilin proteins that assemble in the bacterial periplasm and can extend up to 40 μm into the surrounding environment where it can interact with other bacteria and host cells. Evidence has shown that deletion of bfpA, the gene encoding pilin, abolishes the ability of EPEC to produce BFP and generate microcolonies resulting in dramatic impairment of bacterial adherence and ~200-fold attenuation in virulence. By contrast, upregulation of bfpA can enhance bacterial colonization possibly through binding to phosphatidylethanolamine lipids and/or specific glycosidic residues on host cell membrane. This can be accomplished by exposing EPEC to exponential-phase growth and environmental factors that resemble the luminal milieu within the small intestine such as low ammonium and calcium levels, and 37°C temperature. It is also possible that these factors may influence the expression of other BFP genes. For example, BfpF is a putative ATPase that facilitates the retraction of BFP toward the microbial surface. Recent evidence points to BfpF expression as being an important player for efficient bacterial colonization and effector translocation. Together, these observations are consistent with BFP playing a major role in the persistence of adherent EPEC in the intestinal epithelium and bacterial virulence.

1.2.2. **E. coli common pilus (ECP)**

A number of other EPEC surface molecules have been implicated as adhesin factors in early bacterial-host cell contact, but the details regarding their molecular mechanisms is still lacking. One of these molecules is the bacterial fimbriae known as the *E. coli* common pilus (ECP). As its name suggests, the ECP is found widely among
non-pathogenic as well as pathogenic *E. coli* such as EHEC and ETEC. With EHEC, ECP expression improves its adherence to epithelial cells, while in EPEC bacterial-host cell attachment was only significantly enhanced when BFP is absent \(^{47,56}\). These findings suggest that early attachment of EPEC to host cells is facilitated by BFP, and perhaps ECP as well. Another adhesin molecule that has been proposed is EspA—the major subunit of the type III secretion needle complex. Though there is little evidence to support this claim \(^{47,57}\), the role of EspA as an adhesin appears to be ancillary to its indispensable involvement in facilitating effector translocation.

### 1.2.3. Locus of enterocyte effacement (LEE)

The first pathogenicity island identified in EPEC was a \(~35\text{kb}\) region from the prototypical strain E2348/69 \(^{16}\). This was later designated as the LEE because many of the genes within this gene cluster were found to be indispensable for the A/E phenotype and virulence \(^{16,58}\). In fact, the organization of genes within the LEE appears to be generally conserved among A/E pathogens such as EPEC, EHEC, and *C. rodentium* \(^{16,59,60}\). Since its discovery, much of the research on EPEC has been focused on describing the functions of protein-encoding genes within the LEE \(^{16,61-68}\). In the subsequent sections, I will describe many of the gene products encoded within the LEE that are important for A/E lesion formation and subversion of host cell functions.

### 1.2.4. Type 3 secretion system (T3SS)

The T3SS is a syringe-like structure important for delivering a battery of disease-causing proteins from many pathogenic gram-negative bacteria into eukaryotic cells \(^{69}\). In EPEC and other A/E pathogens, over a dozen genes located within the LEE are known to encode for the T3SS. While many of these genes are generally conserved among A/E bacteria, other genes that encode for type III secreted virulence factors, called ‘effectors’, are more variable \(^{70}\). Various reports have not only characterized the T3SS in detail, but have also examined the intrinsic and extrinsic factors that regulate effector delivery. Research has shown that type III secretion activity is influenced in response to a diverse array of external cues including calcium, ammonium, and temperature \(^{71,72}\). These findings suggest that EPEC can sense its surroundings and promote bacterial colonization when the environmental conditions are favourable \(^{71,73}\).
Though details of how these signals are deciphered at the molecular level are still elusive, mounting evidence has revealed that T3SS assembly and effector translocation occurs in a hierarchical fashion and is regulated through a combination of post-translational and transcriptional factors.

The basal body is the portion of the type III secretion apparatus that spans the bacterial cell wall (Figure 1.2). At the outer membrane, monomeric EscC proteins assemble into a ring-shaped structure in the lipid bilayer. Findings have shown that the outer membrane ring connects to the inner rod of the translocation needle, which is comprised of EscF oligomers. As for the rest of the basal body, evidence suggests that EscJ and EscD form the inner membrane rings. Within the periplasm, Escl oligomerizes to form the inner rod that connects the inner and outer membrane ring assemblies. Taken together, the basal body of the T3SS is a multi-component structure that forms a single conduit for effector translocation through the bacterial cell wall.

The needle complex forms a continuous channel that extends the basal body of the T3SS outwards into the extracellular space. At the proximal end of the needle complex, a short tube is generated through the polymerization of EscF. Once this inner tube is assembled, the needle protein EspA polymerizes in a helical fashion around the EscF filament to form the outer sheath of the needle complex. In vitro experiments have ascertained that the length of the EspA-filament can average from 600 to 6,300 nm long and that the inner core of the EspA-filament has an mean diameter of 12 nm. Given these findings, it is widely perceived that effectors remain un-folded with the aid of molecular chaperones from the time they are manufactured within the bacterial cytoplasm. Molecular chaperones, such as CesT and CesAB, have been shown not only to prevent protein folding, but to also act as guides to direct effectors into the T3SS. Once the protein exits out of the needle complex and in the host cytoplasm, it is presumed that they are then folded into their natural conformations.

It is now clear that certain proteins secreted through the type III needle-complex are responsible for forming the pore complex that spans the host plasma membrane. EspB and EspD are two known translocators delivered through the EPEC T3SS. In
the host cell plasma membrane, oligomers of the hydrophobic protein EspD generate an inner ring complex. Another hydrophobic effector, EspB, assembles around this inner ring and consequently forms the outer portion of the pore complex. Notably, both EspB and EspD are required for effectors to be exported through the target cell membrane. Interactions between the EspB/EspD complex and the EspA-filament has been demonstrated and supports the existing model that describes the translocation needle as a continuous passageway between the bacterium and host cell. In addition to its pore-forming activity, EspB has been implicated to play the role of an effector capable of disrupting actin stress fibres and inhibiting myosin II-dependent bacterial uptake. Binding between EspB and other host molecules (ie. α-catenin and α1-antitrypsin) have also been detected however it is uncertain what biological significance these interactions have on EPEC pathogenesis.

For A/E pathogens, the ATPase EscN is required to generate energy for effector secretion through the T3SS. Similar T3SS ATPases have been identified in other bacterial pathogens such as YscN from Yersinia and InvC from Salmonella enterica. Structural evidence describing EscN suggests that the protein oligomerizes into a hexameric ring in the bacterial cytoplasm just underneath the basal body. A recent study by Chen and co-workers (2013) examining the specific role of EscN found that it can bind to the molecular chaperones CesA/B, but only when CesA/B is bound to its substrate. This finding suggests that interactions between EscN and the chaperone-substrate complex are necessary for effector targeting to the injectosome.

1.2.5. **Intimin**

Intimin is a ~102 kDa outer membrane protein encoded by the LEE-gene, eaeA. Expressed by all A/E bacteria, intimin is the principle bacterial adhesion molecule required for extensive colonization of the intestinal mucosa and A/E lesion formation. Importantly, in vivo experiments revealed that intimin is critical for full virulence. Intimin is a modular protein containing three distinct portions: a N-terminal flexible region, a central membrane-anchoring portion, and a C-terminal translocated intimin receptor (Tir)-binding region. The N-terminal of intimin contains a short signal sequence that targets the adhesin to the inner membrane via the Sec complex. A second targeting signal has also been found within intimin—this one present near the N-
terminus of the central membrane-anchoring region. This signal peptide is recognized by the BAM complex, which folds the central region of intimin into a β-barrel conformation embedded in the outer membrane. This β-barrel anchor domain has the propensity to dimerize with neighbouring intimin molecules along the bacterial surface. Conceivably, this could aid in the clustering of surface receptors on the host plasma membrane.

Whereas the N-terminal region of intimin plays a vital part in its proper localization, it is the C-terminal half that plays a key role in facilitating intimate bacterium-host cell attachment. In this region there are three successive immunoglobulin-like domains followed by a C-type lectin-like domain that faces the furthest away from the bacterial surface. Compelling evidence reveals that the C-type lectin domain by itself can directly bind to the extracellular receptor, Tir, reaffirming that the critical residues involved in Tir-mediated adherence are located in this region of the adhesin. Additional findings from in vivo and ex vivo studies revealed that EPEC and EHEC intimin-deletion mutants were impaired in their ability to adhere to the intestinal epithelium and generate A/E lesions. Thus, it is clear that intimin is a central colonization factor that allows A/E pathogens to remain tightly attached to the intestinal epithelium during the infections.

Sequence analysis showed that intimin molecules in EPEC, EHEC, and C. rodentium share ~90% amino acid similarity. However, the C-terminal 280 amino acids, which contains the residues involved in Tir binding, accounts for most of the genetic diversity among A/E pathogens. As such, it has been proposed that intimin can be categorized into distinct subtypes. Despite the allelic variability, experimental evidence demonstrates that intimin from EPEC, EHEC, and C. rodentium are functionally interchangeable.

Much less is known about the non-Tir intimin partners on the host surface. Cell surface molecules that have been reported to interact with EPEC intimin include nucleolin and β1-integrin, however these interactions do not seem to affect early or intimate EPEC attachment. Interestingly, a murine study with C. rodentium demonstrated that expression of intimin causes colonic hyperplasia and inflammation suggesting that this outer membrane protein may have additional pathogenic functions.
1.2.6. **LEE Encoded effectors**

Effectors encoded within the LEE gene cluster were the first to be identified and are among the most studied in EPEC. It was later discovered that effector-coding genes are also present outside of the LEE, but relatively little is known about them. This section will highlight the pathogenic functions of LEE-encoded effectors Tir, EspF, EspG, EspH, EspZ, and Map.

**Tir**

The translocated intimin receptor, Tir, is a 78kDa protein that acts both as an extracellular receptor for the outer membrane protein intimin and an effector that can trigger host-signalling events\(^{129,130}\). The \(\text{tir}\) gene is encoded within the core of the LEE\(^{130}\). Upon bacterial-host attachment, Tir is among the earliest effectors injected directly into the host cytoplasm via T3SS\(^{74,131}\). It is also suggested that Tir coordinates the type III secretion because EPEC mutants lacking Tir alters the translocation rate of other LEE-encoded effectors (ie. Map, EspH)\(^{79,131}\). Once in the host cytoplasm, Tir inserts its two transmembrane domains, which are flanked by a central extracellular loop, into the host plasma membrane forming a hairpin-shaped structure\(^{132,133}\). Its presence in the plasma membrane then permits the bacterial surface protein intimin to dock onto the intimin-binding domain (IBD), which in \(\text{Tir}_{\text{EPEC}}\) is a 109 amino acid sequence within the central extracellular loop\(^{132}\). This intimin-Tir binding is essential for intimate attachment\(^{41}\) as well as the clustering of Tir along the bacterial-host interface, which is needed to stimulate actin accumulation and ultimately biogenesis of A/E lesions\(^{116,133-135}\).

There are two cytoplasmic domains in Tir each located at the N- and C-termini. Relative to the C-terminal regions little detail is known concerning the N-terminal cytoplasmic domain, which is the shorter of the two regions. A major finding regarding the N-terminus of Tir is that it contains a secretion signal necessary for effector targeting into the T3SS\(^{136}\). However, within the host cell, its pathogenic role is much more obscure than that of the C-terminal region, partly because the N-terminal cytoplasmic domain of \(\text{Tir}_{\text{EPEC}}\) and \(\text{Tir}_{\text{EHEC}}\) are completely dispensable for intimate attachment and actin accumulation beneath the site of bacterial-host contact\(^{134,137}\). Although this segment of Tir is not necessary to induce localized actin polymerization, evidence does suggest that its role may be in assisting with host-cell signalling triggered by the C-
terminal cytoplasmic domain \(^{137-139}\). Host proteins thought to interact with the N-terminal domain of \(\text{Tir}_{\text{EPEC}}\) include \(\alpha\)-actinin, ezrin, and protein 14-3-3 \(^{140,141}\).

The C-terminal cytoplasmic domain of Tir is crucial for actin accumulation beneath the bacteria at points of contact. Compared to the N-terminus, the longer C-terminus of \(\text{Tir}_{\text{EPEC}}\) and \(\text{Tir}_{\text{EHEC}}\) are less conserved with just 41% amino acid identity (vs. 54%) \(^{142,143}\). Substantial evidence has shown that this disparity has functional repercussions at the molecular and cellular levels. An example of this is the distinct mechanisms that \(\text{Tir}_{\text{EPEC}}\) and \(\text{Tir}_{\text{EHEC}}\) employ to induce actin assembly within host cells, both of which have been well characterized \(^{81,144,145}\).

\(\text{Tir}_{\text{EPEC}}\) is the archetype model that has shaped our understanding of how A/E pathogens usurp existing host signalling pathways to promote actin polymerization. The C-terminal cytoplasmic domain of \(\text{Tir}_{\text{EPEC}}\) possesses two tyrosine residues at positions 454 and 474, both of which can become phosphorylated upon intimin-mediated binding and clustering along the bacterial-host interface \(^{143}\). EPEC pedestal assembly essentially requires the phosphorylation of \(\text{Tir}_{\text{EPEC}}\ Y_{474}\), which in turn induces activation of the Arp2/3-mediated actin polymerization complex \(^{142}\). When compared to \(\text{Tir}_{\text{EPEC}}\ Y_{474}\), \(\text{Tir}_{\text{EPEC}}\ Y_{454}\) is phosphorylated but is \(\sim\)13-fold less efficient at inducing localized actin polymerization \(^{146}\). Additional details regarding how Tir induces actin recruitment beneath the site of attachment will be covered later in the “EPEC pedestal formation” section.

\(\text{EspF}\)

The effector EspF is a multifunctional protein that can subvert a wide range of cellular processes in the host cell, but does not appear to play a significant role in adherence to cultured epithelial cells \(^{147}\). Orthologs of EspF have been found across the A/E bacterial family, though their function can differ noticeably between microorganisms due to genotypic variation of \(espF\) (75% identity between EPEC EspF and EHEC EspF) \(^{147}\). Among the most conserved feature of EspF in typical EPEC \(^{147}\), EHEC \(^{148}\), REPEC \(^{149}\), and \(C. \text{rodentium}\) \(^{150}\) are the repeated proline-rich sequences at the C-terminal region of the effector. These proline-rich sequences are also separated by one or more class III PDZ domain binding motifs \(^{149}\). Because many actin-associated proteins expressed in eukaryotes contain proline-rich regions and PDZ-binding domains \(^{151}\), it’s believed that EspF utilizes molecular mimicry to hijack the host actin cytoskeleton. EspF
has been shown to interact with monomeric actin, N-WASP (neural Wiskott-Aldrich syndrome protein), profilin, and the Arp2/3 complex \(^{149,152}\). Interestingly, a study by Janneth Peralta-Ramierez in 2008 revealed that rabbit EPEC (REPEC) lacking EspF generated shorter actin filaments beneath sites of intimate attachment while overexpression of this effector had the opposite effect \(^{149}\). In microvilli-producing epithelial cells, EspF expression contributes to both microvilli effacement and elongation of microvilli surrounding A/E lesions \(^{153,154}\). Together, these data demonstrate that EspF targets various molecules to co-opt actin-related functions in host cells.

EspF contains an N-terminal signalling sequence that is required for mitochondrial targeting \(^{155,156}\). Immunofluorescence labelling of EspF confirmed this as most of the EspF proteins expressed were localized in this organelle \(^{156}\). Within the mitochondria, EspF disrupts the mitochondrial membrane potential in order to activate the intrinsic apoptotic signaling pathway \(^{155,157,158}\). This results in the release of cytochrome c into the cytosol, which causes the activation of apoptosis-mediated cell death \(^{158}\). Indeed, \(\Delta espF\) deletion mutants reduced host cytotoxicity by \(~27\%\) compared to WT EPEC \(^{159}\). By comparison, ectopic expression of EspF in cultured epithelial cells resulted in substantial induction of apoptotic factors \(^{159}\). Mitochondrial localization of EspF is also necessary to induce leakage of the nucleolar protein nucleolin into the cytosol \(^{160}\) though questions still remain as to how these two events are connected.

A fraction of EPEC EspF molecules reside in the host cytoplasm where it is purportedly capable of subverting various cellular functions for example remodelling of the host membrane \(^{154}\), inhibiting SGLT-1 \(^{154}\), hijacking actin cytoskeletal proteins \(^{149}\), and disrupting membrane permeability \(^{161}\). In addition, secretion of EspF by EPEC has been shown to cause noticeable changes in intermediate filament organization within the host cell. Co-immunoprecipitation experiments of lysates from EPEC-infected epithelial cells more specifically reveals that cytoplasmic EspF can form a complex with the intermediate filament protein, cytokeratin 18, and the adapter protein, 14-3-3 \(^{162}\). Whether these interactions contribute to the disruption of intermediate filaments has yet been shown \(^{162}\).

EspF is one of multiple known T3SS effectors that contribute to increased epithelial barrier permeability—a hallmark of diarrhea \(^{163}\). This was first evident in a study
by Gail Hecht’s lab when colon epithelial T84 cells infected with EPEC had a greater decline in transepithelial resistance when EspF expression was induced\textsuperscript{164,165}. This implies that secretion of EPEC EspF can impair tight junctions (TJs) and allow small molecules to leak between adjacent cells\textsuperscript{164}. Furthermore, immunofluorescence data indicates that occludin and claudin along the plasma membrane of adjoining cells are fragmented supporting the notion that TJ complexes are internalized\textsuperscript{164,166}. Moreover both proteins along with the TJ-associated adapter proteins ZO-1 and ZO-2 appear to be recruited toward sites of intimate bacterial-host attachment\textsuperscript{149,167}. This phenotype was not observed when \textit{espF} or even the entire LEE was deleted from EPEC\textsuperscript{164,166}. In agreement with this, findings from \textit{C. rodentium} infected mice have shown that expression of EspF is required for the redistribution of membrane-associated claudin away from the lateral membrane\textsuperscript{163}.

EspF can inhibit bacterial uptake by phagocytic macrophages and M (or ‘microfold’) cells, which naturally engulf antigens from the luminal intestinal surface and transfers them to antigen-presenting cells at the basolateral end of the cell. Using an \textit{in vitro} model containing M cells, EPEC expressing EspF was shown to strongly inhibit bacterial uptake (~2%) in comparison to its isogenic Δ\textit{espF} mutant, which had a substantially higher proportion of intracellular bacteria ~18%\textsuperscript{168}. Well aligned with this observation, expression and translocation of EPEC EspF into phagocytic cells were required to suppress bacterial uptake\textsuperscript{156,169}. However compared to EPEC EspF, the activity of EHEC EspF to prevent bacterial uptake by phagocytic cells and M cells is considerably lower\textsuperscript{169}. For EPEC EspF, the first 101 amino acids are known to be required for its phagocytosis inhibiting activity. Interestingly, ablation of the mitochondrial signaling sequence, which is present within this segment, had no significant affect on bacterial uptake suggesting that its phagocytosis inhibition activity is independent of its mitochondrial localization\textsuperscript{156}. Various studies have found that EPEC EspF perturbs phagocytic uptake of the microbe through inhibiting the molecule phosphatidylinositol 3-kinase (PI3K)\textsuperscript{156,169,170}. EPEC EspF has also been shown to interact with the membrane deformation protein sorting nexin 9\textsuperscript{152,169,171}, however the functional significance of this interaction remains elusive\textsuperscript{152,171}. 

13
Map

Map is a well-conserved effector among A/E pathogens that can regulate various different host cellular processes. Following type III dependent secretion, this effector is targeted to the host cytoplasm and can even translocate into the mitochondria as it contains an N-terminal mitochondrial targeting sequence. The import of Map into the mitochondrial matrix is known to be facilitated by the TOM (translocase of the outer membrane) complex and mtHsp70 (mitochondrial Heat shock protein 70). Studies reveal that mitochondrial localization of Map and amino acid residues between positions 101 and 152 are necessary to cause aberrant organelle morphology associated with the disruption in mitochondrial membrane potential.

Independent of mitochondrial localization and dysfunction, Map induces filopodia formation at EPEC attachment sites during the early stages of infection in cultured cells. The ability of Map to modulate actin dynamics in cultured cells is dependent on the activation of Cdc42, a small regulatory GTPase protein that plays a central role in promoting filopodial generation. Interestingly, a study by Brendon Kenny and his team found that intracellular ΔmapΔtir double mutants and Δmap single mutants had a lower propensity to become intracellular whereas overexpression of Map enhanced its invasiveness. Their findings implicate Map and Tir in promoting EPEC internalization through a small molecule GTPase signaling pathway, though further experiments will be necessary to determine if these results are recapitulated in vivo. It is, however, important to note that multiple EPEC effectors are known to possess anti-phagocytic and phagocytic properties and that normal regulation of their secretion may be what allows EPEC to remain typically extracellular. Although Map was originally thought to mimic Rho family GTPases, more recent data challenges this and provides compelling evidence, both structurally and functionally, implicating Map as a GEF. Other bacterial effectors such as IpgB1 in Salmonella spp. and IpgB1 and IpgB2 in Shigella spp. are known to target different molecules in the GTPase signaling pathway. These proteins do not share any sequence or structural homology with known Rho GEFs, though they do possess a common WxxxE motif that is critical for their GEF-like function.
Ex vivo and in vivo studies with EPEC and C. rodentium, respectively, have revealed that type III secretion of effectors EspF, Map, and Tir influence microvilli destruction. For Map, this process partly involves the sustained activation of Cdc42. Though in the absence of Tir (or intimin) and EspF, EPEC causes extensive hyper-microvilli effacement along the host cell surface implying that other EPEC effectors regulate the GEF activity of Map. Additionally, Map can also bind to the PDZ-domain of ezrin binding protein 50 (Ebp50) aka. Na+/H+ exchanger regulatory factor 1 (NHERF1)—a region of Ebp50/NHERF1 that can couple with ezrin and other ligands within actin-rich microvilli. This interaction appears to involve three core amino acids (Thr-Arg-Lys) at the C-terminus of this effector. Although molecular studies of the PDZ-domain indicate that this region of Ebp50/NHERF1 is critical for microvillar assembly, exactly how Map interferes with Ebp50/NHERF1 to promote microvilli effacement remains elusive.

Other host cell effects caused by Map are the inactivation of SGLT-1 (Sodium-glucose linked transport-1) transporter and destruction of TJs in cultured cells. To determine if Map causes intestinal barrier dysfunction in vivo, Ma and colleagues measured the leakage of mannitol, a molecule that diffuses only via paracellular pathway, from the intestinal lumen. Compared to uninfected mice, animals infected with WT C. rodentium expressing both Map and EspF had significantly higher permeability to mannitol. This implies that EspF and Map contribute to the weakening of the intestinal epithelial integrity. Further evidence, implicate that these two effectors likely work together to disrupt epithelial barrier function as TJ disruption is attenuated in C. rodentium lacking EspF.

EspG

The LEE-gene encoding the effector EspG has been identified in C. rodentium, EPEC, EHEC and REPEC and is attributed to intestinal colonization defects during in vivo infections. In fact, EPEC appears to be the only A/E family member that is known to carry a duplicate EspG-encoding gene referred to as espG2. Albeit only EPEC EspG is encoded within the LEE while its homolog EspG2, which has 62% amino acid similarity, is located in a non-LEE pathogenicity island. A comparison between EHEC EspG and EPEC EspG shows that they are nearly identical (98%). Interestingly,
some clinical EPEC isolates have been identified that carry either espG or espG2, or neither. EspG and EspG2 bear some resemblance to the VirA effector of S. flexneri (40% and 38%, respectively), which is involved in the degradation of microtubules to induce membrane ruffling. Surprisingly, gene complementation with EPEC espG into the invasion deficient S. flexneri ΔvirA mutant enhanced bacterial internalization compared to WT S. flexneri suggesting both bacterial effectors are functionally analogous.

A seminal paper by Matsuzawa et al. in 2004 showed that WT EPEC generated microcolonies more frequently in regions locally depleted of microtubules compared to EspG/G2-deficient mutants. Since then, several subsequent reports have been published that describes a similar phenotype following infection with EspG/G2-expressing EPEC. It is worth mentioning that the lack of quantitative data and negative controls does raise important questions to the validity of their conclusions, as experimental bias can potentially be introduced. Another point that should be raised is that many of the cells that are depicted lacked noticeable filaments resembling microtubules, which suggests a problem with their anti-tubulin staining. In an experiment by Clements and colleagues, the authors noted that cells ectopically expressing EspG had no noticeable degradative effects on the microtubule network. To determine whether EspG/G2 directly interacts with tubulin heterodimers, protein-protein interaction experiments were performed that showed A/E pathogen EspG/G2 homologs, in addition to S. flexneri VirA, can bind to tubulin heterodimers in vitro. Two major questions that have so far not been addressed with absolute certainty are 1) what are the molecular interactions between EspG/G2 and tubulin heterodimers? and 2) how does EspG/G2 destabilize microtubules beneath the site of bacterial attachment during EPEC pathogenesis?

In addition to their purported role in localized microtubule network alterations, various reports have implicated EspG/G2 as a major contributor to intestinal epithelial barrier disruption and production of diarrhea. In cultured epithelial cells, experiments have demonstrated that infection of WT EPEC reduces TER by >75%, whereas infection with the double ΔespG/G2 mutant resulted in a ~30% decrease implying that EspG/G2 promotes paracellular leakage of molecules across the monolayer. Yet, attempts to ectopically express EspG in mammalian cells had no effect on TER possibly because the majority of effector proteins were unable to be translocated out of the Golgi.
apparatus following protein synthesis. Secretion and expression of EspG by EPEC is also known to directly affect TJ-associated proteins. Immunofluorescence data of WT EPEC infections resulted in the mislocalization of occludin, claudin and ZO-1 away from the lateral membrane of polarized epithelial cells. On the contrary, infection with espG/G2 double knockouts had no considerable accumulation of claudin or occludin in the host cytoplasm much like uninfected cells. Although there is no substantial evidence that demonstrates EspG directly interacts with TJ proteins, recent findings have proposed that EspG/G2 interferes with the translocation of occludin to the cell-cell border—a process that is known to be facilitated via microtubule-mediated transport. Whereas the effectors EspG/G2 has only been shown to interfere with the localization of TJ complexes in cultured cells, secretion of EspG and EspF have been found to be associated with the accumulation of aquaporin-2 and aquaporin-3 in the cytoplasm of colonocytes and increased water content in the stool of C. rodentium infected mice. These aquaporins are protein channels responsible for water transport across the plasma membrane. Because aquaporin-2 and aquaporin-3 are respectively localized along the apical and apical/lateral membranes of uninfected colonocytes, it is interesting to speculate that the effects of EspG and EspF on multiple aquaporin isoforms could play a significant role in the pathophysiology of human diarrhea.

**EspH**

EPEC translocates the 20 kDa effector EspH to the plasma membrane of its target cell and consequently causes extensive remodelling of actin filaments. One of two known strategies EspH uses to hijack this cytoskeletal network involves targeting Rho family of GTPases—which is a key regulator of actin dynamics. EspH inhibits Rho signalling through engagement with Rho GEF (Guanine nucleotide exchange factor), rather than Rho GTPase itself. The DH-PH (Dbl-homology and pleckstrin-homology) domain of EspH forms a complex with Rho GEF thereby inhibiting RhoA binding and activation. By targeting the Rho signalling pathway, EspH can promote filopodial retraction and stress fibre assembly, and additionally inhibit phagocytosis in macrophages. The second tactic that EspH employs to subvert the host actin cytoskeleton involves the effector Tir. Findings by Wong and colleagues show that EspH is concentrated to the site of intimate attachment. They further demonstrated that EspH accumulation beneath EPEC required the C-terminal region of
Tir, but is independent of Tir\textsubscript{EPEC} Y\textsubscript{474} and Y\textsubscript{454} phosphorylation as well as Rho GTPase signalling. Although details regarding this Tir-driven mechanism are ill defined, various experiments suggest that EspH expression is important for enhancing for EPEC-induced actin assembly, intestinal colonization, and virulence\textsuperscript{62,201,202}.

\textbf{EspZ}

EspZ is a highly conserved (60-81\% amino identity) effector among A/E pathogens EPEC, EHEC, and \textit{C. rodentium}\textsuperscript{203}. Once injected into the host cell, EPEC EspZ is thought to form a hairpin-shaped structure in the plasma membrane\textsuperscript{204}. Various mechanisms have been proposed detailing its core effector function. Findings from Shames and co-workers (2010) revealed that EspZ interacts with glycoprotein CD98\textsuperscript{205}. Binding of EspZ to CD98 induced phosphorylation of FAK (Focal adhesion kinase), which in turn stimulates the $\beta$1-integrin cell prosurvival pathway\textsuperscript{205}. Another report found that expression of EspZ can also activate prosurvival signals through the EGFR/PI3K/Akt signalling pathway\textsuperscript{206}. Further support for EspZ's function in host cell survival was demonstrated when cultured cells infected with an EPEC $\Delta$espZ mutant resulted in high cytotoxicity and cell detachment\textsuperscript{206}. In these infected cells, substantial mitochondrial cytochrome c was found in the cytosol along with apoptosis and caspase activation\textsuperscript{206,207}. Because cytochrome c is released from the mitochondria, evidence suggests that EspZ may target to the mitochondria and interact with a translocase of inner mitochondrial membrane 17b\textsuperscript{207}. However, this evidence heavily relies on the use of transfected cells expressing HA-tagged EspZ and it is unclear if native EspZ localizes to the mitochondria when secreted by EPEC\textsuperscript{207}. Interestingly, epithelial cells that ectopically express EspZ were resistant to the cytotoxicity upon infection with EPEC $\Delta$espZ as well as treatment with the apoptosis inducing chemical staurosporine suggesting that effector translocation into host cells is necessary for its pathogenic function\textsuperscript{206}.

Another mechanism in which EspZ can perhaps promote cell survival is through regulating effector secretion. A report from Gad Frankel's lab found that EspZ expression prevented hyper-secretion of several type III effectors\textsuperscript{204}. Since EPEC secretion of effectors (\textit{i.e.} EspF, Map) can cause cytotoxicity to the host cell\textsuperscript{155,160,206,208}, it is possible that regulation of effector secretion is another tactic used by EPEC to
modulate host survival and death signals. Another noteworthy finding is that EspF inhibits PI3K signalling pathway, whereas EspZ induces it. This suggests EspZ can counteract the apoptosis activation signals promoted by EspF^{206,208}.

*In vivo* experiments demonstrate that *C. rodentium ΔespZ* mutant is strongly attenuated in virulence and poorly colonizes the intestinal epithelium in mice^{203,205,209}. It is not known how EspZ contributes to virulence *in vivo*, though it has been speculated that *C. rodentium ΔespZ* mutant may cause cell death in the intestinal epithelium resulting in extensive shedding of microbes along with the infected cells in the feces^{205}.

### 1.3. EPEC pathogenesis

In humans, extracellular EPEC preferentially colonizes the small intestine where it causes gross morphological changes to the intestinal epithelium associated with profuse watery diarrhea. Immortalized cell lines have been widely used by researchers to gain detailed insights into molecular interactions that occur during EPEC pathogenesis. Because evidence from standard cultured cell models is often difficult to interpret and do not always reflect EPEC disease phenotypes, more complex model systems are often used to provide greater clarity on the pathophysiological alterations that occur *in vivo*. In this section, I will highlight the *in vivo* and *ex vivo* models that have been applied to study EPEC pathogenesis.

#### 1.3.1. *C. rodentium* mouse model and other *in vivo* systems

EPEC is primarily a human pathogen, but this restriction does not apply to cultured cell infections. Even though the utilization of mammalian cell lines has greatly advanced our knowledge of host-pathogen infections, our understanding the pathophysiological changes associated with EPEC-induced diarrheal disease is hampered due to the lack of a small animal model system. As such, many researchers use *C. rodentium* as a surrogate pathogen to study the pathogenesis of EPEC. *C. rodentium* is a member of the A/E family and a natural mouse intestinal pathogen. This extracellular microbe can also infect cultured cells and induce localized actin assembly beneath sites of intimate contact. Because *C. rodentium* and EPEC both carry similar
genes within the LEE, the *C. rodentium* infection model has been widely used to study the pathogenic functions of effectors encoded within this pathogenicity island. Oral inoculation of mice requires about $10^8$-$10^9$ microorganisms. Within 7 days PI, A/E lesions can be observed on colonocytes in the large bowel of infected mice. It should be noted that EPEC typically colonizes the small intestine in humans, whereas *C. rodentium* is preferentially found in the large bowels and induces colonic hyperplasia. Also during this period, the mean water content in their stool increases from 73% in uninfected mice to 92%; similar to fecal samples taken from diarrheal human patients. Given the conveniences of using conventional laboratory mice, *C. rodentium* is an attractive tool that allows researchers to investigate the many evolutionarily conserved genes shared amongst members of the A/E family.

In addition to mice, researchers have utilized other *in vivo* models to study human EPEC disease. One of the earliest animal EPEC models used colostrum-deprived piglets, which are highly susceptible to infection with EPEC and many other pathogens (*ie.* EHEC and Rotavirus). Another diarrhea model that has been used by many researchers are germ-free gnotobiotic piglets. Regardless of which model used, oral infection with $\sim 10^8$ EPEC microbes is standard, to cause watery diarrhea within 24 h, but is higher than the estimated infectious dose for EPEC in healthy adults ($10^6$ bacteria). In the host, EPEC can intimately adhere to enterocytes and form A/E lesions in the intestine—a phenotype that requires the bacterial adhesins plasmid-encoded BFP and intimin. Other organisms such as *Caenorhabditis elegans* have also been exploited as a model to primarily study innate immunity and gene regulation. This model is, however, rather limited because intestinal colonized EPEC does not induce actin polymerization nor can diarrhea be evaluated in this model. Because human isolates of EPEC have a narrow host range, atypical EPEC strains isolated from animals such as cats, dogs, rabbits, and lambs have also been used for *in vivo* studies—albeit rarely.

### 1.3.2. *Ex vivo* studies

Cell culture infections have been extensively used to investigate the pathogenesis of A/E bacteria. The use of immortalized cell lines offers simplicity and convenience and can be exploited to determine the relationship between the microbe.
and host organism. However, standard tissue cultures do not always accurately replicate primary cells. To try to recapitulate the \textit{in vivo} infections researchers have turned to tissue explants that are freshly harvested from mammals and grown in conditions similar to cultured cells. \textit{Ex vivo} models, sometimes referred to as \textit{in vitro} organ culture (IVOC), offer greater flexibility in terms of spatiotemporal control and are better suited to parallel the normal tissue morphology over that of immortalized cells. As such, a number of publications have used explants to examine various aspects related to host-pathogen interactions including effector function \cite{153,154}, A/E lesion formation \cite{128,224,225}, and colonization factors \cite{96,111,128,226}. Despite the advantages of explants, many labs still use standard cell cultures because IVOC requires fresh tissue from live animals and that requires substantially more time, labour and cost to perform.

1.4. EPEC pedestal formation

Characteristic pillar-like projections that extend from the normal surface of mammalian cells, called pedestals, are the hallmark of A/E pathogenesis. These A/E-induced structures are generated upon microbial attachment and require the secretion of effectors through a bacterial-encoded needle-like T3SS. Once translocated into the host cell, these effectors enable the A/E pathogen to subvert control over various host-signalling pathways. In this section, I will describe the intricate molecular mechanisms that EPEC exploits to hijack the host subcellular components during the pedestal formation process, highlight the differences in the bacterial mechanisms employed by EPEC, EHEC, and \textit{C. rodentium} to induce localized actin assembly, and elaborate on the putative functions of pedestals during the infections.

1.4.1. EPEC Tir Y_{474}-dependent actin assembly

Following injection of Tir\textsubscript{EPEC}, the protein is inserted into the host plasma membrane where it can cluster and act as a cognate receptor for the bacterial surface protein, intimin. Various \textit{in vitro} studies have demonstrated that both the translocation of Tir\textsubscript{EPEC} into the host plasma membrane and subsequent clustering of this extracellular receptor are necessary to induce Tir phosphorylation-dependent signalling \cite{116,130,134}. Phosphorylation of Tir\textsubscript{EPEC} at the site of bacterial attachment is mediated by host kinases.
belonging to the Abl and Src-families \(^{68,227}\). At the C-terminal region of \(\text{Tir}_{\text{EPEC}}\), \(Y_{474}\) is often referred to as the ‘major site of phosphorylation’, because it is the principle residue for pedestal assembly \(^{143}\). Phosphorylation of \(\text{Tir}_{\text{EPEC}}\) \(Y_{474}\) allows the SH2 domain of Nck—a host adapter protein—to dock onto this major site of phosphorylation. By bridging \(\text{Tir}_{\text{EPEC}}\) to other host proteins, the Tir-Nck signaling complex can recruit various molecules towards the site of bacterial adherence and efficiently activate the host actin polymerization machinery.

**N-WASP and Arp2/3 complex**

A principal molecule of the host actin polymerization machinery signalled via \(\text{Tir}_{\text{EPEC}}\):Nck docking is the actin nucleation promoting factor N-WASP. Normally N-WASP adopts an auto-inhibited conformation, which can be reversed by several stimuli including Cdc42 \(^{228}\), Nck \(^{229}\), WIP (WASP-interacting protein) \(^{230}\), Grb2 (Growth factor receptor-bound protein 2) \(^{231}\), and profilin \(^{232}\). This, in turn, exposes the VCA (Verprolin, Cofilin, Acidic) domain of N-WASP allowing the Arp2/3 (actin-related protein 2/3) complex to bind and become activated \(^{66,228,233}\). In fact, N-WASP is indispensible for pedestal formation given its principle role as a potent activator of the Arp2/3 complex \(^{66,139,234}\). The Arp2/3 complex plays a key role in initiating the assembly of new actin filaments as well as binding to branch points where new ‘daughter’ strands are generated at a 70° angle from the existing mother filament \(^{235}\). Consequently, actin assembly within the host cell at sites beneath EPEC forces the underlying plasma membrane to protrude above the normal surface of the cell thereby forming the characteristic pedestal-shaped structure (Figure 1.1).

**WASP Interacting Protein**

The WASP-interacting protein (WIP) acts as a bridge between the adapter Nck and the nucleation promoting factor N-WASP \(^{230,233}\). During EPEC and EHEC infections, the presence of N-WASP appears to be necessary for WIP to accumulate beneath the site of intimate attachment and along the entire pedestal length \(^{236}\). However, the question whether WIP is required for pedestal formation is still contested, as some studies have suggested that it is required for the recruitment of N-WASp \(^{237}\) and actin assembly \(^{236}\), whereas other reports argue that it is dispensable \(^{233}\). Nonetheless, the N-
terminal WH1 domain of N-WASP, which interacts with WIP and other proteins (i.e. CR16, WICH/WIRE), is required for pedestal assembly 236.

**Endocytic proteins**

Clathrin is well known for its involvement in receptor-mediated endocytosis, but only recently has this coat protein been found in protrusive structures called immunological synapses 238. Prior to this discovery, it was already understood that clathrin is exploited by intracellular pathogens *L. monocytogenes* and by the extracellular bacterium EPEC for bacterial invasion and generation of pedestals, respectively 239. During EPEC infections, clathrin is recruited to sites of bacterial contact—a process that requires TirEPEC Y474-phosphorylation dependent signaling 239. The role of clathrin in pedestal biogenesis appears to be an essential one as depletion of this endocytic protein precludes actin nucleation 239. It was further revealed by Pascal Cossart’s lab that clathrin heavy chain molecules localized at pedestals were tyrosine phosphorylated at positions 1477 and 1487 240. Using the pharmacological Src kinase inhibitor PP1, the authors demonstrated that phosphorylation of Y1477 and Y1487 on the clathrin heavy chain are necessary for pedestal formation. Furthermore, they uncovered that GFP-clathrin tyrosine Y1477F/Y1487F mutant, expressed in cells depleted of endogenous clathrin, did not accumulate beneath intimately attached EPEC 240. Together, these studies indicate that EPEC 1) requires clathrin to induce localized actin assembly, and 2) stimulates clathrin tyrosine phosphorylation in order to redistribute the protein directly beneath intimately attached EPEC 240.

Aside from clathrin, studies have found multiple other endocytic proteins associated with endocytic clathrin-coated pits that are also present in non-invasive structures such as EPEC pedestals. For instance, epsin and its binding partner Eps15 are normally involved in membrane deformation of clathrin-coated pits 241,242; but during EPEC infections, epsin and Eps15 accumulate at the apical tip of pedestals and both possess a C-terminus ubiquitin-interacting-motif (UIM), which is the minimal domain necessary to direct both proteins beneath adherent EPEC 243. While it is understood that UIMs can bind to ubiquitinated molecules, evidence have found that TirEPEC is not ubiquitinicted during EPEC infections. Nonetheless, TirEPEC and Y474 phosphorylation are needed for their accumulation 243. Interestingly, the clathrin adapter protein, AP-2,
(accessory protein 2) and the minus-end actin-directed motor, myosin VI, are absent in EPEC pedestals\(^{239,240}\). Since AP-2 (a protein that links the cargo to the clathrin-coated pit) and myosin VI (a principle motor protein that mediates CME) play a central part in CME\(^{244-246}\), it is conceivable that the exclusion of AP-2 and myosin VI at pedestals could be the reason why coated pits are not observed in EPEC pedestals. Furthermore, the indispensability of clathrin\(^{239}\) and its associated proteins CD2AP\(^{210}\), dab2\(^{240}\), dynamin 2\(^{247}\), Eps15\(^{243}\), epsin\(^{243}\), and Hip1R\(^{240}\) for Arp2/3-mediated actin polymerization implies that these components together generate an actin nucleating platform rather than facilitate clathrin-dependent endocytosis\(^{240,247,248}\).

**Cortactin**

Cortactin is a major scaffolding protein that localizes to sites of actin rearrangement generally at the cell cortex and is a regulator of Arp2/3-mediated actin nucleation. In pedestals, some reports describes cortactin as a protein that is localized throughout the entire structure while others have shown it concentrated only at the apical tip\(^{210,249,250}\). The recruitment of cortactin to EPEC pedestals is dependent on several known proteins CD2AP, clathrin, dynamin, N-WASp, and the C-terminus of Tir\(^{210,247,249}\). Using truncation mutants, studies suggest that the N-terminus of cortactin is necessary for its recruitment to pedestals as well as for efficient actin assembly\(^{250}\). Within this region lies the N-terminal acidic and tandem repeat domain of cortactin, both of which are involved in binding and activation of the Arp2/3 complex\(^{250,251}\). This is influenced by tyrosine phosphorylation mediated by Src and Erk, however we do not know precisely if cortactin is post-translationally modified in EPEC pedestals\(^{250,252}\). Moreover, the N-terminal acidic domain of cortactin co-immunoprecipitated with Tir in WASP-deficient cells\(^{252}\) suggesting that cortactin can form a stable complex with Tir. Towards the C-terminus of cortactin is the SH3 domain, which can interact with various proteins such as N-WASP, WIP, Hip1R, and CD2AP\(^{210,240,253}\). This SH3 domain also appears to be necessary for the recruitment of cortactin to pedestals\(^{250}\).

**Non-actin cytoskeletal systems**

The actin cytoskeleton is not the only cytoskeletal system that plays an integral part in the morphogenesis of EPEC pedestals. A published report from Gad Frankel’s lab suggest that EPEC redistributes keratin proteins toward the site of intimate bacterial
contact. In their study, they identified two intermediate filament proteins, cytokeratin-8 (CK-8) and cytokeratin-18 (CK-18), enriched within actin-rich pedestals. The authors also found that gene silencing of CK18 resulted in pedestal production and bacterial adherence to cultured cells being significantly hindered. This suggests that CK-18 is an important component for bacterial-host cell attachment and biogenesis of EPEC pedestals. It is possible that the adapter protein 14-3-3τ, which complexes with Tir, can also interact and facilitate in the recruitment of CK-8 and CK-18. Given the significance of their findings, further work should be performed to further characterize the role of intermediate filament proteins during EPEC pathogenesis.

Recent evidence from our lab demonstrates that the spectrin cytoskeleton plays an integral part in the pathogenesis of EPEC. Within the spectrin cytoskeleton, an extensive meshwork of filaments comprised of spectrin heterodimers are tethered along the cytoplasmic face of the plasma membrane. Using cultured epithelial cells, we demonstrated that depletion of spectrin cytoskeletal components impeded EPEC adherence and completely abolished pedestal formation. Although the involvement of the spectrin cytoskeleton in bacterial-host cell contact requires further investigation, in vitro studies have provided fascinating insight on its arrangement within these microbial-induced structures. Immunolocalization of spectrin, which can oligomerize into filaments, localizes to the apical and lateral membranes of EPEC pedestals as well as the bottom 2/3 of the structures. The arrangement of spectrin suggests that it forms a spectrin cage around the entire actin-rich structure (Figure 1.1). Consistent with this hypothesis, two proteins that link spectrin filaments to integral membrane proteins and the barbed end of actin filaments, protein 4.1 (p4.1) and adducin, were found to colocalize with spectrin along the periphery of actin-rich pedestals though the latter one was absent at the apical tip of the pedestals.

**Distal pedestal proteins**

EPEC-induced pedestals and intestinal microvilli are protrusive actin-rich structures generated along the apical surface of epithelial cells. Even though microvilli are effaced upon contact with EPEC, several proteins situated beneath these hair-like structures are analogously positioned beneath sites of EPEC-induced actin accumulation. These actin-associated proteins include filamin A, conventional non-
muscle myosin II, and tropomyosin. While the role of filamin A and tropomyosin in pedestal biogenesis has yet been explored, evidence show that inhibition of myosin II with 2,3-butadione monoxime (BDM) reduces retrograde actin flow and actin depolymerization resulting in abnormally long EPEC pedestals (up to 50 \( \mu \text{m} \))\(^{258}\). When BDM was removed, these long actin-rich structures returned to their normal length. However, it should be noted that the ATPase specificity of BDM is still in question as there is evidence that suggests BDM has non-specific effects on Ca\(^{2+}\) regulation\(^{259-262}\).

During EPEC infections, ZO-1 and ZO-2—two scaffold proteins that link tight junction transmembrane proteins to the actin cytoskeleton—are redistributed away from tight junctions through a mechanism that is dependent on Nck-dependent Tir\(_{\text{EPEC}}\) signaling\(^{167}\). Since mammalian cells can express multiple ZO isoforms, knockout of a single isoform does significantly impact the TJ integrity\(^{263}\). Conversely, cells that lack all ZO isoforms are incapable of forming TJs\(^{264}\). As TJs are disrupted during EPEC infections, it is possible that the enrichment of ZO-1 and ZO-2 to pedestals is one of several strategies employed by EPEC to disrupt the intestinal epithelial barrier\(^{167}\).

### 1.4.2. EPEC Tir \( Y_{454} \)-dependent actin assembly

Most of our scientific understanding of Tir\(_{\text{EPEC}}\) and its role in pedestal formation is centered on \( Y_{474} \). Yet, several reports have shown that EPEC pedestals can be generated, albeit infrequently, in the absence of Tir\(_{\text{EPEC}}\) \( Y_{474} \) phosphorylation\(^ {146}\). This residual activity of Tir appears to be attributed to the tyrosine residue at position 454, which is inefficiently phosphorylated\(^ {146}\). Phosphorylation of Tir\(_{\text{EPEC}}\) \( Y_{454} \) appears to trigger Arp2/3-mediated actin polymerization through a second separate signaling pathway apart from Tir\(_{\text{EPEC}}\) \( Y_{474} \) that is Nck-independent, but still N-WASP-dependent\(^ {146}\).

### 1.4.3. EHEC pedestal formation

Tir\(_{\text{EHEC}}\) does not rely on tyrosine phosphorylation or Nck to induce actin-rich pedestal assembly\(^ {125,145}\). But rather, the core of this divergent signalling mechanism utilizes a C-terminal sequence between positions 454-463 on Tir\(_{\text{EHEC}}\)\(^ {137,265}\). The residues NPY\(_{458}\) found within this motif are critical for this extracellular receptor to directly engage the host scaffold protein IRSp53 (insulin receptor substrate protein of 53kDa) or its
homologue IRTKS (insulin receptor tyrosine kinase substrate)\textsuperscript{266,267}. Both of IRSp53 and IRTKS contain an N-terminal I-BAR (inverted Bin/Amphiphysin/Rvs) domain that is recognized by Tir\textsubscript{EHEC}\textsuperscript{266,267}. IRSp53/IRTKS are thought to self-dimerize in a parallel fashion allowing two molecules of Tir\textsubscript{EHEC} to be linked together beneath the site of bacterial attachment\textsuperscript{266,268,269}. This Tir\textsubscript{EHEC}:IRSp53/IRTKS complex subsequently triggers the recruitment of a second EHEC effector, called EspF(U)/TccP (\textit{E. coli} secreted protein F-like from prophage U / Tir cytoskeleton coupling protein)\textsuperscript{144,270}. EspF(U)/TccP is not encoded in the LEE but is found in the cryptic prophage CP-933U region of the bacterial chromosome\textsuperscript{144}. EspF(U)/TccP contains multiple highly conserved proline-rich repeats, with each repeat capable of recruiting and activating a single N-WASP molecule\textsuperscript{144,270}. EspF(U)/TccP can also bind to membrane deforming proteins TOCA1, CIP4, and FBP17\textsuperscript{271}. In vitro experiments of these F-BAR (FCH-Bin-amphiphysin-Rvs) sequence-containing proteins further suggests that TOCA1 acts synergistically with its binding partner EspF(U)/TccP to activate the N-WASP/WIP complex, whereas CIP4 and FBP17 inhibit this process\textsuperscript{271}. Once coupling between EspF(U)/TccP and the SH3 (SRC homology 3) domain on IRSp53/IRTKS is established, Tir\textsubscript{EHEC} initiates N-WASP-Arp2/3-mediated actin assembly.

1.4.4. \textit{C. rodentium} pedestal formation

Tir molecules expressed by the mouse pathogen \textit{C. rodentium} and EPEC usurp essentially identical host signalling pathways to induce pedestal assembly. In common with Tir\textsubscript{EPEC}, Tir\textsubscript{C. rodentium} utilizes two tyrosine residues in the C-terminal cytoplasmic domain in order to hijack the host Nck-independent (Y\textsubscript{451}) and Nck-dependent (Y\textsubscript{471}) signalling pathways for pedestal biogenesis. Studies have shown that Tir\textsubscript{EPEC} or Tir\textsubscript{C. rodentium} are functionally interchangeable\textsuperscript{211,218}. By contrast, neither Tir\textsubscript{C. rodentium} or Tir\textsubscript{EPEC} can be substituted for Tir\textsubscript{EHEC}\textsuperscript{211,272}.

1.5. \textit{Francisella tularensis}

\textit{Francisella tularensis} is a facultative intracellular bacterium that causes a potentially fatal disease in humans known as tularemia. \textit{F. tularensis} is also a zoonotic pathogen that can reside within a wide range of host organisms. In fact, reseach
suggests that Francisella has over 250 host species of birds, arthropods, fish, mammals, and reptiles \(^{273-275}\). Despite its persistence in the natural environment, cases of human infections from \(F. \text{ tularensis}\) are rare, though they have been reported in all continents except for Antarctica. Given their global distribution, these gram-negative bacteria have undergone significant genotypic divergence and can be subdivided into three major subspecies (ssp.) based on geographic location and virulence in humans: \(F. \text{ tularensis}\) (Type A), \(F. \text{ tularensis}\) ssp. \(holarctica\) (Type B), and \(F. \text{ tularensis}\) ssp. \(F. \text{ mediasiatica}\). Type A ssp. \(tularensis\) is endemic in North America. The Type A strain, with an LD\(_{50}\) of <10 CFU, is the most highly virulent and has a mortality rate of up to 30-60\% \(^{276,277}\). By contrast, Type B ssp. \(holarctica\) and \(mediasiatica\) have mild virulence in humans (For ssp. \(holarctica\): LD\(_{50}\) <10\(^5\) CFU) and rarely lead to significant mortality \(^{277}\). Despite >97\% genomic identity across the subspecies \(^{278}\), little is known with regards to the virulence mechanisms that make the Type A strain more virulent than the other subspecies. In terms of regional differences, isolates of ssp. \(holarctica\) are often found in Europe but it is thought to have likely originated from Asia \(^{278}\), while ssp. \(mediasiatica\) has so far been restricted to Central Asia.

1.5.1. History, epidemiology, and treatment of \(F. \text{ tularensis}\)

\(F. \text{ tularensis}\) was first isolated in 1911 from ground squirrels that had plague like-disease in Tulare County, CA, U.S.A \(^{279}\). The genus ‘Francisella’ was later named after Edward Francis, who pioneered the research on this microorganism and the disease now known as tularemia \(^{280}\). In the 1930’s and 1940’s, large water-borne outbreaks of \(F. \text{ tularensis}\) occurred in Europe, U.S., and the former Soviet Union prompting research into the creation of a tularemia vaccine \(^{281}\). The first team to achieve this were a group of Soviet scientists who attenuated the virulence of Type B \(F. \text{ tularensis}\) by continually sub-culturing the microbe \textit{in vitro} and subsequently demonstrated the vaccine’s efficacy by immunizing large populations in regions where an outbreak of tularemia had occurred \(^{282}\). By the start of the Second World War, both the U.S. and Russia had established programs aimed at developing a tularemia vaccine as well as for weaponizing \(F. \text{ tularensis}\). By the 1960’s, over 60 million individuals in the former Soviet Union were vaccinated with this live attenuated vaccine. Given its success, a variant of the Soviet strain was brought over to the U.S. in 1959 spurring a limited number of human trials.
Over the subsequent years, those human trials revealed that immunization with *F. tularensis* LVS (Live vaccine strain) conferred only partial protection against the most lethal route of infection—inhaled infection by the highly virulent Type A *F. tularensis*\(^{282}\). In 1969, the U.S. declared an end to its biological weapons program and all stockpiles of biological weapons including *F. tularensis* were destroyed. Rather than follow suit, the former Soviet Union maintained its biological weapons program and by the 1990’s they developed a multi-resistant antibiotic strain of *F. tularensis*. One estimate predicts that 250,000 people would be infected if 50 kg of *F. tularensis* were to be released into the air in a city with a population of 5 million\(^{281}\). Because of its low infectious dose, ease of aerosolization, history as a biological weapon, and potential to cause massive loss of life, *F. tularensis* is designated as a ‘Category A Select Agent’ by the U.S. Centres for Infectious Disease Control and Prevention (CDC).

The most recent report by the U.S. CDC, estimates a total of 1,208 cases of tularemia between 2001 and 2010\(^{283}\). This is slightly less compared than the 1,368 incidences reported from 1990 to 2000\(^{283,284}\). Since 1990, the epicentres where the most frequent outbreaks of tularemia occurred were in Arkansas and Missouri\(^{283,284}\). Because *F. tularensis* is a zoonotic pathogen, these cases likely reflect the persistence of *F. tularensis* in the natural environment (ie. soil, water). Known reservoirs of this microbe and sources of human transmission are rodents, lagomorphs (ie. hares and rabbits), and arthropod/insect vectors\(^{277,285}\). Consequently, individuals who are at significantly higher risk of *F. tularensis* infection live in rural areas and perform activities such as lawn mowing and brush cutting\(^{286}\), making hay\(^{287}\), swimming in a contaminated body of water\(^{288}\), and handling infected carcasses\(^{289}\). Age groups that are most susceptible to tularemia are children (aged 5 to 9 years old) and males over 55 years old\(^{284}\). It is important to note that no cases of human-to-human transmission have been reported.

Given that no effective and safe vaccine is available to the public for tularemia, antibiotics are the only front-line treatment for this disease. Fortunately, *F. tularensis* is susceptible to several classes of antibiotics including doxycycline and ciprofloxacin. These antibiotics are taken orally and can be used for prophylactic measures as well\(^{290}\). Gentamicin or streptomycin are also effective against *F. tularensis*, but their use is typically restrained because aminoglycoside antibiotics are linked to rare neurological and kidney problems\(^{281}\). Whereas these drugs are bactericidal to *F. tularensis*\(^{291,292}\),
other types of antibiotics (ie. β-lactam and macrolides) are not recommended because these drugs are considered to be bacteriostatic as well as the presence of bacterial β-lactamases.

Currently, there are renewed efforts to develop a novel vaccine that confers greater protection against aerosolized transmission of highly virulent Type A F. tularensis. However, given what little we know about the pathogenic mechanisms of this pathogen, the development of a preventative therapy is likely to be decades away. Until then, the live attenuated strain of F. tularensis LVS (Francisella LVS) is the only protective therapy that has been granted approval as an ‘Investigational New Drug’ by U.S. Department of Defense. This ‘Investigational New Drug’ designation means that immunizations with Francisella LVS are limited only to those at high risk of exposure (ie. laboratory workers). As Francisella LVS is the only tularemia vaccine that has been extensively evaluated in humans, there is broad consensus in the scientific community to comprehensively understand the molecular mechanism of attenuation for F. tularensis LVS in order to meet the criteria for a New Drug Application so that it can be utilized as a vaccine in the interim.

Representative strains of Type A and Type B F. tularensis, F. tularensis LVS, F. novicida have all been sequenced and this data has provided researchers with a wealth of information in order to elucidate the genetic mechanisms that contribute to the attenuation of F. tularensis LVS virulence from its parent strain, F. tularensis subsp. holarctica. A number of regions marked by gene deletion events and single nucleotide variations have been identified. Two mutant alleles located within these regions of differences that could contribute to attenuation of F. tularensis LVS are pilA and FTT0918. A report published by Salomonsson and colleagues demonstrated that replacement of both alleles with their wild-type counterparts ameliorated bacterial virulence in mice comparable to its parent strain.

1.5.2. Pathology of tularemia

The clinical manifestations of tularemia can vary depending on the route of entry and the systemic dissemination of the pathogen within the host. The incubation period is typically 3 to 6 days from the time of exposure, but can take as long as 21 days before
the onset of symptoms \textsuperscript{298}. The most common form of tularemia occurs on the skin through a bite from an arthropod vector or open wound \textsuperscript{277,299}. Referred to as glandular tularemia, infection of \textit{F. tularensis} around the surrounding lymph nodes can cause painful swelling. In fact, ulceroglandular and glandular tularemia comprises 75-85\% of all cases \textsuperscript{289}. Besides swollen lymph nodes, an individual can also develop ulceroglandular tularemia, which is characterized by the formation of a skin ulcer at the site of infection. Oculoglandular tularemia is another form of the disease where \textit{F. tularensis} can infect the eyes causing conjunctivitis coinciding with swelling of lymph nodes around the neck and head. Ingestion of contaminated foods or water can cause oropharyngeal/gastrointestinal tularemia. Common signs and symptoms associated with this disease are mild yet persistent diarrhea, intestinal bleeding and ulcers, abdominal pain, and sores in the mouth and throat areas \textsuperscript{277,281}.

\textit{F. tularensis} type A strains are generally more virulent than type B strains. Inhalation of just 10-50 Type A \textit{F. tularensis} bacteria is sufficient for infection of the lungs resulting in pneumonic tularemia \textsuperscript{277}. By comparison, non-airborne routes of entry typically require up to 100 CFU to cause severe disease or \textasciitilde10^3 CFU for the lesser virulent Type B \textit{F. tularensis} strain. Patients that contract pneumonic tularemia usually present symptoms such as dry cough, pleuritic chest pain, and dyspnoea. Fortunately, pneumonic tularemia is rarely acquired in the natural settings. Colonization of the lungs or in other severe cases can lead to \textit{F. tularensis} causing septicaemia (\textit{aka.} typhoidal tularemia). Typhoidal tularemia accounts for 10-15\% of tularemia cases and is characterized by septicaemia, fever, chills, and malaise but is not usually accompanied with lymphadenopathy \textsuperscript{277}. The most lethal forms of the disease are pneumonic and typhoidal tularemia, and they account for approximately 30\%-60\% mortality, if antibiotics are not administered early \textsuperscript{277}. However, early detection combined with the use of antibiotics has significantly reduced the fatality rate to less than 2\% \textsuperscript{281,298}.

1.6. \textbf{Francisella genome}

The \~1.89 Mb genome of \textit{Francisella novicida} and all three \textit{F. tularensis} subspecies (ssp. \textit{tularensis}, ssp. holarctica, ssp. mediasiatica) are nearly identical in size, GC (guanine cytosine) content (\~32\%), and nucleotide sequence (\~97\%) \textsuperscript{278,300,301}. 
Despite these likenesses, sequencing of all three *F. tularensis* strains has identified ~1,400 protein-coding genes, which is substantially less compared to the ~1,700 in *F. novicida* \(^{301}\). A large part of this difference is compensated by the higher abundance of pseudogenes in *F. tularensis*. In fact, approximately 160 functional genes in *F. novicida* are inactivated in *F. tularensis* \(^{300}\). Of these >160 pseudogenes in *F. tularensis*, 41% are predicted to have been at one point involved in amino acid biosynthesis. Also, over half of these pseudogenes are located within 100 bp of genomic breakpoints, which are sites within the chromosome that undergo higher frequency of genetic changes (*ie.* missense mutations, gene inversions, and recombination events) compared to other parts of the genome. A common cause for the loss of gene function is attributed to the transposition of insertional sequence (IS) elements. A comparison of the two *Francisella* species have found more than 50 predicted ISFtu1 type IS elements in the *F. tularensis* genome compared to just a one in *F. novicida* \(^{300,301}\).

The loss of functional genes involved in amino acid biosynthesis is characteristic of an evolutionary process known as gene decay. Often genes that contribute to the microbe’s fitness in the non-host environment, but are antagonistic or dispensable for its ability to establish an infection in the host tissue are lost. Gene decay is considered a pathoadaptive mechanism that some microorganisms have utilized to enhance their pathogenicity. For example, loss of the *cadA* gene in all *Shigella* spp. enhances their virulence because expression of lysine decarboxylase disrupts the microbe’s ability to induce transepithelial migration of PMNs \(^{302-304}\). In contrast to the loss of gene function, *F. tularensis* (Schu S4 strain) appears to have acquired 41 new genes that are not found in *F. novicida* (Utah 112 strain) \(^{300}\). It is conceivable that one or more of these genes could enhance the virulence of *F. tularensis* in humans.

Genomic analysis of *F. tularensis* and *F. novicida* has predicted two potential protein translocation machineries. One of the two multi-component apparatuses identified is the type IV pilus (Tfp) system, which directly facilitates the biogenesis of hair-like fimbria on the bacterial surface \(^{305,306}\). Gene deletion of the *pilA* gene reduces the virulence of *Francisella* \(^{297,307}\). Under specific conditions, *Francisella* appears to generate fibers thought to be Tfp from the surface of the microbe \(^{308}\), though whether they are formed during *in vivo* infections has not been proven.
1.6.1. *Francisella* pathogenicity island (FPI)

The *Francisella* pathogenicity island (FPI) is a ~34 kb region that contains up to 19 virulence-associated genes within the core bacterial chromosome. The entire FPI region is central for not only bacterial replication and survival within professional phagocytic cells, but also infectivity of the host organism (Figure 1.3). Genome sequencing of *F. novicida* has shown that it possesses only a single copy of the FPI, while all Type A and Type B *F. tularensis* strains contain two essentially identical gene clusters. The fact that *F. tularensis* harbours two copies of the FPI has led some to speculate that duplication of this highly conserved region signals the emergence of a highly infectious human pathogen. Whether duplication of the FPI significantly enhances the virulence of *F. tularensis* is an important question that has not been firmly established, however, reports have demonstrated that deletion of a single allele in the FPI does not severely hamper bacterial proliferation within macrophages—which is considered by many to be central for its pathogenicity in mammals. This suggests that a redundant FPI could be the pathogen’s strategy to protect key virulence genes within this region from possible allelic mutations that could detrimentally affect its fitness within the host organism.

The intracellular growth locus (*igl*) and pathogenicity determinant protein (*pdp*) are two major groups of genes in the FPI. It is important to note, however, that despite their names not all genes designated *igl* or *pdp* are important for intracellular replication and maintaining virulence. Various lines of evidence have so far yielded 12 FPI gene products that are required for rapid intracellular proliferation within phagocytic cells and pathogenicity in mice (Table 1.1). These gene deletion experiments also demonstrate that at least 10 genes are important for promoting *Francisella* escape out of the growth-limited phagosomal compartment (Table 1.1). Yet, the exact functions of these proteins are still elusive given the lack of biochemical and molecular data available. So far, only a few FPI genes (*pdpD, pdpE, iglG, and anmK*) appear to be dispensable for bacterial replication within phagocytic cells.
1.7. *Francisella* Type 6 secretion system (T6SS)

Scientific progress to understand the function of the FPI and its encoded proteins has been hampered by the lack of homology with proteins found in other sequenced microorganisms. Nonetheless, bioinformatic analysis of the FPI has uncovered a number of open reading frames (ORFs) that encode proteins similar to those associated with the type VI secretion system (T6SS). Based on the bioinformatic analysis of type VI secretion gene clusters, it is believed that a wide range of non-pathogenic and pathogenic gram-negative bacteria expresses the T6SS. Much of our knowledge regarding the function of this ancient translocation apparatus is derived from the archetype T6SS in *Vibrio cholerae* and *Pseudomonas aeruginosa*\(^\text{317-320}\). Research on their T6SSs has taught us that it is a versatile multi-component machine that can be utilized while in the extracellular milieu for such roles as anti-microbial competitiveness\(^\text{320}\), bacterial cell-to-cell communication\(^\text{318}\), effector and toxin delivery into host cells\(^\text{317,321}\), and cell adhesion\(^\text{320,322}\).

Bioinformatic analysis indicates that the *Francisella* FPI lacks many of the T6SS genes found in gram-negative pathogens such as *V. cholera* and *P. aeruginosa*\(^\text{323}\). In fact, four FPI-encoded proteins are known to be distant homologs of known T6SS components: PdpB (IcmF), IglC (Hcp), VgrG (VgrG), and DotU (TssL). This has led some to speculate that *Francisella* and other microorganisms encode a protein translocation apparatus that is distinctly different from the canonical T6SS model; however, more research is needed to test whether this hypothesis is accurate.

1.7.1. Biochemical and structural highlights on the T6SS

Because there is no microscopic data that confirms the existence of a *Francisella* T6SS, I will provide an overview of the secretion machinery based on published evidence acquired from the well-characterized model in *V. cholerae*. It is broadly accepted that the T6SS functions analogously to the needle complex of a T4 bacteriophage\(^\text{318}\). Within the bacterial cytoplasm, the oligomerization of Hcp assembles to form the long 'needle' filament of the translocation apparatus (Figure 1.4). In *Francisella*, it is surmised that IglC functions analogously to Hcp (Hemolysin co-regulated protein). This is in part based on the orientation of its loci in the relatively
conserved T6SS gene cluster\textsuperscript{324,325}. Additionally, localization experiments imply that IglC is associated with the outer surface, cytosol and bacterial membranes suggesting that its localization is dynamic just like Hcp in \textit{V. cholerae} \textsuperscript{314,326}. This is also supported by crystal structure data of IglC, which appears to demonstrate some resemblance to Hcp, even though their sequence homology is weak\textsuperscript{325}.

Surrounding the T6SS needle is the outer tube, which in \textit{V. cholerae}, is thought to generate the contractile force that drives the needle through the opposing membranes\textsuperscript{327}. The outer tube of the \textit{V. cholerae} T6SS is composed of two major subunits VipA and VipB. Fitting the profile of VipA and VipB, \textit{Francisella} FPI-proteins IglA and IglB can interact with each other \textit{in vitro} to form a heterodimer\textsuperscript{325}. Localization of IglA and IglB in the bacterial cytosol and membrane further implicates that these proteins may assemble to form the outer sheath of the secretion system\textsuperscript{328,329}. Homology between PdpB and the \textit{V.cholerae} protein IcmF predicts that it could be a part of the pore complex that lies in the bacterial membrane\textsuperscript{329,330}. This pore complex provides a conduit for the translocation needle to project through the bacterial membranes toward the adjacent cell. Once the needle punctures the neighbouring cell membrane, part of the needle complex and its payload are released into the target’s cytoplasm. At the tip of the T6SS needle is the capping protein VgrG, which is necessary for effector delivery\textsuperscript{331}. In \textit{Francisella}, its counterpart, also named VgrG, appears to fit the same secretion profile suggesting that both are functionally alike\textsuperscript{330,332}.

\section*{1.7.2. Functional evidence on the T6SS}

So far, there have only been a few published reports investigating the existence of a functional T6SS in \textit{Francisella}. Early research pioneered by the Sjöstedt and Klose labs found that secretion of FPI-encoded proteins VgrG, PdpE, and IglI into the host cytosol is dependent on T6SS gene expression\textsuperscript{333,334}. This was followed by a more comprehensive study by Bröms and colleagues, which involved infecting phagocytic cells with various FPI mutants (each expressing a different FPI protein fused to TEM $\beta$-lactamase) and detecting the translocation of these recombinant proteins in the host cytoplasm by fluorescence of CCF2-AM dye (a substrate of $\beta$-lactamase). This screen of multiple FPI proteins detected the presence of several molecules within the host cell (IglC, IglE, IglI, IglJ, IglF, PdpA, PdpE, and VgrG)\textsuperscript{335}. Moreover, they found that
translocation of these proteins was contingent on the expression of IglG, T6SS components DotU, IglC, and VgrG. Although this method has been used before to measure effector secretion using other microorganisms, a weakness of this study was that most effectors were not detected until 18 h PI. To overcome this issue, a subsequent publication utilized a different approach to assess protein secretion. Using a set of FPI mutants that express FLAG-tagged proteins, Hare and Hueffer (2014) were able to detect the secretion of multiple proteins (IglA, IglB, IglC, IglD, IglE, IglF, IglG, IglH, IglI, IglJ, PdpA, PdpC, PdpE, DotU, and VgrG) in the host cytoplasm as early as 30 min PI. Together, these reports provide substantial evidence to support the existence of a functional Francisella T6SS.

1.8. Pathogenesis of F. tularensis

Research on Type A F. tularensis is challenging given the restrictions limiting its use in facilities with a minimum certification of biosafety level 3 as well as its inclusion as a ‘Tier 1 select agent’ by the US CDC. Additionally, the ease of exposure, high virulence, and lack of a completely effective vaccine are factors that subject laboratory workers to a high level of risk. To compensate these risks, two surrogate models that scientists often use are the murine pathogen F. novicida and LVS (a human attenuated Type B F. tularensis strain). The benefit of these strains is that they require lower biosafety level 2 compliance and both pathogens are not considered virulent to healthy individuals.

The use of F. novicida as a surrogate model has a number of significant advantages over F. tularensis LVS. A chief benefit of using F. novicida is that it is a more tangible model for genetic manipulations. Genomic comparison indicates that F. novicida is >97% identical to F. tularensis and contains nearly all of the same genes within the highly conserved FPI. Having a single copy of this gene cluster (vs. 2 copies in F. tularensis strains) makes it much more amenable to genetic manipulations and less time consuming when investigating the function of FPI genes. Also, whereas the mechanism of attenuation is unclear with F. tularensis LVS, F. novicida remains fully virulent in rodents and is only known to cause illness in human individuals who are immunocompromised or have an underlying chronic liver disease. Another striking resemblance is that both pathogens colonize identical organs and cell types in murine
infection models \(^{290,326,340-343}\) \(^{344}\). Also, \emph{F. tularensis} LVS is a very fastidious microorganism (requiring at least 24-48 h for growth on agar plates and liquid culture before it can be used) and requires expensive components for growth in solid media that must be consumed within a couple of weeks. In contrast, \emph{F. novicida} cultures can be grown overnight using standard bacterial growth media supplemented with L-cysteine. The use of \emph{F. tularensis} strains can also be quite challenging because they can randomly undergo a well-known phenomenon known as phase variation \(^{345}\). Phase variation is a process where the microbe infrequently develops phenotypic changes that can lead to significant variations in the research data.

Despite all the advantages that \emph{F. novicida} offers as a surrogate pathogen, there are some important differences. One is that the LPS O-antigen from \emph{F. tularensis} and \emph{F. novicida} are not antigenically cross-reactive implying that their structures are not identical \(^{346,347}\). While this difference appears to influence the degree in which \emph{F. novicida} and \emph{F. tularensis} are resistant to complement-mediated killing \(^{347,348}\), both microorganisms are just as capable of seeking refuge within various cell types \(^{349}\). Another distinction is that respiratory challenge in mice with \emph{F. novicida} induces ~1,000-fold greater neutrophil infiltration compared to \emph{F. tularensis} LVS \(^{344}\). Whether this could impinge on the ability of \emph{F. novicida} to cause disease is unclear because both microorganisms are capable of infecting neutrophils and interfering with reactive oxygen species production \(^{350}\). Another difference is that \emph{F. tularensis} is able to suppress TLR2 activation \(^{351}\), whereas \emph{F. novicida}, which lacks this particular ability, utilizes a different mechanism to evade detection by inflammasome-mediated pathogen recognition factors within the host cytoplasm \(^{352}\). Studies \emph{in vivo} show that modulation of host inflammasome activation is a central virulence strategy for both microbes \(^{353-355}\).

### 1.8.1. Phagocytic cell infections

In humans and other mammals, \emph{Francisella} is an intracellular pathogen that can replicate and survive within multiple types of cells, but also has a significant extracellular phase in the blood plasma \(^{356}\). These cell types can be segregated into two groups based on their normal phagocytic activity. One group referred to as phagocytic cells can readily phagocytose \emph{Francisella} and other foreign material into the cell. These cells include eosinophils, monocytes, macrophages, dendritic cells, and neutrophils all of
which can be parasitized by *Francisella*. Rather than being phagocytosed and then killed by these phagocytes, *Francisella* has evolved ways to disrupt their bactericidal activity and thrive within these hostile cells. Among these phagocytic cells, macrophages are a major target of *F. tularensis* in the lungs and skin. Given that the pathogenesis of *Francisella* has primarily been studied in phagocytes, I will cover the three key stages of its pathogenic life cycle in this section.

**Phagocytic cell entry**

*Francisella* are readily phagocytosed by macrophages, monocytes, dendritic cells, and neutrophils. Although not required for bacterial uptake, *Francisella* can enhance its internalization through direct recognition with the mannose receptor or indirect interaction with one of several cell surface receptors (ie. complement receptor 3 (CR3), scavenger A receptor (SRA), Fcγ receptor (FcγR), and surface-exposed nucleolin) through a process known opsonization. While phagocytosis is the primary mechanism involved in *Francisella* uptake in macrophages, Clemens and colleagues (2005) have demonstrated that these microbes elicit the formation of spacious pseudopod loops to facilitate their engulfment (via interacting with the C3 receptor).

**Francisella-containing phagosome (FCV) and vacuole escape**

*In vitro* studies have shown that upon internalization, *Francisella* transiently resides within a vacuole and acquires the early endosomal marker EEA1 as early as 15 min post-infection. As the vacuole matures, the *Francisella*-containing vacuole (FCV) acquires late endosomal markers LAMP-1, LAMP-2, and CD63. Although this vacuole normally becomes acidified during phagosomal maturation, evidence suggests that the FCV is either completely resistant or able to delay acidification. Furthermore, it is unclear what the pH level is at the moment of phagosomal escape and whether pH is a key factor in triggering destruction of the FCV membrane. Also, the absence of the luminal hydrolase Cathepsin D and lysosomal tracers suggests that *Francisella* is able to avert fusion with other lysosomes. Macrophages and neutrophils also utilize a defense mechanism known as respiratory burst to complement its microbial killing armaments. As such, the viability of *Francisella* within the FCV and its virulence in mice are dependent on their capacity to combat the production of reactive
oxygen and nitrogen species\(^{343,371}\). One of the most studied bacterial encoded molecule involved in this process is the acid phosphatase AcpA\(^{350,372-374}\), which can counteract intraphagosomal generation of reactive oxygen species (ROS). Detailed biochemical studies demonstrate that AcpA can hydrolyze monoesters within an acidic pH environment and dephosphorylate the components in order to impede NADPH oxidase assembly\(^{350}\)—the core element that facilitates ROS production\(^{343,350,371,373,374}\).

Essentially all of the FCVs within infected phagocytes remain completely intact within the first 2 h of infection\(^{364-366}\). By 4 h PI, microscopic observations reveal that most of the internalized Francisella are free from the FCV and reside within the cytoplasm\(^{364,369,370}\). Coinciding with this, the expression of FPI genes is upregulated\(^{364}\). Although the biochemical mechanisms involved in phagosomal membrane disintegration remains elusive, studies have shown that many FPI genes are central for the microbe’s ability to escape the FCV\(^{375}\) (Table 1.1). Bioinformatic studies of the Francisella genome have not found any relationship with known bacterial proteins possessing membrane-disrupting activity (i.e. listeriolysin O from L. monocytogenes)\(^{376,377}\). Hence, it is possible that Francisella encodes a novel factor or mechanism to rupture the FCV.

**Intracellular replication and re-acquisition of the FCV**

The commencement of bacterial proliferation can be detected within the host cytosol as early as 4 h PI. However, several studies have found that many genes within the FPI are important for their ability to efficiently escape from the FCV and rapidly proliferate in the host cytoplasm (i.e. \(\Delta\text{iglC, iglE, pdpE, iglE}\)\(^{332,370,378,379}\). Francisella can hastily replicate and fill the cytoplasm in approximately 24 h. So far, the only known F. tularensis genes that show only intracellular proliferation defects when deleted are dipA, fopA, and ripA. In murine studies, F. tularensis \(\Delta\text{dipA, fopA, or ripA}\) mutants were completely attenuated for virulence suggesting that their capacity to replicate within host cells are essential for disease\(^{234,380-383}\). Cell fractionation experiments predict that their gene products localize to the bacterial membrane\(^{380,383,384}\), but unfortunately their underlying function has yet to be deciphered.

During the intracellular replication phase, a subset of cytoplasmic bacteria is found encapsulated in double-membrane compartments. Immunolocalization of these late stage FCVs have identified several autolysosomal markers decorated around the
membrane (autophagosomal membrane-associated protein LC3, ubiquitin, SQSTM1, LAMP-1, Cathepsin D)\textsuperscript{384,385}. \textit{Francisella} is unique in that it is the only known pathogen so far that re-enters the vacuolar compartment. It is estimated that 38-46\% of intracellular bacteria are FCV-bound at 24 h PI. By 36 h PI, the proportion of FCV-bound \textit{Francisella} can climb up to \textasciitilde{}81\% \textsuperscript{384,385}. Viability assays suggest that \textit{Francisella} can remain alive within these double-membrane vacuoles \textsuperscript{384}. Treatment of murine macrophages using autophagy inhibitors appears to diminish FCV formation hinting that activation of the host autophagy pathway is required to generate these large spacious vacuoles \textsuperscript{385}. On the contrary, microarray data from two independent labs found that autophagy-related genes in these murine macrophages are not activated, but are instead, down-regulated during the late stages of infection \textsuperscript{368,369}. Contrary to their murine counterparts, both \textit{F. novicida} and \textit{F. tularensis} fails to re-enter autophagosome-like FCVs in human macrophage \textsuperscript{387,388}. A key question that remains is whether these autophagosomal-like FCVs are a ‘bona-fide’ stage in the intracellular life cycle.

### 1.8.1. Epithelial cell infections

In humans and other mammals, \textit{Francisella} can parasitize a diverse range of non-phagocytic cells including alveolar and skin epithelial cells \textsuperscript{389,390}, fibroblasts \textsuperscript{391}, and hepatocytes \textsuperscript{392}. Investigations into non-phagocytic cell infections have frequently focused on lung epithelial cells because respiratory-acquired tularemia is associated with the highest rate of mortality in humans \textsuperscript{276,393,394}. Within the lungs, this microbe frequently colonizes along the outer alveolar wall, 98\% of which is lined with a single layer of epithelial cells \textsuperscript{395,396}. Here, \textit{F. tularensis} can infect lung epithelial cells with a preference towards surfactant secreting alveolar type II cells \textsuperscript{344,389,395} as well as gain access to the pulmonary circulation. It is important to note that \textit{F. tularensis} can enter the bloodstream either directly (\textit{ie.} open wound, bite from arthropod vector) or through other portals of entry, and yet still cause fatal cases of Tularemia \textsuperscript{280,285,394,397}.

The liver is among the most frequently colonized organ in humans and animals infected with \textit{F. tularensis} irrespective of the route of transmission and as such it is considered a major site of histopathology for tularemia \textsuperscript{280,298,340,390,394,398,399}. Bacterial colonization of the liver is accompanied by gross histopathological changes such as necrosis, discoloration, and enlargement \textsuperscript{399-401}. In both humans and rodents, specialized
epithelial cells, called hepatocytes, comprise up to 70-80% of the parenchymal volume whereas resident liver macrophages known as ‘Kupffer cells’ make up ~2% \(^{402-404}\). Within the liver parenchyma, bacterial replication is most evident within large multicellular foci that are predominantly comprised of hepatocytes \(^{404,405}\). Considering that the liver has among the highest bacterial burden in animal models \(^{406-410}\), it is not unexpected that hepatocytes are a major site of bacterial replication.

A few studies have described attempts to elucidate the host or bacterial mechanisms involved in \textit{Francisella} invasion into epithelial cells and its intracellular pathogenesis. Work by Thomas Kawula’s lab proposed that \textit{F. tularensis} LVS invades epithelial cells through a passive mechanism. In their study, they measured the invasion activity for WT and paraformaldehyde-killed bacteria and found that both microbes internalized into murine epithelial TC-1 cells at the equal rates \(^{411}\). A notable concern with this evidence is that the sample was centrifuged and chilled to 4°C which could have artificially forced bacterial uptake and therefore may not be representative of the natural bacterial invasion process. In another set of experiments, it was demonstrated that impairment of actin polymerization or microtubule assembly with with pharmacological inhibitors (cytochalasin D and colchicine, respectively) ablated epithelial cell invasion \(^{411}\). These results paralleled previous findings that used cultured epithelial cell lines A549, Hep-2, and HBE \(^{412}\). The authors also found that wortmannin, a PI3K inhibitor, and genistein, a tyrosine kinase inhibitor, impeded its invasion into TC-1 cells, suggesting that \textit{F. tularensis} could utilize these signalling molecules to induce actin polymerization \(^{411}\). Still, neither concentrated actin filaments surrounding the microbe like that induced by InlA of \textit{L. monocytogenes} \(^{413}\) nor actin ruffles similar to those produced by \textit{Salmonella} spp. \(^{414}\) have been observed. In common with phagocytes, \textit{Francisella} enters a vacuole upon internalization where it transiently accumulates endosomal markers such as EEA-1 and LAMP1 following its internalization into the host cell \(^{411}\). By 2h, 70% of the intracellular bacteria will have ruptured the surrounding vacuolar membrane and would subsequently be able to proliferate within the cytoplasm \(^{395,411}\). We believe that clarification of the internalization mechanisms is needed because epithelial cell invasion is a key stage in the pathogenesis of many intracellular pathogens.
Importance of epithelial cells in disease

The virulence of *Francisella* correlates with its capacity to rapidly proliferate within host cells and overwhelm the host innate immune system. Since *Francisella* can infect a broad range of cell types, it is not well established whether phagocytic and/or non-phagocytic cells represent a vital replicative niche for the progression of Tularemia. In an attempt to delineate this question, seminal work from Horzempa and his associates (2010) examined the pathogenicity of a uracil auxotrophic *F. tularensis* mutant, \( \Delta \text{pyrF} \), in *in vitro* and *in vivo* models. As with other uracil auxotrophic *Francisella* mutants, \( \Delta \text{pyrF} \) is efficiently killed via ROS-mediated response within primary macrophages, yet still remained capable of dividing within non-phagocytic cells. Respiratory infections with Type A *F. tularensis \( \Delta \text{pyrF} \) resulted in the high bacterial burdens in the liver, spleen, and lungs. Also, the \( \Delta \text{pyrF} \) mutant remained highly virulent in mice, and the majority of bacterial replication occurred in the liver. Together, this suggests that replication within non-phagocytic cells is important in contributing to the manifestation of disease.

1.9. Rationale and Research hypothesis

Morphological pedestal-shaped structures generated on the surface of enterocytes are present in all EPEC disease. Because these pedestals are closely associated with the host cell body, molecular probes are typically utilized to uncover novel host constituents in order to better understand their role in pedestal formation and EPEC pathogenesis. Such reagents have identified about 50 pedestal components over the past two decades; however, due to their cost and other factors, these probes are not feasible for use in large-scale screens. Given the limited extent of host proteins that have been investigated so far, I hypothesize that many pedestal components not yet been uncovered and a subset of these molecules could also be important for EPEC pathogenesis.

In contrast to the intestinal bacterium EPEC, *F. tularensis* can enter the host through various routes and subsequently disseminate into different parts of the body. A major site of bacterial replication associated with fatal tularemia disease is the liver. The
liver is predominantly made up of non-phagocytic hepatocytes. Unlike phagocytic cells that can naturally engulf *F. tularensis*, the process in which this intracellular microbe internalizes into these epithelial cells is still obscure. Since many known intracellular pathogens by hijack existing host signaling mechanisms, I hypothesize that *F. tularensis* can subvert cellular components within hepatocytes in order to facilitate its own internalization.
1.10. Figures

Figure 1.1  EPEC and EHEC Tir-induced pedestal formation
A) The hallmark of enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) is their ability to tightly adhere onto the intestinal epithelium, efface the surrounding microvilli, and generate characteristic actin-rich structures called pedestals. B) EPEC- and EHEC-expressed translocated intimin receptor (Tir) induces the host Arp2/3-mediated actin assembly through distinctly separate pathways that ultimately leads to the formation actin-rich pedestals. Both Tir_{EPEC} and Tir_{EHEC} are injected into the host cytoplasm via type III secretion system (T3SS), translocated into the host plasma membrane, and subsequently accumulate beneath the site of bacterial contact. Intimin-mediated clustering of Tir_{EPEC} is necessary the signalling and recruitment of downstream molecules. By contrast, Tir_{EHEC}-mediated signalling is independent of host tyrosine phosphorylation, but instead requires a second effector called EspFu. (Illustration from HT Law and Julian Guttman, 2014 [416]).
Figure 1.2  Illustration of EPEC/EHEC Type III secretion system (T3SS)
An illustration of the needle-like protein translocation machinery and 15 of its core components as represented along the inner and outer bacterial membranes (IM and OM, respectively). Two pore-forming proteins EspD and EspB are inserted into the plasma membrane (EM) of the host cell via the type III secretion. Illustration taken from 417.
The *Francisella* pathogenicity island (FPI) is a ~30kb gene cluster important for intracellular replication, phagosomal escape, and virulence. *F. tularensis* ssp. *tularensis* (Type A), ssp. *holarctica* (Type B) and ssp. *mediasiatica* all possess two copies of the FPI, whereas *F. novicida* contains only a single copy. Image derived from "418.

**Figure 1.3  Diagram of FPI in Type A *F. tularensis* (Schu 4)**

The *Francisella* pathogenicity island (FPI) is a ~30kb gene cluster important for intracellular replication, phagosomal escape, and virulence. *F. tularensis* ssp. *tularensis* (Type A), ssp. *holarctica* (Type B) and ssp. *mediasiatica* all possess two copies of the FPI, whereas *F. novicida* contains only a single copy. Image derived from "418.

Putative components of *Francisella* Type VI secretion system (T6SS)
- Secreted into macrophages
- Other virulence factors
- Open reading frames
Figure 1.4  Model for *Francisella* T6SS effector translocation

The *Francisella* type VI secretion system (T6SS) can translocate bacterial ‘effector’ proteins into host mammalian cells. Based on the effector secretion model from *V. cholerae* T6SS, effectors are thought to be loaded at the needle tip where VgrG is located. This is believed to be followed by nucleation of the inner rod (IglC) and outer sheath (IglA/B) in the bacterial cytoplasm. Compression of the outer sheath forces the inner rod through the bacterial cell wall and into the host cytoplasm where VgrG and other effectors are released. This figure is derived from 319,325. Illustration is drawn by HT Law.
## 1.11. Table

### Table 1.1 Influence of *Francisella* pathogenicity island (FPI) genes on *Francisella* pathogenicity

<table>
<thead>
<tr>
<th>FPI genes:</th>
<th>Rapid intracellular growth</th>
<th>Efficient phagosomal escape</th>
<th>Attenuation of virulence in mice (ID$_{50}$)</th>
<th>Ref.</th>
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<td><em>iglA</em></td>
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<td>Y</td>
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<td>-</td>
<td>-</td>
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<td>Y</td>
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<td>Y / N</td>
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<td>-</td>
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*Putative type VI secretion components. This table is derived from 323.*
Chapter 2.

Mass spectrometry-based identification of proteins in EPEC pedestals

The contents in this chapter have been accepted for publication.


Author contributions: HTL and CM performed the experiments and analyzed the results with the assistance from KMM. HTL wrote the manuscript. JAG, LJF, and MC reviewed the manuscript.
2.1. Abstract

During enteropathogenic *Escherichia coli* (EPEC) infections these attaching and effacing (A/E) microbes co-opt existing host signaling pathways and recruit >55 known host proteins to motile morphological structures, called pedestals, at sites of bacterial attachment. EPEC pedestals are hallmarks of EPEC-based disease and the identification and characterization of the functions of pedestal proteins continue to steadily increase. To identify additional constituents in an unbiased manner we began by artificially elongating EPEC pedestals up to ~25 \( \mu \)m in length through transwell filters and then concentrating them. The harvested pedestal proteins were then analyzed by mass spectrometry (MS)-based proteomics and >90 unique mammalian proteins were identified over multiple experimental trials. 17 novel pedestal proteins from the samples were significantly higher in abundance when compared to both the negative controls and sample means \( (p < 0.05) \). Validation of two novel pedestal proteins (the non-actin associated protein, cyclophilin A, and actin-associated protein, transgelin) by immunolocalization was used to confirm our analysis and both showed enrichment at EPEC pedestals. Moreover, their distribution in these actin-rich structures did not significantly differ in the presence or absence of 2,3-butanedione monoxime (BDM). The EPEC pedestal concentration technique developed here together with the identification of novel pedestal proteins provides not only a resource for the further characterization of molecular components within these structures, but also demonstrates that EPEC pedestals can be used as a model system for the identification of novel functions of proteins not normally thought to be at actin-based structures.

2.2. Introduction

Enteropathogenic *E. coli* (EPEC) causes profuse watery diarrhea and significant mortality of children under 2 years old in developing countries \(^{433}\). EPEC and other diarrheagenic bacteria such as enterohemorrhagic *E. coli* (EHEC) and *Citrobacter rodentium* belong to a family of microbes referred to as 'attaching and effacing (A/E)
bacteria’. The hallmark of A/E pathogens is their ability to generate characteristic pedestal-shaped lesions. These A/E lesions are demarcated by the dissolution of microvilli at the sites of bacterium/host cell contact and the assembly of actin filaments to form a ‘pedestal’ structure that protrudes from the host cell surface just beneath the site of bacterial contact. Several reports have demonstrated that these pedestals enhance colonization of A/E pathogens and are important for full virulence in humans \(^3\) and in animal models \(^{211,217,434,435}\).

EPEC remain primarily extracellular during their pathogenesis and use a type III secretion system to inject bacterial ‘effector’ proteins into host cells. A key effector necessary for pedestal formation is the translocated intimin receptor, Tir. When docked with intimin on the bacterial surface a number of host proteins become recruited to the cytoplasmic regions of Tir, which ultimately direct actin filament assembly beneath the EPEC attachment site \(^{436}\).

Over the past two decades, >50 components have been identified at EPEC pedestals \(^{416}\). Much of this has come from the use of antibodies or molecular probes that localize with their target(s) to these structures. Although this process is important in validating the proper localization of proteins at pedestals, it is a relatively slow process that requires a certain degree of prior knowledge of protein function to determine those to pursue. Thus, to identify novel host proteins within EPEC pedestals in an unbiased manner, we developed an approach to concentrate EPEC pedestals then used those preparations for mass spectrometry analysis. This required the infections of host cells through a transwell filter and the addition of 2,3-butadione monoxime (BDM), a non-muscle myosin II inhibitor, to stimulate the elongation of the pedestals through the filter pores prior to mass spectrometry (MS) analysis. We identified over 90 proteins from these assays that included proteins from various sub-cellular compartments. Further analysis demonstrated that 17 novel proteins were significantly more abundant than in our controls. To validate our findings, we selected an actin-associated protein, transgelin, and a non-actin associated protein, cyclophilin A, from our list of candidates. Using fluorescence microscopy, we demonstrated that both molecules were enriched at both wild-type and BDM-elongated pedestals. This work demonstrates the potential of EPEC pedestals as a system for the identification of novel functions of previously known
eukaryotic proteins at actin-rich structures while concurrently being able to characterize the role of those proteins during EPEC-mediated disease.

2.3. Results

2.3.1. Concentration of elongated EPEC pedestals

To uncover additional proteins at EPEC pedestals in an unbiased manner, we devised a strategy to concentrate these characteristic structures for use as substrates for MS-based proteomics analysis (Figure 2.1). We initially used the technique developed by Shaner and colleagues to generate long pedestals by supplementing BDM onto EPEC pedestals grown on Ptk2 cells (Movie B1). With these epithelial cells, we prepared our pedestal preparation by adhering Ptk2 cells to the bottom of transwell membranes and then subsequently inverting the transwells into their proper orientation and infecting with WT EPEC through the membrane pores on the top (acellular) side of the Transwell membrane. This allowed the bacteria to enter through the 3µm Transwell pores and make contact with the monolayer on the opposite side. Because EPEC pedestals are normally 2-5 µm long and thus not tall enough to span the thickness of the 10µm Transwell membrane, we applied BDM, a non-selective inhibitor of conventional myosin, to promote the elongation of actin-rich pedestals so that they were of sufficient length to protrude from the acellular side of the membrane (Movie B2). The long pedestals were then scraped off from the top of the Transwell and concentrated. Pedestal morphology remained intact after the scraping procedure (Figure 2.2). Identical samples were prepared using an EPEC Δtir mutant defective for pedestal formation to account for any proteins that could have made their way to the top of the Transwell chamber in the absence of EPEC pedestals.

2.3.2. Verification of known host proteins in BDM-treated pedestals

Given that BDM dramatically rearranges the actin cytoskeleton within the cell, we sought to determine whether this pharmacological inhibitor had changed the general localization of known proteins within pedestals. For this, we chose several known
proteins that are well described in terms of their localization at the pedestal tip (eg. clathrin \(^{239}\), CD2AP \(^{210}\), and Nck \(^{142}\)), apical region and stalk (eg. Arp2 \(^{66}\) and cortactin \(^{250}\)), and base (eg. spectrin and p4.1 \(^{256}\)). We found that the distribution profile of these 7 proteins in long BDM-treated pedestals was consistent with their known localization in normal EPEC pedestals (Figure 2.3A).

Because BDM is considered a conventional myosin ATPase inhibitor, we investigated if this chemical affected the recruitment of myosin-9 beneath adherent EPEC. In BDM-treated samples, we found that myosin-9 is enriched at the apex of EPEC pedestals (Figure 2.3B), just the same as in wild-type pedestals. Together, our data indicates that pedestal lengthening by BDM does not significantly alter the localization of known EPEC pedestal proteins.

### 2.3.3. MS-based proteomics of long EPEC pedestals

To screen for novel protein constituents in EPEC pedestals, wild-type EPEC and control samples were chemically conjugated with stable isotope dimethyl labels and analyzed by MS-based proteomics. The data was queried against a mammalian proteomic database because a complete proteome of *Potorous triactylis* does not currently exist. We found 92 unique proteins that were identified repeatedly over four experiments (Table 2.1 and Table C1). Candidates were then scored based on their relative abundance in the wild-type EPEC sample vs. control sample. Our analysis was based on our expectation that pedestal proteins would be enriched, thus we used a one-tailed z-test to determine those that had a significantly higher relative abundance value compared to the sample mean (p < 0.05) (Table 2.2). This resulted in the identification of 11 known components (as denoted by # in Table 2.2) and 17 unique proteins predicted to be significantly more abundant at EPEC pedestals. We also determined using annotated data from the Uniprot repository that many of the 28 proteins were cytoplasmically located while a small proportion had known association with the mitochondria, endoplasmic reticulum, nucleus, and the plasma membrane (Figure 2.4A).
2.3.4. Verification of novels pedestal proteins

To verify that our MS-based proteomics methodology appropriately detected unidentified pedestal components, we localized transgelin, an actin-associated protein involved in cell migration and cyclophilin A, a non-actin associated protein that catalyzes trans to cis isomerization at proline residues. Both proteins were immunolocalized in EPEC infected Ptk2 cells following BDM treatment where we observed both molecules concentrated at actin-rich pedestals (Figure 2.4B). More specifically, we found that transgelin localized more densely throughout the entire pedestal whereas cyclophilin A appeared more concentrated at the apex. In uninfected Ptk2 cells, characteristic actin-rich pedestals were not observed and both molecules were distributed throughout the cytoplasm (Figure 2.5). These observations are consistent with our MS-based proteomic data.

To confirm and examine the location of both proteins in native EPEC pedestals, HeLa and Ptk2 cells were infected with WT EPEC and in the absence of BDM. Using immunofluorescence staining, we discovered that cyclophilin A and transgelin are both redirected beneath EPEC following bacterial attachment (Figure 2.6). Their distributions in EPEC pedestals generated from human and potoroo epithelial cell lines were comparable with that observed in elongated BDM-treated pedestals (Figure 2.4B and Figure 2.5). This validated that cyclophilin A and transgelin are two novel constituents of EPEC pedestals and suggests that our pedestal/MS analysis has generated appropriate findings.

2.4. Discussion

In this study, we devised an approach to concentrate and identify novel host proteins at EPEC pedestals. A key piece of this strategy required that long EPEC pedestals be generated and physically severed from the cell body for MS-based proteomic analysis. As a result, we detected a total of 92 unique proteins in these preparations, of which 28 were enriched to statistically significant levels (11 known, 17 unknown) in samples of concentrated EPEC pedestals. Our findings reveal that cyclophilin A and transgelin, two predicted pedestal proteins, were highly concentrated.
at these sites. Moreover, the general distribution of cyclophilin A and transgelin in long EPEC pedestals appeared to be analogous to normal EPEC pedestals.

We used the general myosin inhibitor BDM to stimulate EPEC pedestal growth well beyond their normal length. This treatment did not alter the general distribution profile of known host proteins recruited beneath the site of bacterial-host attachment including Arp2, clathrin, Nck, and N-WASP; all of which play key roles in pedestal formation \(^{66,142}\). The use of this inhibitor to identify the precise location of proteins in pedestals could be a valuable tool when looking to see if proteins in small wild-type pedestals are in fact co-localized.

A number of inherent assumptions in our analysis could have influenced our findings. Our interpretation was based on the premise that pedestal proteins would be more abundant in the MS analysis as compared to the negative control and to the sample mean. Thus any proteins with relatively low abundance levels or those masked by highly abundant proteins, such as actin, would be more likely to be excluded. Given that a complete *Potorous tridactylus* proteome does not exist, our ability to identify Ptk2 cell proteins is contingent on the sequence conservation of trypsinized peptides against matching records in the mammalian proteomic database. While these drawbacks limited the scope of proteins that could be identified, this approach did provide a clearer overall depiction on the breadth of constituents within EPEC pedestals. We found 27 proteins with either known or novel association with pedestals without having to rely on individual molecular probes. The use of single molecular probes for a screen of this magnitude would not only be costly, but also impractical and would rely on not only the lucky guess that a protein might be present at the sites, but also the availability of good probes for identification. Our verification of two proteins from our list of putative pedestal components is promising. Although it is not surprising that actin-associated proteins are recruited to actin-rich pedestals, the confirmation of cyclophilin A and the identification of other other proteins that are thought not to be associated with actin suggests that those molecules might have additional roles not only at pedestals but in eukaryotic cells in general.

We have devised the first methodology of its kind to concentrate EPEC pedestals and screen for novel proteins within these structures by MS-based proteomic analysis.
Our results identify several known constituents within these actin-rich structures, while also suggesting that many proteins not previously discovered are likely recruited to EPEC pedestals. The groundwork laid here will undoubtedly lead to further discoveries as the roles of these proteins at pedestals and in eukaryotic cells in general are explored.

2.5. Materials and Methods

2.5.1. Bacterial growth conditions

Frozen stocks of wild-type JPN15 strain EPEC and deletion mutant, Δtir, were streaked onto LB agar plates. Following overnight incubation at 37°C, single bacterial colonies were used to inoculate culture tubes containing LB broth, which were incubated (shaking) overnight at 37°C. To prepare EPEC for infection, 1 mL of overnight EPEC culture was added to 4 mL of LB broth and then grown in a 37°C shaker (230 rpm) for 3 h.

2.5.2. Cell culture and infections

*Potorous tridactylus* kidney (Ptk2) and human cervical epithelial (HeLa) cell lines were respectively cultured in F-12/FBS [DMEM/F-12 (1:1) [HyClone, Thermo Scientific] + 10% fetal bovine serum (FBS)] and DMEM/FBS [DMEM containing high-glucose [HyClone, Thermo Scientific] supplemented with 10% FBS]. Both cell lines were grown at 37°C (5% CO₂). Cells were trypsinized with 0.05% Trypsin-EDTA [Gibco, Life Technologies] and seeded onto Transwell membranes or coverslips that were placed into 6-well plates. To adhere Ptk2 cells onto polycarbonate Transwell membranes (3 µm pore size), trypsinized cells were added on the reverse side of the Transwell support [Costar, Corning Inc.] and then the entire sample was placed in a cell culture incubator for 3 h. Next, the Transwell supports were flipped right-side up and placed into 6-well plates. The top (acellular) side of the Transwell chamber was then washed 3X with Dulbecco’s phosphate buffered saline containing Ca²⁺ and Mg²⁺ (PBS [+/+]) [Cellgro, Corning Inc.]. Once the top and bottom chambers were filled with fresh cell culture media, the multi-well plates were immediately placed back into the incubator to allow the
cells to form a confluent monolayer. After 16 h, the top of Transwell supports were scraped with a cell scraper and washed multiple times with PBS to remove any cell protrusions that may have migrated through the pore. Fresh cell culture media was then added back to both compartments in addition to 100 µL of WT EPEC (or for the control sample: EPEC Δtir) to the top of the Transwell chamber. After 3 h of infection, the top and bottom chambers were washed several times with PBS (+/+ ) to remove any non-adherent bacteria. The infection persisted for a total time of 6 h.

2.5.3. Generate long EPEC pedestals

At 3 h post infection, the spent media was removed from the multi-well plates and replaced with 40 mM of BDM [Sigma-Aldrich] in warm F12/FBS media. The samples were placed back into the cell culture incubator for 30 min and cells were re-exposed to fresh BDM for an additional five times (every 30 min).

2.5.4. Collection of proteins from the top Transwell chamber

At the end-point of the infection, the Transwell chambers were thoroughly washed 6 times with Dulbecco’s phosphate buffered saline without Ca²⁺ and Mg²⁺ (PBS [-/-]) to rinse away non-adherent bacteria and residual serum proteins. 100 µL of PBS (-/-) was then added to the top of the Transwell chamber. Using a cell scraper, the top of the Transwell chamber was scraped and transferred into a microcentrifuge tube. Proteins from each sample were then precipitated by adding 4 times the volume of absolute ethanol and 20 µg of glycogen. The solution was brought to 50 mM NaCH₃CO₂ at pH 5.0 and afterwards the sample was allowed to stand at ambient temperature overnight. After the sample was centrifuged for 10 min at 13,000 rpm, the supernatant was removed and the remaining protein pellet was re-suspended with digestion buffer (50 mM of Ammonium bicarbonate, pH 8.0) and stored at -80°C.

2.5.5. Protein determination

The protein concentration of all frozen stocks was determined using a BCA assay kit [Pierce]. Both the pedestal preparation and negative control samples were loaded onto 96-well plates and compared against a bovine serum albumin standard that ranged
from 1 mg/mL to 0.03125 mg/mL. A mixture of solutions A and B were added to each of the wells and the plate was subsequently incubated at 37°C according to the manufacturer’s instructions. The 96-well plates were read at a wavelength of 562nm using a SpectraMax M2e microplate reader [Molecular Devices] and analyzed by SoftMax Pro [Molecular Devices].

2.5.6. Protein digestion and LC-MS-MS

The samples were solubilized in digestion buffer (3% sodium deoxycholate, 50mM NH₄HCO₃) and heated to 99°C for 10 min, mixed with 0.5 μg/μL of DTT in water, incubated for 30 min at 37°C, mixed with 2.5 μg/μL iodoacetamide in water, and placed back into the incubator for 20 min. The samples were then digested overnight with sequence grade trypsin (0.5 μg/μL 1mM HCl) at 37°C after which they were desalted by C18 STAGE-Tip purification as previously described. The eluted peptides were then dried by SpeedVac, re-suspended with 100 mM of TEAB, and sonicated for 5 min. Afterwards, the pedestal preparation and control samples were dimethyl labeled with 200 mM of CH₂O and 200 mM of CD₂O, respectively, and then NaBH₃CN was added to both vials using the previously detailed procedures. Subsequently, both reactions were sonicated for 5 min and left in the dark at ambient temperature for 90 min. To halt the reactions, samples were exposed to NH₄Cl and subsequently acidified below pH 2.5 by adding 4X Sample buffer (12% MeCN, 4% TFA, 2% AcOH). Samples were desalted again by C18 STAGE-Tip protein purification and re-suspend in sample buffer (3% MeCN, 1% TFA, 0.5% AcOH). Lastly, 5 μg of the pedestal and 5 μg of control samples were pooled together. Samples were analyzed using a linear-trapping quadrupole Orbitrap mass spectrometer (LTQ-Orbitrap Velos; Thermo Fisher Scientific) with a nanospray ionization source and coupled to an Agilent 1290 series HPLC using the parameters published in.

2.5.7. Analysis of quantitative proteomics data

A list of proteins was generated from four independent experiments with Proteome Discoverer (version 1.2) and searched using a Mascot algorithm (version 2.4) against the mammalian Uniprot database. Hits against common serum proteins and
contaminants such as keratin were discarded. Each candidate was scored based on relative abundance (R.A.) in the pedestal preparation compared to the negative control sample. R.A. values > 7.00, which are considered less precise, were omitted. Proteins that were identified repeatedly were pooled together and normalized. Each candidate was then ranked by their abundance and only those that returned a p-value of < 0.05 (one tailed Z-test) were considered to be predicted EPEC pedestal components. To determine the general function of proteins in human cells, we referred to the annotated data from Uniprot (http://www.uniprot.org).

2.5.8. Immunofluorescence staining

Transwell membranes were fixed in warm 3% paraformaldehyde for 15 min, washed with PBS, permeabilized with 0.2% Triton-X and stained with Alexa-594 conjugated phalloidin [Life technologies] and/or protein-specific antibodies according to the protocol by Law and colleagues [442]. To localize all proteins, with the exception of transgelin, paraformladehyde-fixed samples were permeabilized in -20°C acetone and then completely air-dried. Alternatively, cells were permeabilized in 0.2% Triton X-100 for the immunostaining of transgelin. Subsequently, these samples were blocked with 5% NGS in PBS, and stained with one of the following antibodies: rabbit anti-cyclophilin A (1:200) [Abnova], rabbit anti-FM22α (transgelin; 1:150) [Abcam], mouse anti-clathrin heavy chain (1:50) [BD] [239], rabbit anti-Nck (1:50) [BD], mouse anti-N-WASp (1:100) [Santa Cruz; sc-100964], mouse anti-Arp2 (1:100) [Abcam; ab56818], rabbit anti-myosin IIA (myosin-9) [Genetex; gtx113236], mouse anti-cortactin (1:100) [Upstate], mouse anti-β-spectrin II [256], or rabbit anti-EBP41 [256]. After multiple washes with TPBS-BSA, Alexa-488 conjugated goat antibodies (1:1000) against either mouse and rabbit IgG were added to the samples. After overnight incubation with the secondary antibodies at 4°C, the samples were washed multiple times with TPBS-BSA, stained with Alexa-594 conjugated phalloidin, and mounted with Prolong Gold containing DAPI.

2.5.9. Fluorescence microscopy imaging

Microscopy images were acquired using a Quorum Angstrom structured illumination device attached to a Leica DMI 4000B microscope fitted with a Hamamatsu
Orca R2 CCD camera run through Metamorph v7.8.3 software. Images were processed using ImageJ and Photoshop without altering the integrity of the data.
2.6. Figures

Figure 2.1  An outline of our experimental approach for screening novel EPEC pedestal components

A monolayer of Ptk2 cells adhered to the reverse side of a 3 µm Transwell membrane. The Transwell was then inverted into the proper orientation and then infected in the well of transwell inserts with either WT EPEC or Δtir EPEC (negative control). Samples were then treated with 2,3-butadione monoxime (BDM) to elongate the pedestals. Pedestals protruding through the transwell membrane pores to the top of the membrane were then scraped off, proteins concentrated, and prepared for MS analysis. The MS data was searched against mammalian proteomic databases using a Mascot algorithm. The resulting quantitative dataset was then normalized and the ‘putative’ pedestal proteins were determined based on relative abundance.
**Figure 2.2  EPEC pedestal preparations**

[a, a'] Fluorescence and phase micrographs showing an individual pedestal that was severed from the host cell body. [b, c] Multiple EPEC pedestals were also observed by fluorescence microscopy from the pedestal preparations (Maximum intensity Z-projection). Samples were stained with phalloidin (F-actin, green) and DAPI (nuclei, blue). White arrows point to EPEC attached to pedestals. Scale bar = 10µm.
Figure 2.3  Distribution profiles of known pedestal proteins are not significantly altered by BDM
A) Apical tip proteins (CD2AP, clathrin heavy chain, Nck), apical region and stalk proteins (Arp2, cortactin) and distal end concentrated proteins (spectrin, p4.1) are localized in long BDM-treated EPEC pede-
stals. B) Myosin-9 is enriched towards the apical tip of elongated (BDM-treated) and normal (untreated) EPEC pedestals. Arrows point to the apex of pedestals. Samples were fixed, permeabilized, and immunolocalized using protein-specific antibodies (green), Alexa Fluor-594 conjugated phalloidin (red), and DAPI (blue). Images were acquired by phase and epifluorescence microscopy. Scale bar = 10 µm.
Figure 2.4 Subcellular location of putative pedestal proteins and immunolocalization of cyclophilin A and transgelin in long EPEC pedestals

A) Annotated data from Uniprot was used to determine the general subcellular location of 28 predicted & known pedestal proteins identified by our MS-based proteomics screen. B) Phase and fluorescence microscopy shows cyclophilin A and transgelin recruited to long EPEC pedestals following BDM treatment. Samples were fixed, permeabilized, and immunolocalized with cyclophilin A and SM22 (transgelin) antibodies (green). Samples were also stained with fluorescent phalloidin (F-actin, red) and DAPI (DNA, blue). Scale bar = 10 µm.
Figure 2.5  Cyclophilin A and transgelin in Ptk2 cells treated with BDM
Potoroo epithelial Ptk2 cells were treated with 2,3-butanedione monoxime (BDM). Epithelial Ptk2 cells were fixed, immunolocalized with α-cyclophilin A and transgelin antibody (green), stained with for F-actin (red) and DNA (blue) and imaged by structured illumination microscopy. Scale bar = 10 µm.
Figure 2.6  Cyclophilin A and transgelin are recruited to normal EPEC pedestals.

Actin-rich pedestals were formed following EPEC infection of human (HeLa) and potoroo (Ptk2) epithelial cell lines. Cells were fixed with paraformaldehyde, permeabilized, and stained for F-actin (red) and DNA (blue). Cyclophilin A and transgelin were immunolocalized using protein-specific antibodies (green). Scale bar = 10 µm.
### 2.7. Tables

**Table 2.1**  Proteins identified in multiple independent experiments using mass spectrometry-based proteomic analysis

# Known EPEC pedestal proteins; Bolded proteins indicate potential novel EPEC pedestal proteins.

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Chapter 3.

Nexilin is a dynamic component of \textit{Listeria monocytogenes} and enteropathogenic \textit{Escherichia coli} actin-rich structures

The contents in this chapter have been published.


† These authors contributed equally

\textbf{Author contributions:} HTL and BM performed the experiments (BM, Fig. 3.1-3.4, 3.7-3.9; HTL, Fig. 3.5, 3.6, 3.10, 3.16) and analyzed the results. HTL and BM wrote the manuscript. JJ contributed to Fig. 3.11. JAG and PC reviewed the manuscript.
3.1. Abstract

The bacterial pathogens *Listeria monocytogenes* and enteropathogenic *Escherichia coli* (EPEC) generate motile actin-rich structures (comet tails and pedestals) as part of their infectious processes. Nexilin, an actin-associated protein and a component of focal adhesions, has been suggested to be involved in actin-based motility. To determine whether nexilin is commandeered during *L. monocytogenes* and EPEC infections, we infected cultured cells and found that nexilin is crucial for *L. monocytogenes* invasion as levels of internalized bacteria were significantly decreased in nexilin-targeted siRNA-treated cells. In addition, nexilin is a component of the machinery that drives the formation of *L. monocytogenes* comet tails and EPEC pedestals. Nexilin colocalizes with stationary bacteria and accumulates at the distal portion of comet tails and pedestals of motile bacteria. We also show that nexilin is crucial for efficient comet tail formation as cells pre-treated with nexilin siRNA generate malformed comet tails, whereas nexilin is dispensable during EPEC pedestal generation. These findings demonstrate that nexilin is required for efficient infection with invasive and adherent bacteria and is key to the actin-rich structures these microbes generate.

3.2. Introduction

*Listeria monocytogenes* and enteropathogenic *Escherichia coli* (EPEC) cause serious gastrointestinal diseases that can be fatal. A common feature shared by these pathogens is their co-opting of the actin cytoskeleton within the cells they infect. To accomplish this, these microorganisms have evolved specialized strategies that allow them to commandeer various cytoskeletal components. *L. monocytogenes* exploits the actin-polymerization machinery for its internalization into host cells and subsequent dissemination into neighbouring cells; whereas EPEC remains extracellular but harnesses actin cytoskeletal dynamics for motility on the top of infected cells.
Listeria monocytogenes interacts with host cells via InlA and InlB, two bacterial surface proteins that recognize the host receptors E-cadherin and Met respectively. The activation of host receptors trigger downstream signalling cascades that lead to localized remodelling of the actin cytoskeleton required for the internalization of bacteria. Once internalized, L. monocytogenes escapes the phagocytic vacuole and uses the bacterial surface protein ActA to recruit the host’s actin polymerizing machinery to generate actin-rich comet tails that propel the bacteria within the host cytoplasm. The motility generated by harnessing actin-based dynamics using comet tails is essential for bacterial spreading to neighbouring cells.

Unlike L. monocytogenes, EPEC is an extracellular pathogen that utilizes a molecular syringe-like type III secretion system (T3SS) to cause disease. Although a number of T3SS effectors have been shown to influence pedestal generation, the effector that is most important for pedestal formation and EPEC-mediated disease is the translocated intimin receptor (Tir). Upon delivery into host cells, Tir becomes phosphorylated at a variety of tyrosine (Y) residues. A key residue at Y474 is needed for efficient pedestal formation as bacteria with Tir474 mutated do not normally generate pedestals. Subsequent to Tir delivery and its docking to the intimin protein on the bacterial surface, actin is recruited beneath the attached bacteria, together with numerous actin-associated components. The assembly of actin filaments leads to the formation of motile actin-rich ‘pedestal’ structures that raise EPEC off of the surface of the host cell, enabling the bacteria to ‘surf’ on the exterior of the cell.

Nexilin is an actin-associated protein that localizes along actin stress fibres and at focal adhesions. Within focal adhesions, nexilin is thought to act as a molecular linker, anchoring the plasma membrane to the actin cytoskeleton. Nexilin has been demonstrated to influence cell motility as the overexpression of nexilin in HeLa cells resulted in an approximate two-fold increase in cell motility and adhesion.

Based on this evidence, we investigated the potential role of nexilin at actin-rich structures generated during bacterial infections. We initially immunolocalized nexilin at sites of actin recruitment during L. monocytogenes and EPEC infections and found that nexilin was recruited to the site of bacterial internalization and was essential for the
uptake of *L. monocytogenes*. At later stages of infection, nexilin was also associated with *L. monocytogenes* comet tails and was required for uniform comet tail morphology. Similarly, during EPEC infections nexilin was localized to pedestals, but showed dynamic motility, being positioned along the entire actin-rich stalk of stationary pedestals, then concentrating at the base of pedestals when in motion. Our findings demonstrate that nexilin is a component of dynamic actin-rich structures triggered by bacterial infections and suggest that nexilin may function as a platform for the actin-based motility of bacterial pathogens.

### 3.3. Results

#### 3.3.1. Nexilin is required for the internalization of *L. monocytogenes* within host cells

A key event in *L. monocytogenes* pathogenesis is the actin-mediated internalization within host cells. This is elicited by the interaction of bacterial surface proteins (InlA and InlB) with signalling receptors at the surface of the host cell (E-cadherin and Met respectively), which trigger downstream signalling pathways leading to rearrangements of the actin cytoskeleton. Early studies had shown that the inhibition of actin polymerization by cytochalasin D completely ablates bacterial internalization.

As nexilin plays an important role in the rearrangements of the actin cytoskeleton at the plasma membrane, we first investigated its localization and role during *L. monocytogenes* infections. HeLa cells infected with *L. monocytogenes* were fixed and processed for immunofluorescence localization where endogenous nexilin was labeled with a specific antibody and the actin cytoskeleton was revealed with fluorescent phalloidin. After 30 min of infection, nexilin was clearly recruited at bacterial entry sites together with actin (Figure 3.1A). Next, HeLa cells were depleted of endogenous nexilin by siRNA treatment (Figure 3.1B) and infected with *L. monocytogenes*. The efficiency of bacterial internalization was assessed by the gentamicin survival assay in mock-treated (control) and nexilin-depleted cells. Remarkably, nexilin depletion reduced *L. monocytogenes* internalization by ~ 90% (Figure 3.1C), which is comparable with previously reported results obtained when actin polymerization is inhibited by cytochalasin D.
3.3.2. **Nexilin localizes at *L. monocytogenes* actin comet tails**

Another key event in *L. monocytogenes* pathogenesis is the harnessing of the host cell actin machinery to form actin comet tails. These structures allow bacteria to move in the host cell cytoplasm and induce the formation of protrusions at the host cell plasma membrane that are functional for the invasion of neighbouring cells. We therefore investigated the role of nexilin in actin-based bacterial motility. HeLa cells were infected with *L. monocytogenes* and fixed at 4 h post infection, which corresponds to the highest frequency of comet tail formation in these cells. Nexilin colocalized with actin around non-motile bacteria (Figure 3.2A arrows) and along the actin comet tails of motile bacteria (Figure 3.2B). Of note, when bacteria had established comet tails, nexilin showed a seemingly higher concentration towards the rear of long tails (Figure 3.2B), whereas it accumulated at the point of origin of short, newly formed tails (Figure 3.2A inset). Similar nexilin recruitment was evident in Jeg3 cells during 5 h infections (Figure 3.7). To further study the accumulation of nexilin during these infections, we generated EGFP and Tomato-tagged nexilin to follow its dynamics in infected cells, either alone or in combination with YFP-tagged actin. The addition of a fluorescent tag at the N-terminal domains of nexilin did not alter its localization in non-infected cells as compared with the immunolabelling of endogenous nexilin (data not shown). Both EGFP- and Tomato-tagged nexilin accumulated around non-motile, actin-positive *L. monocytogenes*, and along comet tails formed by motile bacteria (Movie B3, Movie B4, Figure 3.8 and Figure 3.9). Remarkably, nexilin failed to accumulate at comet tails forming protrusions (Movie B3, Figure 3.8A white arrows and Figure 3.8B arrow 1). We also observed that nexilin-associated fluorescence around non-motile bacteria gradually increased with time (Movie B4 and Figure 3.9B), with a peak of fluorescence occurring shortly before actin tail formation (Figure 3.9B circled area at 6 min). The fluorescence intensity of actin remained constant (Figure 3.9B circled area at 6 min as compared with the same area at 2 min). As the bacteria became motile, nexilin remained concentrated at the point of origin of the tail, whereas a relatively weaker fluorescence signal was observed along the length of the actin tail (Figure 3.9B circled area at 7 min and along the comet tail at 8 min).

To examine the requirement of actin filaments in recruiting nexilin to *L. monocytogenes* we infected cultured cells with a *L. monocytogenes* ΔactA mutant. The
\textit{L. monocytogenes} surface protein ActA is crucial to concentrate actin filaments to the bacteria as \textit{L. monocytogenes ΔactA} fail to recruit actin or generate comet tails. Using this strategy we found that nexilin did not localize at the bacteria (Figure 3.3). This suggested that actin is likely needed at the bacterial surface prior to nexilin. These results suggested that nexilin might play a role in the efficient formation of actin tails. We then determined whether dynamic actin polymerization was needed for nexilin recruitment to comet tails. To assess this we used cytochalasin D to halt actin polymerization in infected cells, following comet tail formation. Although the bacteria became immobile, we found that nexilin remained localized to the stationary bacteria (Movie B5). To further test the role of nexilin in comet tail formation, we followed their morphology in cells depleted of endogenous nexilin by siRNA, which were infected with \textit{L. monocytogenes}. As mentioned above, nexilin depletion strongly reduces bacterial internalization and such analysis was performed on the remaining percentage of invading bacteria. In agreement with our previous observations, bacteria internalized in nexilin-depleted cells formed very short actin comet tails (labelled with fluorescent phalloidin) with an irregular (thinner) morphology as compared with comet tails originating from bacteria invading control cells (Figure 3.4A and Figure 3.4B arrows in panels 4–6). The percentage of internalized bacteria forming actin comet tails slightly decreased (by \textasciitilde 10\%) in nexilin-depleted cells as compared with control cells (Figure 3.4C).

### 3.3.3. Nexilin is recruited to actin-rich pedestals by EPEC and requires Tir

Based on the importance of nexilin in the pathogenesis of \textit{L. monocytogenes}, we expanded our investigation by examining whether nexilin was also involved in EPEC pathogenesis. One of the characteristic phenotypes of EPEC infections is the generation of actin-rich pedestals at the site of bacterial adherence \textsuperscript{65,436}. To determine whether EPEC altered the distribution of nexilin, we immunolabelled EPEC infected HeLa and CaCo-2 cells and found that nexilin colocalized with F-actin that had accumulated beneath the attached bacteria (Figure 3.5A and Figure 3.10). This recruitment did not alter the localization of nexilin at focal contacts or along actin stress fibres (Figure
3.11A); likewise, overall nexilin protein levels remained unchanged during the infections (Figure 3.11B).

To determine whether nexilin recruitment to sites of bacterial docking involved the translocation of effectors or simply bacterial contact, we infected cultured cells with an EPEC \( \Delta \text{escN} \) mutant that lacked the functional ATPase (EscN) required for the delivery of effectors through the T3SS \(^{105}\). During these infections, nexilin was absent from the sites of bacterial contact, indicating that the interaction of EPEC with the host surface alone is not sufficient for nexilin recruitment (Figure 3.5A). Because our data indicated that nexilin is recruited to EPEC pedestals by a process that requires a functional T3SS and is associated with actin at the cell periphery, we examined the involvement of the bacterial effector Tir, as this effector is crucial for the generation of actin-rich pedestals. We infected cells using EPEC mutated in tir (EPEC \( \Delta \text{tir} \)) and immunolocalized nexilin. We found that nexilin was absent from regions of bacteria/host cell contact suggesting that Tir influenced nexilin recruitment and that nexilin was a component of pedestals (Figure 3.5B). This was confirmed by using a \( \Delta \text{tir} \) mutant that was complemented with tir, which rescued the wild-type phenotype (Figure 3.5B).

Tir can be phosphorylated at a variety of different tyrosine residues \(^{130,146}\), however, Y474 has been shown to be the most important for pedestal formation (Kenny, 1999). In order to determine whether this site on Tir was required for nexilin to be present at those structures we infected cells with an EPEC \( \Delta \text{tir} \) mutant that was complemented with tir bearing a point mutation at Y474 (Y474F) \(^{81}\). Immunofluorescent localization of nexilin indicated that Y474 was crucial for nexilin re-localization as nexilin was not recruited beneath the attached EPEC \( \Delta \text{tir} + \text{tirY474F} \) (Figure 3.5B). Although other bacterial effectors have been shown to influence pedestal formation, all effector mutants that were tested did not abrogate the recruitment of nexilin at EPEC pedestals (Figure 3.12).

The generation of EPEC pedestals requires the binding of the host protein Nck directly to Y474 of Tir \(^{142}\). To examine the need of this host protein in recruiting nexilin to pedestals we infected EPEC on \( \text{nck}^-/- \) cells and compared nexilin localization to infections on \( \text{nck}^+/+ \) cells. During \( \text{nck}^-/- \) infections pedestals are not generated and accordingly nexilin did not localize to sites of bacterial contact (Figure 3.13). We then
sought to determine whether the inhibition of filamentous actin in cells treated with cytochalasin D prior to EPEC infection also influenced nexilin recruitment. During these infections we also found that nexilin was not localized to the bacterial contact points, indicating that actin was required for nexilin localization (Figure 3.14).

3.3.4. Dynamics of nexilin recruitment at EPEC pedestals

Because nexilin appears to be recruited to EPEC pedestals, *L. monocytogenes* comet tails, and in both cases exclusively in the presence of actin, we sought to determine the order of nexilin and actin recruitment to pedestals. Using time-lapse imaging coupled with fluorescently tagged mKate-actin and EGFP-nexilin, we found that within 40 min of bacterial attachment to host cells both nexilin and actin were simultaneously recruited to pedestals (Figure 3.15).

To try to detail the precise localization of nexilin within EPEC pedestals we tracked the localization of fluorescently tagged actin and nexilin during infections using the JPN15 strain of EPEC. JPN15 is an EAF plasmid cured strain of the wild-type EPEC (E2348/69). This strain does not have a functional bfp gene cluster and consequently generates large individual pedestals that are easily resolved because they do not form microcolonies. We found that although both proteins colocalized in immobile pedestals (Figure 3.16A), nexilin began to segregate from actin upon pedestal movement and eventually trailed actin at the base of the pedestal (Figure 3.16A and B). Given the requirement of nexilin for the appropriate formation of comet tails and for bacterial invasion during *L. monocytogenes* infections, we used RNA interference (RNAi) to investigate whether nexilin knock-down (KD) alters EPEC pedestal formation. Cells were pre-treated with siRNA targeted against nexilin and then subsequently infected with JPN15 for 6 h, allowing sufficient time for EPEC pedestals to form. In nexilin-depleted cells with no observable cytoplasmic immunostaining of nexilin, EPEC was able to generate actin-rich pedestals that were indistinguishable from those seen in the ‘Control siRNA’ samples (Figure 3.6).
3.4. Discussion

The identification of nexilin as a crucial protein involved in the invasion of *L. monocytogenes* as well as at comet tails and EPEC pedestals in a variety of cell types initially appeared to conform to the localization of numerous other actin-related proteins present at actin-rich structures during these infections. Our evidence that the presence of actin was required for nexilin recruitment to comet tails and pedestals suggest that nexilin binding to the actin-rich structures is likely through the actin-binding domain of nexilin. Routinely during these infections actin-related proteins concentrate along the entire length of either comet tails or EPEC pedestals and if segregation of actin-associated proteins are evident, this usually occurs with the identified protein located near the bacteria, with actin trailing. During the motile phases of these bacterial infections the opposite was found as nexilin trailed actin at these structures. The heavily concentrated nexilin at the base of newly formed *L. monocytogenes* comet tails suggested the possibility that nexilin might act as a platform for the propulsion of the bacteria. Additionally, its general localization suggested that nexilin could stabilize the actin-rich structures, a hypothesis that was partially substantiated when the siRNA-mediated knockdown of nexilin resulted in disorganized comet tails but surprisingly did not influence EPEC pedestals. A potential reason that alterations to EPEC pedestals were not detected could stem from their much shorter stature.

Nexilin appeared to smoothly increase in intensity with a concomitant decrease in F-actin at these structures. This corresponded to an intensified localization of nexilin towards the base of the structures as motility progressed. This localization is contrary to what would be predicted based on the current cell motility literature. In motile cells nexilin localizes to both the leading edge and is also present at focal adhesions where it is thought to act as a linker between the actin filaments and the plasma membrane. Consequently, one would have predicted that especially in the case of EPEC pedestals, nexilin would have interacted where actin and the plasma membrane are most closely associated. Additionally, even though nexilin was needed for efficient invasion of *L. monocytogenes*, nexilin recruitment was only evident when the bacteria were away from the plasma membrane as *L. monocytogenes* forming listeriopods were deficient in nexilin recruitment.
Taken together our evidence demonstrates that both invasive and extracellular bacteria use nexilin as part of their infectious processes and provide the first evidence of a dynamic protein at the base of their actin-rich structures. The lack of nexilin at sites of *L. monocytogenes* protrusion to neighbouring cells as well as the involvement of nexilin during *in vivo* infections will be examined in upcoming research.

### 3.5. Materials and Methods

#### 3.5.1. Chemicals, reagents and antibodies

Unless otherwise indicated, all reagents used in the study were obtained from Sigma Aldrich Canada (Mississauga, Ontario, Canada). Sixteen per cent Paraformaldehyde was acquired from Canemco (Canton de Gore, QC, Canada), Triton X-100 was purchased from Ricca Chemical (Arlington, TX) and Tween-20 was from MP Biomedicals (Solon, OH).

#### 3.5.2. Bacterial strains and growth conditions

*Listeria monocytogenes* (EGD BUG600) were grown in brain heart infusion (BHI) broth at 37°C. GFP-*L. monocytogenes* (BUG1908) and Δ*actA* GFP-*L. monocytogenes* (BUG3011) were grown as above in the presence of erythromycin. EPEC bacterial strains used in this study included: EPEC (strain E2348/69), E2348/69 mutants (ΔescN, Δtir, Δmap, ΔespF, ΔespG, ΔespH, ΔsepZ), EPEC (strain JPN15), JPN15 mutant (Δtir) and JPN15-complemented strains (Δtir complemented with tir, Δtir complemented with tirY474F). The JPN15 strain is an EAF plasmid-cured strain of E2348/69, which lacks functional bundle-forming pilus (*bfp*) gene expression. All EPEC strains were grown in LB broth (MP Biomedicals, Solon, OH) at 37°C overnight as standing cultures.

#### 3.5.3. Cell culture

HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing high glucose (Thermo Fisher Scientific, Nepean, ON, Canada). Although HeLa cells were very useful for initial screening and were amenable to numerous cell
manipulations, they are not derived from an organ system readily accessible to EPEC or L. monocytogenes, consequently other cell types were used to confirm our findings. Vero cells were grown RPMI. Jeg3 and CaCo-2 cells were grown in DMEM supplemented with 1% non-essential amino acids. Murine fibroblasts (parental strain and nck-/-) were grown in DMEM. Ptk2 cells were cultured in DMEM/F-12 media (Thermo Fisher Scientific, Nepean, ON, Canada). DMEM, DMEM/F-12 and RPMI media were supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich Canada, Oakville, ON, Canada). Cells were seeded onto coverslips, tissue culture treated multi-well plates (Corning, NY) or collagen-coated transwell inserts (BD Biosciences, NY) and maintained in a cell culture incubator (37°C, 5% CO2).

3.5.4. Infections

Unless otherwise indicated, cultured cells were grown to 70–100% confluency for all infections. For L. monocytogenes studies, overnight cultures were diluted 10X, grown as a shaking culture until (Abs$_{600}$ = 0.8), and infected at a multiplicity of infection (moi) of 50:1 at 37°C for either 30 min to study bacterial internalization or 4–5 h to follow actin comet tail formation. For EPEC studies, cells were infected for 6 h at 37°C at an moi 10:1.

3.5.5. Immunolocalization

Cells were fixed with 3% paraformaldehyde for 15 min at room temperature (RT), which was followed by three consecutive washes with PBS (Fisher Scientific, Fairlawn, NJ). With the exception of the murine fibroblasts, cells were permeabilized with 0.1% Triton X-100 at RT in PBS for 5 min, then washed three additional times with PBS (10 min each wash). Murine fibroblasts were treated with -20°C acetone for 10 min and air dried. All samples were blocked with 5% normal goat serum (NGS) in PBS for 20 min at which point a rabbit anti-human nexilin antibody (Sigma Life Sciences, St. Louis, MO), used at 3.7 mg ml$^{-1}$, or a mouse anti-nck antibody used at 0.02 mg ml$^{-1}$ (BD Biosciences, San Jose, CA) was prepared in TPBS/BSA (0.5% Tween-20, 0.1% BSA in PBS) + 1% NGS and incubated with the samples overnight at 4°C. Subsequently, the cells were rinsed three times (10 min each wash) with TPBS/BSA and treated with secondary antibodies in TPBS/BSA for 2 h at RT. For the secondary antibodies, goat anti-rabbit IgG
conjugated to AlexaFluor-488 and/or goat anti-mouse IgG conjugated to AlexaFluor-594 (Invitrogen, Eugene, OR) was used at 10 mg ml\(^{-1}\). To visualize filamentous actin, samples were stained using phalloidin conjugated to AlexaFluor-594 (Invitrogen, Eugene, OR) in TPBS/0.1% BSA. Following three additional washes in PBS for 10 min each wash, coverslips were then mounted onto glass slides with Prolong Gold containing DAPI (Invitrogen, Eugene, OR).

Immunofluorescence controls were prepared identically to experimental samples, except for the substitution of the primary antibody for rabbit IgG (‘primary’ control) and the omission of the primary antibody (‘secondary’ control). Controls did not show any specific staining (data not shown).

3.5.6. Microscopy and live cell imaging

A Leica DMI4000B inverted fluorescent microscope equipped with a Hamamatsu Orca R2 CCD camera (Hamamatsu, Japan) was used to acquire wide-field images for all live cell imaging and immunostained samples. These images were evaluated using Metamorph Imaging System software (Universal Imaging Corp., Downingtown, PA). For live cell imaging, a Chamlide IC top stage incubator system was used to maintain a constant temperature (37°C) and 5% humidified CO\(_2\) gas in air.

3.5.7. DNA constructs

To generate GFP-tagged nexilin, nexilin was PCR-amplified from a plasmid containing a mouse nexilin open reading frame (OriGene, Rockville, MD) using the primers Fwd: GAG TCA GCT AGC CAT GAA TGA CGT TTC GCA AAA G and Rev: TGA CTC CTC GAG CGG CCG CGT ACG C, which contained the NheI and XhoI restriction sites respectively. These enzymes were used to subclone the nexilin ORF into a pEGFP-N2 vector (Invitrogen). To generate Tomato-tagged nexilin, the EGFP ORF was excised from the above vector and replaced by the Tomato ORF using the AgeI and XbaI restriction enzymes. YFP-tagged actin was obtained from Seveau and colleagues\(^{455}\). The mKate-actin construct was obtained from Evrogen (Moscow, Russia).
3.5.8. **Cell culture transfections**

All constructs were transfected into cultured cells using the jetPRIME transfection reagent according to the protocol established by the manufacturer (Polyplus Transfection, Illkirch, France). Cells were incubated at 37°C for 24 h to allow the expression of the respective gene products.

3.5.9. **Nexilin RNAi knock-downs**

Nexilin siGenome siRNA and siGenome non-targeting siRNA (Thermo Scientific, Chicago, IL) were transfected into cells using INTERFERin (Polyplus transfection, Illkirch, France) according to the protocol established by the manufacturer. To allow for optimal knock-down of gene expression, cells required a 96 h incubation.

3.5.10. **Gentamicin survival assay**

*Listeria monocytogenes* were grown at 37°C to an OD$_{600}$ of 0.8–1.0. Before infection, bacteria were washed twice in PBS, diluted in DMEM and incubated with HeLa cells at an MOI of 50. After 1 h of incubation at 37°C the cells were washed with complete culture medium and incubated for 2 h at 37°C in complete culture medium containing gentamicin (10 mg ml$^{-1}$). Cells were washed and lysed in 0.2% Triton X-100. The number of viable bacteria was assessed by titring on agar plates.

3.5.11. **Cytochalasin D assays**

Cultured cells were either pre-treated with 2.5 µm Cytochalasin D for 30 min prior to infecting the cells with EPEC for 6 h or infected with *L. monocytogenes* for 5 h then treated with Cytochalasin D. EPEC samples were fixed prior to staining for nexilin and F-actin whereas *L. monocytogenes* infections were imaged live using fluorescently tagged nexilin and actin. DMSO (carrier buffer controls) infected cells resembled infected cells that were untreated (Data not shown).
3.5.12. Lysate preparation and Western blotting

Cells were washed three times in PBS followed by a 5 min treatment with RIPA lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA, 1% Nonidet P-40, 1% deoxycholic acid, 10% SDS) on ice. Protein concentrations were determined by bicinchoninic acid (BCA) assays. Prior to loading, protein lysates were prepared in Laemmlli buffer and denatured by boiling the samples for 10 min. Equal amounts of protein were loaded and separated onto 10% SDS-polyacrylamide gels, then transferred onto a nitrocellulose membrane using a semi-dry transfer apparatus (Bio-Rad Laboratories, Mississauga, ON). The blots were washed three consecutive times with TBST (0.05% Tween-20 in TBS) for 10 min each, blocked with 4% Blotto (Santa Cruz Biotechnology, Santa Cruz, CA) and treated with primary antibodies overnight at 4°C. Blots were washed three additional times with TBST and incubated at RT with secondary antibodies for 1 h. Primary and secondary antibodies were prepared in a TBS-T (0.05% Tween-20 in TBS) + 1% BSA solution and consisted of a rabbit anti-human nexilin antibody (Sigma Life Sciences, St. Louis, MO) used at 0.37 mg ml⁻¹ as well as goat anti-rabbit IgG conjugated to HRP (Cell Signaling Technology, Danvers, MA) used at 10 mg ml⁻¹. For chemiluminescence detection, Western Lightning-ECL (PerkinElmer, Waltham, MA) was added to the blots exposed on Kodak BioMax Light Film (Carestream Health, Rochester, NY). To ensure proper loading, the blots were then stripped with stripping buffer (2% SDS, 12.5% Tris pH 6.8, 0.8% b-mercaptoethanol) for 45 min at 50°C and re-probed with a mouse α-tubulin antibody (Developmental Studies Hybridoma Bank, Iowa City, IA) used at 0.478 mg ml⁻¹ or with a mouse anti-actin antibody (Clone AC-15 Sigma) used at 0.5 mg ml⁻¹, which were incubated overnight at 4°C. This was followed by incubations with secondary HRP-conjugated goat anti-mouse antibodies for 1 h at RT and chemiluminescent detection.
3.6. Figures

Figure 3.1  Nexilin is recruited to Listeria monocytogenes entry sites.
A. HeLa cells infected with L. monocytogenes were labelled with the anti-nexilin antibody (red) and AlexaFluor coupled phalloidin to reveal F-actin (green). Arrows and inset mark bacteria surrounded by actin and nexilin. Scale bar = 10 µm. B. HeLa cells were treated with control or nexilin-targeted siRNA sequences and whole cell lysates probed for endogenous nexilin. C. Control and nexilin-depleted cells were incubated with L. monocytogenes and the efficiency of bacterial internalization was determined by gentamicin survival assays. Values are means ± standard deviation of four independent experiments. Asterisks represent P-values (***P ≤ 0.001, Student’s t-test).
Figure 3.2  Nexilin localizes at L. monocytogenes actin comet tails.
A and B. HeLa cells infected with L. monocytogenes for 4 h were labelled with anti-nexilin antibody (red) and AlexaFluor coupled phalloidin to reveal F-actin (green). Arrows in A point at nexilin accumulation around non-motile bacteria. The inset in (A) focuses on nexilin accumulation at the point-of-origin of the actin comet. Arrows in (B) point at nexilin accumulation along actin comet tails. Scale bars = 10 µm.
Figure 3.3  Nexilin recruitment at *L. monocytogenes* requires ActA.
Vero cells were transfected with Tomato-tagged nexilin (purple) and infected with wt GFP-*L. monocytogenes* (green) (A) or with the GFP-*L. monocytogenes ΔactA* mutant (B) for 5 h. Cells were then fixed and processed for fluorescence microscopy. Actin (blue) was labelled with Cy5-coupled phalloidin. Full arrows point at sites of nexilin and actin colocalization around non-motile bacteria, light arrow point at colocalization of nexilin and actin at a *Listeria* comet tail. Scale bars = 10 µm.
Figure 3.4  Nexilin depletion perturbs actin comet tails formation.
A. HeLa cells were treated with non-targeting control (Control) or nexilin-targeted siRNA (siRNA Nexilin) sequences and infected with *L. monocytogenes* for 4 h to allow comet tails formation. Cells were labelled with phalloidin to reveal F-actin (green), anti-nexilin antibody (red) and DAPI (blue). B. Magnification of the corresponding boxed comet tails in (A). C. HeLa cells were treated as in (A) and the efficiency of comet tails formation was quantified as the ratio of bacteria forming actin tails versus the total number of bacteria recruiting actin per cell. Scale bars = 10 µm.
Figure 3.5 Recruitment of nexilin to EPEC pedestals requires a functional type III secretion system (T3SS) and phosphorylation of tyrosine-474 on Tir.

A. Nexilin (green), F-actin (red) and DNA (blue) were immunolocalized in HeLa cells infected with wild-type (WT) EPEC and T3SS mutant (EPEC ΔescN).

B. Infection with JPN15 and mutants (Δtir, Δtir + tir, Δtir + tirY474F) illustrates that phosphorylation of Tir at tyrosine-474 is necessary for both the recruitment of nexilin and formation of actin-rich pedestals. Arrowheads indicate the regions depicted in the inset. Scale bar = 5 μm.
Figure 3.6   EPEC JPN15 infection of nexilin-siRNA depleted HeLa cells.
Knock-down of nexilin does not impede the formation of actin-rich pedestals. Cells were
immunolocalized with antibodies against nexilin (green) as well as probed for F-actin (red) and
DNA [DAPI] (blue). Arrowheads indicate the location of EPEC pedestals depicted in the insets.
Scale bar = 5 μm.
Figure 3.7  Nexilin is recruited to *Listeria* actin-rich structures during Jeg3 cell infections.

Jeg3 cells were infected with *L. monocytogenes* for 5 h, fixed and processed for immunofluorescence. Endogenous nexilin was labelled with a specific antibody (red) and actin was labelled with TRITC-coupled phalloidin (green). Full arrows point at sites of nexilin and actin colocalization around non-motile bacteria, light arrow points at colocalization of nexilin and actin at a *Listeria* comet tail. Scale bar = 10 μm.
Figure 3.8  Dynamics of EGFP-nexilin comet tails.
Ptk2 cells were transfected with EGFP-nexilin (green), infected with L. monocytogenes for 4 h and imaged using an epifluorescence microscope. Red arrows point at intracellular motile bacteria, white arrows point at bacteria in protrusions of the host-cell plasma membrane. B. Magnification of the inset in A shows a bacterium protruding from the host-cell membrane (arrow 1) and a nearby bacterium moving within the cytoplasm (arrow 2). Scale bar = 10 µm.
Figure 3.9  Dynamics of nexilin and actin at comet tails.  
A and B. Ptk2 cells were transfected with Tomato-nexilin (red) and YFP-actin (green), incubated with *L. monocytogenes* for 4 h and imaged using an epifluorescence microscope. B. Magnification of the inset in (A) illustrating the successive steps of nexilin and actin recruitment during comet tail formation. Scale bar = 10 µm.
Figure 3.10  Nexilin localizes to EPEC pedestals during non-polar and polar CaCo-2 cell infections.

Non-polar (A) and polar (B) CaCo-2 cells infected with EPEC recruit nexilin to their pedestals. Arrowheads in the phase-contrast and DAPI images indicate the location of some of the bacteria. DAPI (blue) also labels the host cell nuclei. Insets indicate some of the bacteria with F-actin (red) and nexilin (green) colocalized at pedestals. Scale bar = 5 µm.
Figure 3.11  **Nexilin localization and expression in HeLa cells**
A. Nexilin (green) is localized at focal contacts towards the ends of actin stress fibres (red). Arrowheads indicate focal contacts as shown in the inset. B. Cell lysates from EPEC-infected cells were analysed by Western blotting and probed for nexilin. Staining with α-tubulin served as a loading control. Scale bar = 5 µm.
Figure 3.12 EPEC effectors known to influence pedestal formation are not responsible for nexilin accumulation to EPEC pedestals. HeLa cells were infected with WT EPEC, ΔespF, ΔespG, ΔespH, Δmap and ΔsepZ prior to being fixed and probed for nexilin (green), F-actin (red) and DNA [DAPI] (blue). Arrows indicate attached EPEC as shown in the insets. Scale bar=5µm.
**nck +/+ cells**

A.  
WT EPEC  
Uninfected

B.  
WT EPEC  
Uninfected

**nck -/- cells**

C.  
WT EPEC  
Uninfected

D.  
WT EPEC  
Uninfected
Figure 3.13  Nck is required for nexilin recruitment to sites of EPEC attachment. EPEC infected and uninfected nck+/+ (A, B) and nck-/ cells (C, D) were fixed and stained for nexilin (green) and F-actin (red). Phase images indicate the location of the bacteria in conjunction with white arrowheads. Scale bars = 5 µm.
Figure 3.14  Nexilin localization at EPEC/host cell contact points requires filamentous actin.

Cells were pre-treated with cytochalasin D then infected with EPEC. The samples were then labelled with nexilin (green), actin (red) and DAPI (blue) as well as imaged with phase microscopy. Arrowheads point to some of the attached bacteria. Scale bars = 5 µm.
Figure 3.15  Nexilin and actin are simultaneously recruited beneath EPEC upon attachment to host cells.

Co-transfected Ptk2 cells ectopically expressing EGFP-nexilin and mKate-actin were infected with EPEC JPN15. Live cell imaging commenced 1.5 h post inoculation. We denote ‘-20 min’ as the time point without docked bacteria and ‘0 min’ as the time point of bacterial attachment. Large and small arrows indicate the location of each bacterium. Scale bar = 2 µm.
### A.

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Figure 3.16  Re-distribution of nexilin within EPEC pedestals
A. Nexilin segregates from actin and remains at the base of the structure upon EPEC pedestal elongation. Live cell imaging began 1.5 h after co-transfected Ptk2 cells (mKate-actin, EGFP-nexilin) were infected with EPEC JPN15. We denote ‘0 min’ as the time at which nexilin and actin colocalize beneath a stationary bacterium. Phase images show the site of bacteria-host contact (indicated by arrowheads). B. Nexilin is localized at the base of motile pedestals. Ptk2 cells were transfected with mKate-actin and EGFP-nexilin. Time-lapse images of the infections were captured 1.5 h post infection with EPEC JPN15. Arrow indicates the location of motile EPEC propelled by the actin-rich pedestal. (A) Scale bar = 2 μm, (B) scale bar = 10 μm.
Chapter 4.

*Francisella tularensis* uses Cholesterol and Clathrin-based Endocytic Mechanisms to Invade Hepatocytes

The contents in this chapter have been published.


† These authors contributed equally

**Author contributions:** HTL and AEL performed the experiments (HT, Fig. 4.1-4.3, 4.9; AEL, Fig. 4.6-4.8) and analyzed the results. AEL wrote the manuscript. BQ contributed to Figure 4.1. YK contributed to Fig. 4.4-4.6. FEN reviewed the manuscript. HTL and JAG were involved in data analysis and manuscript preparation.
4.1. Abstract

*Francisella tularensis* are highly infectious microbes that cause the disease tularemia. Although much of the bacterial burden is carried in non-phagocytic cells, the strategies these pathogens use to invade these cells remains elusive. To examine these mechanisms we developed two *in vitro* *Francisella*-based infection models that recapitulate the non-phagocytic cell infections seen in livers of infected mice. Using these models we found that *Francisella novicida* exploit clathrin and cholesterol dependent mechanisms to gain entry into hepatocytes. We also found that the clathrin accessory proteins AP-2 and Eps15 co-localized with invading *Francisella novicida* as well as the *Francisella* Live Vaccine Strain during hepatocyte infections. Interestingly, caveolin, a protein involved in the invasion of *Francisella* in phagocytic cells, was not required for non-phagocytic cell infections. These results demonstrate a novel endocytic mechanism adopted by *Francisella* and highlight the divergence in strategies these pathogens utilize between non-phagocytic and phagocytic cell invasion.

4.2. Introduction

The potentially fatal disease tularemia is caused by the pathogenic microbe *Francisella tularensis* subspecies *tularensis* (*F. tularensis*). These bacteria are highly virulent as exposure to as few as 10 organisms can result in 30–35% mortality if left untreated. *F. tularensis* enter hosts through a variety of routes including inhalation, ingestion, abrasion and transmission through arthropod vectors. Within their hosts these microbes colonize a variety of organs including the lungs, spleen, and liver. *F. tularensis* encode a secretion system that shares homology with type VI secretion gene clusters, as well as crucial genes needed for intracellular growth and virulence on a region of their bacterial chromosome called the *Francisella* Pathogenicity Island. Much of the work examining the genetics, biochemistry and cell biology of *F. tularensis* exploit two closely related surrogate *F. tularensis* subspecies: *Francisella tularensis* subspecies *novicida* (*F. novicida*); a murine pathogen that does not normally cause disease in healthy humans but colonizes identical organs as *F. tularensis* during in vivo murine infections and infects both phagocytic and non-phagocytic cells.
F. novicida and F. tularensis share 97% of their DNA sequence and both have homologous virulence factors. The second surrogate commonly used is F. tularensis subspecies holarctica Live Vaccine Strain (Francisella LVS); an attenuated strain that can infect human and murine cells.

Microbes often utilize multiple strategies to gain entry into cells. The process of endocytosis encompasses both the internalization of extracellular particles into phagocytic cells [by phagocytosis] and non-phagocytic cells [referred to as pinocytosis]. These entry methods center around the use of the large GTPase, dynamin II, in releasing the endocytic vesicles from the invaginating membrane at the final stage of endocytosis (scission). Dynamin dependent pinocytosis is sub-divided into two categories; clathrin-mediated and caveolin-mediated pinocytosis. Clathrin-mediated pinocytosis uses the coat protein clathrin and a number of accessory proteins to create a structure known as a clathrin-coated vesicle (CCV) for internalization. Although the mechanism of pinocytosis was thought to be restricted to the internalization of vesicles ranging from 30 nm to 150 nm in diameter, recent work has demonstrated that bacteria and ligand coated beads up to 5.5 µm are readily internalized into cells via clathrin-mediated endocytosis. Caveolin-mediated endocytosis utilizes caveolin-1, a cholesterol-binding integral membrane protein, to form 50–80 nm flask-shaped membrane invaginations called caveolae. Caveolae are also associated with sphingolipid and cholesterol-rich sub-domains, commonly called lipid rafts. Consequently, perturbation of cholesterol or lipid rafts often impede caveolin-dependent endocytosis. Dynamin-independent pinocytosis has emerged as an internalization strategy utilized by many microbes. This type of pinocytosis can be separated into three general divisions: 1) non-clathrin/non-caveolin dependent pinocytosis, 2) macropinocytosis, and 3) lipid raft-mediated pinocytosis.

Like many invasive pathogens, F. tularensis gain entry into host cells as an initial step of infection. Efficient internalization of F. tularensis in macrophages has been shown to require complement. Additionally, through the use of the cholesterol sequestering agent Methyl-β-cyclodextran in combination with a plasma membrane marker, it has been suggested that cholesterol-rich microdomains of the plasma membrane, also known as lipid rafts, are also involved in F. tularensis internalization into phagocytes. These factors together with the endocytic protein caveolin-1 are involved.
thought to synergistically participate in the invasion of *Francisella* in phagocytic cells \(^{465}\). Although *Francisella* has been known to invade non-phagocytic cells \(^{326,411,458}\), the mechanism of *Francisella* entry into these types of cells has not yet been elucidated. In order to examine the sub-cellular mechanisms *Francisella* use to invade hepatocytes, we developed two *F. novicida* infection models using two different non-phagocytic murine hepatocyte cell lines, NMuLi and BNL CL.2 cells. Through the coupling of our *in vitro* systems with pharmacological inhibitors and RNA interference (RNAi), we demonstrate that the clathrin-associated machinery together with cholesterol are vital for efficient *F. novicida* invasion into non-phagocytic cells, and that this internalization is independent of caveolin.

4.3. Results

4.3.1. Murine hepatocytes, a target of *F. novicida*.

*F. tularensis* are known to infect both phagocytic \(^{290,341-343}\) and non-phagocytic host cells \(^{326,344,467}\); however studies examining non-phagocytic epithelial cells during these infections have trailed those of phagocytes. In order to generate a baseline for comparison we initially examined *F. novicida* infections occurring within murine hepatocytes by using the common *F. novicida* infection strategy of intraperitoneally infecting mice with 50 *F. novicida* \(^{406,430}\). At 48 h post-infection, livers were collected from mice and subsequently co-stained with anti-albumin antibodies, a classical marker of hepatocytes \(^{468-470}\) and anti-*F. novicida* antibodies. Cells that stained positive for albumin were typically infected in clusters, and varied from a few bacteria within a single cell to an unmeasurable number of *F. novicida* that completely filled the host cytoplasm (Figure 4.1A). By using microscopy and manually enumerating cells infected with *F. novicida* in liver cross sections, we found that 11.3% of albumin-positive cells and 1.1% of albumin-negative cells (those that did not react with anti-albumin antibodies) were infected (Figure 4.1B). This work, thus, sets a benchmark for the establishment of our epithelial cell culture models using *F. novicida* and suggests that the majority of infected cells within the livers of *F. novicida* infected mice were hepatocytes.
To generate cell culture models that would be useful for studying *F. novicida* hepatocyte infections, we selected two non-phagocytic hepatocyte cell lines, BNL CL.2 and NMuLi cells. We confirmed their hepatocyte characteristic by immunolocalizing albumin in those cell lines and found that both cell lines contained the classical albumin staining present in hepatocytes (Figure 4.2). Following that verification, both cell types were infected at a multiplicity of infection (MOI) of 100 for 24 h. During these infections the number of intracellular bacteria varied from 10 to hundreds of bacteria within the cytosol of a single cell (Figure 4.3A). Approximately 15.8% of BNL CL.2 and 25.4% of NMuLi cells became infected with *F. novicida* (Figure 4.3B) and the bacterial clustering patterns observed *in vivo* were also evident in the *in vitro* results (Figure 4.1A, Figure 4.3A). Next, we examined the colonization levels of *F. novicida* during shorter infection durations and coupled microscopic examinations with invasion assays using both cell lines. We found a modest level of invasion at 4 h and 8 h (10^4 CFU/mL), whereas the highest level of intracellular bacteria were detected at 24 h of infection (10^6 CFU/mL) (Figure 4.3A), suggesting that the bacteria had invaded the hepatocytes and likely replicated within them.

### 4.3.2. *Francisella* enters non-phagocytes through a clathrin and cholesterol-dependent mechanism.

Diverse endocytic strategies are used by microbes to gain entry into non-phagocytic cells 471. To evaluate the internalization mechanism(s) that promote *F. novicida* invasion of hepatocytes, we used invasion assays together with pharmacological inhibitors that disrupt specific cellular components to block particular endocytic functions. Clathrin-mediated endocytosis (CME) is a classical pathway often used by pathogens to gain entry into non-phagocytic cells (extensively reviewed by others 472 and our lab 471). To determine whether CME was involved in *F. novicida* invasion, we analyzed levels of bacterial internalization in the presence of the chemical inhibitors monodansylcadaverine (MDC) and chlorpromazine (CPZ), which are frequently used to study the role of CME during pathogen invasion 473,474. MDC is a transglutaminase inhibitor that prevents the assembly of clathrin-coated pits (CCP) at the plasma membrane, whereas CPZ functions as a clathrin-sequestering agent, inhibiting endosomal recycling of clathrin. We pre-treated both NMuLi and BNL CL.2 cells with
these reversible inhibitors at various concentrations for 30 minutes prior to infections with *F. novicida* and analyzed their effects on bacterial invasion. *F. novicida* internalization into host cells was reduced at 80 mM MDC (by 60%) and 5 mM CPZ (by 40–50%) as compared to untreated cells (Figure 4.4A, Figure 4.5A). Neither condition caused any detectable cytotoxicity to host cells or bacteriocidal effects as drug treated cells remained morphologically viable and intact when observed by phase contrast microscopy (data not shown). Because these drugs are well-known inhibitors that are specific for blocking CME, the loss of bacterial invasion in drug treated cells suggested that *F. novicida* invasion likely occurred through a CME pathway.

Despite the presence of the CME blocking agents, *F. novicida* still exhibited the ability to enter epithelial cells (Figure 4.4A, Figure 4.5A), suggesting that *F. novicida* likely use alternative route(s) in addition to CME for entry. To investigate how *F. novicida* could invade epithelial cells with dysfunctional clathrin machinery, we examined clathrin-independent endocytic mechanisms, such as caveolin and lipid-raft dependent endocytosis, both of which require cholesterol-rich domains. Extraction of cholesterol from the plasma membrane using methyl-β-cyclodextrin (MβCD), a potent inhibitor that disrupts lipid composition and thus cholesterol-based endocytosis, caused a statistically significant reduction, of 60%, in *F. novicida* invasion (Figure 4.4B, Figure 4.5B). We performed additional tests using a combination of progesterone (a cholesterol-synthesis inhibitor) and nystatin (a cholesterol-sequestering agent) to validate the importance of lipid raft domains during these invasion events. Interruption of synthesis and transport of cholesterol to membrane lipid-raft domains by these drugs caused a moderate (30%), yet statistically significant inhibition in *F. novicida* uptake in epithelial cells (Figure 4.5D). This data suggests that the presence of cholesterol at the plasma membrane is necessary for efficient invasion given that removal of cholesterol caused more prominent defects in invasion as compared to lipid-raft disruption through cholesterol binding. The importance of cholesterol at the plasma membrane for *F. novicida* invasion was further emphasized when the invasion levels were almost restored to those of untreated cells when cells that were pre-treated with MβCD were supplemented with excess cholesterol prior to the infections (Figure 4.4B). By contrast, replacement of cholesterol failed to enhance *F. novicida* invasion in MDC-treated cells. To further analyze the importance of cholesterol and CME during *F.
*F. novicida* invasion, a combination of MβCD and MDC were used concurrently to deplete both cholesterol and clathrin in host cells, thus blocking both CME and cholesterol-dependent endocytosis. This further exaggerated the inhibitory effect of *F. novicida* internalization when compared to a single inhibitor treatment, as the invasion level was diminished by 80% (Figure 4.4C). We also explored other possible endocytic pathways such as caveolin-dependent endocytosis and/or macropinocytosis that might be used by *F. novicida*. To accomplish this, we infected cells in the presence of filipin, a cholesterol-sequestering agent that impairs the membrane invagination of caveolae and consequently prevents caveolin-based endocytosis. *F. novicida* invasion appeared to be resistant to filipin treatment as it did not significantly impede invasion levels in either BNL CL.2 or NMuLi hepatocytes (Figure 4.6A). Further analysis by immunolocalization also revealed a normal pattern of caveolin at the cell periphery and within the cytoplasm that resembled untreated cells; the cytoplasmic caveolin did not accumulate near *F. novicida* throughout different stages of infection [at 8 h, 16 h or 24 h post-infection] (Figure 4.6B). These results strongly indicate that caveolin likely does not participate in *F. novicida* invasion of hepatocytes. To assess whether *F. novicida* engages in macropinocytosis during invasion, we examined cellular invasion in the presence of amiloride, an inhibitor of the Na⁺/H⁺ exchange channel at the plasma membrane known to specifically block internalization via macropinocytosis. Treatment with amiloride did not cause any notable changes in *F. novicida* invasion, especially when compared to *Salmonella Typhimurium* invasion, which uses well-established macropinocytosis strategies to invade their host’s cells (Figure 4.6C). This result is consistent with the lack of actin membrane ruffles (a characteristic of macropinocytosis) in the vicinity of *F. novicida* during the invasion process (Figure 4.3A), thus strengthening the evidence that *F. novicida* does not utilize macropinocytosis during its invasion process of hepatocytes.

Having demonstrated that clathrin and cholesterol inhibitors significantly attenuate *F. novicida* invasion, we extended our study by immunolocalizing clathrin and cholesterol to assess whether they became re-distributed during this process. To visualize clathrin localization during invasion, BNL CL.2 cells were infected with *F. novicida* for 8 h and co-localized with anti-*F. novicida* and anti-clathrin antibodies. We chose the 8 h timepoint in an attempt to identify regions of bacterial invasion without
significant cytoplasmic bacterial replication so that the precise localization of the proteins could be identified at sites of bacterial contact. We found that while the overall protein levels of clathrin remained unchanged (Figure 4.4E), clathrin localized strongly at sites where F. novicida interacted with the host cells (Figure 4.4F). This finding was also evident in BNL CL.2 cells during Francisella LVS invasion as clathrin was found to associate closely with invading Francisella LVS following 8 h infections (Figure 4.7). To examine the distribution of cholesterol, we took advantage of the cholesterol binding property of the fluorescent probe filipin\textsuperscript{475,480}. Similar to clathrin staining, we observed an accumulation of filipin at sites of F. novicida invasion (Figure 4.4F). Altogether, our data suggest that F. novicida invades epithelial cells at cholesterol-rich domains, in a clathrin-dependent manner.

4.3.3. Clathrin accessory proteins Eps15 and AP-2 are recruited and required for efficient Francisella invasion.

To investigate whether F. novicida utilize the protein assembly of classical-CME machinery for cell invasion, we examined the roles of CME adaptor proteins during F. novicida invasion of non-phagocytic cells. Using a similar approach as aforementioned, we immunolocalized Eps15 and AP-2 (housekeeping components that typically participate in cargo recognition and CCP formation to facilitate the progression of CME\textsuperscript{481}) 8 hours post-infection and found that both Eps15 and AP-2 were recruited to sites of invasion and co-localized with F. novicida in a similar pattern as was found with clathrin (Figure 4.8A, Figure 4.8D). Identical results were also found during Francisella LVS infections as bacteria were co-localized with cellular Eps15 and AP-2 after 8 h infections (Figure 4.7). Eps15 or AP-2 protein expression levels remained unchanged in cells infected with F. novicida when compared to uninfected cells (Figure 4.8B, Figure 4.1E). Because both AP-2 and Eps15 are major constituents of CCPs and govern the progression of CME, recruitment of these molecules to sites of Francisella interaction with host cells strongly supports the notion that Francisella exploit CME as a route for epithelial cell entry.

We continued our study by investigating the importance of Eps15 and AP-2 in F. novicida invasion. Using RNAi, we knocked down both Eps15 and AP-2 (a-adaptin subunit) to undetectable levels (Figure 4.8B, Figure 4.8E). Depletion of these proteins
have been shown to impede CME of many extracellular cargos, including microbes. We found that loss of Eps15 caused a significant inhibition in clathrin-dependent internalization of *F. novicida*, resulting in only 20% invasion relative to cells treated with non-targeting control pool (CP) siRNA (Figure 4.8C). To confirm the importance of clathrin-coat formation at the plasma membrane during the invasion process, we knocked down AP-2, which is known to cause an interruption in CCP assembly. Consistent with results from Eps15 RNAi, AP-2 depleted cells also exhibited a significant decrease in *F. novicida* invasion with 40% of invasion remaining when compared to untreated cells (Figure 4.8F). Collectively, these data show that the clathrin adaptors AP-2 and Eps15 are essential for clathrin-dependent uptake of *F. novicida*.

Disruption of actin polymerization interferes *F. novicida* internalization. The cytoskeleton is known to be an integral element necessary for functional and efficient endocytosis. Using the cytoskeletal targeting drugs, cytochalasin-D (Cyt D) and colchicines, Craven and co-workers had previously demonstrated that both actin filaments and microtubules were used by *Francisella* LVS to efficiently invade pulmonary epithelial cells. To assess whether *F. novicida* required these filament systems for efficient invasion in hepatocytes, we utilized the cytoskeletal inhibitors Cyt D and latrunculin A (LN A) to block actin filament polymerization. Additionally, we used nocodazole, a pharmacological drug that interferes with microtubule polymerization. Consistent with a previous *Francisella* LVS report in alveolar epithelial cells, we found that cells pre-treated with the actin-disrupting agents exhibited a significant defect in *F. novicida* invasion in hepatocytes. Cells pre-treated with Cyt D or LN A blocked 60% of bacterial entry as compared to untreated cells (Figure 4.4D, Figure 4.5D). By contrast, nocodazole treated cells did not exhibit any significant defects in internalizing *F. novicida* (Figure 4.4D). Together, these results suggest that the actin machinery, but not microtubules, is needed for efficient *F. novicida* invasion into hepatocytes. To further delineate the role of actin during *F. novicida* invasion, we immunolocalized actin filaments at 8 h and 24 h of infection and detected no direct association of actin and *F. novicida* at either time point (Figure 4.3). Consequently, it appears that *F. novicida* either uses actin for invasion via an indirect manner or could use it as part of the CME process, as the actin cytoskeleton has been described to play an important role in facilitating CME.
4.4. Discussion

Over the past number of years a familiar theme of endocytic protein hijacking by microbial pathogens has developed. Although *Francisella* has been identified as an invasive intracellular pathogen\(^3\)\(^9\)\(^2\)\(^,\)\(^4\)\(^0\)\(^5\), a general lack of knowledge of its internalization process and lifestyle in non-phagocytic cells remains. Most of the previous *Francisella* invasion studies into non-phagocytic cells have been focused on examinations of the *Francisella* LVS. One study demonstrated that the *Francisella* LVS can invade and replicate within murine alveolar cells *in vivo*, as well as in TC-1 and MLE12 murine lung epithelial cell lines and human alveolar type2 cells A549\(^4\)\(^1\)\(^1\). Another study demonstrated that the *Francisella* LVS can infect Hep-2 and human bronchial epithelial [HBE] cells *in vitro*; invasion assays showed relatively low levels of *Francisella* LVS invasion in these cells 5 hours post-infection, as approximately \(5 \times 10^3\)-\(10^4\) CFU were recovered, representing 0.05%–0.1% of the initial inoculum\(^4\)\(^1\)\(^2\). Although these important studies demonstrated the ability of *Francisella* LVS to replicate within these cell types, the internalization strategies utilized by these microbes during their invasion events still required further investigation. Because of the fastidious nature of the *Francisella* LVS, we opted to primarily use *F. novicida* as a model of *F. tularensis* infections. Using these bacteria we demonstrated a high level of colonization in murine hepatocyte *in vivo* and to elucidate their mechanisms of entry we developed 2 robust *in vitro* hepatocyte infection models.

We initiated our study by utilizing a panel of drugs that target specific endocytic pathways; none of the drugs used in the study caused adverse effects in bacterial growth, which suggested that the inhibitory effects we observed specifically impeded bacterial entry instead of bacterial replication. Although centrifugation of bacteria onto the host cells is a strategy that some laboratories use to enhance host cell contact with the microbes, in our experiments we allowed the bacteria to attach and colonize onto the surface of host cells without external forces to decrease the external influences during the infections. After monitoring levels of *F. novicida* infection in the hepatocytes throughout a 24 h period, we found a moderate degree of bacterial containment within the hepatocytes at the 8 h time point and not surprisingly a much higher level of invasion 24 h post-infection. Unlike many fast-invading bacteria such as *Salmonella*
Typhimurium, which invade within 30 minutes, F. novicida required an extended amount of time to initiate invasion into host cells for suitable detection and analysis. As a result, we chose to primarily monitor F. novicida invasion for up to 24 h infections to have ample numbers of internalized bacteria for comparison. Extended infection durations are not uncommon, as Helicobacter pylori invasion into cultured cells occurs at 24 h post-infection and Chlamydia trachomatis invasion is often assessed between 24 h to 72 h post-infection. Using MDC and CPZ, we first found that the loss of functional CME caused a significant decrease in F. novicida invasion when assessed at 24 h. Furthermore, we identified clathrin and major associated proteins at the Francisella entry sites after 8 h of infection. Taken together, these results suggest that CME is a key pathway targeted by these microbes, regardless of the endpoint assayed.

CME is a complex and multi-step process that involves many clathrin accessory proteins to complete the process. Among the ~20 accessory proteins, Eps15 and AP-2 have been shown to be important for efficient internalization of molecules in general. Our finding of impaired F. novicida invasion following Eps15 and AP-2 siRNA treatment highlights the importance of these clathrin-mediated endocytic proteins and CME as a whole during Francisella invasion.

Because F. novicida retained some capacity to invade these epithelial cell lines in the absence of functional CME, we postulated that another strategy must also be involved. Using the strong cholesterol-binding agent MβCD we extracted cholesterol from the membrane and found a significant decrease in F. novicida uptake. This phenotype reverted when the samples were supplemented with excess cholesterol. These findings coupled with the observation of cholesterol at F. novicida entry sites is reminiscent of cholesterol recruitment seen during Shigella flexneri invasion of HeLa cells.

Because phagocytic cells have been shown to use caveolin during F. novicida invasion events, we also studied its involvement and found that caveolin was not required for hepatocyte invasion, despite our findings that invasion requires cholesterol. Such dependence on cholesterol-rich domains in the absence of caveolin is not exclusive to F. novicida as it has been previously demonstrated during the invasion of...
viral (i.e., Simian virus 40) \(^{489}\) bacterial (i.e., *Brucella abortus*) \(^{490}\), as well as a much larger parasitic (*Toxoplasma gondii*) \(^{491}\) pathogens.

Recruitment of actin filaments at the site of internalization has been recognized as important processes during endocytosis, as polymerization of actin creates mechanical force to promote membrane curvature and invagination \(^{492}\). In this study, we have shown that *F. novicida* invasion is severely inhibited in the presence of the actin inhibitor cytochalasin D without detecting any notable membrane ruffling as seen during *Salmonella* invasion; similar results were previously described in a *Francisella* LVS invasion study using HEP-2 cells \(^{412}\) and murine alveolar cells TC-1 \(^{411}\). While it is likely that *F. novicida* invasion does not induce massive actin reorganization at the invasion sites, it has also been challenging to detect the small amounts of actin recruited to sites of endocytosis using fluorescent microscopy. Based on the involvement of actin in endocytosis \(^{484},^{492}\), we suspect that the inhibition of invasion through the impairment of the actin cytoskeleton likely comes from the association of the actin meshwork and endocytic events \(^{484}\). While the same *Francisella* LVS studies also suggested *Francisella* entry into TC-1 lung epithelial cells and human HEP-2 epithelial cells required microtubule \(^{411},^{412}\), we did not detect any notable defects in *F. novicida* invasion into either NMuLi or BNL CL.2 cells when infected with *F. novicida* in the presence of the microtubule disrupting drug nocodazol. This discrepancy could be due to the difference in the *Francisella* strain and host cell types used.

Collectively, we have demonstrated *F. novicida* uses multiple strategies for efficient internalization into hepatocytes, which involves functional clathrin-mediated endocytosis and cholesterol-rich microdomains.

### 4.5. Materials and Methods

#### 4.5.1. Bacteria and growth conditions.

*F. tularensis* subsp. *novicida* strain U112 was grown on trypticase soy agar (TSA) or broth (TSB) supplemented with 0.1% \(\text{L}-\text{cysteine}\) at 37°C. The bacteria were grown to stationary phase for 18 h at 37°C with shaking at 220 rpm. *Salmonella*
Typhimurium (strain SL1344) was grown using the same parameters on Luria Bertani (LB) agar or broth.

4.5.2. Animal infections, tissue preparation and tissue immunolocalization.

All procedures and husbandry of the animals was done in accordance with standards set by the Canadian Council of Animal care and all protocols were approved by the animal care committee of SFU. Female BALB/C mice aged 6–8 weeks (Charles River Laboratories, Quebec, Canada) were left undisturbed to recover from transport for at least 4 days. Infections were performed by intraperitoneal injection of $5 \times 10^5$ F. novicida bacteria in 100 ml of TSB + 0.1% \( \text{L-} \)cysteine. Immediately following the infection, the identical volumes of the bacterial culture were spread onto TSA-C plates. Colony forming units (CFUs) were enumerated after plates were incubated overnight at 37°C. Following 48 h infections, mice were euthanized and livers extracted then submerged into 3% paraformaldehyde (room temperature) in phosphate-buffered saline (PBS; 150 mM NaCl, 5 mM KCl, 0.8 mM KH\(_2\)PO\(_4\), 3.2 mM Na\(_2\)HPO\(_4\), pH 7.3) for 3 h. After 3 successive 10 min PBS washes, fixed organs were frozen (using liquid nitrogen) and attached to an aluminum stub by OCT compound (Sakura Finetek USA, Torrance). 5 mm frozen sections were cut and attached to Superfrost (VWR) glass slides that were pre-coated with 0.01% poly-L-lysine solution (Sigma). Slides with attached tissue sections were immediately plunged into -20°C acetone for 5 min, air-dried, then blocked with 5% “Blotto” non-fat dry milk (Santa Cruz Biotechnology) in PBS for 20 min. The slides were then incubated overnight at 4°C with a 1:1000 dilution of rabbit anti-F. novicida antibodies \(^{456,493}\) and 20 \( \mu \)g/\( \mu \)l goat anti-mouse albumin (Bethyl Laboratories Inc.) antibodies diluted in PBS containing 1% Tween-20 and 1% non-fat milk. The following day the sections were washed 3 times for 10 min each with TPBS/non-fat milk (1% Tween-20 and 0.1% non-fat milk in PBS) before being incubated for 2 h with 20 \( \mu \)g/\( \mu \)l Alexa Fluor 594 donkey anti-goat antibodies (Invitrogen) then with 20 ng/\( \mu \)l Alexa Fluor 488 goat anti-rabbit antibodies for 1 h (Invitrogen). After the last set of washes, coverslips were mounted using Prolong Gold (Invitrogen). Images were acquired using a Leica DMI4000B inverted fluorescent microscope equipped with a Hamamatsu Orca R2 CCD camera (Hamamatsu, Japan) and Metamorph Imaging System software (Universal
Imaging Corp., Pennsylvania). The presence of albumin in cells was assessed by examining samples and those that did not react were considered absent of albumin. In all of those instances positively reacting cells were also present in the samples, which acted as positive controls for the experiment. For antibody specificity controls, primary antibodies were replaced with normal immunoglobulin from the host animal species at identical concentrations to the primary antibodies.

4.5.3. **Cell lines and culture model infection parameters.**

Cultured BNL CL.2 (TIB-73) and NMuLi (CRL-1638) murine hepatocytes (ATCC) were grown in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) (Thermo Scientific) supplemented with 10% Fetal Bovine Serum (FBS) at 37°C and 5% CO₂. Cells were grown to confluence in 6- or 24-well cell culture plates overnight in 10% FBS in DMEM before switching to 10% FBS in DMEM supplemented with 0.1% L-cysteine prior to infection.

4.5.4. **Cultured cell invasion assays and inhibitor studies.**

Inhibitors targeting different endocytic pathways were used to evaluate levels of *F. novicida* invasion. The duration of treatments and concentrations were determined and optimized according to previous studies as well as cell viability assays. BNL CL.2 or NMuLi cells were seeded at a concentration of $\sim 1.5 \times 10^5$ cells/mL in 24-well culture plates and used 24 h after seeding. Monolayers of confluent cells were pre-incubated at 37°C with the following inhibitors for 30 min: 5 µM chloropromazine, 5 µM cytochalasin D, 2.5 µM latrunculin A, 2.5 mM MβCD, 80 µM MDC, 2 µg/mL progesterone combined with 10 µM nystatin. Cells were pre-treated for 2 h with 2.5 µM nocodazole and 15 min with 5 mM amiloride at 37°C prior to infection. For the cholesterol supplementation study, 1 mM solubilized cholesterol in chloroform was added to MβCD treated cells at a final concentration of 2 mM for 30 min prior to infection. All compounds (purchased from Sigma) were maintained in the media during infections except amiloride, which was removed prior to infections. Untreated controls are only treated with carrier buffer (*i.e.*: DMSO) in the absence of inhibitors. Following pre-treatment, cells were infected with *F. novicida* at an MOI of 100. At 22 h post-infection, cells were washed three times with
warm DMEM 10% FBS and treated with media containing 100 µg/ml gentamicin for 2 h at 37°C to kill extracellular bacteria. For amiloride studies, cells were infected with *F. novicida* at an MOI of 100 for 6 h followed by 2 h gentamicin treatment. As a positive control, HeLa cells were infected with *Salmonella* Typhimurium at an MOI of 100 for 30 min followed by 1 h gentamicin treatment. After 3 PBS washes, cells were lysed either mechanically using a 27" gauge needle or chemically using 1% Triton X-100 in PBS. The released internalized bacteria were immediately serially diluted and plated on TSA-C for colony-forming unit (CFU) enumeration to quantify levels of internalization.

### 4.5.5. Cultured cell immunolocalization.

Cells were grown to 80% confluency on sterile No.1.5 glass coverslips in 6-well culture plates. Prior to infection, media was replaced with DMEM 10% FBS with 0.1% cysteine. After 8 h, 16 h or 24 h infections at an MOI of 100, cells were fixed in 3% paraformaldehyde in PBS for 15 min, washed three times with PBS, incubated with 0.2% Triton X-100 and treated with 5% NGS following chemical reagent or antibody treatments in processes mentioned above. Cholesterol localization was determined using a UV fluorescent cholesterol binding fungal toxin, filipin, which binds cholesterol and emits blue fluorescence. Cells were incubated with filipin (50 µg/mL) for 30 min at RT, washed three times with PBS, then co-localized with *F. novicida*. Clathrin, Eps15, AP-2 and caveolin-1 localization was performed using mouse anti-clathrin heavy chain antibodies at 15.6 µg/ml (BD Biosciences), mouse anti-Eps15 (clone 17) antibodies at 16.67 µg/ml (Santa Cruz Biotechnology), mouse anti-AP2 a-adaptin (3B5) at 10 µg/ml (BD Biosciences), or mouse anti-caveolin-1 (clone 2297) antibodies at 16.67 mg/ml (BD Biosciences). Prior to staining with clathrin, Eps15 and AP-2 antibodies, the antibodies were pre-cleared against *F. novicida* to ensure no cross reactivity with the bacteria. To accomplish this, 1 ml of live *F. novicida* was pelleted, fixed for 20 min with 500 ml of 3% paraformaldehyde in PBS at RT then washed 6 times with PBS at 15 min intervals. *F. novicida* was pelleted and re-suspended overnight at 4°C with the antibody, prepared at the concentrations described above, then used for immunolocalization.

BNL CL.2 cells were grown on 150 mm tissue culture dishes and infected at an MOI 100 with *F. novicida* for 24 h. Cells were washed 3 times with PBS containing 1 mM CaCl$_2$ and 1 mM MgCl$_2$ followed by treatment with RIPA lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4, 5 mM EDTA, 1% Nonidet P-40, 1% Deoxycholic acid, 10% SDS) for 10 min on ice. Western blotting was performed according to Guttman and colleagues $^{199}$. Briefly, equal amounts of total protein was loaded and separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked with 5% non-fat milk and washed in Tris-buffered saline with 0.1% Tween-20 (TBST) 3 times for 5 min. A primary rabbit anti-Eps15 antibody (Santa Cruz Biotechnology, CA) was used at 1.0 mg/ml, a mouse anti-AP2 α-adaptin (3B5) antibody (Trevor Williams lab, Developmental Studies Hybridoma Bank, IA) was used at 0.725 µg/ml, and a mouse anti-clathrin heavy chain antibody (BD Biosciences) was used at 0.25 µg/ml. Antibodies were prepared in TBST supplemented with 1% BSA. After washing, a horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology) was used as previously described $^{199}$. Signals were detected by enhanced chemiluminescence (Perkin Elmer) and exposed on Kodak BioMax light film.

4.5.7. RNA interference.

Small interfering RNA (siRNA) ON-TARGETplus SMART pools were synthesized and purchased from Dhharmacon, targeting Eps15 (Catalog: 004005) and AP-2 A1 α-adaptin (Catalog: 11771). For RNAi controls, equal amount of siGENOME Non-Targeting siRNA Pool (Catalog: D-001206-13) was used. BNL CL.2 cells were grown in 24 well plates to 40% confluency and transfected with 50 nM of the siRNA pool using the jetPRIME transfection reagent or INTERFERin (Polyplus transfection) according to the manufacturer’s procedures. At 48 h post-transfection, cells were either treated with RIPA lysis buffer supplemented with a protease inhibitor cocktail (Roche) for cell lysates, or infected with *F. novicida* for 24 h before being processed for invasion assays.
4.5.8. **Statistical analysis.**

All experiments were performed at least in triplicate during a single run, and repeated a minimum of three times. For enumerating infected murine liver cells, phase and fluorescent images of liver cells stained for albumin (or F-actin), *F. novicida*, and DNA were overlayed and tallied using Fiji software (http://fiji.sc/wiki/index.php/Fiji). Localization of *F. novicida* within the cytoplasm of albumin-positive and negative cells were considered ‘infected’, whereas cells that show no *F. novicida* localization within the cell periphery were counted as ‘uninfected’. Each field of view was acquired as a stack under both phase-contrast and fluorescent microscopy. Student’s t-test was applied to determine statistical significance between the two experimental groups. For inhibitor studies, results were expressed as the means ± standard deviation (s.d.) from repeated independent experiments. Differences between untreated and inhibitor treated samples were compared using One-way ANOVA followed by Dunnett’s Multiple Comparison Test (P, 0.05). Differences between control pool and RNAi treatments were compared using Student’s t test. (P, 0.05).
4.6. Figures

Figure 4.1  *F. novicida* colonization of murine hepatocytes.
(A) Immunofluorescent and phase micrographs showing wild-type *F. novicida*, albumin, and DNA [DAPI] localization in infected liver tissue section harvested from mice challenged via the intraperitoneal route. Arrows indicate uninfected albumin-negative cells, large arrowheads point to infected albumin-positive cells containing uncountable levels of *F. novicida* and the small arrowhead points to an albumin-positive cells cells with few internalized bacteria, respectively. Scale bar = 10µm. (B) Quantification of cells infected with *F. novicida* based on immunofluorescent images of tissue sections stained with anti-mouse albumin and anti-*F. novicida* antibodies. N (below) is defined as the number of tissue section regions collected in which image stacks were taken (each image stack represents between 500 to 1500 cells). Mouse 1 (n=17), Mouse 2 (n=21), and Mouse 3 (n=17). *** P<0.0001.
Figure 4.2  Albumin labeling of BNL CL.2 and NMuLi cells.
BNL CL.2 and NMuLi cells were probed for albumin (red) using an antimouse albumin antibody, F-actin (green), and DNA [blue, DAPI]. Scale bar = 10µm.
Figure 4.3  *F. novicida* invades and replicates within BNL CL.2 and NMuLi hepatocytes.

(A) Immunofluorescent images of *F. novicida* infections in cultured hepatocytes. Hepatocytes were fixed following 24 h *F. novicida* infections with both BNL CL.2 and NMuLi cells. Samples were labelled with anti-*F. novicida* antibodies (green), fluorescent phalloidin [to indicate the cell boundaries] (red) and DAPI (blue). Arrowheads indicate some of the bacteria within the infected cells. Scale bar = 10µm. (B) Quantification of the proportion of infected hepatocytes as assessed by microscopic examination. NMuLi (n=17) and BNL CL.2 (n=16). (C) Titre of intracellular *F. novicida* at various timepoints following infection of BNL CL.2 and NMuLi cell infections by invasion assay (n=3).
Figure 4.4  Internalization of *F. novicida* into BNL CL.2 cells is a clathrin and cholesterol dependent process that requires actin

(A-D) BNL CL.2 cells were pre-treated with the indicated drugs and incubated with *F. novicida* for 22 h followed by a 2 h gentamicin treatment for CFU enumeration. (A) Clathrin inhibitors: 80µM monodansylcadaverine (MDC) or 5.0µM chlorpromazine (CPZ). (B) Cholesterol inhibitors: 2.5mM MβCD or 80µM MDC supplemented with 1mM cholesterol. (C) Individual or a combination of 2.5mM MβCD and 80µM MDC. (D) Cytoskeleton inhibitors: 5µM cytochalasin D (Cyt D), 2.5µM latrunculin A (LN A) or 2.5µM nocodazole (ND), * P<0.01. ** P<0.05. (E) Western blot shows that the overall level of clathrin heavy-chain (HC) protein remains unaltered after 24 hour *F. novicida* infections of BNL CL.2 cells compared to uninfected cells. For immunolocalization experiments, untreated cells were infected with *F. novicida* for 8 h, fixed and stained with (F) an anti-*F. novicida* antibody (green), anti-clathrin HC (red) and DAPI or (G) an anti-*F. novicida* antibody (green) and filipin (blue) as well as phase contrast. Arrowheads indicate some of the bacteria that co-localize with the other labels. Scale bar= 5µm.
Figure 4.5 Internalization of *F. novicida* into NMuLi cells is a clathrin- and cholesterol dependent process that requires actin.

NMuLi cells were pre-treated with the indicated drugs and incubated with *F. novicida* for 22 h followed by a 2 h gentamicin treatment for CFU enumeration. (A) Clathrin inhibitors: 80µM monodansylcadaverine (MDC) or 5.0µM chlorpromazine (CPZ). (B) Cholesterol inhibitors: 2.5mM MβCD or 80µM MDC supplemented with 1mM cholesterol. (C) Individual or combination of 2.5mM MβCD and 80µM MDC. (D) Cells were treated simultaneously with 2µg/mL progesterone and 10µM nystatin (Prog/Nys). Cytoskeleton inhibitors: 5µM cytochalasin D (Cyt D), 2.5µM latrunculin A (LN A) * P<0.01, ** P<0.05.
Figure 4.6 *F. novicida* entry does not utilize caveolin-dependent or macropinocytosis pathways.
(A) BNL CL.2 and NMuLi cells were treated with the caveolin-inhibitor filipin and infected with *F. novicida* for 22 h followed by 2 h gentamicin treatment for invasion assays. (B) Immunolocalization of caveolin-1 (green) and DAPI (blue) of uninfected BNL CL.2 cells or following 8, 16, and 24 h infections with *F. novicida* showed no co-localization between *F. novicida* and caveolin-1. Arrowheads indicate the localization of *F. novicida*. Scale bar = 5µm. (C) 5µM of amiloride was used to pre-treat BNL CL.2 cells and HeLa cells for 15 min prior to infection with *F. novicida* and *Salmonella* Typhimurium (SL1344), respectively. Untreated cells were treated with media containing DMSO. Cells infected with *F. novicida* for 6 h followed by 2 h gentamicin treatment did not show a decrease in invasion while cells infected with *S. Typhimurium* for 30 min followed by 1 h gentamicin treatment showed a significant decrease in invasion *P<0.01.*
Figure 4.7 Recruitment of clathrin and its accessory proteins during *Francisella* Live Vaccine Strain (LVS) invasion into non-phagocytic cells.

BNL CL.2 cells were infected with the Francisella LVS for 8 h. To detect *Francisella* localization, an anti-*Francisella* LVS antibody (green) was used. To detect clathrin-associated proteins, anti-clathrin HC or anti-Eps15, or anti-AP2 (red) were used to visualize their recruitment to sites of internalization. DAPI was used to stain both bacterial and host cell DNA. Arrowheads indicate some of the bacteria that co-localize with the other labels. Scale bar = 10µm.
Figure 4.8  Clathrin-associated adaptor proteins Esp15 and AP-2 are crucial for *F. novicida* invasion into non-phagocytic cells.

(A, D) BNL CL.2 cells were infected with *F. novicida* for 8 h and immunolocalized with anti-*F. novicida* together with (A) anti-Eps15 (red) or (D) anti-AP-2 α-adaptin (green) antibodies. Arrowheads indicate bacteria and protein co-localization. Scale bar = 5µm. (B and E). Western blots show the overall protein levels of (B) Eps15 (145kDa) or (E) AP-2 α-adaptin (50kDa) in 8 h *F. novicida* infected cells (untreated with RNAi) remained unchanged compared to uninfected control. Cells transiently transfected with siRNA (48 h) showed a significant reduction in the targeted Eps15 or AP-2 α-adaptin proteins as compared to cells transfected with siRNA control pool (CP). (C and F) Reduced Eps15 and AP-2 α-adaptin lead to a significant defect in *F. novicida* internalization 24 h post-infection. * P< 0.05.
Chapter 5.

IglC and PdpA are important for promoting *Francisella* invasion and intracellular growth in epithelial cells

The contents in this chapter have been published.


† These authors contributed equally

**Author contributions:** HTL designed and performed all experiments. AS and FC contributed to experiments in (Fig. 5.1). EBN and FEA provided bacterial mutants. HTL and JAG analyzed the results and wrote the manuscript.
5.1. Abstract

The highly infectious bacteria, *Francisella tularensis*, colonize a variety of organs and replicate within both phagocytic as well as non-phagocytic cells, to cause the disease tularemia. These microbes contain a conserved cluster of important virulence genes referred to as the *Francisella* Pathogenicity Island (FPI). Two of the most characterized FPI genes, *iglC* and *pdpA*, play a central role in bacterial survival and proliferation within phagocytes, but do not influence bacterial internalization. Yet, their involvement in non-phagocytic epithelial cell infections remains unexplored. To examine the functions of IglC and PdpA on bacterial invasion and replication during epithelial cell infections, we infected liver and lung epithelial cells with *F. novicida* and *F. tularensis* ‘Type B’ Live Vaccine Strain (LVS) deletion mutants (Δ*iglC* and Δ*pdpA*) as well as their respective gene complements. We found that deletion of either gene significantly reduced their ability to invade and replicate in epithelial cells. Gene complementation of *iglC* and *pdpA* partially rescued bacterial invasion and intracellular growth. Additionally, substantial LAMP1-association with both deletion mutants was observed up to 12 h suggesting that the absence of IglC and PdpA caused deficiencies in their ability to dissociate from LAMP1-positive *Francisella* Containing Vacuoles (FCVs). This work provides the first evidence that IglC and PdpA are important pathogenic factors for invasion and intracellular growth of *Francisella* in epithelial cells, and further highlights the discrete mechanisms involved in *Francisella* infections between phagocytic and non-phagocytic cells.

5.2. Introduction

The intracellular bacteria *Francisella tularensis* cause the zoonotic disease in humans and other mammals, called tularemia. *F. tularensis* have multiple subspecies including ssp. ‘type A’ *tularensis*, ssp. ‘type B’ *holarctica* and ssp. *mediasiatica*. Though *F. novicida* has been considered a fourth subspecies because of its high genomic identity (>97%) with *F. tularensis*300,349,457,494, this taxonomic classification remains controversial due to the distinct evolutionary and ecological profiles between the two bacteria301,495,496. Both *F. novicida* and *F. tularensis* can enter hosts through multiple routes including the intestinal and respiratory tracts as well as through exposure from
infected animals or arthropod vectors. Once internalized, these bacteria colonize various organs, including the lungs and liver. F. tularensis cause disease from exposure to as few as 10 bacteria, which can result in mortality rates as high as 60% if left untreated whereas F. novicida causes human-like tularemia in mice with as few as 50 bacteria. F. tularensis LVS can causes disease in mice and can infect human cells in culture. All of these microbes infect phagocytic and non-phagocytic cells, and contain similar sets of virulence factors. Because F. tularensis has the potential for aerosolization and is classified by the USA Centers for Disease Control and Prevention as a ‘Category A’ select agent, requiring BCL-3 facilities for experimentation, much of the research on Francisella have used F. novicida and F. tularensis LVS (BCL-2) as surrogate pathogens to investigate the genetics, biochemistry and pathogenesis of Francisella.

The Francisella pathogenicity island (FPI) is a highly conserved ~31 kb region, comprising 16 to 19 protein-coding genes, within the bacterial chromosome. It is thought to exist as a single copy in F. novicida, but is duplicated in all F. tularensis species. Evidence has shown that deletion of a single FPI gene in F. tularensis does not significantly diminish its virulence suggesting that both alleles are phenotypically alike. Though most FPI genes are unique to the Francisellaceae family and remain poorly defined, a subset of genes, including iglA, iglB, dotU, and vgrG, share limited homology with core structural components of a Type VI secretion system (T6SS). A thorough study by Bröms and colleagues examining dotU, iglC, iglG, and vgrG suggest that all four genes are needed for the delivery of virulence factors into host macrophages.

A commonly associated function of the FPI is its involvement in the replication of Francisella within host macrophages. During the initial intracellular stage of these infections, Francisella transiently reside within phagosomes and associate with early and late stage endosomal markers. In order to evade lysosome-mediated killing, Francisella can escape these vacuoles in as little as 15 min; by 4h, ~90% of the bacteria are already present in the host cytosol. Several studies have demonstrated that most FPI-encoded proteins, including IgIC and PdpA, are essential for bacterial escape from the phagosomal compartment and/or bacterial replication. Although we have yet to fully understand how these proteins
interact with host cells, recent evidence suggests that IglC, PdpA, and six other FPI-encoded proteins are translocated into the cytoplasm of phagocytes and their delivery is dependent on the expression of *Francisella* T6SS genes \(^{335}\). Despite the importance these T6SS genes play in intracellular replication, their expression does not appear to significantly influence the uptake of *Francisella* into macrophages \(^{333,419,425,456}\).

Non-phagocytic epithelial cell infections are considered to be important for the progression of disease as *Francisella* colonize and replicate within epithelial cells both in vitro and in vivo \(^{281,395,404,407,412}\). Although several organs are known to be colonized by these bacteria \(^{298,390,401,408,497-499}\), two primary sites of *Francisella* colonization are the lungs and liver. The lungs are susceptible to infection through aerosol delivery, requiring the low infectious doses to trigger disease \(^{276}\). Once inside the lungs, *F. tularensis* can infect alveolar type II epithelial cells, which are considered well positioned for the pathogen to enter the circulation as they reside near the endothelium \(^{395,411}\). The livers of *Francisella* infected animals have been known for decades to be sites of bacterial colonization \(^{282,510}\). Within the livers of infected mice, \(~12\%\) of the cells are colonized by these microbes; >90\% of which are hepatocytes \(^{500}\). We have previously shown that *Francisella* predominantly invade hepatocytes by usurping the host clathrin-mediated endocytic (CME) machinery \(^{500}\). Although the precise bacterial mechanisms that *Francisella* use to hijack the host endocytic machinery has remained elusive, viruses \(^{511-513}\) and other invasive pathogens including *Listeria monocytogenes* and *Yersinia pseudotuberculosis* rely on virulence factors encoded within clusters of pathogenic genes to trigger bacterial internalization via CME \(^{239,514}\).

Despite recent work that has alluded to the importance of non-macrophage infections in the *Francisella* disease process \(^{312,391}\), the study of *Francisella* remains primarily focused on phagocytic cell infections. Given that a previous report has indicated that the FPI likely plays a predominant role in the pathogenesis of *Francisella* in epithelial cells \(^{375}\), we examined the involvement of two FPI genes in *Francisella* invasion and replication during liver and lung epithelial cell infections. We report that *iglC* and *pdpA* are required for efficient bacterial invasion and intracellular proliferation during non-phagocytic epithelial cell infections.
5.3. Results

5.3.1. IglC and PdpA are required for efficient epithelial cell invasion

The involvement of the FPI genes *iglC* and *pdpA* has been examined during macrophage infections and was shown to be important for intracellular replication, but not for the uptake of *Francisella* into those cells\(^{323,425,456}\). To begin to investigate whether *iglC* and *pdpA* play a role in bacterial entry into epithelial cells, we evaluated the internalization of wild-type *F. novicida*, deletion mutants (\(\Delta iglC\), \(\Delta pdpA\)), and their respective gene complemented strains during murine liver BNL CL.2 cell infections using gentamicin protection/invasion assays. These assays are common microbiological experiments used to quantify the amount of intracellular bacteria in an entire sample. Because minimizing the amount of intracellular bacterial replication is a general concern when performing these assays\(^{366,385,412}\), we allowed *F. novicida* to contact and invade BNL CL.2 cells for 3 h before treating the samples with gentamicin for 1 h to kill the extracellular microbes. This time-point was selected because there are extremely few bacteria that are detectable by plating during infections lasting <3 h (unpublished data). Thus, at 4 h PI, host cells were lysed and intracellular bacteria were plated onto solid media. Using this procedure, we found a significant reduction of intracellular bacteria in samples infected with \(\Delta iglC\) [20.4\%] and \(\Delta pdpA\) [19.9\%] when normalized against wild-type *F. novicida* invasion [100\%] (Figure 5.1). Invasion was significantly restored for both \(\Delta iglC::iglC\) [64.9\%] and \(\Delta pdpA::pdpA\) [65.2\%] when genes were re-introduced back into the microbe (Figure 5.1). Our bacterial deletion data at this early time-point suggest that IglC and PdpA influence *F. novicida* invasion into epithelial cells.

To determine whether these findings were *F. novicida* specific or if they were more broadly applicable to *Francisella* in general we ran similar assays using *F. tularensis* LVS infected A549 epithelial cells. We chose human lung A549 cells over BNL CL.2 cells for this assay primarily because of the low infections rates BNL CL.2 cells show at 4 h when infected with *F. tularensis* LVS. We found that *F. tularensis* LVS \(\Delta iglC\) and \(\Delta pdpA\) infected at a rate of 63.3\% and 46.6\% as compared to wild-type [100\%] (Figure 5.2). Although this was significantly lower than wild-type *F. tularensis* LVS, it was not as dramatic as the decrease seen with *F. novicida* (Figure 5.1). A significant
improvement in the numbers of intracellular bacteria was found when complemented strains were used (Figure 5.2), thus supporting our hypothesis that IglC and PdpA both play a role in the epithelial internalization process.

Given that our evidence showed that gene deletion of *iglC* and *pdpA* decreased the levels of intracellular bacteria, we then further studied this phenotype with *F. novicida* using fluorescence microscopy. These infections were performed for 24 h, instead of 4 h, because short infections resulted in <1% of total colonized hepatic cells when examined by microscopy. To distinguish infected and uninfected cells, hepatocytes were infected separately with wild-type *F. novicida*, deletion mutants (Δ*iglC* and Δ*pdpA*) and their respective complement strains. Samples were then fixed and labeled using differential bacterial staining, which allows extracellular (Figure 5.3; arrow) and intracellular bacteria (Figure 5.3; arrowhead) to be distinguished. After enumerating >1,000 cells, we found that wild-type *F. novicida* infected approximately 28.7% of BNL CL.2 cells (Figure 5.4), which is in-line with previous reports 500,508. The microscopic images point to both invasion and bacterial replication deficiencies during these mutant *F. novicida* infections, as extremely few bacteria were found in the cells in general. In cells that had intracellular bacteria, clusters of *F. novicida* that would be expected if bacterial replication were functional were not observed. Additionally, the population of cells colonized by Δ*iglC* [4.1%] and Δ*pdpA* [12.4%] mutants were significantly lower as compared to wild-type (Figure 5.4). Gene complementation of *pdpA* back into its respective deletion mutant significantly increased colonization [21.2%]. Although we observed a similar doubling in bacterial colonization for Δ*iglC::iglC* [8.3%] over the mutant strain, this increase was not significant (Figure 5.4). Together, our 24 h microscopic data provided similar results to the *F. novicida* gentamicin protection/invasion assays taken at 4 h, suggesting that *iglC* and *pdpA* genes are involved in both bacterial invasion and early stage replication within epithelial cells.

To further study the influence that *iglC* and *pdpA* have on intracellular replication, we infected mouse BNL CL.2 cells with wild-type *F. novicida* and mutants (Δ*iglC*, Δ*iglC::iglC*, Δ*pdpA*, and Δ*pdpA::pdpA*) and allowed them to invade for 3 h. The hepatocytes were then kept in gentamicin-containing media for up to 24 h, when the total intracellular bacterial loads were measured. We found that *F. novicida* deletion mutants
ΔiglC and ΔpdpA did not show significant growth over the course of the infection (Figure 5.5), but rather, the population of intracellular ΔiglC and ΔpdpA declined marginally as early as 8 h PI (Figure 5.5). By contrast, wild-type F. novicida grew very rapidly from 4 to 8 h (Figure 5.5), with an average doubling time of 1.16 h, which slightly outpaced that of ΔiglC::iglC [1.53 h] and ΔpdpA::pdpA [1.41 h] (Table 5.1). By 12 h, the live growth was about 3-fold less and the doubling time for wild-type F. novicida, ΔiglC::iglC, and ΔpdpA::pdpA respectively increased to 4.44, 4.49, and 5.05 h (Table 5.1). These results demonstrate that the early stage replicative deficiencies seen with F. novicida ΔiglC and ΔpdpA at 4 h PI were maintained up to 24 h following epithelial cell infections.

We also examined whether IgIC and PdpA were important for bacterial replication during the late phase of its intracellular lifecycle. To test this, we compared the bacterial burden of F. novicida in BNL CL.2 cells at 24 h post-inoculation with a ‘prolonged’ 48 h infection in the absence of additional bacterial internalization by treating the cells with fresh media containing 100 μg mL⁻¹ of gentamicin at the 22 h time-point, which was followed and maintained by 10 μg mL⁻¹ of gentamicin from 24-48 h. We found that the amount of internalized wild-type F. novicida had increased by ~100-fold in the final 24 h of infection (by the 48 h time-point) (Figure 5.6). During the same period, the ΔiglC and ΔpdpA F. novicida mutants both did not have a replicative burst (Figure 5.6). By contrast, gene complementation of ΔiglC::iglC and ΔpdpA::pdpA replicated to nearly wild-type F. novicida levels (Figure 5.6). Concurrently, we qualitatively assessed intracellular bacterial replication by fluorescence microscopy. Clusters of bacteria were found in colonized BNL CL.2 cells infected with wild-type F. novicida, as well as ΔiglC::iglC and ΔpdpA::pdpA complement strains; many of which were completely filled with bacteria Figure 5.7). By contrast, F. novicida-filled cells were rarely (if ever) observed when hepatocytes were infected with ΔiglC and ΔpdpA deletion mutants and most infected cells contained only a small number of bacteria (Figure 5.7).

To determine whether this late-stage replicative phenotype was F. novicida specific, we performed identical assays on F. tularensis LVS-infected A549 cells and found similar results that F. tularensis LVS ΔiglC and ΔpdpA were both attenuated in their abilities to rapidly grow within infected cells at 48 h as compared to wild-type Francisella LVS (Figure 5.8). Interestingly, we noticed a gain in the amount of
intracellular ΔiglC (Figure 5.8), which was not apparent when assayed with \textit{F. novicida} (Figure 5.6). Increased bacterial levels were again seen with the gene complement strains ΔiglC::iglC and ΔpdpA::pdpA (Figure 5.8). Taken together this supporting evidence indicates that iglC and pdpA genes are important for efficient bacterial proliferation during all stages of epithelial cell infection.

5.3.2. IglC and PdpA are crucial virulence factors for intracellular proliferation and LAMP1-positive FCV dissolution

Following bacterial entry in macrophages, \textit{Francisella} are found enclosed within a membrane-bound compartment referred to as the \textit{Francisella}-containing vacuole (FCV) \textsuperscript{385}. The bacteria can reside within FCVs for up to few hours \textsuperscript{366} until they escape into the cytoplasm where it is favorable for replication \textsuperscript{385,425}. Previous phagocytic cell studies have demonstrated that proficient escape from FCVs requires the presence of IglC \textsuperscript{369,424}. Yet, it is not known whether the same is true for PdpA. LAMP1 is a commonly used marker of FCVs and its presence or absence is indicative of the maintenance or dissolution of the FCV. Given that iglC and pdpA influence intracellular bacterial growth in both macrophages \textsuperscript{309,515} and epithelial cells, and because it is known that replication occurs after \textit{Francisella} escape from the FCVs during macrophage infections \textsuperscript{366}, we investigated the temporal dynamics of LAMP1 around intracellular \textit{F. novicida} at 4, 8, 12 and \textit{24 h PI} in order to determine whether iglC and pdpA affected its localization. To investigate this, we infected BNL CL.2 cells for 3 h with wild-type \textit{F. novicida} as well as iglC and pdpA mutants and their complemented strains. Extracellular bacteria were then washed away and exposed to gentamicin for up to 24 h then processed for bacterial and LAMP1 localization.

We found that there was a significant increase in the number of bacteria associated with LAMP1 when ΔiglC [33.9\%, 4 h; 25.9\%, 8 h; 18.3\%, 12 h] was compared to wild-type \textit{F. novicida} [12.8\%, 4 h; 4.0\%, 8 h; 2.4\%, 12 h] up to 12 h PI (Figure 5.9). Similarly ΔpdpA (33.6\%, 8 h; 23.1\%, 12 h) showed a significant increase in localization events around \textit{F. novicida} between 8 and 12 h PI when compared to wild-type \textit{F. novicida} (Figure 5.9). Although there was a delay in the phenotypic effects of the pdpA complementation until the 8 h time-point, the observation of increased association
of LAMP1 around the mutant *F. novicida* suggests that those microbes were impaired in their ability to break out of the FCV, thus remaining in the vacuolar compartment.

### 5.4. Discussion

Research on the sub-cellular events underlying *Francisella* pathogenesis has primarily concentrated on phagocytic cell infections, while those involved in non-phagocytic epithelial cell infections have remained largely unexplored. This has occurred despite epithelial cells being a primary site of infection and carrying a considerable bacterial load. Given the FPI’s importance for phagocytic cell infections, we began by investigating whether two of the FPI components, IgIC and PdpA, affected bacterial entry and replication in epithelial cells. In order to examine this, we used iglC and pdpA deletion mutants from 2 *Francisella* bacteria; *F. novicida* and *Francisella* LVS. We initially found that both iglC and pdpA were required for bacterial internalization into liver and lung epithelial cells. To investigate a potential mechanism responsible for the replication impedance that the ΔiglC and ΔpdpA mutants showed, we turned to the FCVs. *Francisella* is known to rapidly replicate in the cytosol of macrophages and both IgIC and PdpA are needed for that process to occur efficiently. By using a common marker for FCVs, LAMP1, we found that LAMP1 localization to ΔiglC and ΔpdpA *F. novicida* was maintained long after the wild-type bacteria had shed the LAMP1 protein, suggesting that the FCVs were maintained for a longer duration during the mutant infections as compared to the wild-type bacteria. This is consistent with past epithelial and phagocyte studies. The increased time housed within the FCVs could conceivably impede replication during that time, thus contributing to the decreased bacterial levels seen with the mutants. Although LAMP1 localization was essentially gone by 24 hours in all samples, the delay in FCV breakdown could explain the significant differences seen throughout the 48 h time-points. If FCV escape is inhibited there is a possibility that gentamicin that was added to kill the extracellular *Francisella* could have accumulated within endosomes and fused with the FCVs to kill the FCV contained bacteria. However in other systems where the influence of gentamicin on epithelial cell infections had been assayed this was found to not be the case. In a study that looked directly at this possibility Martinez-Moya and co-workers found that in
epithelial cells infected with *Salmonella* Typhimurium for up to 72 hours and had gentamicin in the extracellular milieu, gentamicin did not accumulate in intracellularly.\(^{516}\). This was contrary to phagocytic dendritic cells, which did have gentamicin accumulation.\(^{516}\)

*Francisella* use different strategies to gain entry into non-phagocytic and phagocytic cells. During phagocytic cell (macrophage) infections, *Francisella* are engulfed by host cells through phagocytic mechanisms.\(^{343,362,465}\). By contrast to macrophage invasion, we previously found that *F. novicida* and *F. tularensis* LVS utilize clathrin-dependent mechanisms together with cholesterol- to enter hepatocytes.\(^{500}\) The additional strategy of macropinocytosis has been met with controversy as one group claims an involvement.\(^{517}\), whereas others exclude the possibility.\(^{411,500}\) Worth noting is that clear actin-based membrane ruffling at sites of bacterial entry that are required for macropinocytosis have never been documented.

During *Francisella* infections, nearly all of the genes within the FPI are necessary for full virulence *in vivo* as well as for replication within macrophages,\(^{309,310,323,333,378,410,420,427,430,505}\), but are not required for entry during phagocytic cell infections.\(^{325}\). We speculate that the FPI does not significantly influence the phagocytic process and thus does not alter other bacterial surface molecules recognized by host phagocytic receptors. Conversely, when *Francisella* invade epithelial cells, our findings suggest that both IglC and PdpA are required for efficient *Francisella* internalization, this further suggests that these components could somehow influence the clathrin-based internalization processes occurring in the host. Whether this alters bacterial ligands, host receptors, or intracellular host internalization mechanisms remains to be elucidated. IglC has been implicated as a component of the *Francisella* T6SS itself\(^{325}\) and evidence from other systems has indicated that the T6SS can influence bacterial invasion. Studies on *Campylobacter jejuni* and *Pseudomonas aeruginosa* have shown that these microbes utilize T6SSs to enhance bacterial invasion into epithelial cells.\(^{518,519}\). Because IglC and PdpA have been detected within the cytoplasm of macrophages, it is conceivable that they could also act as T6SS-dependent effectors.\(^{335}\). However, the binding partners and precise functions of those factors remain unknown. Because ΔiglC and ΔpdpA mutants retained some ability to invade, we surmise that *Francisella* employ additional mechanisms for entry into epithelial cells.
An inconsistency that we are faced when we examined bacterial invasion was
that while the \( \Delta \text{pdpA}::\text{pdpA} \) and \( \Delta \text{iglC}::\text{iglC} \) \( F. \text{novicida} \) both had about 3 times the CFUs
at 4 h over the mutant strains (Figure 5.1) and about double the number of cells invaded
when examined microscopically, the \( \Delta \text{iglC}::\text{iglC} \) complement did not increase invasion
levels significantly over \( \Delta \text{iglC} \) when tested by differential staining. What factors could
have influenced this? Potential reasons for this could lie in the abundance, proper
localization or proper orientation of IgIC in the microbe when ectopically expressed.
These features could have influenced individual invasion events, while not impeding
replication once the bacteria had invaded. Because the 4 h CFU-based invasion assays
assessed both the invasion and early stages of replication while the 24 h microscopic
assays only examined the number of invaded cells, without addressing the number of
individual bacterial cells within each host cell, this could have also compounded our
differing complementation results.

Even though others have used centrifugation to force the attachment of
\( \text{Francisella} \) to the cell surface thereby allowing bacterial invasion to be assessed at
earlier time-points and to "synchronize" the infections \[^{370,385,411,412}\], we would prefer to not
introduce additional variables into the experiments and thus not force bacterial contact
with the host cells if this can be avoided. Consequently, we allowed \( F. \text{novicida} \) to
naturally contact the cell surface and elicit its own internalization. We are confident that
the vast majority of the bacteria we measured at the 4 h time-point are those of invaded
bacteria, because significant bacterial replication is not detected until \( >4 \) h post-infection,
even when centrifugation is used \[^{366,385,412}\]. However, when we used this infection
approach on \( F. \text{tularensis LVS} \), we were unable to detect \( F. \text{tularensis LVS} \) invasion at 4
h. Thus, in order to obtain sufficient invasion, we found that we needed to centrifuge the
bacteria onto the cells. To ensure that only intracellular bacteria were counted, we
performed concurrent infections in order to detect that no \( \text{Francisella} \) appeared on plates
in the absence of epithelial cells (to mimic the extracellular bacteria present during the
invasion assays). This indicated to us that the gentamicin was effective at killing any
extracellular \( \text{Francisella} \) (data not shown).

Our work studying the influence of two of the FPI genes, IgIC and PdpA, during
\( \text{Francisella} \) epithelial cell infections has highlighted important differences in epithelial
versus macrophage internalization, while demonstrating that \( \text{Francisella} \) replication is
likely governed through similar mechanisms regardless of the cell-type infected. This study provides a framework for elucidating the detailed roles of individual FPI components in both the internalization and replicative phases of the bacterial lifecycle and will ultimately demonstrate how host epithelial cell processes can be subverted by foreign proteins.

5.5. Materials and Methods

5.5.1. Bacterial and growth conditions.

_F. novicida_ strain Utah 112, deletion mutants ΔiglC, ΔpdpA, and their gene complements (ΔiglC::iglC-FLAG KmR [ΔiglC::iglC], ΔpdpA::pdpA-FLAG KmR [ΔpdpA::pdpA]) as well as _F. tularensis_ ssp. _holarctica_ Live vaccine strain [LVS], ΔpdpA, and ΔpdpA::pdpA HygR were supplied by Dr. Francis Nano from the University of Victoria. The detailed description of the _F. tularensis_ LVS ΔpdpA mutant will be described elsewhere (Nix et al, in preparation). In brief, the two _pdpA_ alleles in _F. tularensis_ LVS were sequentially removed using the approach previously used to remove the one _pdpA_ allele from _F. novicida_ [427]. Genetic complementation was accomplished by introducing a copy of _pdpA_ on a derivative of plasmid pMP831 [520]. _F. tularensis_ LVS ΔiglC and its complement _F. tularensis_ LVS ΔiglC::iglC KmR were kindly provided by Dr. Anders Sjostedt. All bacteria were grown according to the procedures described previously [329,367,456,467].

5.5.2. Bacterial titre.

To estimate the amount of bacterial growth at 24 h post-infection (PI), 10-fold serial dilutions of the _Francisella_ cultures were made in Tryptic soy broth (TSB) supplemented with 0.1% L-cysteine (TSBC). These were performed by plating 10 μL of diluted cultures that ranged from 10⁻¹ to 10⁻¹⁰ onto Tryptic soy agar (TSA) supplemented with 0.1% L-cysteine (TSAC) plates (for _F. novicida_) or Chocolate blood agar [TSAC and 5% defibrinated horse blood] (CBA) plates (for _F. tularensis_ LVS). When appropriate, bacteria were grown in media containing 10 or 15 μg mL⁻¹ of kanamycin or 100 μg mL⁻¹
of hygromycin B. Plates were incubated overnight at 37°C (for *F. novicida*) or for up to 3 days (for *F. tularensis* LVS), at 37°C prior to counting colony forming units (CFUs).

### 5.5.3. Cell cultures and infections.

Murine hepatocytes, BNL CL.2 cells (ATCC; TIB-73) and human lung epithelial A549 cells (ATCC; CCL-185) were respectively cultured in high-glucose Dulbecco’s Modified Eagle’s Medium (DMEM) [Thermo] and Kaghn’s F-12 media [Thermo] both containing with 10% fetal bovine serum (FBS) and incubated at 37°C with 5% CO₂. These cells were seeded into 24-well plates or onto #1.5 glass coverslips, grown to confluence, then infected with *F. novicida* at a multiplicity of infection (MOI) of 100:1 for up to 48 h as done previously ⁵⁰⁰. For *F. tularensis* LVS studies, the cells were infected at an MOI of 200:1 and immediately centrifuged for 5 min (300 x g) at room temperature (RT), as previous described ⁵²¹. In this manuscript this is referred to as the 0 h time-point. Antibiotic selection was maintained over the course of the infection by supplementing the cell culture media with appropriate antibiotics. All infections proceeded in a humidified chamber at 37°C with 5% CO₂ and were only placed at RT during inoculation, washes, and at the experimental endpoint. At the appropriate time-points, samples were washed 6 times with PBS +/- (Dulbecco’s PBS containing 0.0133% calcium and 0.01% magnesium) [Sigma] both before and after extracellular bacterial-killing using fresh infection media supplemented with 30 (for LVS) or 100 (for *F. novicida*) µg mL⁻¹ of gentamicin ⁴¹¹,⁵⁰⁰. When indicated, a lower concentration of gentamicin (10 µg mL⁻¹) was subsequently applied to prevent the growth of extracellular bacteria. Before commencing gentamicin protection assays or immunofluorescence staining, all wells were washed 3 times with PBS +/-.

### 5.5.4. Gentamicin protection assays.

Gentamicin protection assays (4 h invasion assays) were performed following the procedures previously used by our laboratory ⁵⁰⁰. To summarize, the samples were washed 6 times with PBS +/- then incubated with gentamicin at 3 h post inoculation (PI) to kill any extracellular bacteria. At all of the experimental endpoints, uninfected and *F. novicida* infected cells were lysed with 1% Triton-X in PBS. For *F. tularensis* LVS
infections, cells were lysed with sterile ddH$_2$O because the lysis reagent prohibited the formation of visible colonies. The lysates were serially diluted with TSBC and then plated onto either TSAC (for *F. novicida*) or CBA (for *F. tularensis* LVS). Solid agar plates containing appropriate antibiotics were used when necessary and were subsequently incubated at 37°C.

To compare the intracellular growth profiles of wild-type and mutant *Francisella* between 4 h and 24 h PI, we allowed *Francisella* to invade epithelial cells for 3 h, washed the samples multiple times and treated them with gentamicin for 1 h. The samples were then either lysed immediately, to measure the initial amount of intracellular bacteria, or replaced with fresh media containing gentamicin (10 µg mL$^{-1}$), thus permitting the invaded bacteria to replicate for up to 24 h PI. Bacterial replication was measured during the late stages (24-48 h PI) of infection by allowing *Francisella* to infect epithelial cells for 22 h, washing the samples with PBS, then adding gentamicin to kill uninvaded bacteria for 2 h. At 24 h PI, samples were either lysed to quantify intracellular bacteria or allowed to replicate further within epithelial cells for an additional 24 h in the presence of low concentrations of gentamicin.

5.5.5. **Differential bacterial immunolabeling.**

The method used for differential (inside/outside) bacterial labeling was based on previously published work $^{508}$. To immunolocalize extracellular bacteria, 3% paraformaldehyde-fixed/non-permeabilized samples were washed with PBS, blocked with 5% normal goat serum (NGS) at RT for 20 min, and stained using a 1:1000 dilution of rabbit anti-*Francisella* antibody $^{493}$ in PBS containing 0.1% BSA (PBS/BSA) supplemented with 1% NGS for 1 h at 37°C. Subsequently, the samples were washed with PBS/BSA and treated with 1 µg mL$^{-1}$ of Alexa Fluor 594 goat anti-rabbit IgG (H+L) antibody (in PBS/BSA + 1% NGS) for 1 h at 37°C. After washing with PBS/BSA, the samples were permeabilized with 0.2% Triton X-100 in PBS for 15 min, washed with PBS/BSA, and blocked with 5% NGS for an additional 20 min at RT. The samples were then treated with the primary rabbit anti-*Francisella* antibody [diluted 1:1000 in PBS/BSA containing 0.05% Tween-20 (TPBS/0.1% BSA) and supplemented with 1% NGS] for 1 h, washed with TPBS/BSA, incubated with 1µg mL$^{-1}$ Alexa Fluor 488 conjugated goat anti-
rabbit IgG (H+L) antibodies prepared in TPBS/BSA + 1% NGS for 1 h at 37°C, washed with TPBS/BSA, and mounted using ProLong Gold antifade reagent with DAPI. This procedure labels intracellular bacteria green and extracellular bacteria (red and green in merged panels). Unless otherwise stated, all washes were repeated 3 times and lasted a total of 30 min.

To quantify the proportion of intracellular bacteria and lysosomal-associated membrane protein 1 (LAMP1), all samples were infected for 3 h then treated with gentamicin for 1 h. For 8, 12, and 24 h time-points, the infections were allowed to proceed in the presence of gentamicin (10µg mL⁻¹) until the experimental endpoints. Localization of Francisella required the use of mouse anti-Francisella 2H1 antibodies (1:100) [Immunoprecise Antibodies] in combination with Alexa Fluor 350 and 594 goat anti-mouse IgG (H+L) antibodies to discriminate between intracellular (red and blue overlap) and extracellular (blue) bacteria. Following permeabilization of cell membranes, rat anti-LAMP1 1D4B antibodies (1:50) [Developmental studies hybridoma bank] were used in combination with either Alexa Fluor 488 goat anti-rat (1:100) to localize LAMP1. These coverslips were mounted with the ProLong Gold antifade reagent.

5.5.6. Image and statistical analysis.

A Leica DM4000B inverted fluorescence microscope attached to an Angstrom Grid Confocal system [Quorum Technologies] was used to view the samples and Metamorph Software was used to capture the images. ImageJ version 1.44i (http://imagej.nih.gov/ij) was used to count the number of invaded bacteria from images captured by either epifluorescence or structured illumination microscopy. Adobe Photoshop CS5 was used to process immunofluorescence images without changing the integrity of the data. To calculate the population of cells in each image, we semi-automatically measured the number of DAPI-stained cell nuclei using the “nucleus counter” plug-in, which is part of the McMaster Biophotonics Facility ImageJ bundle (http://www.macbiophotonics.ca/downloads.htm). In the nucleus counter plug-in, the following parameters were adjusted to tally the number of nuclei in each image [taken at 40X magnification]: 2000, ‘Smallest Particle Size’; 6000, ‘Largest Particle Size’, Mean 3x3, ‘Smooth method’; enabled, ‘Watershed filter’. In case there were any miscounted nuclei or multi-nucleated cells, each image was further reviewed by overlaying the DAPI-
stained image with the phase-contrast image. Within the population of adherent hepatocytes, infected and uninfected cells were manually tallied. Each infected cell was given a score of ‘1’ based on the presence of *F. novicida* within the cell boundaries, whereas each uninfected cell that had an absence of intracellular *F. novicida* was given a score of ‘0’. The proportion of infected cells was calculated based on the number of infected cells divided by the total number of cells in each image. To determine whether *Francisella* was within a LAMP1 containing vacuole, we looked for bacteria that were at least 50% surrounded or completely co-localized with LAMP1.

Statistical analysis was performed using Graphpad Prism 6 software. Statistical significance was calculated using one-way ANOVA followed by Bonferroni multiple comparison test for data in Figures 5.1, 5.2 and 5.3. Data in Figures 5.4 and 5.7 were analyzed by Two-way ANOVA followed by Bonferroni multiple comparison test, whereas for Fisher’s LSD test was used for data in Figure 5.8.
5.6. Figures

![Graph showing relative internalized bacteria](image)

**Figure 5.1** Deletion of genes encoding IglC and PdpA perturb *F. novicida* invasion.

Murine BNL CL.2 hepatocytes were infected with wild-type *F. novicida*, deletion mutants (ΔiglC and ΔpdpA) as well as complement strains (ΔiglC::iglC and ΔpdpA::pdpA) for 3 h. Subsequently, samples were washed and treated with gentamicin for 1 h. At 4 h PI, lysates were plated onto agar-containing media and bacterial colonies were enumerated the following day. Error bars, S.E.M. (n = 4)
Figure 5.2  Internalization of *F. tularensis* LVS ΔiglC and ΔpdpA mutants in A549 human lung epithelial cells

Bacteria were centrifuged onto human A549 cells and allowed to invade for 3 h. To determine the amount of invaded bacteria, gentamicin protection assay (invasion assay) was performed at 4 h PI and bacterial titre was measured after a 3-day incubation. Error bars, S.E.M. (n = 3)
Figure 5.3  Phase and fluorescence micrographs were taken of uninfected and *F. novicida* infected hepatocytes at 24 h PI.

Mouse BNL CL.2 cells were infected with wild-type *F. novicida* and mutants ΔiglC, ΔiglC::iglC, ΔpdpA and ΔpdpA::pdpA for 22 h. Afterwards, samples were washed, treated with gentamicin for 2 h, and then fixed with 3% paraformaldehyde. Fixed samples were prepared using an immunolocalization technique that can differentiate extracellular (green and red co-localization, arrows) and intracellular bacteria (green only, arrowheads). Each fluorescence image represents a superimposed ‘maximum intensity’ Z-projection image around the cell nucleus (blue). Scale bar = 10µm.
Figure 5.4  *F. novicida* lacking either IglC or PdpA reduces liver epithelial cell colonization.

Samples were fixed at 24 h PI, differentially stained for intracellular and extracellular bacteria, and then visualized by fluorescence microscopy. The proportion of infected cells was tallied from over 1,000 cells. Cells containing one or more intracellular bacteria are considered 'infected'. Error bars, S.E.M. (n = 3)
Figure 5.5 Intracellular growth kinetics of *F. novicida* mutants during hepatocyte infections.
BNL CL.2 cells were infected with wild-type *F. novicida*, deletion mutants (ΔiglC and ΔpdpA) and their respective complements. Bacteria were allowed to invade for 3 h after which extracellular bacteria were rapidly washed with PBS and killed with 100 µg mL⁻¹ of gentamicin for 1 h. Low concentrations of gentamicin (10 µg mL⁻¹) remained in the media (to inhibit extracellular bacteria) until experimental endpoint. Intracellular bacteria were then released by lysing host cells, diluted with TSBC, and plated for bacterial enumeration. Error bars, S.E.M. (n = 3)
Intracellular bacterial replication is severely compromised when genes encoding \textit{iglC} and \textit{pdpA} are deleted. 24 h and 48 h gentamicin protection assays were performed on liver BNL CL.2 cells infected with wild-type \textit{F. novicida}, deletion mutants (\textit{ΔiglC} and \textit{ΔpdpA}), and their respective complement strains (\textit{ΔiglC::iglC} and \textit{ΔpdpA::pdpA}). Samples were then treated with gentamicin starting from 22 h post-inoculation until the experimental endpoint. After host cells were lysed, the released bacteria were diluted and plated for CFU enumeration. Error bars, S.D. (\textit{n} = 4)
Figure 5.7 IglC and PdpA are essential for robust *F. novicida* growth within hepatocytes.

Phase and fluorescence microscopic images were taken of BNL CL.2 cells infected with wild-type *F. novicida*, deletion mutants (ΔiglC and ΔpdpA), and complement strains (ΔiglC::iglC and ΔpdpA::pdpA) for 48 h. At 22 h post-inoculation, the samples were washed with PBS and replaced with media containing gentamicin to prohibit further bacterial invasion. *F. novicida* (green) and DNA (blue, DAPI) were stained in the fixed samples. Each image represents a ‘maximum intensity’ Z-projection comprising a stack through the cell body. Images taken by fluorescence and phase microscopy were merged together to illustrate the cell borders. Scale bar = 10µm.
During the late intracellular phase, IgIC and PdpA are necessary for efficient proliferation in lung epithelial cells. Human A549 cells were infected by wild-type *F. tularensis* LVS, ΔigIC, ΔpdpA, ΔigIC::igIC and ΔpdpA::pdpA. Intracellular bacteria were enumerated at 24 and 48 h time-points using gentamicin protection assay. At 24 h PI, the sample was switched to a low gentamicin concentration (10 µg mL⁻¹) in order to inhibit growth of extracellular microbes. Intracellular bacteria were titred after they were released from host cells and serial diluted onto agar-containing media. Error bars, S.E.M (n = 3)
Figure 5.9  Proportion of *F. novicida* associated with LAMP1 during murine hepatocyte infections.

(A) wild-type *F. novicida*, deletion mutants (Δ∗pdpA, Δ∗iglC) and complements strains (Δ∗pdpA::pdpA, Δ∗iglC::iglC) invaded BNL CL.2 cells for 3 h, after which extracellular bacteria were washed off and then killed with gentamicin (100 µg mL⁻¹). Subsequently, samples were exposed to low gentamicin concentration until the experimental endpoint was reached. Image stacks were assembled and used to determine the frequency of LAMP1-associated bacteria. For time-points 4, 8 and 12 h, between 30 and 50 intracellular bacteria were counted. For the 24 h time-point, more than 50 intracellular bacteria were counted. Error bars, S.E.M. (n = 3)
5.7. Tables

Table 5.1  Bacterial doubling time during the early intracellular stages in hepatocytes

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>4 to 8h</th>
<th>8 to 12 h</th>
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<tbody>
<tr>
<td>WT <em>F. novicida</em></td>
<td>1.16</td>
<td>1.61</td>
<td>4.44</td>
</tr>
<tr>
<td>Δ<em>pdpA</em></td>
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<td>-5.38</td>
<td>-14.26</td>
</tr>
<tr>
<td>Δ<em>iglC</em></td>
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<td>-185.02</td>
<td>-8.91</td>
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<tr>
<td>Δ<em>pdpA::pdpA</em></td>
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<td>1.76</td>
<td>5.05</td>
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<tr>
<td>Δ<em>iglC::iglC</em></td>
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<td>1.96</td>
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</tbody>
</table>

Average doubling time (h)
Chapter 6.

General Discussion

6.1. Enteropathogenic *E. coli* (EPEC)

The mechanisms utilized by EPEC to generate pedestals have been well characterized, but key questions still remain regarding the exact composition of these actin-rich structures and the role of each constituent in its pathogenesis. A section of my PhD dissertation has been concentrated on the development of a method to identify novel pedestal components in an unbiased manner. In another section I characterized the function of a novel pedestal protein called nexilin.

To identify novel pedestal proteins I devised a unique approach to concentrate EPEC pedestals then analyzed these samples using high-throughput MS-based proteomics. Using this screening strategy, I identified 17 novel pedestal proteins and used two proteins (cyclophilin A and transgelin) to verify the MS results. Because the target is not pre-determined, as is the case with antibody-based localization techniques, my approach allowed me to detect pedestal proteins that would not be predicted to be at these actin-rich structures. Because we do not know how many components are actually in EPEC pedestals, the strategy used in this study could help us more expeditiously identify all proteins in EPEC pedestals. One of the proteins from my list of candidate pedestal proteins that I believe warrants further investigation is cyclophilin A. Cyclophilin A is often referred to as peptidyl-prolyl isomerase (PPIase) because it can catalyze proline isomerization between the *cis* and *trans* conformation, which is intrinsically a slow reaction. Various studies have implicated this PPIase in facilitating protein transport, influencing cytoskeletal rearrangement, and acting as a chemoattractant. At this time we’ve only begun to functionally examine the role of this PPIase in EPEC pathogenesis, but I speculate that its presence in pedestals could be associated
with the polymerization of actin filaments beneath the site of bacterial-host cell attachment. The relationship between cyclophilin A and the actin cytoskeletal network could be interconnected through the nucleation promoting factor N-WASp. Findings from a published report have implicated that cyclophilin A can bind to N-WASp and inhibit its degradation in mammalian cells. Because N-WASp is needed for EPEC to induce actin polymerization, cyclophilin A may promote pedestal assembly by retaining N-WASp molecules at the pedestal apex. Our results show that cyclophilin A is distributed at the apical tip as well as along the entire pedestal structure, where N-WASp is absent. Because cyclophilin A and other members of the immunophilin family can catalyze protein folding directed at specific proline-containing sequences, it may be able to also interact with other substrates, which may explain its localization beneath the apical region of the structures. During EPEC infections, it’s conceivable that cyclophilin A could also facilitate the folding of specific effectors to their native conformation. These T3SS effectors are generally thought to arrive in the host cytoplasm in a linearized state. Research from Brian Staskawicz’s lab has implicated cyclophilin A in the folding and activation of the T3SS effector AvrRpt2 from Pseudomonas syringae. By redistributing cyclophilin A to EPEC pedestals, I believe that it would be ideally located to catalyze folding of effectors to their functional conformation as they translocate to their intended subcellular target.

Several other non-actin associated proteins scored highly as EPEC pedestal proteins: i) Endoplasm (aka. Gp96) is a molecular chaperone that normally resides in the endoplasmic reticulum (ER), yet during certain conditions, such as cell activation, infections, and necrotic cell death this heat shock protein is also trafficked to the cytosol and host cell surface. Given that endoplasm is predicted in EPEC pedestals, this would imply that EPEC infection induces the release of endoplasm from the ER. It would be interesting to determine the exact cellular localization of endoplasm in EPEC-infected cells. If in fact endoplasm is present on the surface of pedestals, it could interact with an EPEC surface ligand. Cell surface endoplasm is commonly exploited by diverse pathogens for their internalization, cell adherence, and endocytosis of endotoxins. Two surface ligands OmpA from neonatal meningitis E. coli strain K1 (NMEC) and Vip from L. monocytogenes can interact with endoplasm to induce invasion in an actin-dependent manner, yet their cellular signalling mechanisms appear
to be dissimilar\textsuperscript{534,540}. These examples demonstrate that endoplasmin is capable of being exploited to induce actin polymerization. Interestingly, studies have also demonstrated that endoplasmin is important in inhibiting internalization of \textit{Staphylococcus aureus} and \textit{Neisseria gonorrhoeae}\textsuperscript{538,541}. Because EPEC co-opts the host endocytic machinery to induce actin polymerization while remaining extracellular, cell surface localization of endoplasmin could have a significant impact on its pathogenesis. ii) Sodium/potassium-transporting ATPase subunit alpha-1 (\textit{Na}\textsuperscript{+}/\textit{K}\textsuperscript{+} ATPase) is important in maintaining the electrochemical gradient of \textit{Na}\textsuperscript{+} and \textit{K}\textsuperscript{+} ions across the plasma membrane\textsuperscript{542}. It’s unclear how EPEC enriches \textit{Na}\textsuperscript{+}/\textit{K}\textsuperscript{+} ATPase to the pedestal periphery, but a previous study suggests that this ion pump is stimulated by direct interaction filamentous actin\textsuperscript{543}. Since EPEC pedestals are densely packed with actin filaments, I suspect that EPEC could be creating a microenvironment where there is lower concentration of \textit{Na}\textsuperscript{+} ions and ATP and proportionally higher concentration of \textit{K}\textsuperscript{+} ions and ADP within the pedestal compared to the rest of the cell. As the number of EPEC pedestals rise it is likely that this microenvironment will intensify and in which case could ultimately exhaust the reserve of ATP within the host cell. Furthermore, I predict that ATP synthase subunit beta, which was also scored highly in our screen, could be a host mechanism to counteract for escalation of ATP hydrolysis in EPEC pedestals. iii) Stathmin can sequester tubulin dimers thereby reducing the amount of free tubulin available for polymerization, in addition to its capacity to destabilize microtubules preferentially at the positive end\textsuperscript{544,545}. Its presence in EPEC pedestals could explicate the absence of microtubules in EPEC pedestals. Extracellular EPEC also encodes bacterial effectors EspG/G2 that can bind to tubulin and cause microtubules to disassemble throughout entire host cell\textsuperscript{188,189}. The utilization of stathmin and EspG/G2 suggests that EPEC employs two distinct mechanisms to destabilize the host microtubule network. I believe that future investigations directed at characterizing the function of these novel pedestal components will greatly enhance our understanding of their normal cellular function as well as during EPEC-induced diarrhea.

Given that an EPEC pedestal contains a dense network of actin filaments, many pedestal components discovered to date have been actin-associated proteins. However, only a subset of actin-associated proteins (\textit{ie.} N-WASp, Arp2/3, cortactin) found within EPEC pedestals are critical for their biogenesis. An actin-associated protein that we
have confirmed in our screen is transgelin. Transgelin is a calponin-like molecule that cross-links actin normally and localizes to both actin stress fibres and leading edge of migrating cells \(^{546}\). Transgelin depletion studies suggest that it may suppress cell migration \(^{547,548}\), though whether it does this by stabilizing actin filaments as previously purported is unclear. The localization of transgelin in actin-rich pedestals would seem to support the idea that it can interact with branched actin filaments along the entire stalk. My research on nexilin is a notable example of how we can exploit these pathogenic structures to help define the cellular function of previously characterized proteins. From this published study, we show that nexilin is concentrated at the apical tip in stationary pedestals but it can redistribute to the base when it becomes motile. Similarly, nexilin is concentrated at the rear of \(L.\) monocytogenes motile comet tails where it is necessary for elongation of its actin-rich structure. Because EPEC pedestals and \(Listeria\) comet tails both exploit nexilin and lengthen when they transition from stationary to motile states, I believe that nexilin forms a platform that is integral for EPEC pedestal motility. As I reach the end of my PhD, I consider the research that I have accomplished to appreciably heighten our understanding of EPEC pedestals with regards to their composition and pathogenic function.

### 6.2. \(F.\) tularensis

Irrespective of the route of infection, the liver is a primary site of histopathology associated with tularemia. Within the liver, \(F.\) tularensis can enter and rapidly divide within the cytoplasm of phagocytic Kupffer cells and specialized epithelial cells called hepatocytes, which can make up >80% of cells in this organ. As phagocytic cells naturally engulf microbes that are extracellular, they are substantially easier to infect \textit{in vitro} compared to non-phagocytic epithelial cells. Much of our knowledge regarding host-pathogen interactions during \textit{Francisella} infections is based off of phagocytic cell infections. This despite the fact that \(F.\) tularensis can parasitize hepatocytes and several other host cell types that are not normally phagocytic. For these key reasons and recent evidence that has emerged suggesting that non-phagocytic cells are important for its disease outcomes, I developed a robust \textit{in vitro} infection model and utilized it as a tool to investigate the pathogenesis of \(F.\) tularensis in hepatocytes with an emphasis on elucidating the internalization pathway. From my work, we ascertained that the clathrin-
endocytic machinery and cholesterol are central host factors that mediate bacterial invasion and that *Francisella* IglC and PdpA are pathogenic factors essential for enhancing host cell entry and rapid intracellular growth in hepatocytes.

Three other liver epithelial models have been utilized by others to examine *F. tularensis* LVS, but not *F. novicida*. A major strength of my infection model is that the infections we perform with *F. tularensis* LVS are just as robust as other *in vitro* models (HH4 and FL83B cells) but utilizes an inoculum size that is 5-fold lower (MOI of 200 vs. 1000). Another *in vitro* model that has been developed uses the HepG2 cell line. These cells have a tendency to form aggregates, which can be fairly problematic when examining sub-cellular alterations microscopically. By contrast, BNL CL.2 cells do not have this issue and can form a fully confluent monolayer. Additionally, my model provides other distinct advantages for researchers who intend to use *F. novicida* as a surrogate model for Type A *F. tularensis*, but also have the option to validate their findings in *F. tularensis* LVS. A major reason for selecting *F. novicida* over *F. tularensis* LVS is because the former microbe is substantially more amenable to genetic manipulations and is non-festitdious despite both having nearly identical genomes. Significant advances have made it possible for the utilization of Tn5-based transposon libraries and targeted gene inactivation/complementation in *F. tularensis*. Tn5-based transposon libraries have been a very useful approach to screen for protein function. The most comprehensive *Francisella* transposon library that has been published contains ~16,000 individual *F. novicida* mutants that covers 84% (or ~1,400) of predicted genes. In comparison, the largest transposon library reported in *F. tularensis* LVS contains 1,500-2,000 individual mutants. Given that *F. tularensis* contains a duplicate FPI, transposon-mediated gene disruptions within this region will at most only affect one of the two alleles. Taken together, I believe that my BNL CL.2 model will provide an efficient and straightforward approach to investigate various research hypotheses related to epithelial cell infections with either *Francisella* species.

Using my model, we revealed for the first time that specific factors expressed by the host epithelial cell and *Francisella* can modulate bacterial internalization. My most recent data has implicated a novel role for IglB, IglC, PdpA, and VgrG in enhancing *Francisella* invasion into epithelial cells, in addition to their known involvement in intracellular survival and replication (Figure A1, Figure A2). Research detailing their
specific pathogenic function within the bacterium and host cell is still in its infancy. Though recently, evidence supporting the existence of a *Francisella* T6SS, including putative structural components IglB, IglC, and VgrG, in effector delivery has been mounting. The importance of these four FPI proteins for invasion and completion of its intracellular lifecycle suggests that they may collectively work in unison. Because there are 16-19 protein-encoding genes within the FPI, I believe other molecules expressed from this gene cluster could play a significant role in governing critical stages of its pathogenesis. Among the four FPI proteins that I've studied, only IglB is not secreted into host cells. IglB along with its binding partner IglA are thought to assemble and form the outer tube of the *Francisella* T6SS as they have significant homology to *V. cholerae* VipB and VipA, respectively. It is generally accepted that the T6SS outer tube contracts, and thus, forces the ejection of the needle and its cargo through the secretion machinery and into the neighbouring cell. Given that IglB is a key constituent of the T6SS, it is possible that this FPI protein may indirectly modulate epithelial cell invasion. As for IglC, PdpA, and VgrG, recent data have implicated that these virulence factors are secreted into the host cytoplasm through a mechanism that is *Francisella* T6SS-dependent, which raises the prospect that they may convey effector functions. It is also conceivable that FPI-encoded proteins could be secreted into the extracellular milieu, which would make them directly accessible to host surface molecules. Since host binding partners to IglC, PdpA, and VgrG remain elusive, I believe that a suitable starting point for future investigations would be to determine if any of the three virulence proteins can subvert subcellular components within the host cell that we know are important in facilitating the entry of *Francisella* into epithelial cells (actin, AP-2, clathrin, Eps15, and cholesterol). Within the host cell, IglC, PdpA, and VgrG could directly interact with these host proteins or perhaps even activate existing signal transduction pathways to recruit these components toward the site of invasion. Because gene deletion studies of IglB, IglC, PdpA, and VgrG only dampened *Francisella* internalization, I believe that *Francisella* may utilize a sophisticated strategy to invade epithelial cells that involves a bacterial surface ligand. Intracellular pathogens commonly induce clathrin-dependent internalization through bacterial ligand-host receptor recognition. Host receptors known to be utilized for bacterial invasion include E-cadherin and Met (which are recognized by *L. monocytogenes* InlA and InlB, respectively), β1 integrin (which can interact with *Yersinia pseudotuberculosis* invasin
and α3β1 integrin (which is the cognate receptor of UPEC FimH adhesin of type I pili). Normally, these cell surface molecules cluster into lipid rafts where they elicit signals in the host cytoplasm and recruit the clathrin endocytic machinery to facilitate their internalization. As we revealed in our initial Francisella study, bacterial invasion is severely hampered upon disruption of clathrin and clathrin adapter proteins (Eps15, and AP-2) suggesting that entry into hepatocytes occur primarily through a clathrin-dependent mechanism. In addition, we discovered that actin is critical for Francisella invasion. This, to us, is not surprising because clathrin-mediated endocytosis is thought to generally be an actin dependent mechanism. The notion that Francisella employs its type VI secretion and/or bacterial surface ligand to induce its own internalization is a fascinating thought. Given that we’ve made considerable progress since the development of my in vitro system, I believe this Francisella infection model will be pivotal in further dissecting the epithelial cell entry process.
Chapter 7.

Future Directions

7.1. Determine the molecular functions of nexilin

One question that I had insufficient time to fully address was whether nexilin affects the motility of *Listeria* comet tails, and not just its organization. To accomplish this, gain/loss of function experiments will need to be performed to examine the impact of nexilin on the comet tail length and comet tail velocity using live cell microscopy. Ideally, stable cells lines that ectopically express nexilin and have nexilin knocked out would be generated. The use of stable cell lines would enable the homologous expression of nexilin in all cells in the sample. Because *Listeria* and EPEC exploit Arp2/3-induced actin assembly, I anticipate that the presence of nexilin at comet tails and pedestals will enhance their motility. Therefore, I would recommend that the same parameters be used to quantify intracellular *Listeria* motility for the assessment of static and motile pedestals.

The presence of two highly conserved actin binding domains (ABDs) suggests that nexilin may function as an actin cross-linker. To confirm this assertion, I propose that microfilaments be constructed *in vitro* using purified fluorescent actin monomers. Once these microfilaments are assembled, their stability will be visually monitored in the presence of nexilin as well as in its absence. Another test that I believe should be examined is the impact of this protein on actin stability. In this study, the resistance of *Listeria* comet tails against Latrunculin A, which induces actin depolymerization, would be compared in nexilin+/+ cells vs. nexilin−/− cells. One advantage of using the *in vitro* assay is that nexilin truncation mutants containing no ABD, just one, or both, can be more readily tested.

Previous studies have shown that the ABDs of nexilin are necessary for the molecule to localize to actin filaments. Nexilin also contains a coiled-coil domain,
which can bind to IRS1 (insulin receptor substrate 1)—a molecule involved in promoting actin-rich membrane ruffles and a C-terminal immunoglobulin superfamily class domain. For future experiments, it will be worth investigating whether these well-conserved regions influence Listeria-induced actin motility. An initial approach will involve testing a set of fluorescently tagged truncation mutants by ectopically expressing each of them in nexilin+ cells. These cells will then be infected and examined by fluorescence microscopy. Consequently, this will allow us to determine the regions of the protein that significantly affect the generation of long actin-rich comet tails. Once that is accomplished, the second step would be to identify its binding partners. This will involve taking lysates of infected cells and pulling down nexilin (full-length and truncation mutant) and components of the interaction complex. These proteins can be separated by SDS-PAGE gel and the individual identity of each protein can be verified by mass spectrometry along with any post-translational modifications. Further studies will be conducted to determine how the protein interactions induce lengthening of comet tails.

Nexilin is highly expressed in the heart and skeletal muscles, but is produced in low levels in lung, liver, and kidney tissues. Whether nexilin is expressed at all in the intestinal epithelium in mammals has not been reported. So far, our only hint that nexilin is expressed by intestinal epithelial cells comes from the human immortalized intestinal cell line, Caco-2. To determine expression of nexilin and its other isoforms, tissues from mice will be collected and then analyzed for nexilin expression by Western blot/Real-Time PCR analysis and immunohistochemistry. By performing this, I believe we will be able to better gauge the relevance of nexilin on disease.

### 7.2. Characterization of proteins identified in BDM-treated EPEC pedestals

One of my major research accomplishments was the development of the first successful strategy to enrich EPEC pedestals and identify multiple novel protein constituents within these structures. The outcome of this work in the EPEC field has much potential as we have found 17 novel EPEC pedestal constituents with high statistical confidence. Also within this list, there are several proteins such as cyclophilin A that are not classically considered as actin-associated and therefore would unlikely be
predicted to be present within these actin-rich structures. It would be particularly interesting to examine the remaining candidates and determine if they significantly influence EPEC pathogenesis. Subsequent work will be directed at detailing how EPEC hijacks their normal cellular function.

7.3. Investigating the effector functions of IglC and PdpA during epithelial cell infections

Various reports have investigated the pathogenic role of IglC and PdpA in phagocytic cell infections and have found that both bacterial proteins do not significantly contribute to host cell entry. In contrast, our research directed at epithelial cell infections has yielded results that suggest IglC and PdpA play novel roles in stimulating bacterial invasion. The aim now will be to determine how these bacterial-encoded proteins usurp control over host cells. Reports have been mounting that support the notion that IglC and PdpA are translocated into the host cytoplasm—a process that seems to require the expression of Francisella T6SS genes. However, a major drawback regarding these experiments is that the detection of these proteins occurs in the late stages of its intracellular lifecycle. Moreover, these experiments were performed using cells that can naturally phagocytose Francisella. Because of this limitation and given that phagocytes do not reflect the ability of Francisella to internalize into non-phagocytic cells, I propose that a different approach be applied to detect the secretion of effectors into epithelial cells. This method will utilize a BlaM reporter-based FRET (fluorescence resonance energy technique) assay based on a seminal paper by the Rosenshine lab to measure the secretion kinetics of extracellular EPEC and invasive Salmonella Typhimurium in real-time. For this, bacterial expression plasmids for these BlaM-effector fusions will need to be constructed. The wild-type gene will then be replaced with a recombinant gene via a double cross-over event, a procedure invented by Igor Golovliov and his team. After confirming its proper expression by Western blot using an α-BlaM antibody, the FRET signal emitted by the two molecules will then be quantified using a spectrophotometer over the course of the infection. The FRET assay will involve the addition of a CCF2-AM, a membrane permeable molecule, that will be loaded into epithelial cells prior to infection and where it will be used detect for secretion of BlaM-effector fusion proteins. Consequently, this
approach should provide better sensitivity and temporal resolution enabling us to detect the secretion of *Francisella* T6SS effectors at time-points coinciding with bacterial invasion and phagosomal escape.

To understand how IglC and PdpA subvert the host cell for its internalization into epithelial cells and intracellular pathogenesis, I recommend that an in-depth study be conducted to determine their molecular targets. A typical approach would be to perform *in vitro* protein-protein interaction assays where the sample would be separated by SDS-PAGE gel and analyzed for potential binding partners using mass spectrometry. Once host proteins have been identified, subsequent experiments should look extensively at the biochemical functions of IglC and PdpA to determine how they exactly exploit the host cell and if they do this by commandeering existing host signalling pathways.
Chapter 8.

Conclusion

The research presented in my thesis underscores the discrete virulence factors employed by the intracellular pathogen *F. tularensis* and the extracellular bacterium EPEC to subjugate control over host epithelial cells. I have found that certain host cellular machinery are recurrently targeted and repurposed to further the pathogenesis of various microbes. The significant advances that I have made will help investigators better construct tangible inferences between these pathogenic mechanisms and their involvement in human disease.
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Appendix A:

Supplementary Figures

Figure A1. Invasion assays taken at 4h post-infection showed that deletion mutants lacking the *Francisella* T6SS-encoding genes (*iglB*, *iglC*, *vgrG*) and *pdpA* gene had significantly lower invasion potential compared to wild-type (WT) *F. novicida*. (n=3)
Figure A2. Internalized *F. novicida* expressing T6SS components and PdpA proliferated within hepatocytes. Intracellular growth was measured by gentamycin protection assay at 24 and 48 h PI (n=3). In both experiments, extracellular bacteria were killed with the antibiotic gentamicin (100 µg/mL) and removed by washing with PBS at 22 h PI. For the prolonged infection, a lower concentration of gentamicin (10 µg/mL) was used to inhibit extracellular bacteria.
Appendix B:

Supplementary Movies

**Movie B1.** 3D reconstruction of long EPEC pedestals formed on the host cell surface
Long actin-rich pedestals are generated following BDM-treatment of EPEC infected Ptk2 cells. Paraformaldehyde-fixed cells were stained with fluorescent phalloidin (F-actin, red) and DAPI (nuclei, blue).

**Movie B2.** Fluorescence microscopy of pedestals emerging from the transwell membrane.
Actin-rich pedestals protrude out from 3.0 µm diameter pores embedded in a polycarbonate membrane. Beneath the translucent membrane are adherent Ptk2 cells as recognized by their large nuclei and the actin cytoskeleton. Alexa-594 conjugated phalloidin was used to stain for F-actin, which is also concentrated in EPEC pedestals (red). DAPI is used to stain host cell and bacterial nuclei (blue).

**Movie B3.** Distribution of nexilin during *Listeria* comet tail formation and motility
EGFP-nexilin transfected Ptk2 cells were infected with *L. monocytogenes* for 4 h and imaged using an epifluorescence microscope. Phase image is on the left, EGFP-nexilin is on the right side.

**Movie B4.** *Listeria* comet tails and listeriopods formed in tomato-nexilin and YFP-actin expressing cells
Tomato-nexilin and YFP-actin transfected Ptk2 cells were incubated with *L. monocytogenes* for 4 h and imaged using an epifluorescence microscope. YFP-actin is on the left, Tomato-nexilin is on the right side.

**Movie B5.** Nexilin distribution in motile EPEC pedestal
Ptk2 cells transfected with Tomato-tagged nexilin and YFP-actin were infected with *L. monocytogenes* for 5 h. Cells were then imaged using an epifluorescence microscope and treated with Cytochalasin D after 5 min of acquisition.
Appendix C:

Supplementary Table

Table C1. Compilation of raw data from mass spectrometry-based proteomics for screening novel pedestal proteins

Samples from four independent experiments were prepared and subsequently analyzed by mass spectrometry-based proteomics. Relative abundance is measured based on quantification of peptides from the pedestal preparation, labelled with 'medium' isotopes, and compared against the negative control sample, labelled with 'light' isotopes. The mass spectrometry data here does not include the experiment that identified nexilin, because raw data from that pilot study was not recoverable.