Identification and characterization of bacterial and host epithelial factors altered during *Edwardsiella* infections

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Abstract

Bacteria in the genus *Edwardsiella* cause hemorrhagic septicemia in fish hosts, and severe diarrhea in immunocompromised humans. These phenotypes generally require effector proteins secreted through type III and type VI secretion systems. In this thesis, I investigate 2 common bacterial targets, cytoskeleton and intercellular junctions, in epithelial cells to understand the sub-cellular alterations caused by *Edwardsiella* during these infections. Using two robust epithelial infection models (HeLa and Caco-2 cells) I show that host microtubules are destroyed during the progression of *Edwardsiella* infections, while the actin and intermediate filaments remain unaltered. My evidence points to the host microtubule severing enzymes as key players in the microtubule disassembly event as katanin A1 and the katanin A1 subunit-like 1 proteins both localize to the microtubule cut-sites. The novelty of this phenotype extends to the bacteria, as this phenotype is independent of both type III and type VI secretion systems. Negative screening using an *E. piscicida* transposon insertion mutant library identified 15 bacterial genes needed for host microtubule severing event. The thesis concludes with preliminary examination of host intercellular junction alterations where I demonstrate that the tight junction protein claudin-3 is dissociated from cell peripheries in infected cells. In conclusion, I uncovered two steps of *Edwardsiella*'s infectious process where host structural components are targeted—leading to phenotypes observed during edwardsiellosis.

Keywords: ***Edwardsiella*; edwardsiellosis; cytoskeleton; microtubules; disassembly; epithelial; katanin; claudin-3**

Dedication

I would like to dedicate this thesis to my mother for having endless faith in me, and for always standing by me during difficult times. You inspire me every day through your patience, love, and a fighting spirit.

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List of Acronyms

T3SS	Type III secretion system
T6SS	Type VI secretion system
AJ	Adherens junctions
Arp2/3	Actin-related protein 2/3
BSA	Bovine serum albumin
Caco-2	Colorectal adenocarcinoma epithelial cells
CAR	Coxsackie adenovirus receptor protein
Cdc42	Cell division central protein 42 homolog
C. trachomatis	*Chlamydia trachomatis*
CRP	cAMP-activated global transcriptional regulator
Dsc	Desmocollin
Dsg	Desmoglein
DP	Desmoplakin
DS	Desmosome
E. anguillarum	*Edwardsiella anguillarum*
E. hoshinae	*Edwardsiella hoshinae*
E. ictaluri	*Edwardsiella ictaluri*
E. piscicida	*Edwardsiella piscicida*
E. tarda	*Edwardsiella tarda*
EB3	End-binding protein 3
EdwG	*Edwardsiella* genotype
EPC	Epithelioma papillosum of carp
EPEC	Enteropathogenic *Escherichia coli*
Esc	*Edwardsiella* secretion system chaperone
Ese	*Edwardsiella* secretion system effector
Esp	Enteropathogenic *E.coli* secreted protein
Esr	*Edwardsiella* secretion system regulator
ETA	Exfoliative toxin A
Evp	*E. tarda* virulence protein
F-actin	Filamentous actin
FG-9307	Flounder gill cell line
FIGN	Fidgetin

FIGN-like-1	Fidgetin-like-1
FIGN-like-2	Fidgetin-like-2
FKC	Formalin killed cells
FlgD	Flagellar basal-body rod modification protein
FUCCI	Fluorescence ubiquitination cell cycle indicator
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GUK	Guanylyl kinase
Hcp	Haemolysin co-regulated protein
HCV	Hepatitis C virus
HeLa	Human cervical epithelial cells
HSP	Hereditary spastic paraplegia
InlA	Internalin A
J774A.1	Mouse macrophage cell line
JAM	Junctional adhesion molecule
Jnk pathway	Jun N-terminal kinase pathway
KATNA1	Katanin A1 subunit
KATNAL1	Katanin A1 subunit-like 1
KATNAL2	Katanin A1 subunit-like 2
KATNB1	Katanin B1 subunit
KATNBL1	Katanin B1 subunit-like 1
LD_{50}	Median lethal dose
MAPs	Microtubule associated proteins
MDCK cells	Madin-Darby canine kidney cells
MOI	Multiplicity of infection
MTs	Microtubules
Mv	Microvilli
NGS	Normal goat serum
NP	Nasopharyngeal
P. aeruginosa	*Pseudomonas aeruginosa*
PAAR	Proline-alanine- alanine-arginine repeat proteins
PAK	p21-activated kinase
PG	Plakoglobin
PsaA	Pneumococcal surface adhesin A
Rac	Rho-activated protein

RAW264.7	Mouse macrophage cell line
Rho	Ras homology proteins
ROCK	Rho-associated protein kinase
RT	Room temperature
S. pneumoniae	*Streptococcus pneumoniae*
S. Typhimurium	*Salmonella* Typhimurium
S2 cell line	Schneider-2 cell line
Sct	Secretion and cellular translocation protein
SPI	*Salmonella* pathogenicity island
sp.	species
spp.	subspecies
Sse	*Salmonella* secretion system effector
TJ	Tight junctions
TSB	Tryptic soy broth
TssB & TssC	Contractile sheath proteins
U-2 OS	Human bone osteosarcoma epithelial cells
ULF	Unit length filament
V. cholerae	*Vibrio cholerae*
V. parahaemolyticus	*Vibrio parahaemolyticus*
VgrG	Valine–glycine repeat protein G
WASP	Wiskott Aldrich Syndrome proteins
ZO	Zonula occludens

Chapter 1.

Introduction

Opportunistic bacteria from genus *Edwardsiella* belong to the family of *Enterobacteriaceae*, and have emerged as fish pathogens that cause massive economic losses to aquaculture industries worldwide through a systemic disease called edwardsiellosis (1). Members of this genus are associated with freshwater animals such as fish, amphibia, and reptiles (1,2). Currently, the *Edwardsiella* genus consists of five species- *Edwardsiella tarda*, *E. hoshinae*, *E. ictaluri*. *E. piscicida*, and *E. anguillarum*, which are characterized based on various phenotypic and genetic differences (3). These microbes occupy a wide ecological niche and host range. Consequently, bacteria from the genus *Edwardsiella* pose a serious threat to the environment as well as public health (4).

1.1. Classification of *Edwardsiella* species

Of the five known *Edwardsiella* species, *E. ictaluri* has been exclusively isolated from channel catfish, which caused enteric septicemia in those animals (5). *E. hoshinae* on the other hand, has been isolated from birds, lizards, and water but does not display pathogenic properties (6). Unlike *E. ictaluri* and *E. hoshinae*, *E. tarda* is a versatile pathogen that can infect various hosts including fish, reptiles, and terrestrial mammals including humans (1,4,7). Since 2012, a few changes have been made in the taxonomy of genus *Edwardsiella* (8). Yang and colleagues reported that strains collectively referred to as *E. tarda* were grouped into two highly divergent genomic types, EdwGI (*Edwardsiella* genotype I) and EdwGII (*Edwardsiella* genotype II) (8). The former group represented fish-pathogenic isolates containing 1 type III secretion system (T3SS) and 1 type VI secretion system (T6SS), which were recently classified under a novel species called *E. piscicida* (9) with ET-883 being the type strain for this species. After further investigation, genome-level comparisons of EdwGI strains showed that a strain from diseased Japanese eel from Fujian, China contained multiple sets of type III and type VI secretion systems (3). This group was later proposed as a new species- *Edwardsiella anguillarum* with ET-080813 being the type strain (3). Microbes from *E. anguillarum*

species contain at least 2 sets of T3SS and 3 sets of T6SS gene clusters (3). The human-pathogenic isolates lacking T3SS or T6SS were clustered under the EdwGII genotype and retained the old species name, *E. tarda* (3). In terms of characterization of *E. tarda* species, ATCC15947 is used as the type strain, which was isolated from a human in Kentucky, USA (3). The focus of this study is on *E. piscicida*. There are two strains that have been studied in-depth; *E. piscicida* EIB202—isolated from a diseased turbot (*Scophthalmus maximus*) and *E. piscicida* PPD130/91—isolated from *Serpae tetra* (10,11).

1.2. Epidemiology and treatment

Because *E. piscicida* had been classified as *E. tarda* until recently, most of the older epidemiological studies refer to fish isolates collectively as *E. tarda*. *E. tarda* is referred to as an intracellular, peritrichously flagellated, motile, gram-negative facultative anaerobe that causes edwardsiellosis (4). Edwardsiellosis disease characteristics in fish hosts (12) include hernia, exophthalmia, liver granuloma, necrotic abscesses in the musculature, swollen internal organs, and hemorrhagic septicemia (Figure 1.1). Edwardsiellosis has been widely reported in various economically important fish such as Japanese Eel, Red Sea Bream, Yellowtail, Channel Catfish, Turbot, and more recently, Whitefish (1,13,14). *E. tarda* is an opportunistic and zoonotic pathogen in humans (4,15). In immunocompromised people, *E. tarda* causes a wide spectrum of gastrointestinal and extra-intestinal diseases such as myonecrosis, bacteremia, septic arthritis, and wound infections (7,16,17). However, the hallmark of extraintestinal diseases in humans caused by *E. tarda* is bacterial sepsis with a mortality rate near 44% (18).

1.2.1. Vaccine treatment against *E. tarda*

Vaccination and immunization are the conventional treatments used to reduce losses due to edwardsiellosis in the aquaculture industry. To date, an inactivated vaccine (formalin-killed *E. tarda*) has been commercialized in Korea due to its low cost (19). Wide array of vaccines such as ghost *E. tarda* vaccine (20), live attenuated *E. tarda* (21), heat- or formalin-killed *E. tarda* (19), and DNA vaccines (22), have been reported against edwardsiellosis. Treatment of diseased fish with formalin-killed cells (FKC) has been controversial as some studies have shown that FKC are ineffective in protecting

against *E. tarda* infections (23). To increase the efficacy of FKC to treat edwardsiellosis, a recent study has suggested the use of adjuvants or immunostimulants in combination with FKC (23). Indeed, higher survival rate was observed in diseased turbot treated with combination of formalin-killed *E.tarda* vaccine and low dose of the *E. tarda* flagellar protein FlgD as an adjuvant (23).

1.2.2. Antibiotic treatment against *E. tarda*

In cases of human extra-intestinal infections such as septicemia, patients have been treated with combinations of antibiotics including β- lactam antibiotics (cephalosporins and carbapenems) and aminoglycosides (17,24,25). Recently, a liver metastatic gastric cancer patient with *E. tarda* bacteremia was treated successfully with cefmetazole, a second-generation cephalosporin (17). However, overuse of antibiotics poses a risk of the emergence of antibiotic resistant *E. tarda*. Numerous studies have highlighted the presence of plasmids containing resistance genes against sulphonamide, kanamycin, streptomycin, tetracycline, and chloramphenicol in *E. tarda* (26). Although there have been recent advances in the field of vaccine and antibiotic development against *E. tarda*, a profound understanding of molecular aspects of *E. tarda* pathogenesis is important. To understand the virulence mechanisms that *Edwardsiella* employs to cause disease, researchers commonly use *E. piscicida*, as it employs both type III and type VI secretion systems to cause disease in fish hosts (27,28).

1.3. *E. piscicida* virulence studies / pathogenesis

Although the pathogenic mechanism of *E. piscicida* are not fully known, some virulence properties have been reported; production of siderophores and hemolysins (6), resistance to serum and phagocytic killing (29), and epithelial cell invasion (10,30). Intracellular survival of *E. piscicida* has been observed in both human and fish non-phagocytic epithelial cell lines including: HEp-2 (30), HeLa (31), epithelioma papillosum of carp (EPC) (10) and flounder gill cell lines (FG-9307) (32).

1.3.1. Type VI secretion system and type III secretion system

A crucial step in understanding the mode of pathogenicity of a microbe is in the identification and characterization of its virulence factors. The T3SS and T6SS are major

delivery apparatuses of *E. piscicida*'s virulence arsenal in both host phagocytes and epithelial cells (27,28). In general, both T3SS and T6SS are known to form specialized needle apparatuses to inject bacterial effectors directly into the target cell (33,34).

The T6SS forms a contractile nanomachine to puncture target cell upon contact and deliver toxins directly form the bacterial cytoplasm into the target cell's cytoplasm (35). The main components of an active T6SS are: valine–glycine repeat protein G (VgrG), proline-alanine- alanine-arginine repeat proteins (PAAR), haemolysin co-regulated protein (Hcp), and the contractile sheath proteins (TssB and TssC) (35). Various structural and bioinformatic analyses have reported similarity between T6SS and T4 contractile phage tail components; VgrG forms a cell-puncturing tip (spike), PAAR proteins form a sharp extension on VgrG spike to help effector domains attach to the spike (34), Hcp forms a tail-like tube structure through which effector proteins travel, and TssB and TssC form a sheath that contracts to provide energy for efficient translocation of bacterial effectors (Figure 1.2). Earliest reports on T6SS suggested its role in host virulence (36). For instance, in *Vibrio cholerae*, a gram-negative pathogen known to cause cholera, T6SS effector has been shown to protect *V. cholerae* from amoeba predation (37), and impair phagocytosis by inducing rounding of macrophages through actin cross-linkage (38). More recently, studies have suggested the role of T6SS in inter-bacterial competition (39). The first insight into T6SS being used for inter-bacterial competition came from the gram-negative pathogen, *Pseudomonas aeruginosa*(39). *P. aeruginosa* studies showed that the T6SS is used to antagonize bacterial competitors in close proximity, providing fitness advantage to these microbes (39). In this way, the T6SS has been shown to play an important role in anti-bacterial competition (40) and virulence to eukaryotic hosts (36).

Similar to the T6SS, the T3SS also forms a molecular syringe to inject bacterial effectors into the eukaryotic host's cytoplasm (33). Core component of T3SS is the needle complex that spans the bacterial envelope and is primarily composed of a basal body embedded in the bacterial envelope (41). The secretion and cellular translocation (Sct) prefix has been recently accepted as unified nomenclature for conserved components of T3SSs according to Portaliou et. al. (42). The T3SS basal body itself is a series of ring structures with SctJ and SctD forming concentric rings in the inner membrane, connected to secretin SctC which forms the outer membrane ring (Figure 1.3). After basal body assembly, early bacterial substrates (SctF and SctI) are passed

through an export apparatus made up of five membrane proteins- SctR, SctS, SctT, SctU and SctV (43). Additionally, a substrate sorting platform is located directly beneath the export apparatus, composed of a C-ring (SctQ) and an ATPase complex (SctN) (43). The T3SS needle itself is formed by self-oligomerization of SctF protein while SctI, the inner rod protein, helps in anchoring the needle into basal body (42). The needle complex (SctF and SctI) serves as a channel for passage of translocators (SctE and SctB) as well as bacterial effectors (43). The translocators form pores in the host membranes, allowing effective delivery of bacterial effectors into host cytoplasm (42). This dynamic T3SS nanomachine is used by many intracellular pathogenic *Salmonella* (41), *and Shigella* (44) species that require T3SSs to invade host epithelial cells and build a safe intracellular niche. *Salmonella enterica* serovar Typhimurium (*S.* Tymphimurium), the causative agent of gastroenteritis in humans, is known to have two pathogenicity islands (SPI 1 & 2) encoding T3SSs (41,45). SPI-1 contains genes required for invasion of epithelial cells while SPI-2 contains genes that enable bacterial survival in macrophages, thereby promoting systemic virulence (41,45).

1.3.1.1. *E. piscicida* type VI secretion system

The T6SS in *E. piscicida* was identified as the EVP (*E. tarda* virulence protein) gene cluster and consists of 16 components (*evpA–evpP*) (28). *E. piscicida* secretes three type VI effectors- EvpC, EvpI and EvpP (28). The EvpI homolog VgrG has been studied in *V. cholerae*, and is involved in the formation of the trimeric complex used to build the needle structure between the bacterial outer membrane and the host cell membrane, which acts as a puncturing device of the T6SS machinery (28,46). EvpP is a T6SS effector that plays a role in *E. piscicida* epithelial invasion since an *E. piscicida* deletion mutant of EvpP showed lower *E. piscicida* internalization rate in EPC cells (47). A recent study reported that EvpP is involved in the evasion of the host innate immune system by inhibiting the Ca^{2+}-dependent MAPK-Jnk Pathway (Jun N-terminal kinase pathway), thus preventing activation of inflammasomes in the host cell (48). Secretion of EvpP depends on other T6SS proteins such as EvpC, which is homologous to the Hcp1 (hemolysin coregulated protein 1) protein found in T6SS of *Pseudomonas aeruginosa* (49). In *P. aeruginosa*, Hcp1 forms hexameric rings, which assemble to form phage tail spike-like nanotubes (Figure 1.2) on the bacterial surface to transport effectors directly into the host cytoplasm (49). Jobichen and co-workers (2010) further validated the homology between Hcp1 and EvpC as EvpC also forms hexameric rings that stack

together to form a tube structure (49). In *E. piscicida*, EvpB and EvpC T6SS proteins are needed for virulence in fish hosts (50). According to Rao and co-workers (2004), deletion mutations of EvpB and EvpC have been reported to significantly increase the lethal dose (LD_{50}) levels in blue gourami *in vivo* (50).

1.3.1.2. *E. piscicida* type III secretion system

T3SSs are contact-dependent translocation systems found in many gram-negative bacteria and are an assembly of many components—secretion and translocon apparatuses—used to inject bacterial effectors directly into host cells (33). The *E. piscicida* T3SS gene cluster contains 35 genes (27). *In vivo* studies show that deletion of single T3SS genes in *E. piscicida*, such as *escC*, *eseB*, *eseD*, or *escA*, reduces virulence in blue gourami fish model (51,52). Three chaperones have been reported for *E. piscicida*- EscA (51), EscB (53), and EscC (52). EscB is a chaperone for an effector called EseG (53). EscC is the chaperone for EseB and EseD (52), whereas EscA is the chaperone for EseC (51). EseB, EseC, and EseD, form components of the *E. piscicida* translocon pore complex, and are homologous to *Salmonella* SseB (SctA), SseC (SctE), and SseD (SctB), respectively (Figure 3) . The SseB, SseC, and SseD proteins are secreted by the T3SS of *Salmonella* pathogenicity island 2 (SPI-2) and assemble into complexes that function as a translocon for effector proteins (45). The *E. piscicida* T3SS, in this way, has almost a full set of genes that are homologous to those of SPI-2 (27,45).

Only three *E. piscicida* effectors- EseG (53), EseJ (54), and EseH (55) have been identified. EseJ is a novel effector that has been reported to aid in intracellular bacterial replication in both murine macrophages and fish epithelial cells (EPC cells) (54). EseG shares homology with two *Salmonella* effectors, SseG and SseF(53). In *E. tarda*, EseG overexpression in a transfection study has been reported to cause microtubule disassembly *in vitro* and *in vivo* (53). Another recent study has provided evidence for internalization-dependent secretion and localization of EseG in the membrane of *E. piscicida* containing vesicles (56). The most recently discovered T3SS effector for *E. piscicida* is EseH—an enzyme that belongs to the family of phosphothreonine lyases that are known for their inactivation of MAPK signalling cascades involved in host immune responses (55). To further support this finding, *E. piscicida* deficient in EseH was reported to attenuate virulence in zebrafish infection model by enhancing cytokine expression (55).The T3SS is also essential for the

intracellular replication of *E. piscicida* in murine macrophages (J774A.1 macrophages) (29,57)and for its intracellular growth in human epithelial cells (HEp-2 cells) (58).

Using comparative proteomic analysis of *E. piscicida*, anti-phagocytic killing has been attributed to the presence of both T3SSs and T6SSs (27,29,47). Reduction of virulence in blue gourami fish, and impaired replication in phagocytes of fish and mice has been observed in T3SS (27,54,59) and T6SS (50) mutants of *E. piscicida*.

1.3.2. *E. piscicida* species Infection models

To gain a thorough understanding of the subcellular events that are triggered in the host epithelium following *E. piscicida* infections, we require robust *in vitro* infection models. Although many studies have investigated *E. piscicida* pathogenesis using phagocytic cells (29,60), only few have examined *E. piscicida* pathogenesis in epithelial cells (53,56).

1.3.2.1. Phagocytic infection models

It has been suggested that phagocyte-mediated killing is the first defence mechanism in fish against bacterial invaders (61); however, *E. piscicida* has found ways to survive and replicate within fish macrophages to escape the host innate immune system(62). *In vitro* studies have showed *E. piscicida* adhering, surviving, and replicating within primary blue gourami macrophages (63); reducing the amount of reactive oxygen intermediates produced within these macrophages (63). Although primary fish macrophage models shed light on *E. piscicida* pathogenesis, the difficulty of maintaining these cells and their inability to survive for long periods limits the usefulness of that model. An additional challenge when using fish derived cell are that anti-fish antibodies are generally not commercially available. For these reasons, recent studies have utilized murine macrophage cell lines such as J774A.1 (54) or RAW264.7 (60) as host cells because of their rapid growth rate, ease of culturing, and the availability of numerous murine-specific host antibodies (37,60).

1.3.2.2. Non-phagocytic infection models

Examination of the alterations to epithelial cells during *Edwardsiella* infections has lagged that of phagocytic cells for a variety of reasons. The most commonly used epithelial cells for *E. piscicida* infections are EPC (54) and FG-9307 cells (32). EPC cells

as they are extremely small in size, have low bacterial internalization rates (32) and are difficult to analyze due to lack of availability of fish-specific antibodies. Similarly, FG-9307 cells are fish-derived and display extremely low infection rates (32). To develop an efficient and generally useful *E. piscicida* infection model, mammalian epithelial cells have been proposed. HeLa and HEp-2 cells have been used as mammalian infection models to study *E. piscicida*; however, very low (<1%) invasion levels were reported for both cell lines (18,19).

1.4. Host cytoskeletal and junctional alterations by bacterial pathogens

The vertebrate epithelium, relies heavily on two important structural components to maintain its integrity and maintenance: cytoskeletal filaments (microfilaments, microtubules and intermediate filaments) and intercellular junctions (tight junctions, adherens junctions, and desmosomes). For decades, both the eukaryotic cytoskeleton and intercellular junctions have been identified as targets for many bacterial pathogens (64–67).

1.4.1. Microfilaments (filamentous actin)

Microfilaments are formed by globular (G)-actin monomers that hydrolyze ATP energy to drive filamentous-actin (F-actin) polymerization (68,69). Actin filament assembly and disassembly is affected by several actin-binding proteins. Nucleation factors such as the actin-related protein 2/3 (Arp 2/3) complex together with Wiskott Aldrich Syndrome (WASP) proteins carry out actin nucleation, filament elongation and actin branching (68,69). Formins on the other hand, generate unbranched long actin bundles (69).Several other proteins such as profilin and cofilin regulate the speed of actin polymerization (69). Rearrangements in the actin cytoskeleton take place through the activity of Rho-GTPase signalling cascades (70). The Rho family of small GTP-binding proteins (Rho, Rac, and Cdc42) has been shown to regulate actin microfilament dynamics during various cellular processes such as cell migration (70). In the GTP-bound activated state, these Rho GTPases bind to a variety of effector proteins that include Rho-associated protein kinase (ROCK) and PAK (p21-activated kinase) to regulate the assembly or disassembly of F-actin (70).

1.4.1.1. Microfilament manipulation by pathogens

The dynamic process of actin polymerization is manipulated by several bacterial pathogens. Both intracellular (e.g. *Shigella flexneri* (71), *Listeria monocytogenes* (72), and *S.* Typhimurium (73)) and extracellular pathogens (enteropathogenic *Escherichia coli* (71)) modulate the actin cytoskeleton during their infectious processes. *S.* Typhimurium exploits actin dynamics primarily during its internalization process as it delivers T3SS virulence factors directly into epithelial cells that control the host GTPases to form actin-rich membrane ruffles that engulf the bacteria (74). Many other bacterial pathogens such as *S. flexneri* and *L. monocytogenes* also utilize actin polymerization for their internalization, but do so through the combined actions of actin and the endocytic machinery (72). Once intracellular, those microbes continue to hijack the actin cytoskeleton as they generate actin-rich comet tails (75), which they use for intracellular bacterial propulsion and cell-to-cell spreading. Studying the manipulation of host actin by pathogens has improved our understanding of bacterial pathogenesis while simultaneously providing general cell biological insight into actin-based cell motility.

1.4.2. Microtubules

Microtubule networks play essential roles in chromosome segregation during mitosis (76), organelle and vesicular movement (77), stimulating signal transduction and modulating actin dynamics (78). Microtubules are composed of heterodimers of α- and β-tubulin that polymerize to form linear protofilaments that self-assemble to generate 25nm tubes with hollow cores (79). The individual microtubule filaments resemble dynamic nature of actin filaments as they undergo continual assembly and disassembly (Figure 1.4). This growth and retraction is commonly referred to as dynamic instability and has been observed *in vitro* (79).

To ensure proper regulation of this dynamic process, numerous regulatory proteins bind to and alter individual microtubules. Three major classes of proteins are associated with microtubules; microtubule-associated motor proteins (80), the classical microtubule associated proteins (MAPs) (79,81) and microtubule severing enzymes (82). The well-studied molecular motors, dyneins and kinesins, utilize ATP hydrolysis to drive the transport of vesicles and organelles within the cell using microtubules as tracks (77). MAPs stabilize and promote microtubule assembly (83) while depolymerizing kinesins

(84) and microtubule severing enzymes shorten microtubules (85). At various stages of eukaryotic cell division, depolymerizing kinesins use ATP to uncap microtubules at the ends and carry out depolymerization in a length-specific manner (84). Severing enzymes, on the other hand, remove dimers from the middle of the filament and create internal breaks in the microtubule (82).

1.4.2.1. Microtubule severing enzymes

During eukaryotic cell division (76), morphogenesis (86), cell motility and signaling (86), microtubules undergo continual remodelling to meet the changing needs of the cell. Over the past few years, it has become clear that microtubule-severing enzymes have emerged in a variety of cellular activities such as cell division (82), cilia biogenesis (87), and cell motility (88). Thus, this group of enzymes can be considered as an important class of microtubule regulators (82). Even though *in vitro* studies show that severing enzymes cause complete destruction of microtubules (85), some studies suggest that microtubule severing enzymes can also be utilized for constructive processes such as microtubule movement (89). Because the long microtubule filaments are immobile, severing of these filaments into shorter fragments makes microtubules mobile and available for rearrangements within the cell (89). To date, three classes of microtubule-severing enzymes have been identified, katanin, spastin and fidgetin, which are all categorized as AAA ATPase protein superfamily members (85).

Katanin

Katanin was the first microtubule severing protein discovered in 1993 and originally derived its name from the Japanese sword “katana” (90). Katanin is composed of a catalytic p60 subunit (A1 subunit, KATNA1) that severs microtubules using ATP hydrolysis, and a regulatory p80 subunit (B1 subunit, KATNB1) that binds microtubules and controls the severing activity of the A1 subunit (90). Later studies proved that two additional A1-like katanins (KATNAL1 and KATNAL2) (91) and one additional B1 like katanins (KATNBL1) are present in mammalian genome (91).

Various studies have examined the molecular mechanisms involved in katanin-induced microtubule severing (82,92,93). It has been demonstrated that monomeric katanin subunits in the ADP-bound state exchange their bound ADP for ATP and oligomerize (93). Oligomerization leads to ATP hydrolysis through katanin–katanin

contacts (93). This conformational change in the oligomer leads to a push or pull force on the underlying tubulin subunits and breaks tubulin–tubulin contacts (93). More recently, studies have described this severing mechanism further by stating that the katanin oligomer binds and simultaneously pulls carboxy-terminal tails of tubulin heterodimer (92), releasing the tubulin heterodimer due to physical stress imposed on the heterodimer, therefore weakening inter-tubulin bonds (Figure 1.5). In eukaryotic cell division, katanin localizes at centrosomes and is involved in the active microtubule disassembly near their centrosomal attachment points (94).Being localized at the spindle poles, katanin plays a significant role during anaphase chromosome segregation, by severing the spindle microtubules at their plus ends (82).

Katanin isoforms

KATNA1 and KATNAL1 both contain a MIT (microtubule interacting and trafficking) domain, an AAA domain (severing domain), and a C-terminal VPS4 (Vacuolar protein sorting-associated protein 4) oligomerization domain (95). KATNAL1 also contains a short domain at the N-terminus, which displays 68% identity (95) to the p80 microtubule-binding domain of canonical katanin p60 (Figure 1.6). An *in vitro* study showed the complete dissociation of the microtubule network in human bone osteosarcoma epithelial cells (U-2 OS cell line) when KATNAL1 was overexpressed (95), confirming the fact that KATNAL1 severs microtubules in a fashion similar to other severing proteins (Figure 1.7). Hexamerization is also essential for the severing activity of this protein. During cell division, KATNAL1 protein was also identified as a novel regulator of the mitotic spindle (95). KATNAL1 depletion by siRNA led to a decrease in microtubule density at the spindle poles, an increase in centrosomal γ-tubulin distribution, and an increase in spindle length *in vitro* (95).

Surprisingly, only minor changes are observed in spindle size and mitotic phenotypes, when one of the two human severing enzymes (KATNA1 or KATNAL1) is mutated alone (95,96). Other than a minor delay in metaphase-anaphase transition, inhibition of KATNA1 *in vitro* had very little effect on metaphase and anaphase spindles (96). Thus, it has been speculated that multiple katanins must be involved in regulating spindle size in mammals. Additionally, a study focusing on katanin binding partners showed that KATNAL1 can interact with KATNB1, KATNBL1, and KATNA1, suggesting

that multiple katanin p60/p80 heterodimers might regulate microtubule-severing phenomena (91).

In terms of structure, the N-terminal region of KATNB1 contains a WD40 domain and a proline-rich region, while the C-terminal region has a conserved con80 domain(90). Even though KATNB1 does not have microtubule severing activity on its own, it plays a significant role in enhancing KATNA1's severing activity as well as targeting KATNA1 to the centrosomes in mammalian cells (97). The microtubule binding con80 domain of KATNB1 is able to interact with N-terminal domain of KATNA1, which increases microtubule affinity for KATNA1 (97). In 1998, Hartman and co-workers stated that the WD40 domain of KATNB1, specifically, helps in targeting KATNA1 to centrosomes (90). Contrary to KATNB1, KATNBL1 is missing WD40 domain, but contains the proline-rich region (91).

Like KATNA1 & KATNAL1, KATNB1 has been reported to play various roles in the regulation of the cell cycle as mutations in this gene lead to abnormalities in spindle formation (98). A recent study showed a concentration dependent effect of KATNBL1 on the severing activity of KATNAL1; at a specific concentration, KATNBL1 enhances KATNAL1 severing activity (91). In addition to regulation of KATNAL1 activity, KATNBL1 has also been shown to compete with KATNB1 for binding to KATNA1 or KATNAL1 (91). The abundant cross-talk between the different katanin subunits suggests redundancy in cellular severing activity (91). This further displays the complexity of the microtubule severing enzyme repertoire.

Spastin

Another class of severing enzymes came into spotlight by discovery of Spastin, which was so named because it is the "most frequently mutated gene in the adult-onset neuromuscular disease of hereditary spastic paraplegia (HSP)" (99). A later study suggested a link between spastin dysfunction in HSP and damaged microtubule tracks, which leads to disrupted axonal transport (100). A combination of *in vitro* and *in vivo* studies followed that further identified various roles for spastin.

Similar to katanin, spastin is a member of the AAA ATPase superfamily with a microtubule severing domain and a microtubule interacting domain (MIT) (99). Three highly conserved pore loops project into spastin's hexameric ring, which are critical for

substrate remodeling activity (101). ATP hydrolysis in the highly conserved C-terminal AAA ATPase domain, leads to microtubule disassembly by spastin (101). The mode of severing also resembles that of katanin as spastin oligomerizes into a hexamer using ATP hydrolysis and pulls on the carboxy terminal tail (101) of the tubulin subunit, passing the unfolded tubulin monomer through the spastin central pore, and releasing the tubulin monomer from the microtubule filament (Figure 1.8). However, it has not been established whether carboxy tails of both monomers in the heterodimer are recognized by spastin. *In vitro* studies revealed that overexpression of wild-type spastin (102) caused microtubules to disassemble (Figure 1.9). Overexpression of spastin in larval *Drosophila* muscle fibers erased the whole microtubule network (103). To support this *in vivo* finding, spastin-null *Drosophila* flies showed severe movement defects and shorter lifespans (103).

Fidgetin

Fidgetin is the most recently identified microtubule severing enzyme (104). Similar to katanin and spastin, sequence analysis shows that fidgetin contains an AAA ATPase domain and thus belongs to the AAA superfamily (104). ATP hydrolysis triggers oligomerization of fidgetin proteins as well to form hexamers (82). Unlike *C. elegans* or *D. melanogaster*, which contain a single FIGN ortholog, mammals have three: the canonical FIGN, FIGN-like 1 (105) and FIGN-like 2 (106). Recently, FIGN-like 2 has been localized to cell cortex where it regulates microtubule network organization (106). Like other severing enzymes, fidgetin also localizes to mitotic centrosomes (Figure 1.10) and plays an important role in eukaryotic cell division by persistently depolymerizing spindle pole associated microtubule minus-ends, to carry out chromatid-to-pole movement during anaphase A (104). The impact of fidgetin-based severing activity is more pronounced at the minus ends of microtubules, suggesting a possible role in preventing microtubules from attaching to centrosomes (104).

In vitro studies have confirmed fidgetin's ability to bind microtubules and cause microtubule severing (104). Evidently, purified baculoviral-expressed human FIGN can sever taxol-stabilized rhodamine-labeled MTs *in vitro* (104). Another study shows that overexpression of *Drosophila* fidgetin leads to microtubule severing in the *Drosophila* Schneider-2 (S2) cell line (104).

Interestingly, fidgetin has been linked to mammalian embryonic development as *in vivo* mouse studies show that mutations in the fidgetin gene lead to developmental ear, eye, or skeletal defects in mice (108). A recent study suggests that mutated FIGN can cause developmental defects by affecting spindle positioning and cytokinesis (104). In humans, defective human FIGN leads to aneuploidy or polyploidy, which are leading causes of tumorgenesis (104). Thorough understanding of the interaction of all three classes of severing enzymes, katanin, spastin, and fidgetin, with *E. piscicida* may reveal the molecular aspect of the microtubule severing phenotype caused by *E. piscicida* in epithelial cells.

1.4.2.2. Microtubule manipulation by pathogens

Many bacterial pathogens can manipulate host microtubule network through their virulence proteins. The *S. flexneri* T3SS effector VirA disassembles microtubules in host cells, thereby enhancing the invasive properties of *Shigella* (44). *S. flexneri* is known to invade host cells via micropinocytosis, thus through formation of actin-rich membrane ruffles (44). The effector VirA destabilizes microtubule beneath the membrane ruffles, leading to successful internalization of the bacteria (44). Another T3SS effector found in enteropathogenic *E. coli*, known as EspG, has also been reported to disassemble microtubules in fibroblasts and non-polarized epithelial cells (109). Similarly, the T3SS effector of *E. piscicida*, EseG has also been reported to disassemble microtubules when overexpressed in HeLa cells (53). Recently, EseG was reported to localize in the membranes of *Edwardsiella*-containing-vesicles (56), but the exact mechanism of microtubule disassembly and whether microtubule break-down occurs during normal infections remains unexplored.

1.4.3. Intermediate filaments

The third major class of cytoskeletal filaments involves intermediate filaments, which are 8-12nm wide rope-like filaments (111). The wide array of intermediate filament proteins share common structural organization with a central coiled-coil rod domain, flanked by a head domain (N-terminal) and a tail domain (C-terminal) (111). These filaments are built from a precise arrangement of protein subunits. The first step of intermediate filament formation involves twisting of two coiled coil domains to form dimers, which form tetramers by aligning in a staggered orientation (111). These

tetramers then aggregate laterally to form short unit length filaments (ULFs) (111). End-to-end annealing of these ULFs forms thick mature intermediate filaments (111). Based on sequence homology, the intermediate filaments can be divided into five main types-keratins (type I and type II), desmins and vimentins (type III), neurofilaments (type IV) and nuclear lamins (type V) (113). Additionally, intermediate filaments show specific localization patterns—type I and type II keratins are found in epithelial cells—desmins are found in muscle cells—neurofilaments are found in neurons—lamins are found in the cell nucleus (113). Other than providing cellular structural integrity, and maintaining cellular organization of organelles, intermediate filaments are involved in complex signal transduction pathways by communicating with other cytoskeletal proteins (113,114).

1.4.3.1. Intermediate filament manipulation by pathogens

Like other cytoskeletal filaments, intermediate filament proteins can also contribute to bacterial pathogenesis through the establishment of replicative niches for numerous pathogens. *Chlamydia trachomatis*, which causes chlamydia infection as well as pneumonia, targets the host intermediate filaments to facilitate its entry into the mammalian host (115). More specifically, *C. trachomatis* rearranges three major intermediate filaments—vimentin, cytokeratin 8 and cytokeratin 18 to form a safe enclosure for its replication (115).

1.5. Junctional alterations by bacterial pathogens

The epithelial monolayer is well equipped to act as a barrier against microbial invaders (67). These cells display polarity and adhere to one another via intercellular junctional complexes (67). In vertebrates, epithelial cells are connected to each other by three major types of intercellular junctions: tight junctions, adherens junctions and desmosomes (Figure 1.11). Tight junctions act as the seal between adjacent cells in the epithelium to regulate passage of small molecules between cells (116). Another class that mechanically attach neighboring cells are the adherens junctions (118). Both tight junctions and adherens junctions are attached to the actin cytoskeleton of neighbouring cells (117,118). Third major class of junction complex is comprised of desmosomes, where ropelike intermediate filaments of adjacent cells adhere (120). Despite the presence of these defensive systems, bacterial pathogens have evolved strategies to breach the epithelial lining leading to an assortment of host disease phenotypes.

1.5.1. Tight junctions

Tight junctions are located most apically in the lateral membranes of the epithelial monolayer(116). As a striking characteristic, tight junction complexes can be seen as “kissing-points” (116) where the adjacent cell membranes meet (Figure 1.11). Contrary to adherens junctions and desmosomes, the morphological space between neighboring cells is completely eliminated at these kissing points (116). The tight junctions display selective permeability based on ion size and type (116). Other than the barrier function, tight junctions have long been proposed to act as a fence by functionally segregating the apically expressed membrane proteins from those expressed on the basolateral membrane of polarized epithelial cells (117). However, this premise was challenged when cells without tight junctions still maintained their apical/basolateral protein segregation in mouse epithelial cell lines (122). Epithelial tight junctions are primarily composed of three components: transmembrane proteins—occludin, claudin, junctional adhesion molecules (JAMs), and coxsackie adenovirus receptor protein (CAR) —cytoplasmic adaptors proteins—zonula occludens (ZO) 1, 2, and 3 and the cytoskeletal protein—actin (116,119). Among these, the claudin family membrane proteins are key components for the structure and function of TJs (119). Claudin molecules interact with each other between cells to generate TJ strands, while occludins are incorporated into or localized very close to claudin-based TJ strands (116). In addition, JAM and CAR proteins are also located at tight junctions. JAMs belong to the immunoglobulin (Ig) gene superfamily, and form both homophilic and heterophilic interactions between variety of cells (122). Similarly, the extracellular Ig domains of CAR proteins are involved in formation of homophilic interactions between cells (119). Both JAMs and CARs are exploited by viruses for gaining entry into host cells (65, 119,123). On the cytoplasmic side of TJ, zonula occludens proteins bind to the C-terminal cytoplasmic domain of claudins, occludin, and JAM proteins (119).

1.5.1.1. Occludin

Occludin, the first identified transmembrane TJ protein, is an approximately 65 kDa protein with tetraspanning transmembrane domains (122). It also forms two extracellular loops, an intracellular turn, as well as cytoplasmic N- and C- termini (122). Along with another important tight junctional protein called claudin, freeze-fracture studies suggest that overexpression of occludin in cultured monolayers of Madin-Darby

Canine Kidney (MDCK) cells leads to an increase in the number of tight junction fibrils (117). Interestingly, occludin knockout mice do not show altered TJ structures or functions (125). Further studies showed that tight-junction strands can also be formed without occludin (125). This evidence suggests that occludin is not the only integral membrane protein in the tight junctional complex.

1.5.1.2. Claudin

An additional family of integral membrane proteins known as the claudins was later identified as two proteins- claudin 1 and claudin 2 co-purified with occludin from biochemical fractionation of junction-enriched membranes from chicken liver preparations (126). These proteins also have four transmembrane domains, but do not show any sequence similarity to occludin (126). Claudins are the major regulators of paracellular permeability at tight junctions and multiple claudin variants can be expressed simultaneously at the tight junctions (127). These dynamic proteins can copolymerize in a heteromeric fashion to form single non-paired tight junction strands in an individual cell (117). Moreover, between two paired strands, claudins can form both homotypic and heterotypic interactions depending on the right combination of claudin variants (117). Claudins also interact with other transmembrane tight junction proteins, such as occludin and the cytoplasmic adaptor ZO proteins (117).

Thus far, 27 different mammalian claudins have been found, based on sequence and dynamic properties (127). Claudins that qualify as pore-forming claudins are: claudin-2, -10b, and -15 (cation pores) and claudin-10a and -17 (anion pores) (127). Claudins have been reported to form pores only in specific combinations of claudin isoforms (127). Several *in vivo* studies also reflect on the importance of claudins in tight junction complexes. For instance, claudin-1 plays an important role in the skin barrier as it is expressed in the epidermis (127). Claudin-1 knockout mice die due to dehydration 1 day post birth; as water evaporation through their skin leads to severe dehydration (128). Several other studies also pinpoint importance of claudins; the claudin-16 mutation has been associated with chronic renal failure in patients (129), and claudin-14 knockout mice suffer from deafness due to degeneration of cochlear outer hair cells (130).

1.5.1.3. ZO-1

Many cytosolic proteins have been reported to associate with the cytoplasmic surface of tight junctions, which mediate interaction with the actin cytoskeleton (130). The first tight junction protein to be identified, was zonula occludens 1 (ZO-1); a 220 kDa peripheral membrane protein (130). Based on sequence similarity, zonula occludens has three isoforms: ZO-1, ZO-2, and ZO-3 (121). These contain three PDZ DOMAINS (PDZ1, PDZ2 and PDZ3), one SH3 domain, and one guanylyl kinase-like (GUK) domain (121). The PDZ domains bind to the carboxy termini of claudins, while the GUK domains bind carboxy-terminal tails of occludins (121). However, the interaction between zonula occludens and occludin might be dispensable as ZO-1, ZO-2, and ZO-3 localize to tight junctions even in occludin-deficient mice (124). To connect the junctional complex to cytoskeleton, ZO-1 forms a complex with ZO-2 through PDZ2/PDZ2 interaction and binds directly to actin filaments at their carboxy terminals, while ZO-3 has been reported to only associate with ZO-1 not ZO-2 (121). According to an *in vitro* study, ZO-1, and ZO-2 can also dictate claudin polymerization at tight junction strands (121).

1.5.1.4. Tight junctions altered by pathogens

There has been considerable evidence suggesting a link between tight junctions and disease, as multiple pathogenic bacteria target tight junctions during their infectious processes (65). Examples of this include enteropathogenic *E. coli,* which uses three T3SS effectors called EspF, EspG and Map to disrupt the tight junction barrier (131). Additionally, during *Vibrio parahaemolyticus* infection, which is a leading cause of seafood-borne gastroenteritis worldwide, ZO-1 and occludin are extensively disrupted in the host cells (132). Tight junction proteins can also act as entry cofactors for certain viruses (133), as reported for Hepatitis C virus (HCV). HCV, known to cause chronic hepatitis and cirrhosis in humans, gains entry into epithelial cells via various claudins such as claudin-1, 6, and 9 (133).

1.5.2. Adherens junctions

The adherens junction (AJ) is another intercellular junction in which cadherin receptors connect neighboring plasma membranes (118) via their homophilic interactions (Figure 1.12). Adherens junctions resemble tight junctions as cadherins communicate with the actin cytoskeleton via different cytoplasmic proteins such as α-

catenins and β-catenins (134). The catenin proteins mediate the interaction between cadherins and actin filaments (134). Another class of cytoplasmic AJ proteins is p120 catenins, which have been reported to stabilize cadherin (E-cadherin) proteins at cell-cell contacts (134). The p120-catenin protein also indirectly facilitates cross-talk between actin cytoskeleton and AJs as Rho GTPases can interact with cytoplasmic p120 catenins (135). In addition to cell-cell linkages, AJs help in tissue and organ remodelling by displaying cell sorting properties (134,136).

1.5.2.1. E-Cadherin

One of the major groups of transmembrane proteins that form adherens junctions are classical cadherins. Some of the commonly studied cadherin proteins are – E-cadherin, N-cadherin and P-cadherin, each of which have distinct tissue distribution patterns (134). Unlike claudins at tight junctions, cadherins are regulated by calcium levels and contain a highly conserved calcium sensitive domain (118). Presence of Ca^{2+} triggers adhesive homotypic interactions between cadherins on adjacent cells (134). Based on the extracellular domain sequence of cadherin molecules, the binding partner specificity differs; E-cadherin preferentially binds E-cadherin, and N-cadherin binds to N-cadherin (118). As mentioned earlier, the cytoplasmic domains in classical cadherins bind to p120 and catenins (α- & β-catenin) (Figure 1.12). These catenins in turn communicate with variety of other molecules, including actin filaments and their regulators (118). In this way, junctional E-cadherin–catenin complexes are critical for the proper functioning of epithelia.

1.5.2.2. Adherens junctions altered by pathogens

Some bacterial pathogens utilize adherens junction proteins for host invasion. A classic example of bacterial manipulation of adherens junctions comes from *L. monocytogenes* (138). During these infections, E-cadherin acts as the receptor for the internalin A ligands on the surface of *L. monocytogenes* (138). This InlA-E-cadherin interaction is vital for internalization of *L. monocytogenes* directly into epithelial cells (138). Another example of manipulation of adherens junction comes from *Streptococcus pneumoniae***,** which causes pneumonia (139). Colonization of the nasopharyngeal (NP) epithelial cells is the first step in causing disease by this bacterium, and it is accomplished by pneumococcal surface adhesin A (PsaA) (139). Similar to *Listeria*, E-

cadherin acts as a receptor on human epithelial cells for the PsaA, and contributes to *S. pneumoniae* virulence (139).

1.5.3. Desmosomes

Third major class of intercellular junctions, known to provide robust structural integrity to epithelial monolayers, are desmosomes (120). Figure 1.11 shows the presence of desmosomes along the lateral membranes of intestinal epithelial cells. These strong adhesive junctions are generated by desmosomal cadherins in conjunction with other proteins that bolt neighboring cells together in the epithelia by linking their respective intermediate filament networks (120). Thus, making the epithelial cells highly resistant to mechanical stress (120). Due to the strong mechanical nature of these junctional complexes, desmosomes are highly concentrated in areas of high mechanical stress such as epidermis and myocardium (140). Other than cell-adhesion, desmosomes are also involved in variety of cell signalling pathways (141). Desmosomes are composed of three main classes of proteins- desmosomal cadherins, armadillo proteins, and plakins (120).

1.5.3.1. Desmosomal cadherins

Desmosomal cadherins share sequence homology with classic cadherins and include two major protein subtypes: desmogleins and desmocollins (120). In presence of calcium, desmogleins and desmocollins mediate cell-cell interactions in both heterophilic and homophilic manners (120). Both desmosomal cadherins have different isoforms- Desmoglein 1-4 (Dsg 1-4) and Desmocollin 1-3 (Dsc 1-3) (140). Differential patterns are observed for the desmosomal cadherins in various tissues (120). On the cytoplasmic side of the desmosomes, the desmosomal cadherins interact with intermediate filaments via armadillo and plakin proteins (120).

1.5.3.2. Armadillo family proteins and desmoplakins

The armadillo proteins derive their name due to the presence of a central “armadillo” domain , which is flanked by N- and C-termini (120). The first member of the armadillo family of proteins is plakoglobin, which is highly homologous to β-catenin as they both contain the armadillo repeat domains (120). The gap between the intermediate filament network and desmosomal cadherins is filled by plakoglobin (PG); PG binds to

cytoplasmic tails of both desmogleins and desmocollins and thereby communicates with intermediate filaments via interactions through the N-terminus of desmoplakin (120). Another armadillo protein, plakophilin (120), works in a similar fashion to plakoglobin (Figure 1.13). The plakin family of desmosomal proteins is comprised of various proteins such as plectin, periplakin, envoplakin, and most importantly, desmoplakin (142). Desmoplakin is a critical component of desmosomes as it mediates the interaction between desmosomal cadherins and the intermediate filament network; the N-terminal of desmoplakin interacts with desmosomal cadherins via plakoglobin and plakophilin while the C-terminal interacts with intermediate filaments (120).

1.5.3.3. Desmosomes altered by pathogens

The first link between desmosomes and disease came from pemphigus, an autoimmune skin blistering disease (120). Although many studies suggest that desmogleins are affected during skin disease, recent study identified Dsg2 as a receptor for adenoviruses (serotypes 3, 7,11, and 14) (140). These viruses cause respiratory and urinary tract infections (140). Evidence of bacterial exploitation of desmosomes comes from a toxin produced by the *Staphylococcus* bacteria that causes bullous impetigo-exfoliative toxin A (ETA) (143). ETA is a serine protease known for cleavage of Dsg1 proteins (143). This cleavage damages cell-cell adhesion in the epidermis and results in focal lesions (143). In this way, robust adhesion of adjacent cells due to desmosomes can also be compromised by pathogens.

1.6. Rationale and research hypothesis

E. piscicida is an enteric intracellular pathogen, known to cause hemorrhagic septicemia in fish hosts as well as gastroenteritis and extra-intestinal diseases in immunocompromised humans (4). Invasion and replication within epithelial cells that are useful to researchers has been challenging as most have used fish cells or cells that are too small to see cytoplasmic structures. To overcome this, I set out to initially generate reliable and reproducible epithelial *E. piscicida* infection models and use that model to study subcellular alterations within the host cells. Because host tissue is disintegrated during *E. piscicida* infections and because the cytoskeleton and intercellular junctions are altered by many bacterial pathogens **I hypothesize that *E. piscicida* alters the host cytoskeleton and intercellular junctions during its infectious process.** Using

mammalian epithelial infection models, I analyzed the effects of *E. piscicida* infection on various host cytoskeletal structures (actin, microtubules, and intermediate filaments) and intercellular junctions (tight junctions, adherens junctions, and desmosomes) in epithelial cells and found that the microtubule cytoskeleton and intercellular tight junctions are targets of *E. piscicida*.

1.7. Figures

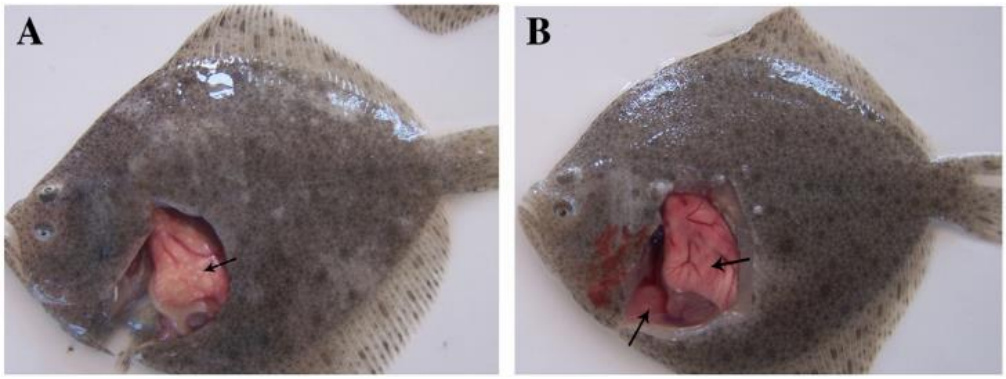

Figure 1.1: Diseased turbot showing signs of edwardsiellosis
A) The arrow is pointing to liver granulomas; B) Left arrow points to swollen heart, and right arrow points to swelling in the liver. Reprinted from Aquaculture, 431, Xu T and Zhang XH, *Edwardsiella tarda*: an intriguing problem in aquaculture, 129-135, 2014, with permission from Elsevier.

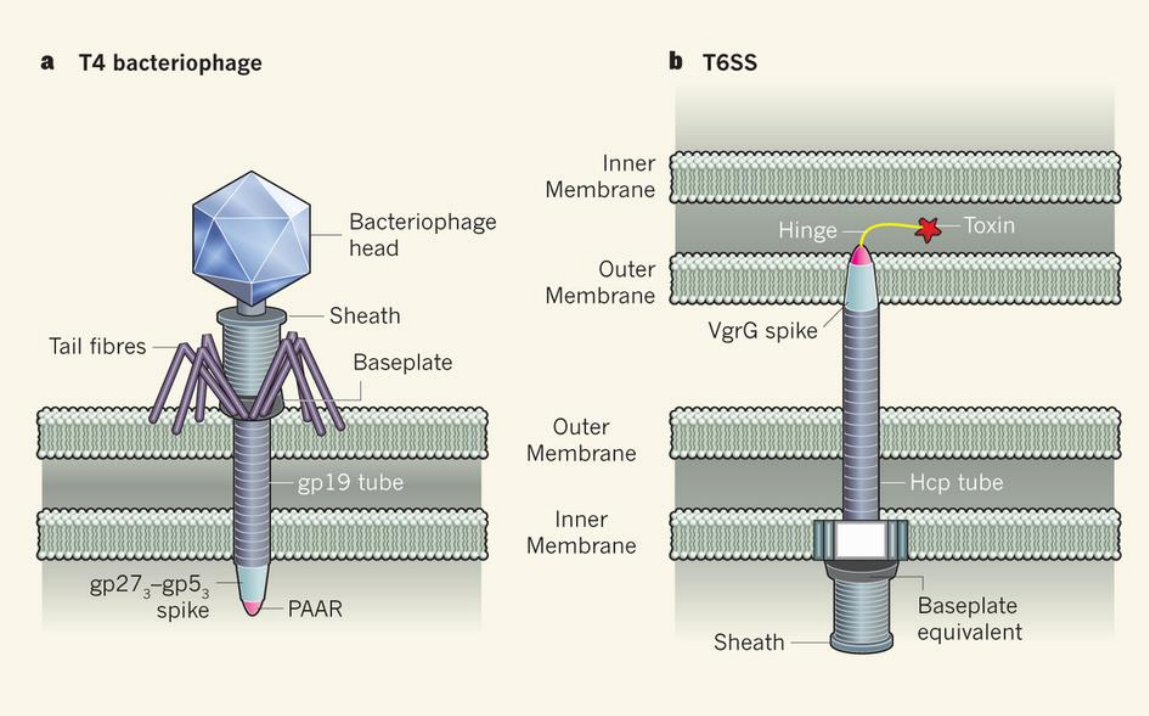

Figure 1.2: Comparison between structure of T4 bacteriophage viruses (a) and bacterial type VI secretion system (b)

The tube of hexameric rings is found in both systems- Hcp in T6SS and gp19 in the T4 bacteriophage. VgrG spike of T6SS is structurally similar to the trimeric gp273/gp53 cell-puncturing complex of bacteriophage T4. A contractile sheath surrounds the tube in both systems- TssB and TssC in T6SS and gp18 in the bacteriophage. PAAR proteins are highlighted in pink in both systems, forming a sharp extension on the spike proteins. In case of T6SS, the hinge may be involved in connecting the toxin to the PAAR proteins. Reprinted by permission from Macmillan Publishers Ltd: Nature (34), copyright 2013.

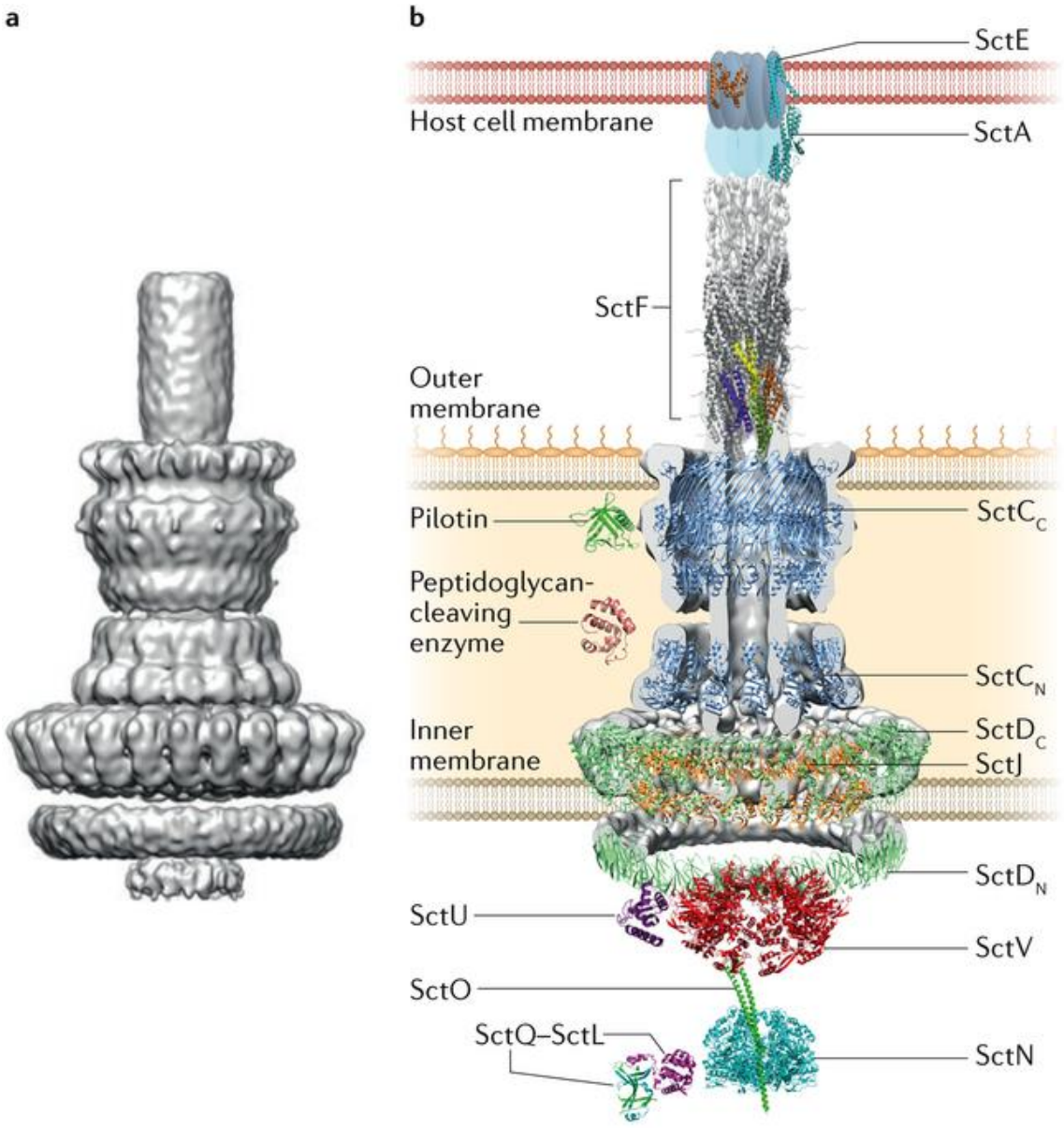

Figure 1.3: Diagram of the T3SS needle complex
a) Cryo-electron microscopy reveals overall structure of the basal body, needle as well as the inner rod in *S.* Typhimurium SPI-1 T3SS. b) Solved structures of T3SS components obtained from various bacterial species. Reprinted by permission from Macmillan Publishers Ltd: Nature (43), copyright 2017.

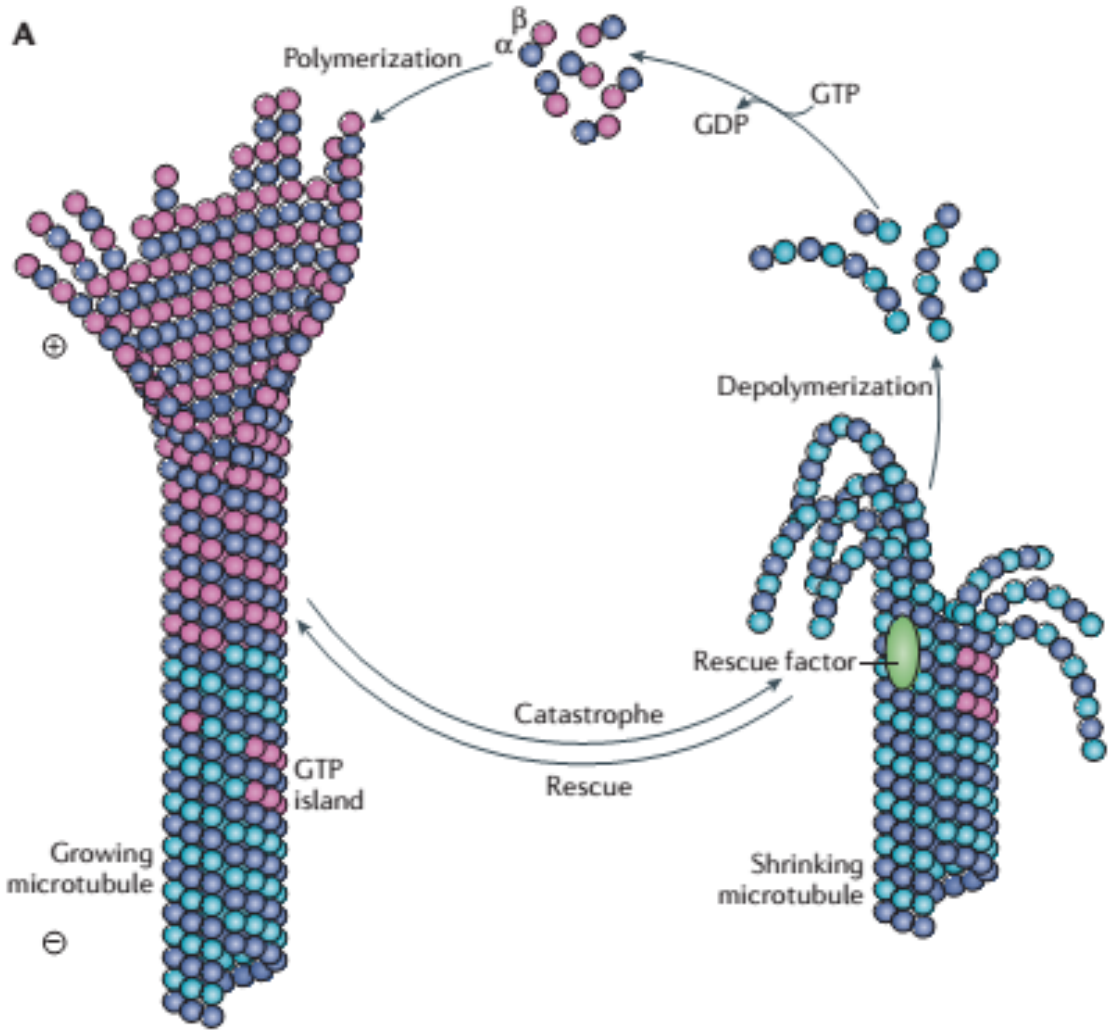

Figure 1.4: Diagram displaying dynamic instability of microtubule filaments. GTP-bound tubulin heterodimers are added on to the polymerizing end of the microtubule filament. Due to slight delay in GTP-hydrolysis, the polymerizing (+) end of microtubule filament maintains a GTP-cap. Catastrophe or depolymerization event occurs in response to loss of the GTP-cap, while rescue event can take place due to presence of various rescue proteins or a "GTP-island" (patch of GTP-bound tubulin dimers) in the microtubule lattice. These rescue factors can recruit GTP-bound tubulin heterodimers to the shrinking microtubule filament. Reprinted by permission from Macmillan Publishers Ltd: Nature (79), copyright 2015.

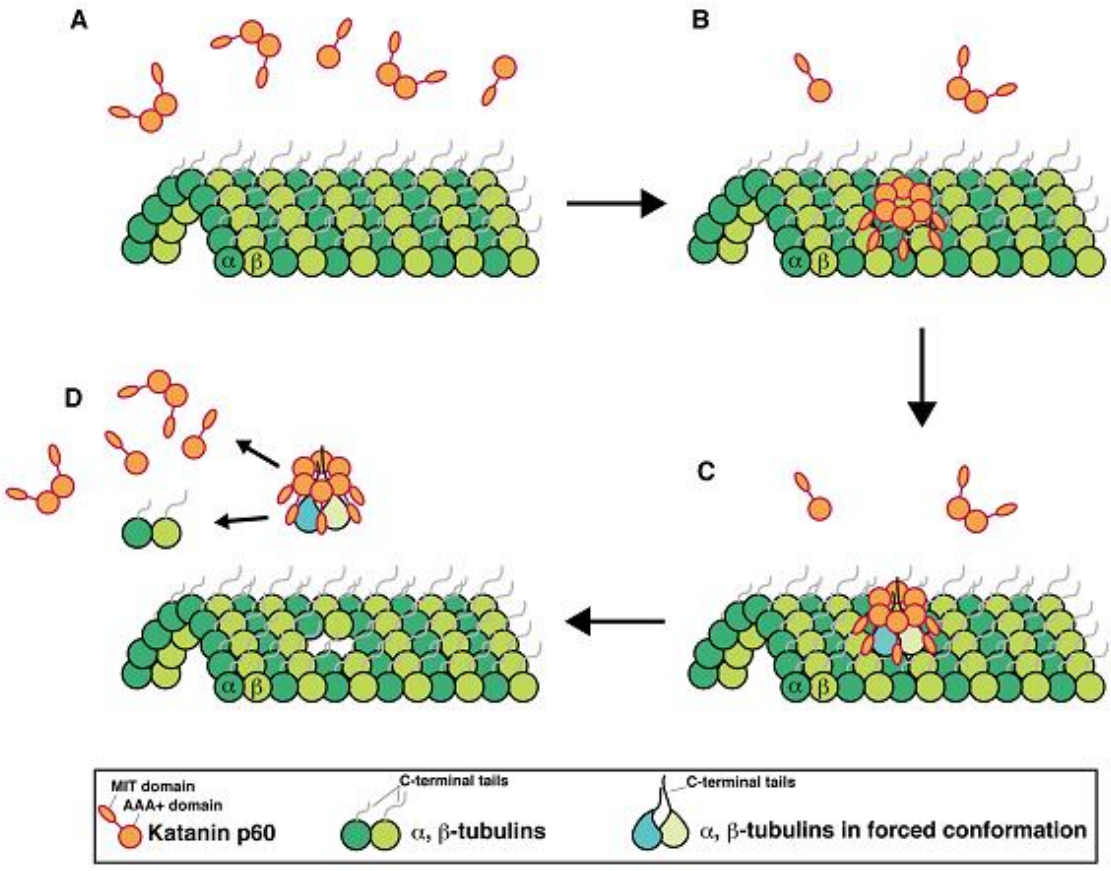

Figure 1.5: Proposed mechanism for MT severing by katanin p60.
A) and B) show katanin p60 oligomerization on the microtubule filament. C) the oligomer pulls on both C-terminal tails of α- and β-tubulins, imposing mechanical stress on the tubulin dimer, and destabilizes inter-tubulin contacts. D) due to the mechanical tension, the tubulin dimer is released from the microtubule filament. Reuse permission granted by Journal of Biological Chemistry (92).

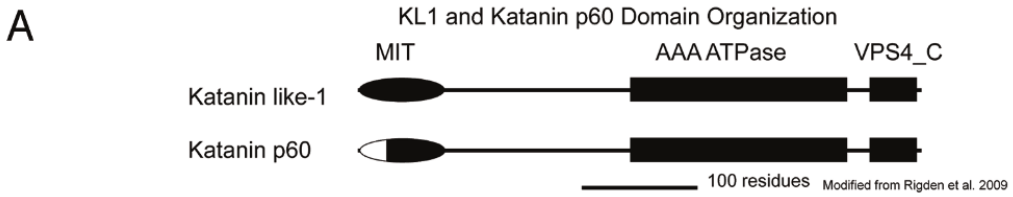

Figure 1.6: Linear maps of KATNAL1 and human katanin p60 polypeptides displaying similar domain organization.
Katanin p80 binding domain is shown as a white region in the microtubule binding domain. Reuse permission granted by Taylor and Francis Group (95).

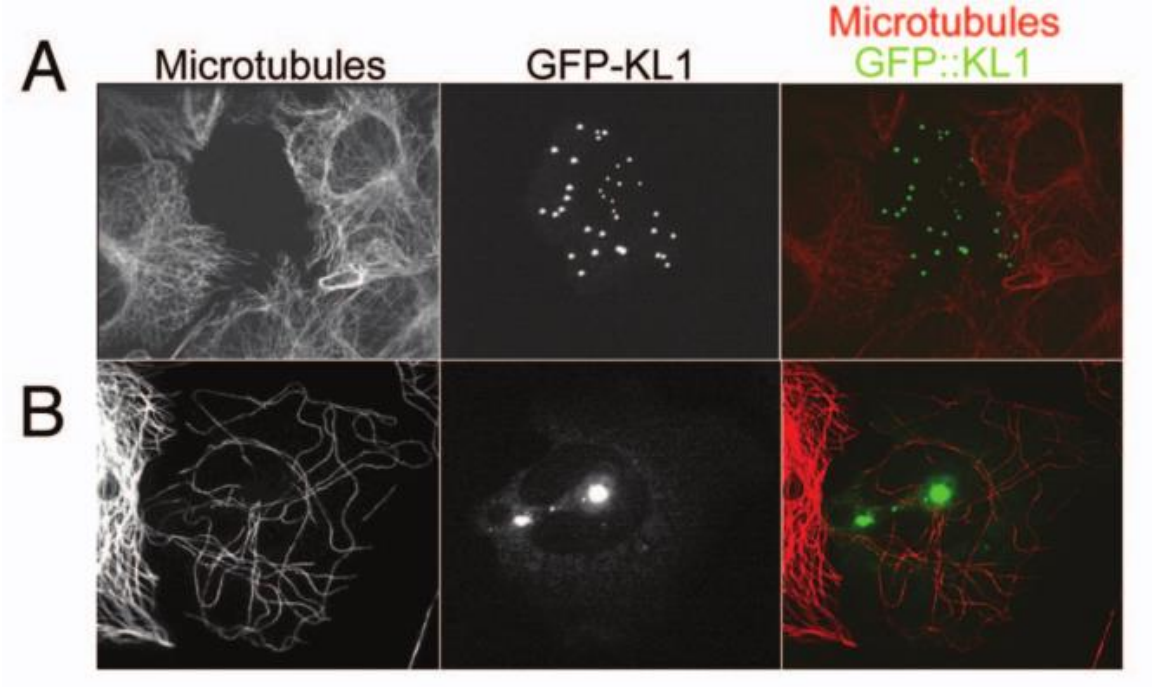

Figure 1.7: KATNAL1 (KL1) disassembles microtubules in U-2 OS (human bone osteosarcoma) cells.

A) Microtubules are shown in red while U-2 OS cells are expressing GFP-tagged KATNAL1 in green. After 24 hours, cells overexpressing KATNAL1 show completely severed microtubules. B) At 15-hour timepoint, cells overexpressing KATNAL1 show partial microtubule severing. Reuse permission granted by Taylor and Francis Group (95).

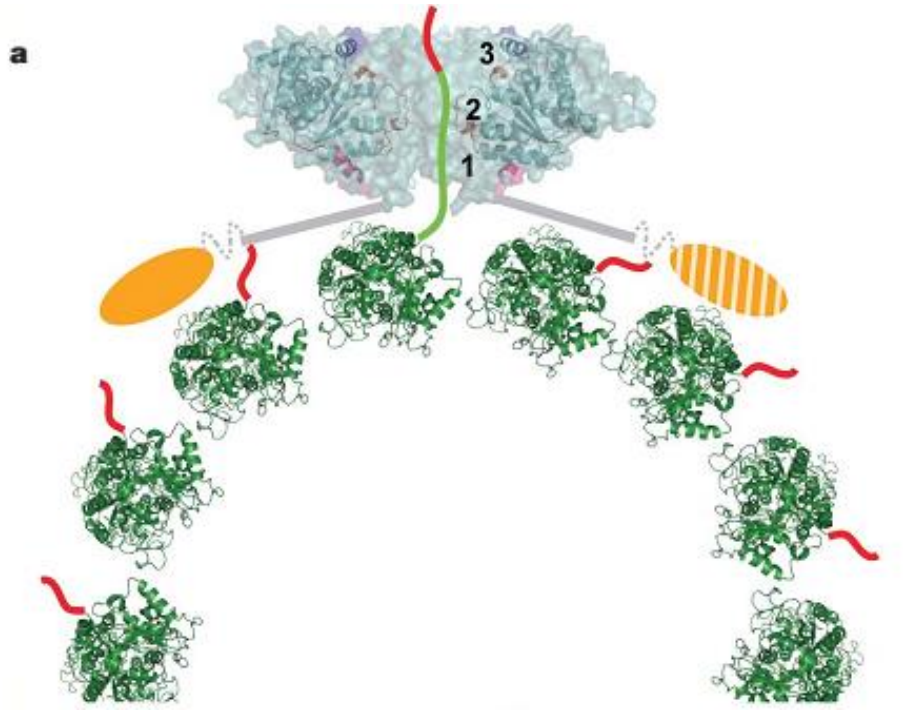

Figure 1.8: Putative mechanism of severing by spastin.
The spastin AAA core is shown in cyan and pore loops 1, 2 and 3 are numbered in the figure. The MIT domains are represented as gold ovals and to consider the possibility of an unengaged MIT domain, a hatched oval is shown. Tubulin heterodimers are shown as green ribbon drawings, while C-terminal tubulin tails are shown in red. Reprinted by permission from Macmillan Publishers Ltd: Nature (101), copyright 2008.

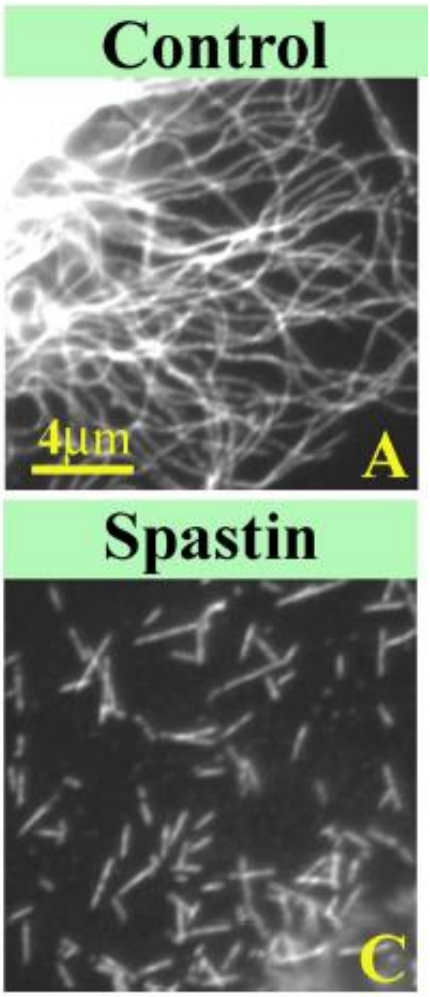

Figure 1.9: Microtubule-severing observed in rat lung RFL-6 fibroblasts due to overexpression of spastin.

A) Untreated RFL-6 cells displayed intact microtubules. C) Overexpression of EGFP-spastin severed microtubules. Reuse permission granted by Molecular Biology of the Cell (102).

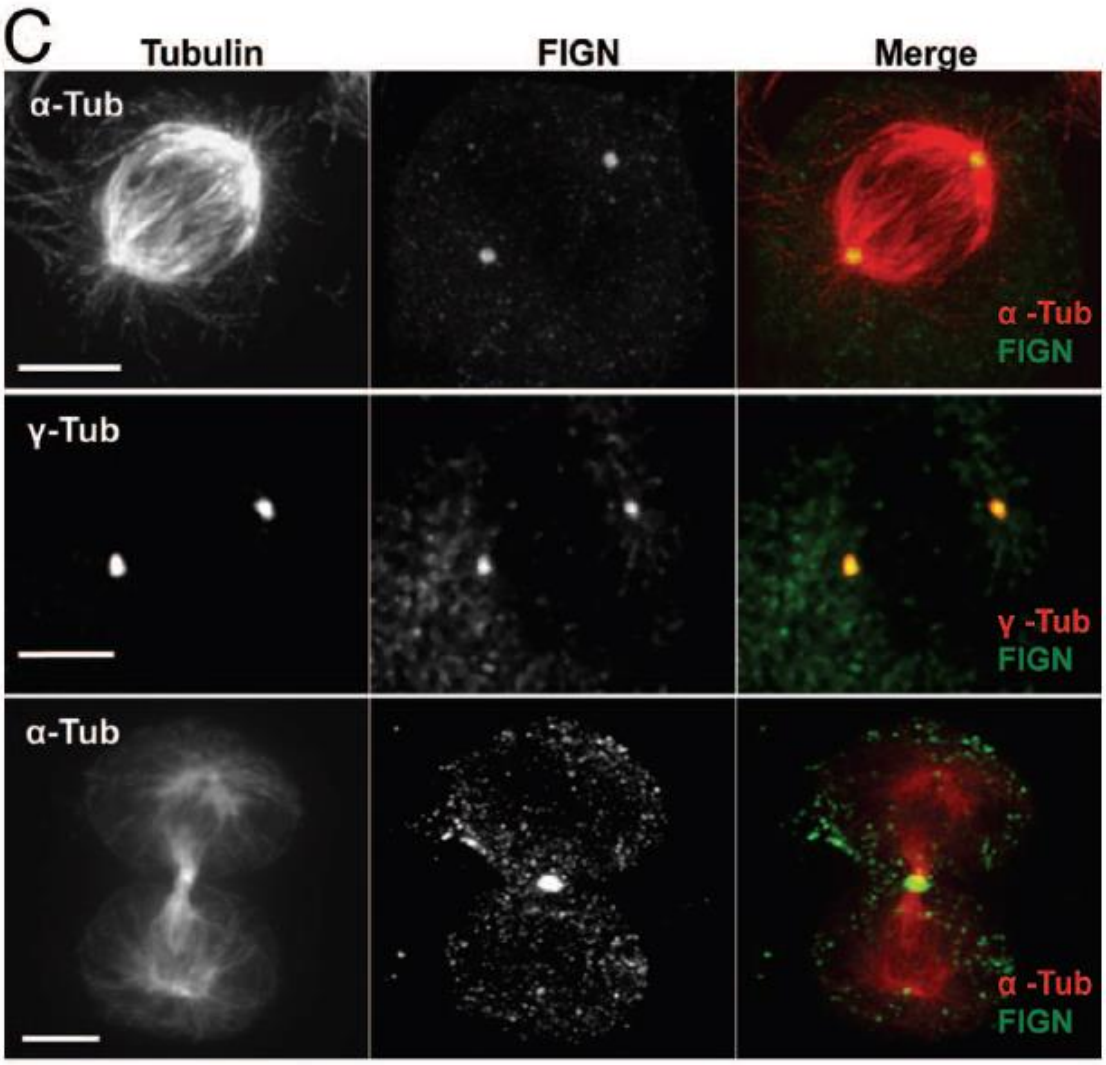

Figure 1.10: Immunofluorescence micrographs showing human FIGN localized to mitotic centrosomes in human U-2 OS cell line.

FIGN (green) and α-tubulin (red) immunolocalized in top and bottom panel showing metaphase and telophase cells, respectively. Middle panel shows staining for FIGN (green) and γ-tubulin (red) during metaphase. Reuse permission granted by Taylor and Francis Group (104).

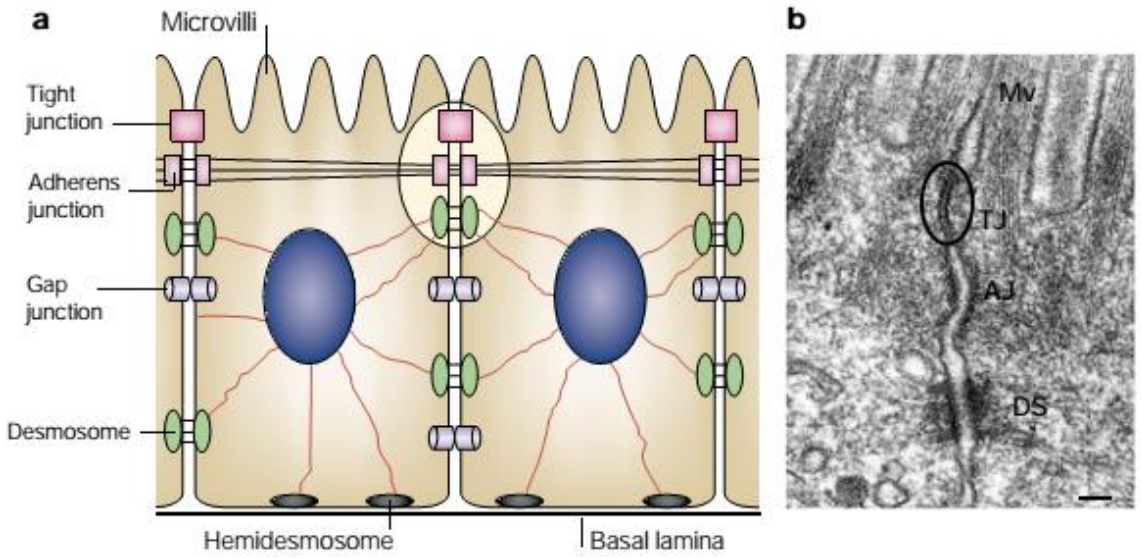

Figure 1.11: Epithelial intercellular junctions.
A) Schematic drawing of intestinal epithelial cells highlighting three types of junctions in a circle, located apically at the lateral membrane of adjacent cells. B) Electron micrograph of intercellular junctions in mouse intestinal epithelial cells, with a circle around the tight junction. (Mv, microvilli; TJ, tight junction; AJ, adherens junction; DS, desmosome). Scale bar, 200nm. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews (116), copyright 2001.

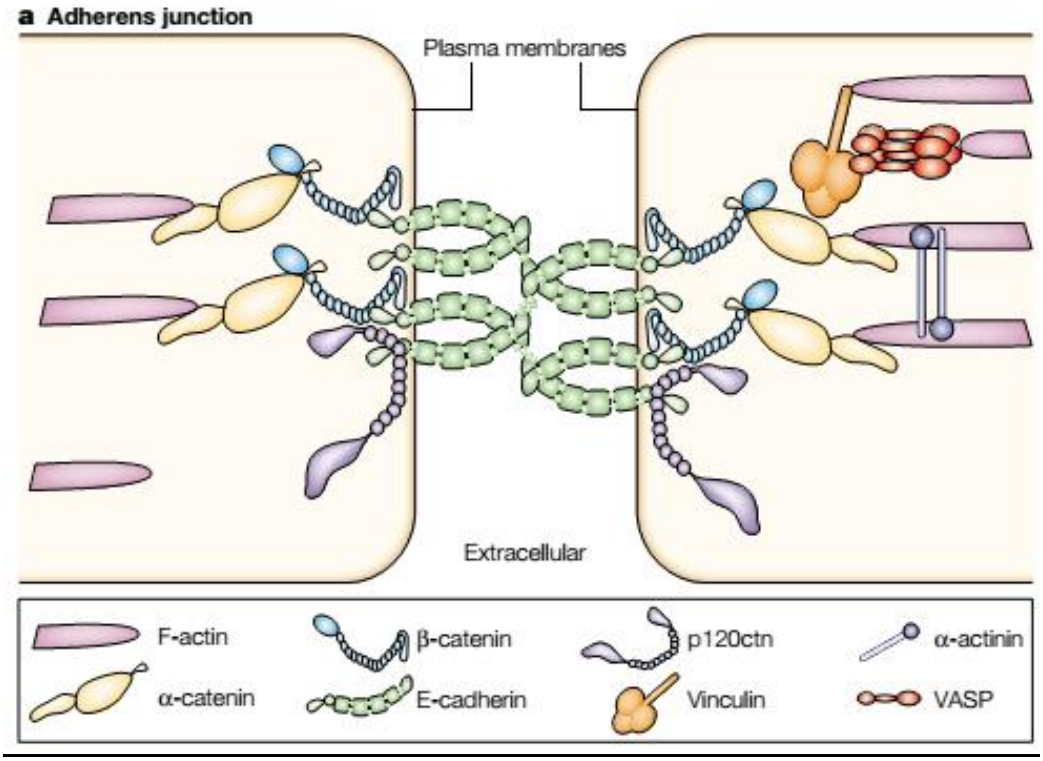

Figure 1.12: Adherens junction in the epidermis.
The adherens junctions form a bridge between the actin cytoskeleton of neighboring cells through association between transmembrane E-cadherin proteins. Catenin proteins connect the E-

cadherin proteins to actin cytoskeleton. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics (137), copyright 2002.

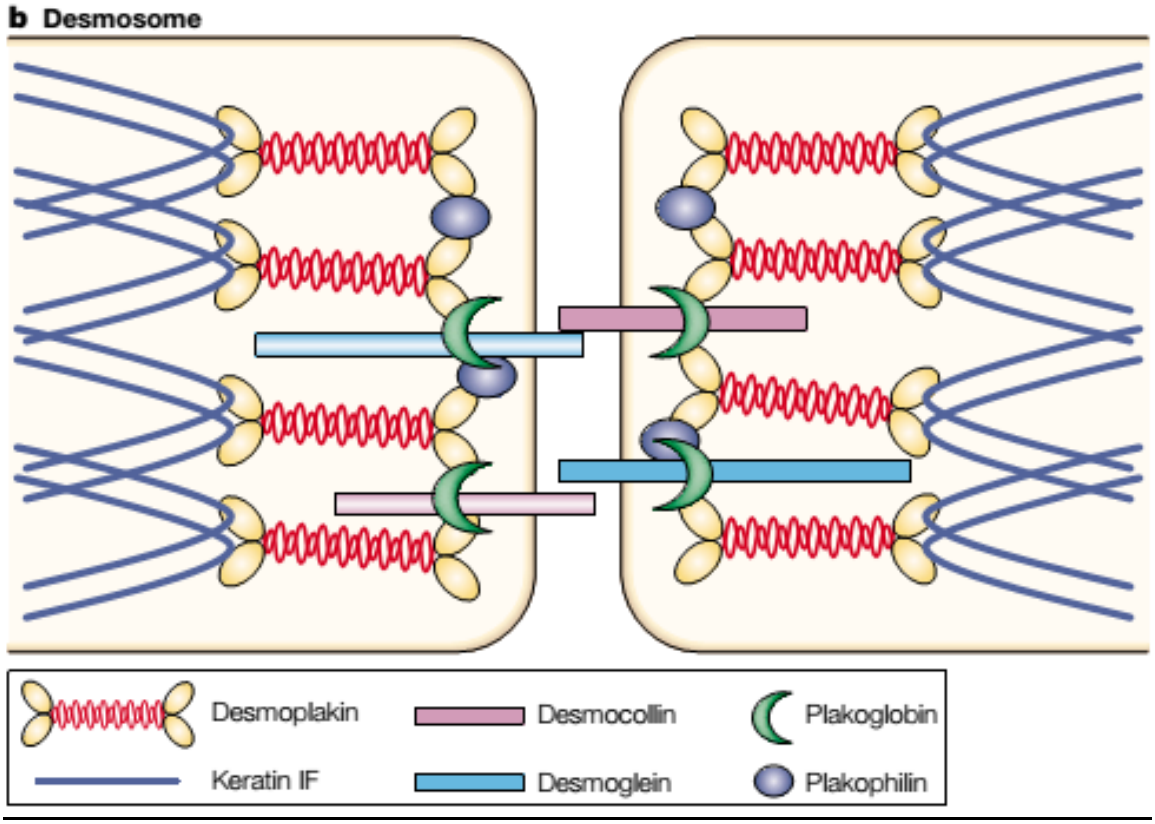

Figure 1.13: Simplified model of desmosomes in the epidermis.
Desmogleins (blue) and desmocollins (pink) form the core of desmosomes, plakoglobin (green) and plakophlin (purple) connect the desmogleins and desmocollins to desmoplakin (red). Desmoplakins connect the core of desmosomes to intermediate filaments (keratin filaments appear blue). Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics (137), copyright 2002.

1.8. References

1. Mohanty BR, Sahoo PK. Edwardsiellosis in fish: a brief review. J Biosci. 2007;32:1–14.

2. Janda MJ, Abbott SL. Infections associated with the genus *Edwardsiella*: the role of *Edwardsiella tarda* in human disease. Clin Infect Dis. 1993;17:742–8.

3. Shao S, Lai Q, Liu Q, Wu H, Xiao J, Shao Z, et al. Phylogenomics characterization of a highly virulent *Edwardsiella* strain ET080813(T) encoding two distinct T3SS and three T6SS gene clusters: Propose a novel species as *Edwardsiella anguillarum* sp. nov. Syst Appl Microbiol. 2015;38:36–47.

4. Park S Bin, Aoki T, Jung TS. Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish. Vet Res. 2012;43:1-11.

5. Hawke JP, Mcwhorter AC, Steigerwalt AG, Brenners DJ. *Edwardsiella ictaluri* sp. nov., the causative agent of enteric septicemia of catfish. Int J Syst Bacteriol. 1981;31:396–400.

6. Janda JM, Abbott SL, Kroske-Bystrom S, Cheung WKW, Powers C, Kokka RP, et al. Pathogenic properties of *Edwardsiella* species. J Clin Microbiol. 1991;29:1997–2001.

7. Yousuf RM, How SH, Amran M, Hla KT, Shah A, Francis A. *Edwardsiella tarda* septicemia with underlying multiple liver abscesses. Malays J Pathol. 2006;28:49–53.

8. Yang M, Lv Y, Xiao J, Wu H, Zheng H, Liu Q, et al. *Edwardsiella* comparative phylogenomics reveal the new intra/inter-species taxonomic relationships, virulence evolution and niche adaptation mechanisms. PLoS One. 2012;7:1-15.

9. Abayneh T, Colquhoun DJ, Sorum H. *Edwardsiella piscicida* sp.nov., a novel species pathogenic to fish. J Appl Microbiol. 2013;114:644–54.

10. Ling SHM, Wang XH, Xie L, Lim TM, Leung KY, Ling H. M. S. Use of green fluorescent protein (GFP) to study the invasion pathways of *Edwardsiella tarda* in *in vivo* and *in vitro* fish models. Microbiology. 2000;146:7–19.

11. Wang Q, Yang M, Xiao J, Wu H, Wang X, Lv Y, et al. Genome sequence of the versatile fish pathogen *Edwardsiella tarda* provides insights into its adaptation to broad host ranges and intracellular niches. PLoS One. 2009;4:1-16.

12. Xu T, Zhang XH. *Edwardsiella tarda*: An intriguing problem in aquaculture. Aquaculture. 2014;431:129–35.

13. Shafiei S, Viljamaa-Dirks S, Sundell K, Heinikainen S, Abayneh T, Wiklund T. Recovery of *Edwardsiella piscicida* from farmed whitefish, *Coregonus lavaretus* (L.), in Finland. Aquaculture. 2016;454:19–26.

14. Vandepitte J, Lemmens P, De Swert L. Human edwardsiellosis traced to ornamental fish. J Clin Microbiol. 1983;17:165–7.

15. Wang IK, Kuo HL, Chen YM, Lin CL, Chang HY, Chuang FR, et al. Extraintestinal manifestations of *Edwardsiella tarda* infection. Int J Clin Pract. 2005;59:917–21.

16. Tamada T, Koganemaru H, Matsumoto K, Hitomi S. Urosepsis caused by *Edwardsiella tarda*. J Infect Chemother. 2009;15:191–4.

17. Nishida K, Kato T, Yuzaki I, Suganuma T. *Edwardsiella tarda* bacteremia with metastatic gastric cancer. IDCases. 2016;5:76–7.

18. Hirai Y, Asahata-Tago S, Ainoda Y, Fujita T, Kikuchi K. *Edwardsiella tarda* bacteremia. A rare but fatal water- and foodborne infection: review of the literature and clinical cases from a single centre. Can J Infect Dis Med Microbiol. 2015;26:313–8.

19. Park S Il. Disease Control in Korean Aquaculture. Fish Pathol. 2009;44:19–23.

20. Wang X, Lu C. Mice orally vaccinated with *Edwardsiella tarda* ghosts are significantly protected against infection. Vaccine. 2009;27:1571–8.

21. Lan MZ, Peng X, Xiang MY, Xia ZY, Bo W, Jie L, et al. Construction and characterization of a live, attenuated *esrB* mutant of *Edwardsiella tarda* and its potential as a vaccine against the haemorrhagic septicaemia in turbot, *Scophthamus maximus* (L.). Fish Shellfish Immunol. 2007;23:521–30.

22. Jiao X, Zhang M, Hu Y, Sun L. Construction and evaluation of DNA vaccines encoding *Edwardsiella tarda* antigens. Vaccine. 2009;27:5195–202.

23. Liu X, Zhang H, Jiao C, Liu Q, Zhang Y, Xiao J. Flagellin enhances the immunoprotection of formalin-inactivated *Edwardsiella tarda* vaccine in turbot. Vaccine. 2017;35:369–74.

24. Mizunoe S, Yamasaki T, Tokimatsu I, Matsunaga N, Kushima H, Hashinaga K, et al. A case of empyema caused by *Edwardsiella tarda*. J Infect. 2006;53:10–3.

25. Clarridge JE, Musher DM, Fainstein V, Wallace RJ. Extraintestinal human infection caused by *Edwardsiella tarda*. J Clin Microbiol. 1980;11:511–4.

26. Yu JE, Cho MY, Kim J woo, Kang HY. Large antibiotic-resistance plasmid of *Edwardsiella tarda* contributes to virulence in fish. Microb Pathog. 2012;52:259–66.

27. Tan YP, Zheng J, Tung SL, Rosenshine I, Leung KY. Role of type III secretion in *Edwardsiella tarda* virulence. Microbiology. 2005;151:2301–13.

28. Zheng J, Leung KY. Dissection of a type VI secretion system in *Edwardsiella tarda*. Mol Microbiol. 2007;66:1192–206.

29. Okuda J, Arikawa Y, Takeuchi Y, Mahmoud MM, Suzaki E, Kataoka K, et al. Intracellular replication of *Edwardsiella tarda* in murine macrophage is dependent on the type III secretion system and induces an up-regulation of anti-apoptotic NF-κB target genes protecting the macrophage from staurosporine-induced apoptosis. Microb Pathog. 2006;41:226–40.

30. Janda JM, Abbott SL, Oshiro LS. Penetration and replication of *Edwardsiella spp.* in HEp-2 cells. Infect Immun. 1991;59:154–61.

31. Strauss EJ, Ghori N, Falkow S. An *Edwardsiella tarda* strain containing a mutation in a gene with homology to *shlB* and *hpmB* is defective for entry into epithelial cells in culture. Infect Immun. 1997;65:3924–32.

32. Wang B, Yu T, Dong X, Zhang Z, Song L, Xu Y, et al. *Edwardsiella tarda* invasion of fish cell lines and the activation of divergent cell death pathways. Vet Microbiol. 2013;163:282–9.

33. Galán JE, Lara-Tejero M, Marlovits TC, Wagner S. Bacterial type III secretion systems: specialized nanomachines for protein delivery into target cells. 2014; 68:415–38.

34. Filloux A. Microbiology: a weapon for bacterial warfare. Nature. 2013;500:284–5.

35. Basler M, Pilhofer M, Henderson GP, Jensen GJ, Mekalanos JJ. Type VI secretion requires a dynamic contractile phage tail-like structure. Nature. 2012;483:182–6.

36. Wan B, Zhang Q, Ni J, Li S, Wen D, Li J, et al. Type VI secretion system contributes to enterohemorrhagic *Escherichia coli* virulence by secreting catalase against host reactive oxygen species (ROS). PLoS Pathog. 2017;13:1–28.

37. Pukatzki S, Ma AT, Sturtevant D, Krastins B, Sarracino D, Nelson WC, et al. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. Proc Natl Acad Sci. 2006;103:1528–33.

38. Ma AT, Mekalanos JJ. *In vivo* actin cross-linking induced by *Vibrio cholerae* type VI secretion system is associated with intestinal inflammation. Proc Natl Acad Sci USA. 2010;107:4365–70.

39. Hood RD, Singh P, Hsu F, Güvener T, Carl MA, Trinidad RRS, et al. A type VI Secretion system of *Pseudomonas aeruginosa* targets a toxin to bacteria. Cell Host Microbe. 2010;7:25–37.

40. Hachani A, Lossi NS, Hamilton A, Jones C, Bleves S, Albesa-Jové D, et al. Type VI secretion system in *Pseudomonas aeruginosa*: secretion and multimerization of VgrG proteins. J Biol Chem. 2011;286:12317–27.

41. Ellermeier JR, Slauch JM. Adaptation to the host environment: regulation of the SPI1 type III secretion system in *Salmonella enterica* serovar Typhimurium. Curr Opin Microbiol. 2007;10:24–9.

42. Portaliou AG, Tsolis KC, Loos MS, Zorzini V, Economou A. Type III secretion: building and operating a remarkable nanomachine. Trends in Biochemical Sciences. 2016;41:175–89.

43. Deng W, Marshall NC, Rowland JL, McCoy JM, Worrall LJ, Santos AS, et al. Assembly, structure, function and regulation of type III secretion systems. Nat Rev Microbiol. 2017;15:323–37.

44. Yoshida S, Katayama E, Kuwae A, Mimuro H, Suzuki T, Sasakawa C. *Shigella* deliver an effector protein to trigger host microtubule destabilization, which promotes Rac1 activity and efficient bacterial internalization. EMBO J. 2002;21:2923–35.

45. Kuhle V, Hensel M. Cellular microbiology of intracellular *Salmonella enterica*: functions of the type III secretion system encoded by *Salmonella* pathogenicity island 2. Cellular and Molecular Life Sciences. 2004;61:2812–26.

46. Joshi A, Kostiuk B, Rogers A, Teschler J, Pukatzki S, Yildiz FH. Rules of engagement: the type VI secretion system in *Vibrio cholerae.* Trends Microbiol. 2017;25:267–79.

47. Wang X, Wang Q, Xiao J, Liu Q, Wu H, Xu L, et al. *Edwardsiella tarda* T6SS component *evpP* is regulated by *esrB* and iron, and plays essential roles in the invasion of fish. Fish Shellfish Immunol. 2009;27:469–77.

48. Chen H, Yang D, Han F, Tan J, Zhang L, Xiao J, et al. The bacterial T6SS effector *evpP* prevents NLRP3 inflammasome activation by inhibiting the Ca^{2+}-dependent MAPK-Jnk pathway. Cell Host Microbe. 2017;21:47–58.

49. Jobichen C, Chakraborty S, Li M, Zheng J, Joseph L, Mok YK, et al. Structural basis for the secretion of *evpC*: A key type VI secretion system protein from *Edwardsiella tarda*. PLoS One. 2010;5:1–10.

50. Srinivasa Rao PS, Yamada Y, Yuen PT, Ka YL. Use of proteomics to identify novel virulence determinants that are required for *Edwardsiella tarda* pathogenesis. Mol Microbiol. 2004;53:573–86.

51. Wang B, Mo ZL, Mao YX, Zou YX, Xiao P, Li J, et al. Investigation of EscA as a chaperone for the *Edwardsiella tarda* type III secretion system putative translocon component EseC. Microbiology. 2009;155:1260–71.

52. Zheng J, Li N, Tan YP, Sivaran J, Mok YK, Lan Mo Z, et al. EscC is a chaperone for the *Edwardsiella tarda* type III secretion system putative translocon components EseB and EseD. Microbiology. 2007;153:1953–62.

53. Xie HX, Yu HB, Zheng J, Nie P, Foster LJ, Mok YK, et al. EseG, an effector of the type III secretion system of *Edwardsiella tarda*, triggers microtubule destabilization. Infect Immun. 2010;78:5011–21.

54. Xie H-X, Lu J-F, Zhou Y, Yi J, Yu X-J, Leung KY, et al. Identification and functional characterization of the novel *Edwardsiella tarda* effector EseJ. Infect Immun. 2015;83:1650–60.

55. Hou M, Chen R, Yang D, Núñez G, Wang Z, Wang Q. Identification and functional characterization of EseH, a new effector of the type III secretion system of *Edwardsiella piscicida*. 2016;19:1-11.

56. Fang S, Zhang L, Lou Y, Yang D, Wang Q, Zhang Y, et al. Intracellular translocation and localization of *Edwardsiella tarda* type III secretion system effector EseG in host cells. Microb Pathog. 2016;97:166–71.

57. Xie HX, Lu JF, Rolhion N, Holden DW, Nie P, Zhou Y, et al. *Edwardsiella tarda*-induced cytotoxicity depends on its type III secretion system and flagellin. Infect Immun. 2014;82:3436–45.

58. Okuda J, Kiriyama M, Yamanoi E, Nakai T. The type III secretion system-dependent repression of NF-κB activation to the intracellular growth of *Edwardsiella tarda* in human epithelial cells. FEMS Microbiol Lett. 2008;283:9–14.

59. Zhou Y, Liu LY, He TT, Laghari ZA, Nie P, Gao Q, et al. *Edwardsiella tarda* EsaE (Orf19 protein) is required for the secretion of type III substrates, and pathogenesis in fish. Vet Microbiol. 2016;190:12–8.

60. Qin L, Sun Y, Zhao Y, Xu J, Bi K. *In vitro* model to estimate *Edwardsiella tarda*-macrophage interactions using RAW264.7 cells. Fish Shellfish Immunol. 2017;60:177–84.

61. Blazer VS. Piscine macrophage function and nutritional influences: a review. J Aquat Anim Health. 1991;3:77–86.

62. Ishibe K, Osatomi K, Hara K, Kanai K, Yamaguchi K, Oda T. Comparison of the responses of peritoneal macrophages from Japanese flounder (*Paralichthys olivaceus*) against high virulent and low virulent strains of *Edwardsiella tarda*. Fish Shellfish Immunol. 2008;24:243–51.

63. Rao PSS, Tit Meng Lim, Ka Yin Leung. Opsonized virulent *Edwardsiella tarda* strains are able to adhere to and survive and replicate within fish phagocytes but fail to stimulate reactive oxygen intermediates. Infect Immun. 2001;69:5689–97.

64. Stevens JM, Galyov EE, Stevens MP. Actin-dependent movement of bacterial pathogens. Nat Rev Microbiol. 2006;4:91–101.

65. Guttman JA, Finlay BB. Tight junctions as targets of infectious agents. Biochim Biophys Acta - Biomembr. 2009;1788:832–41.

66. Yoshida S, Sasakawa C. Exploiting host microtubule dynamics: A new aspect of bacterial invasion. Trends Microbiol. 2003;11:139–43.

67. Ashida H, Ogawa M, Kim M, Mimuro H, Sasakawa C. Bacteria and host interactions in the gut epithelial barrier. Nat Chem Biol. 2011;8:36–45.

68. Dosremedios CG, Chhabra D, Kekic M, Dedova I V, Tsubakihara M, Berry DA, et al. Actin binding proteins: regulation of cytoskeletal microfilaments. Physiol Rev. 2003;83:433–73.

69. Pollard TD. Actin and actin-binding proteins. Cold Spring Harb Perspect Biol. 2016;8.

70. Hanna S, El-Sibai M. Signaling networks of Rho GTPases in cell motility. Cellular Signalling. 2013;25:1955–61.

71. Rottner K, Lommel S, Wehland J, Stradal TEB. Pathogen-induced actin filament rearrangement in infectious diseases. J Pathol. 2004;204:396–406.

72. Cossart P. Actin-based motility of pathogens: The Arp2/3 complex is a central player. Cell Microbiol. 2000;2:195–205.

73. Hayward RD, Koronakis V. Direct modulation of the host cell cytoskeleton by *Salmonella* actin-binding proteins. Trends Cell Biol. 2002;12:15–20.

74. Patel JC, Galán JE. Manipulation of the host actin cytoskeleton by *Salmonella* - all in the name of entry. Curr Opin Microbiol. 2005;8:10–5.

75. Gouin E, Gantelet H, Egile C, Lasa I, Ohayon H, Villiers V, et al. A comparative study of the actin-based motilities of the pathogenic bacteria *Listeria monocytogenes*, *Shigella flexneri* and *Rickettsia conorii*. J Cell Sci. 1999;112:1697–708.

76. Kline-Smith SL, Walczak CE. Mitotic spindle assembly and chromosome segregation: refocusing on microtubule dynamics. Molecular Cell. 2004;15:317–27.

77. Caviston JP, Holzbaur ELF. Microtubule motors at the intersection of trafficking and transport. Trends Cell Biol. 2006;16:530–7.

78. Preciado López M, Huber F, Grigoriev I, Steinmetz MO, Akhmanova A, Koenderink GH, et al. Actin-microtubule coordination at growing microtubule ends. Nat Commun. 2014;5:4778.

79. Akhmanova A, Steinmetz MO. Control of microtubule organization and dynamics: two ends in the limelight. Nat Rev Mol Cell Biol. 2015;16:711–26.

80. Caviston JP, Holzbaur ELF. Microtubule motors at the intersection of trafficking and transport. Trends in Cell Biology. 2006;16:530–7.

81. Maiato H, Sampaio P, Sunkel CE. Microtubule-associated proteins and their essential roles during mitosis. International Review of Cytology. 2004;241:53–153.

82. Sharp DJ, Ross JL. Microtubule-severing enzymes at the cutting edge. J Cell Sci. 2012;125:2561–9.

83. Cassimeris L, Spittle C. Regulation of microtubule-associated proteins. International Review of Cytology. 2001;210:163–226.

84. Walczak CE, Gayek S, Ohi R. Microtubule-Depolymerizing Kinesins. Annu Rev Cell Dev Biol. 2013;29:417–41.

85. Roll-Mecak A, McNally FJ. Microtubule-severing enzymes. Curr Opin Cell Biol. 2010;22:96–103.

86. Rodriguez OC, Schaefer AW, Mandato C a, Forscher P, Bement WM, Waterman-Storer CM. Conserved microtubule-actin interactions in cell movement and morphogenesis. Nat Cell Biol. 2003;5:599–609.

87. Sharma N, Bryant J, Wloga D, Donaldson R, Davis RC, Jerka-Dziadosz M, et al. Katanin regulates dynamics of microtubules and biogenesis of motile cilia. J Cell Biol. 2007;178:1065–79.

88. Baas PW, Sharma V. Cell migration: Katanin gives microtubules a trim. Curr Biol. 2011;21.

89. Baas PW, Karabay A, Qiang L. Microtubules cut and run. Trends in Cell Biology. 2005;15:518–24.

90. Hartman JJ, Mahr J, McNally K, Okawa K, Iwamatsu A, Thomas S, et al. Katanin, a microtubule-severing protein, is a novel AAA ATPase that targets to the centrosome using a WD40-containing subunit. Cell. 1998;93:277–87.

91. Cheung K, Senese S, Kuang J, Bui N, Ongpipattanakul C, Gholkar A, et al. Proteomic analysis of the mammalian katanin family of microtubule-severing enzymes defines KATNBL1 as a regulator of mammalian katanin microtubule-severing. Mol Cell Proteomics. 2016;15.5:1658-69.

92. Johjima A, Noi K, Nishikori S, Ogi H, Esaki M, Ogura T. Microtubule severing by katanin p60 AAA+ ATPase requires the C-terminal acidic tails of both alpha- and beta-tubulins and basic amino acid residues in the AAA plus ring pore. J Biol Chem. 2015;290:11762–70.

93. Ghosh DK, Dasgupta D, Guha A. Models, regulations, and functions of microtubule severing by katanin. ISRN Mol Biol. 2012;2012:1–14.

94. McNally FJ, Okawa K, Iwamatsu a, Vale RD. Katanin, the microtubule-severing ATPase, is concentrated at centrosomes. J Cell Sci. 1996;109:561–7.

95. Yoshida S, Katayama E, Kuwae A, Mimuro H, Suzuki T, Sasakawa C, et al. KL1 is a novel microtubule severing enzyme that regulates mitotic spindle architecture. Cell Cycle. 2015;2:2546–61.

96. Buster D, McNally K, McNally FJ. Katanin inhibition prevents the redistribution of gamma-tubulin at mitosis. J Cell Sci. 2002;115:1083–92.

97. McNally KP, Bazirgan O a, McNally FJ. Two domains of p80 katanin regulate microtubule severing and spindle pole targeting by p60 katanin. J Cell Sci. 2000;113:1623–33.

98. O'Donnell L, Rhodes D, Smith SJ, Merriner DJ, Clark BJ, Borg C, et al. An essential role for katanin p80 and microtubule severing in male gamete production. PLoS Genet. 2012;8:1-12.

99. Errico A, Ballabio A, Rugarli EI. Spastin, the protein mutated in autosomal dominant hereditary spastic paraplegia, is involved in microtubule dynamics. Hum Mol Genet. 2002;11:153–63.

100. Kasher PR, De Vos KJ, Wharton SB, Manser C, Bennett EJ, Bingley M, et al. Direct evidence for axonal transport defects in a novel mouse model of mutant spastin-induced hereditary spastic paraplegia (HSP) and human HSP patients. J Neurochem. 2009;110:34–44.

101. Roll-Mecak A, Vale RD. Structural basis of microtubule severing by the hereditary spastic paraplegia protein spastin. Nature. 2008;451:363–7.

102. Yu W, Qiang L, Solowska JM, Karabay A, Korulu S, Baas PW. The Microtubule-severing proteins spastin and katanin participate differently in the formation of axonal branches. Mol Biol Cell. 2008;19:1485–98.

103. Sherwood NT, Sun Q, Xue M, Zhang B, Zinn K. *Drosophila* spastin regulates synaptic microtubule networks and is required for normal motor function. PLoS Biol. 2004;2:2094-2111.

104. Mukherjee S, Diaz Valencia JD, Stewman S, Metz J, Monnier S, Rath U, et al. Human fidgetin is a microtubule severing enzyme and minus-end depolymerase that regulates mitosis. Cell Cycle. 2012;11:2359–66.

105. Peng W, Lin Z, Li W, Lu J, Shen Y, Wang C. Structural insights into the unusually strong ATPase activity of the AAA domain of the *Caenorhabditis elegans* fidgetin-like 1 (FIGL-1) protein. J Biol Chem. 2013;288:29305–12.

106. Charafeddine RA, Makdisi J, Schairer D, O'Rourke BP, Diaz-Valencia JD, Chouake J, et al. Fidgetin-like 2: a microtubule-based regulator of wound healing. J Invest Dermatol. 2015;135:2309–18.

107. Zhang D, Rogers GC, Buster DW, Sharp DJ. Three microtubule severing enzymes contribute to the "Pacman- flux" machinery that moves chromosomes. J Cell Biol. 2007;177:231–42.

108. Yang Y, Mahaffey CL, Bérubé N, Nystuen A, Frankel WN. Functional characterization of fidgetin, an AAA-family protein mutated in fidget mice. Exp Cell Res. 2005;304:50–8.

109. Tomson FL, Viswanathan VK, Kanack KJ, Kanteti RP, Straub K V., Menet M, et al. Enteropathogenic *Escherichia coli* EspG disrupts microtubules and in conjunction with Orf3 enhances perturbation of the tight junction barrier. Mol Microbiol. 2005;56:447–64.

110. Furuse M. Molecular basis of the core structure of tight junctions. 2010;2:1-14.

111. Goldman RD, Grin B, Mendez MG, Kuczmarski ER. Intermediate filaments: versatile building blocks of cell structure. Current Opinion in Cell Biology. 2008;20:28–34.

112. Çolakoğlu G, Brown A. Intermediate filaments exchange subunits along their length and elongate by end-to-end annealing. J Cell Biol. 2009;185:769–77.

113. Herrmann H, Aebi U. Intermediate filaments: structure and assembly. Cold Spring Harb Perspect Biol. 2016;8:1-22.

114. Margiotta A, Bucci C. Role of intermediate filaments in vesicular traffic. Cells. 2016;5:20.

115. Kumar Y, Valdivia RH. Actin and intermediate filaments stabilize the *Chlamydia trachomatis* vacuole by forming dynamic structural scaffolds. Cell Host Microbe. 2008;4:159–69.

116. Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol. 2001;2:285–93.

117. Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol. 2001;2:285–93.

118. Meng W, Takeichi M. Adherens junction: molecular architecture and regulation. Cold Spring Harb Perspect Biol. 2009;1:1–13.

119. Fanning a S, Mitic LL, Anderson JM. Transmembrane proteins in the tight junction barrier. J Am Soc Nephrol. 1999;10:1337–45.

120. Desai B V, Harmon RM, Green KJ. Desmosomes at a glance. J Cell Sci. 2009;122:4401–7.

121. Umeda K, Ikenouchi J, Katahira-Tayama S, Furuse K, Sasaki H, Nakayama M, et al. ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. Cell. 2006;126:741–54.

122. Zihni C, Mills C, Matter K, Balda MS. Tight junctions: from simple barriers to multifunctional molecular gates. Nat Rev Mol Cell Biol. 2016;17:564–80.

123. Coyne CB, Bergelson JM. CAR: A virus receptor within the tight junction. Advanced Drug Delivery Reviews. 2005;57:869–82.

124. Schulzke JD, Gitter AH, Mankertz J, Spiegel S, Seidler U, Amasheh S, et al. Epithelial transport and barrier function in occludin-deficient mice. Biochim Biophys Acta- Biomembr. 2005;1669:34–42.

125. Saitou M, Fujimoto K, Doi Y, Itoh M, Fujimoto T, Furuse M, et al. Occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions. J Cell Biol. 1998;141:397–408.

126. Furuse M, Fujita K, Hiiragi T, Fujimoto K, Tsukita S. Claudin-1 and -2: Novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. J Cell Biol. 1998;141:1539–50.

127. Günzel D, Yu ASL. Claudins and the modulation of tight junction permeability. Physiological reviews. 2013;93:525-69.

128. Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y, et al. Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. J Cell Biol. 2002;156:1099–111.

129. Hou J, Renigunta A, Gomes AS, Hou M, Paul DL, Waldegger S, et al. Claudin-16 and claudin-19 interaction is required for their assembly into tight junctions and for renal reabsorption of magnesium. Proc Natl Acad Sci USA. 2009;106:15350–5.

130. Ben-Yosef T, Belyantseva IA, Saunders TL, Hughes ED, Kawamoto K, Van Itallie CM, et al. Claudin 14 knockout mice, a model for autosomal recessive deafness DFNB29, are deaf due to cochlear hair cell degeneration. Human Molecular Genetics. 2003;12:2049–61.

131. Glotfelty LG, Hecht GA. Enteropathogenic *E. coli* effectors EspG1/G2 disrupt tight junctions: new roles and mechanisms. Ann N Y Acad Sci. 2012;1258:149–58.

132. Lynch T, Livingstone S, Buenaventura E, Lutter E, Fedwick J, Buret AG, et al. *Vibrio parahaemolyticus* disruption of epithelial cell tight junctions occurs independently of toxin production. Infect Immun. 2005;73:1275–83.

133. Meertens L, Bertaux C, Cukierman L, Cormier E, Lavillette D, Cosset F-L, et al. The tight junction proteins claudin-1, -6, and -9 are entry cofactors for Hepatitis C virus. J Virol. 2008;82:3555–60.

134. Baum B, Georgiou M. Dynamics of adherens junctions in epithelial establishment, maintenance, and remodeling. Journal of Cell Biology. 2011;192:907–17.

135. Noren NK, Liu BP, Burridge K, Kreft B. p120 catenin regulates the actin cytoskeleton via Rho family GTPases. J Cell Biol. 2000;150:567–80.

136. Halbleib JM, Nelson WJ. Cadherins in development: cell adhesion, sorting, and tissue morphogenesis. Genes and Development. 2006;20:3199–214.

137. Fuchs E, Raghavan S. Getting under the skin of epidermal morphogenesis. Nat Rev Genet. 2002;3:199–209.

138. Bonazzi M, Lecuit M, Cossart P. *Listeria monocytogenes* internalin and E-cadherin: from structure to pathogenesis. Cellular Microbiology. 2009;11:693–702.

139. Anderton JM, Rajam G, Romero-Steiner S, Summer S, Kowalczyk AP, Carlone GM, et al. E-cadherin is a receptor for the common protein pneumococcal surface adhesin A (PsaA) of *Streptococcus pneumoniae*. Microb Pathog. 2007;42:225-36.

140. Kottke MD, Delva E, Kowalczyk AP. The desmosome: cell science lessons from human diseases. J Cell Sci. 2006;119:797–806.

141. Broussard JA, Getsios S, Green KJ. Desmosome regulation and signaling in disease. Cell and Tissue Research. 2015;360:501–12.

142. Sonnenberg A, Liem RKH. Plakins in development and disease. Exp Cell Res. 2007;313:2189–203.

143. Amagai M, Yamaguchi T, Hanakawa Y, Nishifuji K, Sugai M, Stanley JR. Staphylococcal exfoliative toxin B specifically cleaves desmoglein 1. J Invest Dermatol. 2002;118:845–50.

Chapter 2.

Identification and characterization of bacterial and host epithelial factors involved in microtubule disassembly in epithelial cells during *Edwardsiella* infections

Abstract

Edwardsiella bacteria cause economic losses to a variety of commercially important fish including the Japanese Eel, Channel Catfish and Turbot. Human infections are relatively rare and result in a gastroenteritis-like illness. Although macrophages are by far the best-studied cell types during these infections, epithelial cells are prominently infected, but their study during these infections has been sparse. Because these bacteria are evolutionarily related to other enteric pathogens and because the host cytoskeleton is a common target of enteric pathogens, we hypothesized that similar infections could be established to evaluate host actin, intermediate filament and microtubule alterations. In this study, we used HeLa and Caco-2 cells for *Edwardsiella* infection models and showed that microtubules were initially severed then completely disassembled during the infections, leaving the actin filaments and intermediate filaments unaltered. Immunolocalization experiments showed the katanin subunit A1 and katanin subunit AL1 at microtubule cut sites, suggesting their involvement in the microtubule disassembly event. To identify bacterial components involved in this phenotype, we screened a 2,758 mutant *Edwardsiella piscicida* transposon insertion library and found that 15 genes/proteins are key players in causing microtubule disassembly in epithelial cells. This work not only provides the first evidence of host cytoskeletal alterations during *Edwardsiella* infections but also provides a resource for further characterization of molecular components involved in microtubule disassembly in general.

2.1. Introduction

Enteric bacteria from the genus *Edwardsiella* cause hemorrhagic septicaemia, petechial hemorrhage and holes in the bodies of an array of commercially important fish

such as the Japanese Eel, Red Sea Bream, Yellowtail, Channel Catfish, and Turbot (1,17). Of the five known species within the *Edwardsiella* genus (*E. tarda*, *E. piscicida*, *E. anguillarum*, *E. ictaluri*, and *E. hoshinae*), only *E. tarda* is pathogenic to humans; causing various gastrointestinal or extraintestinal diseases in immunocompromised people (18–20). *E. tarda* does not have a type III secretion system (T3SS) or type VI secretion system (T6SS) (20). However, the related *Edwardsiella* species, *E. piscicida*, contains 1 T3SS and 1 T6SS and *E. anguillarum* has 2 T3SSs and 3 T6SSs (20). *E. piscicida* has been shown to invade and replicate in epithelial cells such as human epithelial type-2 cells (HEp-2) (6), and fish epithelioma papillosum of carp (EPC) cells (21), as well as phagocytic murine macrophages (J774A.1 and RAW264.7) (3,4), and fish primary macrophages (22).

Cytoskeletal systems are crucial for maintaining tissue integrity, but are also common targets of bacterial pathogens (12–14). Consequently, intracellular (e.g. *Shigella flexneri*, *Listeria monocytogenes*, and *Salmonella enterica* serovar Typhimurium) as well as extracellular pathogens (enteropathogenic *Escherichia coli*) hijack the actin filaments, microtubules and intermediate filaments networks during their infectious processes (13,23–25).The close evolutionary relationship amongst many of those bacteria to *Edwardsiella* (10,11) suggested that *Edwardsiella* may target similar molecular components. Thus, we hypothesized that host cytoskeletal structures might be altered by these microbes.

In this study, we used two epithelial cell culture models to study the host cytoskeletal organization during *Edwardsiella* infections. We show that although actin filaments and intermediate filaments remain morphologically unchanged, host microtubules are severed, then completely disassembled by these microbes. This severing is concurrent with the localization of the Katanin A1 and Katanin A subunit-like-1 (KATNAL1) microtubule-severing proteins at the microtubule cut-sites (18). Interestingly, the bacterial T3SS and T6SS are not required for MT severing to occur. To decipher which bacterial genes were required for the MT break-down phenotype, we screened an *E. piscicida* EIB 202 whole-genome transposon insertion mutant library (26) and found 15 mutants that lost their microtubule-severing abilities. These mutants had defects in F-type ATPase, nucleobase biosynthesis, metabolism, or transcription.

2.2. Results

2.2.1. Microtubules are severed during *Edwardsiella* infections

Key to studying host proteins during bacterial infections is our ability to view the proteins at endogenous levels. Although the use of fish-based cell lines would be ideal to study the infections, antibodies that are commercially available rarely cross-react with fish proteins. Because *E. tarda* also infects humans (27), we opted to develop and use human cell culture infection models. HeLa and Caco-2 cells both showed high bacterial loads, with bacterial replication clearly present within the monolayers (Figure 2.1). This was evident with 4 different *Edwardsiella* strains [EIB202 (*E. piscicida* strain, 1 T3SS and 1 T6SS), ATCC15947 (*E. tarda* type strain, no T3SS or T6SS), PPD130/91 (*E. piscicida* strain, 1 T3SS and 1 T6SS), and ET080813 (*E. anguillarum* type strain, 2 T3SSs and 3 T6SSs)] at MOIs ranging from 5-25 in both HeLa and Caco-2 cells (Figure 2.1).

Using these infection models, we immunolocalized filamentous actin, microtubules and intermediate filaments. Alterations were not observed with actin or intermediate filament cytoskeletons during the infections (Figure S1 & S2). However, microtubules were dramatically changed (Figure 2.2a, b), as microtubules appeared severed within cultured cells throughout the entire dish, including those cells not in the vicinity of the bacteria. This held true for all strains tested (Figure 2.2a, b). This phenotype was validated by live cell imaging, as microtubules appeared simultaneously cut at various locations within the same host cell which was followed by microtubule network disintegration (S1 video).

Our evidence using the *E. tarda* ATCC15947 strain that naturally lacked both T3SSs and T6SSs strongly indicated that effectors released by those secretion systems were not needed for the observed microtubule disassembly events (Figure 2.2). However, there is a T3SS effector (EseG) that has been shown to disassemble microtubules when overexpressed in transfected host cells (8). To further confirm that T3SS or T6SS bacterial effectors were not responsible for the observed microtubule severing we infected cultured cells with mutated *E. piscicida* EIB202 strains (ΔT3SS, ΔT6SS, ΔT3SS/ ΔT6SS, and Δ*esrB*), which all showed identical microtubule severing phenotypes (Figure 2.3).

2.2.2. Microtubule severing enzymes are present at microtubule cut-sites in host cells

Using antibodies to the 3 different classes of microtubule-severing proteins (katanins, spastin and fidgetin) (28) we found that two katanin proteins—katanin A1 and katanin A-like-1—localized to the microtubule cut-sites during *E. piscicida* EIB202 infections (Figures 2.4a, 2.5). Being able to catch this event was difficult as katanins rapidly release from the microtubules once severing is completed (16,29,30). The localization of these 2 proteins on the microtubules suggests that redundant mechanisms could be involved to ensure microtubule severing during *Edwardsiella* infections (Figures 2.4a, 2.5). Despite their localization, levels of katanin A1 proteins were decreased during *E. piscicida* infections (Figure 2.4b), whereas katanin A1 subunit-like 1 protein levels remain unaltered (Figure S3).

2.2.3. Bacterial genes involved in the microtubule severing phenotype

To determine the bacterial factor(s) needed for the microtubule-severing phonotype that we observed during the epithelial cell infections we screened an *E. piscicida* EIB202 transposon-insertion mutant library that covered ~70% of the genome (26). This negative screening approach was used to find the *E. piscicida* mutants that did not cause microtubule disassembly in the host. The candidates were scored based on the amount of disassembly observed at a specific time-point: high disassembly (>80% disassembly), low disassembly (<5%), and no disassembly. We marked the wells with no disassembly as those with bacteria harbouring mutations in potential gene(s) responsible for the host cell microtubule-severing event (Figure 2.6). We identified 15 bacterial candidates in the screen, 4 were components of an F-type H^+-transporting ATPase, 3 were involved in the biosynthesis of purines or pyrimidines, 3 were metabolic enzymes and 1 was a cAMP-activated global transcriptional regulator (CRP) (Table 2.1). These results suggest that although these enzymes are needed to ultimately activate the microtubule severing proteins to disassemble the host cell microtubules, there is likely a target that those enzymes are working on which will be the terminal activator.

2.3. Discussion

Pathogens often target the host cytoskeleton as part of their disease processes (23,31). By coupling an assortment of *Edwardsiella* species with host cell infections we demonstrate that microtubules, but not actin filaments or cytokeratin are destroyed. This microtubule alteration appears to have occurred through the activation of katanin and katanin-like proteins, which are known host microtubule severing proteins (16,32), as these proteins localized precisely at microtubule cut-sites. This disassembly event did not require bacterial invasion or T3SS effectors as host cells located far from the regions of bacterial contact also showed the microtubule severing. How can this occur? Our working hypothesis is that the bacteria release a factor (either a small molecule, protein or protein fragment) into the supernatant that ultimately either enters the host cell or activates a receptor to ultimately activate the katanin proteins to cut the host microtubules. To test for this hypothesis, spent bacterial infection supernatant was filtered and used to infect HeLa cells. Although, no MT disassembly was observed due to the bacterial supernatant, suggesting inactivation of the hypothetical protein or an alternative MT disassembly pathway. Using our negative selection approach we were able to identify 15 genes within *E. piscicida* that were needed for microtubule severing. However, we feel that those enzymes are likely not the key proteins used to trigger the event. Consequently, the use of a positive selection strategy may be needed as any redundancies *E. piscicida* utilizes to ensure host cell microtubule dismantling would unfortunately not be caught using our negative screen.

Why would *Edwardsiella* disassemble the host microtubules of all cells in the sample? Potential reasons may lie with the functions of microtubules as tracts for vesicle movement, maintenance of cell structure and cell motility as shutting down those processes leaves the cells more vulnerable to *Edwardsiella* attack as host mechanisms of bacterial control would be rendered non-functional. Although examined in human cells and not fish cells the observed phenotypes of infected fish with holes in their tissues could be a resulting phenotype of these cytoskeletal alterations as host cells would be hampered in their tissue repair and protein targeting mechanisms.

Microtubule severing is a novel strategy used by microbes to dismantle the host cytoskeletal structures. By identifying the key bacterial proteins involved and crucial steps in the katanin-regulatory pathway our work will enhance not only the bacterial

pathogenesis field but also the general field of cell biology. This work sets the foundation for our ultimate goal of blocking the key pathways used by *Edwardsiella* and to halt these infections.

2.4. Materials and methods

2.4.1. Bacterial Growth Conditions

Bacterial strains used in this study included wild type *Edwardsiella* strains: EIB202 (*E. piscicida*), ATCC15947 (*E. tarda*), and ET080813 (*E. anguillarum*) and mutants of EIB202 strain including Δ*eseBCD*/ΔT3SS (deletion of *eseB-eseD*), Δ*evpAB*/ΔT6SS (deletion of *evpA-evpB*), Δ*esrB* (in-frame deletion of *esrB*), and ΔT3SS/ΔT6SS (deletion of *eseB-eseD* and *evpA-evpB*). All strains were grown using standard tryptic soy broth (TSB) (BD Biosciences), supplemented with appropriate antibiotics. The EIB202 transposon-insertion mutant library was also cultured in TSB with appropriate antibiotics.

2.4.2. Cell Culture and Infections

Human colon adenocarcinoma (Caco-2) and human cervical epithelial (HeLa) cell lines were cultured in DMEM/FBS [DMEM containing high-glucose (HyClone, Thermo Scientific) supplemented with 10% FBS and 1% non-essential amino acids (Gibco, Life Technologies)) and DMEM/FBS [DMEM containing high-glucose (HyClone, Thermo Scientific) supplemented with 10% FBS] respectively. HeLa cells were selected as the epithelial cell model for much of this study due to their epithelial-like appearance. Caco-2 cells proved to be a useful model due to their ability to form intercellular junctions. Both cell lines were grown at 37 °C (5% CO_2). Cells were trypsinized with 0.05% Trypsin-EDTA (Gibco, Life Technologies) and seeded onto grass coverslips (Fisher Scientific) that were placed into 6-well plates (Corning, Fisher Scientific).

2.4.3. Infections

For standard immunofluorescence experiments, 2×10^5 HeLa cells were seeded on 22mm×22 mm square glass coverslips in 6-well plates containing 2 ml of DMEM + 10 % FBS two days prior to infection. For the Caco-2 infection model, 2.5×10^5 cells were

seeded on 22mm×22 mm square coverslips in 6 well plates three days prior to infection. Two days before infecting the cultured cells, *Edwardsiella* strains were streaked on TSA plates and grown for 24 hours at 30°C. Single colonies were picked from agar plates the following day and grown as standing cultures of 2 ml TSB at 30 °C for 16 hours.

As HeLa cells approach 80% confluence on infection day, the culture media was replaced with 2mL of infection media. For all the fish isolates, both HeLa and Caco-2 cells were infected with 25µL of overnight culture for 6 hours, while for the human isolate, 3 µl of overnight inoculum was used to infect for a period of 3 hours.

2.4.4. Bacterial supernatant infections

2×10^5 HeLa cells were seeded on 22mm×22 mm square glass coverslips in two 6-well plates containing 2 ml of DMEM + 10 % FBS two days prior to infection. Two days before infecting the cultured cells, EIB202 (*E. piscicida*) *was* streaked on TSA plate and grown for 24 hours at 30°C. Single colonies were picked from agar plate the following day and grown as standing cultures of 2 ml TSB at 30 °C for 16 hours. On infection day, the culture media in both 6-well plates was replaced with 2mL of infection media each. HeLa cells from one dish were infected with 25µL of overnight culture of EIB202 (*E. piscicida*) for 6 hours. 1mL of spent bacterial supernatant from this infection was filtered through 0.2µm pore size using acrodisc syringe filters (Pall Canada Cat. # 4614). The filtered supernatant was used to infect a fresh well in the second dish containing HeLa cells. These HeLa cells were infected with the filtered supernatant for 6 hours.

2.4.5. Immunofluorescence Staining

At the completion of infections, the samples were washed three times with phosphate-buffered saline (PBS-/-) without magnesium and calcium then fixed with 37°C 3 % paraformaldehyde for 15 min. Both PBS−/− and paraformaldehyde were warmed to 37 °C prior to use. Samples were then washed three times with PBS −/−. After the washes, cells were permeabilized using PBS−/− with 0.2 % Triton-X-100 for 5 min at room temperature (RT), and then washed three times with PBS −/−. The samples were then blocked with 5 % normal goat serum (NGS) (Life Technologies) for 20 min. and stained with primary antibodies overnight at 4°C. Primary antibodies consisted of: mouse anti-alpha tubulin (1:50) (Developmental studies hybridoma bank, catalog #12G10, 1

µg/mL), mouse anti-cytokeratin peptide 18 (Sigma, 1.5 µg/mL), rabbit anti-Katanin A1 (Sigma, 2.5 µg/mL) and rabbit anti-Katanin AL1 (Sigma, 2.5 µg/mL). The primary antibodies were pre-cleared prior to staining procedures. After multiple washes with TPBS-0.1% BSA, Alexa-488 and Alexa-594 conjugated goat antibodies (1:1000) against mouse and rabbit IgG were added to the appropriate samples and incubated for 2 hours at RT. After the secondary antibody incubation, the samples were washed multiple times with TPBS-0.1%BSA and mounted with Prolong Gold containing DAPI. Actin staining was done using Alexa Fluor™ 594 Phalloidin (Life technologies).

2.4.6. Fluorescence Microscopy Imaging

A Leica (Wetzlar, Germany) DMI4000b inverted fluorescent microscope with a Hamamatsu Orca R2 CCD camera (Hamamatsu, Japan) and Leica ×10 HI Plan 0.25 Phase 1, ×40 HCX Plan APO 0.75 Phase 2, and ×100 HCX Plan APO 1.40 oil Phase 3 CS objectives connected to a PC with Metamorph Imaging System software (Molecular Devices, Sunnyvale, CA, USA) was used for data visualization and analysis. Data integrity was maintained while images were processed on ImageJ (NIH, Bethesda, MD, USA) and Inkscape (USA).

2.4.7. Cell culture transfection and live cell imaging

HeLa cells were transfected with a mKate2-EB3 (Evrogen catalogue no. FB316) construct at a concentration of 2µg to label microtubules. Transfection was done using the jetPRIME transfection reagent according to manufacturer's protocol (Polypus Transfection, Illkirch, France). Cells were incubated at 37°C for 24 hours to allow expression of EB3 (end-binding 3) protein. For live cell imaging, a Chamlide IC top stage incubator system was used to maintain a constant temperature of 35°C and 5% humidified CO_2 gas in air. HeLa cells were infected with 3uL of *E. tarda* ATCC 15947 and incubated at 35°C for 1 hour and 30 minutes. Then, images were captured every 2 minutes.

2.4.8. Cell lysate preparation and western blotting

HeLa cells were grown in 6-well tissue culture dishes and infected at an MOI of 25-35 with *E. piscicida* for 6 hours. Cells were washed 3 times with PBS+/+ containing 1

mM CaCl2 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. 25µg of total protein was loaded on 10% SDS-polyacrylamide gels. After separation of protein bands, semi-dry transfer to nitrocellulose membranes (Bio-Rad Laboratories) was performed. 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. Primary antibodies consisted of rabbit anti-Katanin A1 (Sigma, 0.25 µg/mL), rabbit anti-Katanin AL1 (Sigma, 0.25 µg/mL), and mouse anti-GAPDH (Developmental studies hybridoma bank, 0.5 µg/mL). Antibodies were prepared in TBST containing 1% BSA. Membranes were incubated in primary antibody overnight at 4°C. After washing, membranes were incubated in horseradish peroxidase (HRP) - conjugated secondary antibody (Cell Signaling Technology) for two hours at RT. Signals were detected by enhanced chemiluminescence (Perkin Elmer) and images were captured by a Fujifilm LAS4000 chemiluminescent scanner.

2.4.9. Screening of the Transposon insertion mutant library

30,000 HeLa cells were seeded into 96-well glass bottom microtitre plates containing 75µL DMEM+10%FBS media. The next day, each of the mutant library strains were inoculated into 96-well dilution blocks and grown statically overnight for 16 hours at 30°C under gentamicin (15µg/mL) and colistin (20µg/mL) selection. Inoculations were done using a 48 Pin metal multi-blot replicator. The following day, 11µl of the overnight inoculum was used to infect HeLa cells in 96-well glass bottom microtitre plates for 3.5 hours at 35°C. Cells were fixed with 37 °C 3 % paraformaldehyde for 15 min, then washed three times with PBS without magnesium or calcium (PBS −/−). To permeabilize the samples, the cells were incubated with PBS with 0.2 % Triton-X-100 for 5 min at room temperature (RT), then washed three times with PBS −/−. The samples were then blocked with 5 % normal goat serum (NGS) (Life Technologies) for 20 min and stained with mouse anti-alpha tubulin antibody (Developmental studies hybridoma bank, catalog #12G10, 1 µg/mL) overnight at 4°C. After multiple washes with TPBS-BSA, Alexa-488 conjugated goat antibody (1:1000) against mouse IgG was added to the samples and incubated for 2 hours at RT. Following the secondary antibody incubation, the samples were washed multiple times with TPBS-0.1% BSA and viewed under the

microscope to image. This previously described protocol was repeated twice for all 2758 mutants.

The next two screens were performed in 24-well plates using the optimized optical density (HeLa cells were infected with identical OD for each mutant) for all library mutants. Individual mutants were streaked on tryptic soy agar supplemented with 15µg/mL of gentamicin and of 20µg/mL colistin. Single colonies were inoculated into 2mL tryptic soy broth containing both selection antibiotics for 16 hours. 60,000 HeLa cells were seeded into 24-well plates containing 1mL DMEM+10%FBS media. Infections were carried out at MOI of 15 for 6 hours. After 6 hours, cells were fixed, permeabilized, and blocked with NGS according to the protocol above and stained for microtubules using anti-alpha tubulin antibody (Developmental studies hybridoma bank, catalog #12G10, 1 µg/mL). Following the primary antibody staining, cells were stained with secondary Alexa-488 conjugated goat antibody (1:1000) against mouse anti-alpha-tubulin and mounted using Prolong Gold containing DAPI.

2.5. Figures

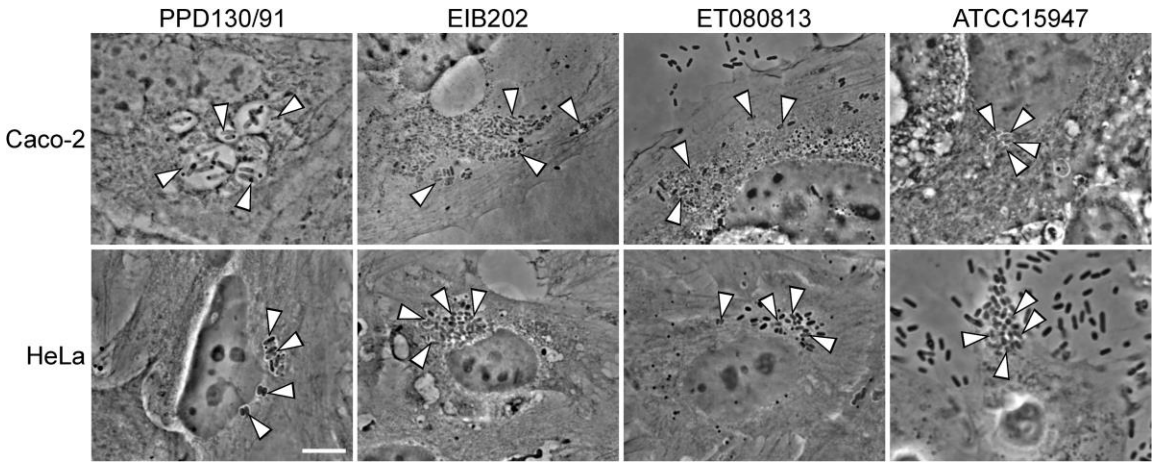

Figure 2.1: HeLa and Caco-2 cell models showing successful internalization of different *Edwardsiella* isolates.

E. piscicida PPD130/91 EIB202*, E. anguillarum* ET080813, and *E. tarda* ATCC 15947, were used to infect both HeLa and Caco-2 cells. Arrowheads point towards internalized bacteria. Scale bar, 10 µm.

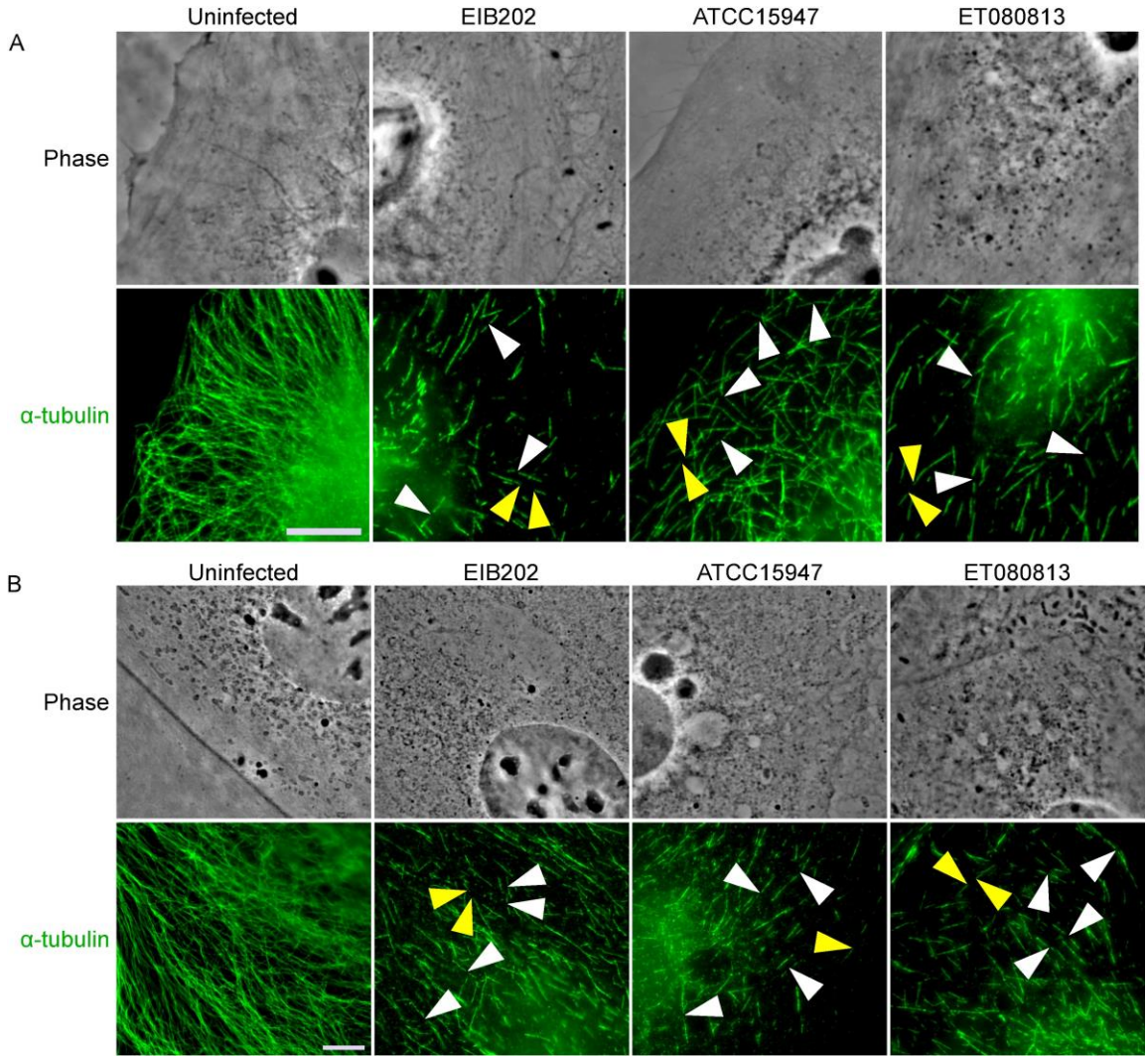

Figure 2.2: Host microtubule severing phenotype observed during *Edwardsiella* infections.

A) HeLa cells showing microtubule disassembly during infection with *E. piscicida* EIB202, *E. tarda* ATCC15947, and *E. anguillarum* ET080813. B) Caco-2 cells showing microtubule disassembly during infection with *E. piscicida* EIB202, *E. tarda* ATCC15947, and *E. anguillarum* ET080813. White arrowheads point to cuts in microtubules in both host cell types, and yellow arrowheads point to larger breaks in host microtubules. Scale bar, 10 µm.

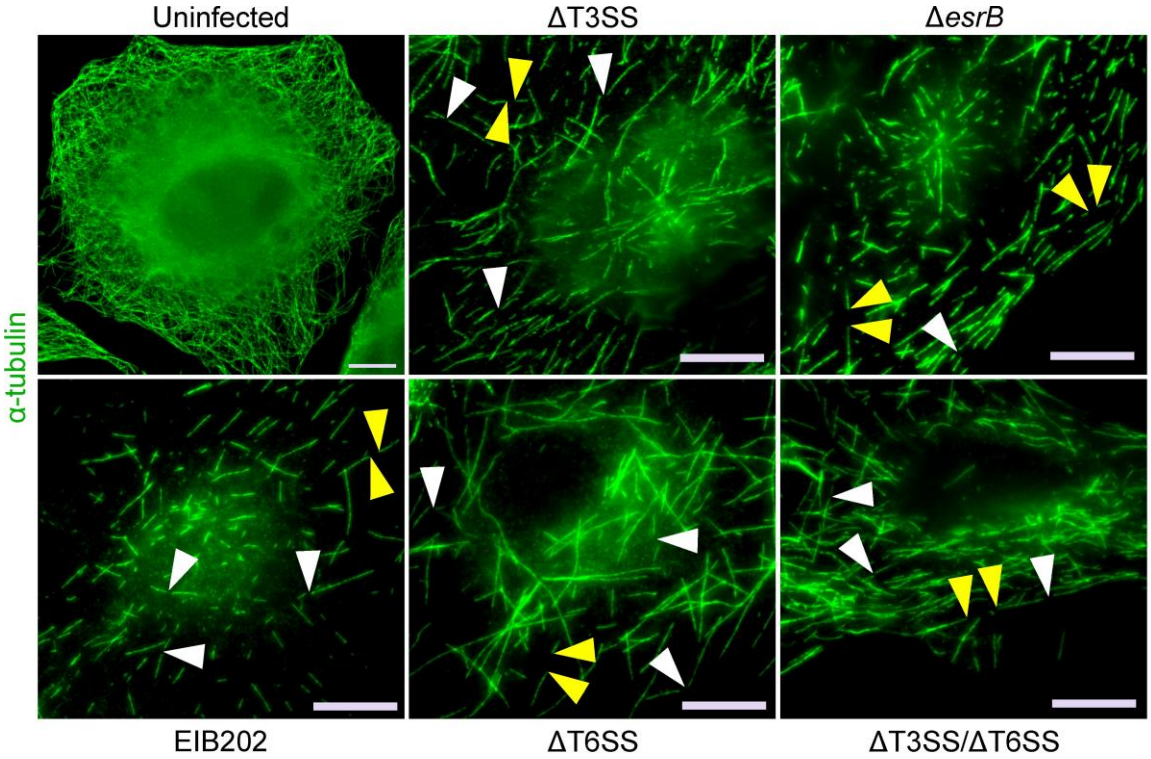

Figure 2.3: *E. piscicida* EIB202 T3SS and T6SS mutants sever microtubules in HeLa cells.

White arrowheads point to cuts in microtubules in both host cell types, and yellow arrowheads point to larger breaks in host microtubules. Scale bar, 10 µm.

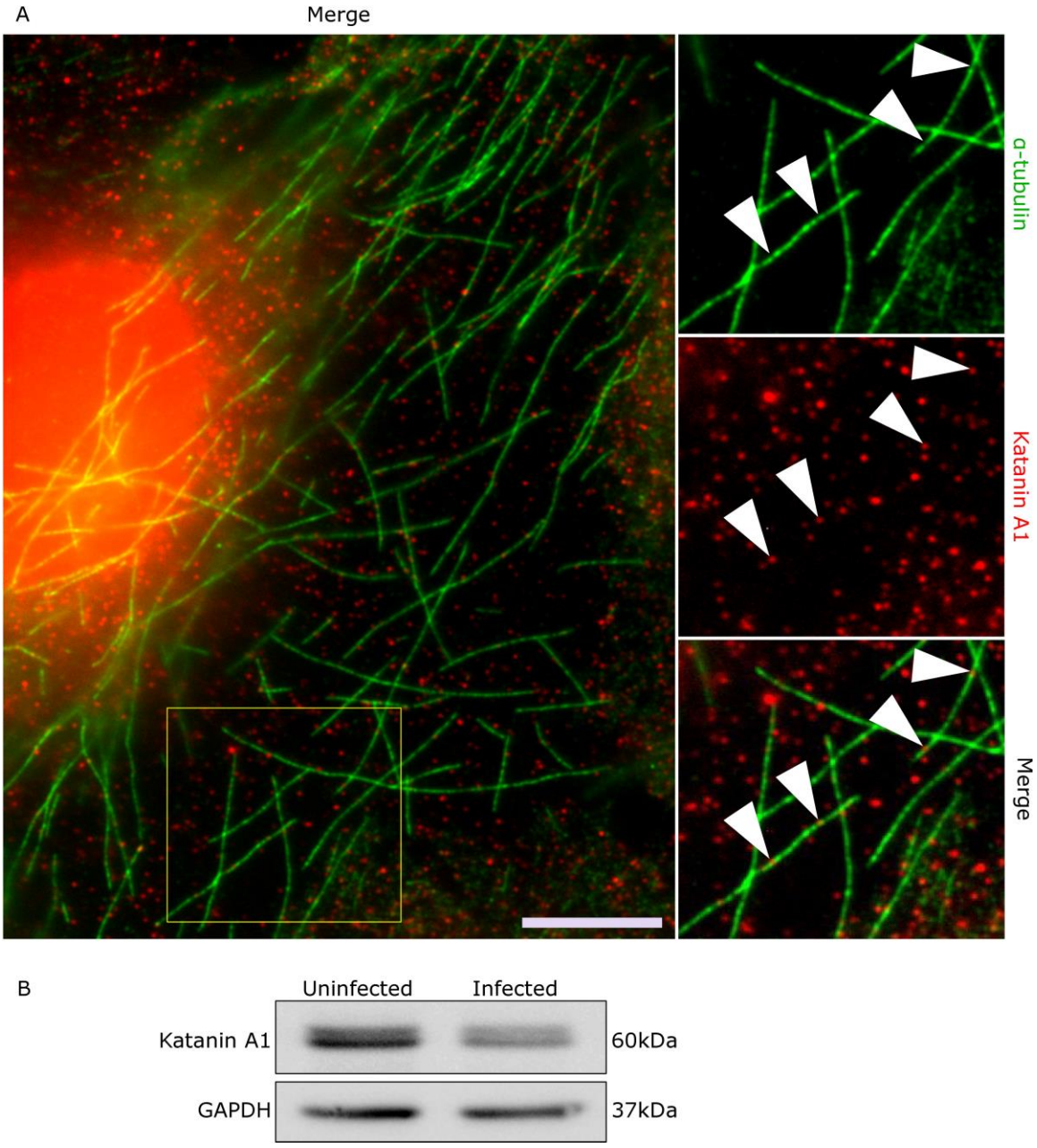

Figure 2.4: Katanin A1 is involved in microtubule severing during *Edwardsiella* infections.

A) Katanin A1 localizes at microtubule cut-sites in HeLa cells post 6-hour infection with *E. piscicida* EIB202. Arrowheads point to cellular localization of katanin A1 at the cut-sites. B) Whole-cell lysates of HeLa were analyzed by Western blot, using antibody recognizing Katanin A1 after infection with *E. piscicida* EIB202 for 6 hours. GAPDH was used as a loading control. Scale bar, 10 µm.

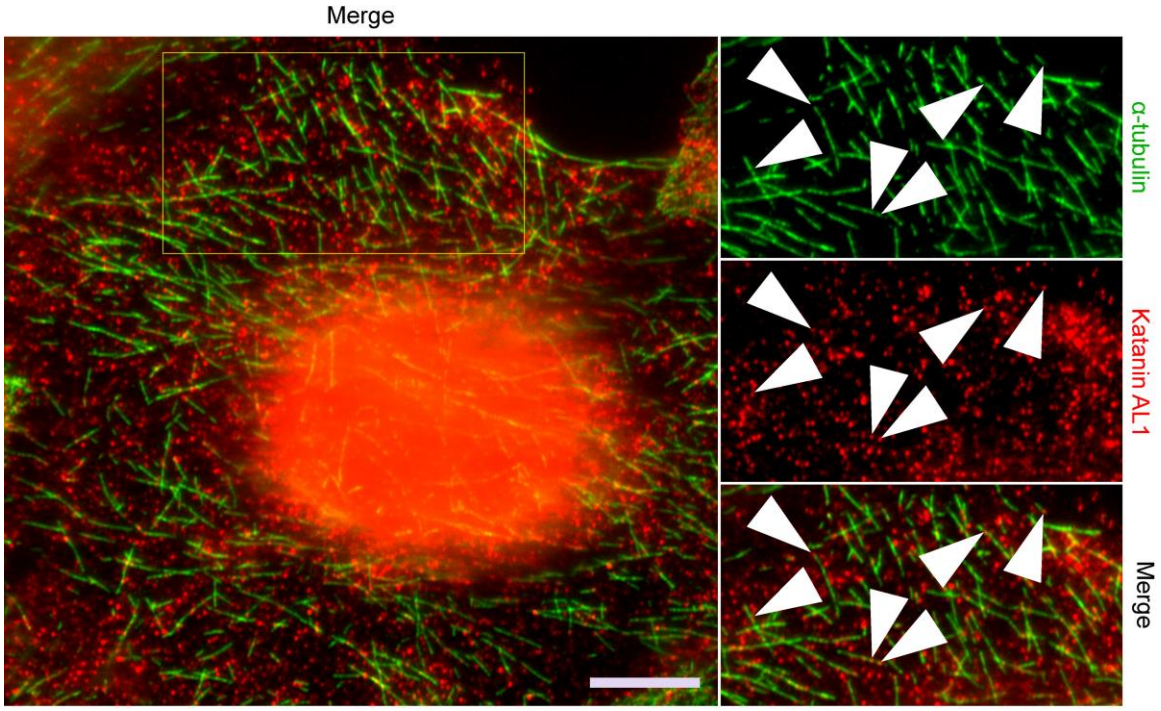

Figure 2.5: Katanin AL1 is involved in microtubule severing during *Edwardsiella* infections.

Katanin AL1 localizes at microtubule cut-sites in HeLa cells 6 hours post infection with *E. piscicida* EIB202. Arrowheads point to the cellular localization of katanin AL1 at the cut-sites. Scale bar, 10 µm.

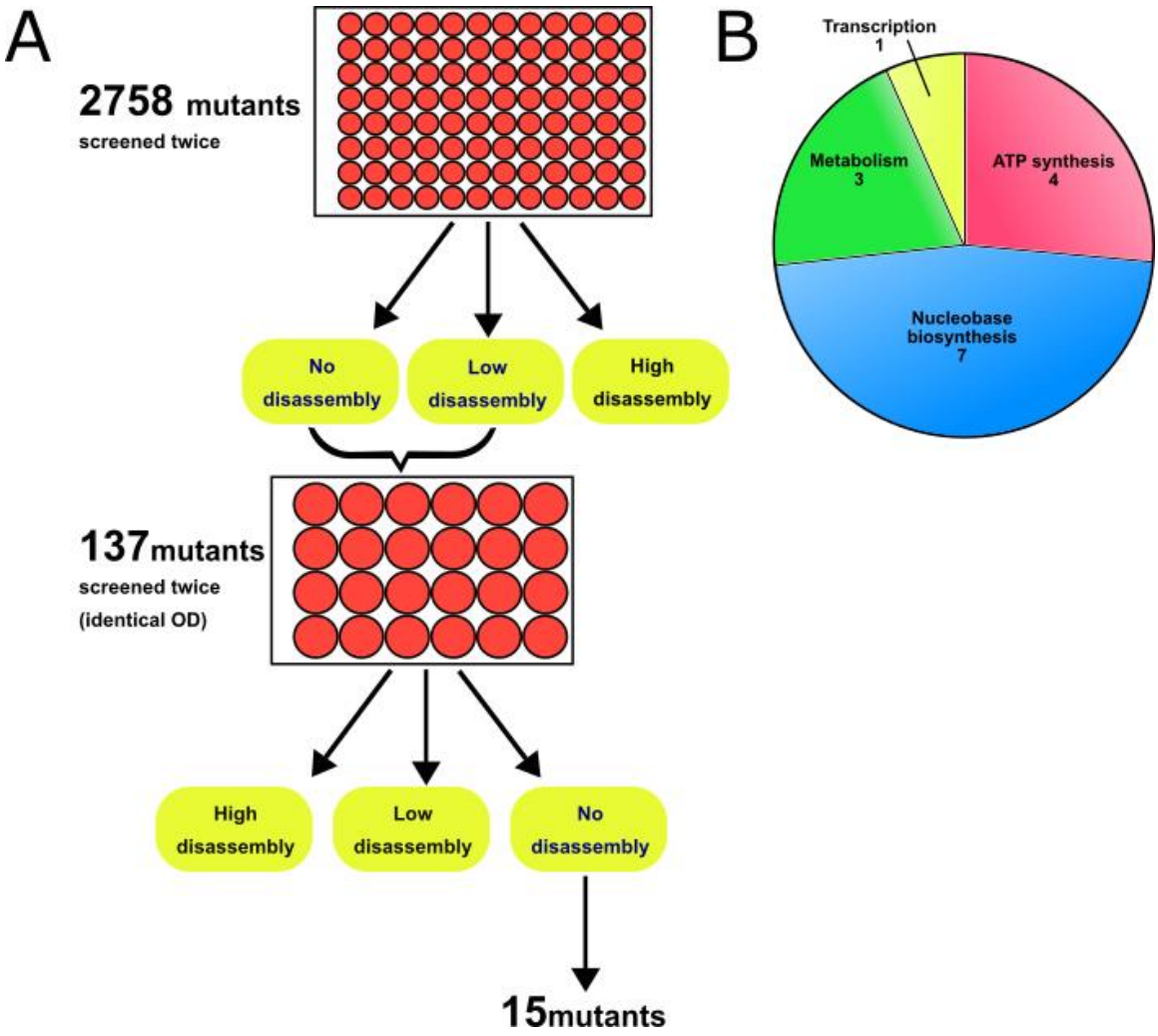

Figure 2.6: Negative screening approach and results.
A) Flowchart diagram depicting the screening approach used for identifying *E. piscicida* EIB202 genes responsible for MT disassembly. B) The 15 gene candidates obtained from the EIB202 library screen were categorized based on their cellular functions.

2.6. Table

Table 2.1: ***E. piscicida*** **EIB202 transposon insertion mutant library candidates involved in inducing microtubule disassembly in HeLa cell line during** ***Edwardsiella*** **infections.**

Category	Accession number	Gene name	Annotation
ATP synthesis	ETAE_3530	*atpH*	F-type H+-transporting ATPase delta chain
ATP synthesis	ETAE_3531	*atpA*	F-type H+-transporting ATPase alpha chain
ATP synthesis	ETAE_3529	*atpF*	F-type H+-transporting ATPase beta chain
ATP synthesis	ETAE_3532	*atpG*	F-type H+-transporting ATPase gamma chain
Nucleobase Biosynthesis	ETAE_0358	*purA*	Adenylosuccinate synthase
Nucleobase Biosynthesis	ETAE_1098	*purC*	Phosphoribosylaminoimidazole-succinocarboxamide synthase
Nucleobase Biosynthesis	ETAE_2409	*purF*	Amidophosphoribosyltransferase
Nucleobase Biosynthesis	ETAE_0774	*purI*	Phosphoribosylformylglycinamidine synthase
Nucleobase Biosynthesis	ETAE_2056	*purB*	Adenylosuccinate lyase
Nucleobase biosynthesis	ETAE_0598	*apaH*	Diadenosinetetraphosphatase
Nucleobase biosynthesis	ETAE_3125	*pyrB*	Aspartate carbamoyltransferase
Metabolism	ETAE_1486	*pncA*	Nicotinamidase/pyrazinamidase
Metabolism	ETAE_1655	*prc*	Carboxy-terminal protease
Metabolism	ETAE_0709	*ptrA*	Protease III precursor
Transcription	ETAE_3299	hypothetical protein	cAMP-activated global transcriptional regulator (CRP)

2.7. References

1. Mohanty BR, Sahoo PK. Edwardsiellosis in fish: a brief review. J Biosci. 2007;32:1–14.

2. Michael J, Abbott SL. Infections associated with the genus *Edwardsiella*: The role of *Edwardsiella tarda* in human disease. Clin Infect Dis. 1993;17:742–8.

3. Qin L, Sun Y, Zhao Y, Xu J, Bi K. In vitro model to estimate *Edwardsiella tarda*-macrophage interactions using RAW264.7 cells. Fish Shellfish Immunol. 2017;60:177–84.

4. Okuda J, Arikawa Y, Takeuchi Y, Mahmoud MM, Suzaki E, Kataoka K, et al. Intracellular replication of *Edwardsiella tarda* in murine macrophage is dependent on the type III secretion system and induces an up-regulation of anti-apoptotic NF-kB target genes protecting the macrophage from staurosporine-induced apoptosis. Microb Pathog. 2006;41:226–40.

5. Xie H-X, Lu J-F, Zhou Y, Yi J, Yu X-J, Leung KY, et al. Identification and functional characterization of the novel *Edwardsiella tarda* effector EseJ. Infect Immun. 2015;83:1650–60.

6. Janda JM, Abbott SL, Oshiro LS. Penetration and replication of *Edwardsiella spp*. in HEp-2 cells. Infect Immun. 1991;59:154–61.

7. Strauss EJ, Ghori N, Falkow S. An *Edwardsiella tarda* strain containing a mutation in a gene with homology to shlB and hpmB is defective for entry into epithelial cells in culture. Infect Immun. 1997;65:3924–32.

8. Xie HX, Yu HB, Zheng J, Nie P, Foster LJ, Mok YK, et al. EseG, an effector of the type III secretion system of *Edwardsiella tarda*, triggers microtubule destabilization. Infect Immun. 2010;78:5011–21.

9. Wang B, Yu T, Dong X, Zhang Z, Song L, Xu Y, et al. *Edwardsiella tarda* invasion of fish cell lines and the activation of divergent cell death pathways. Vet Microbiol. 2013;163:282–9.

10. Nakamura Y, Takano T, Yasuike M, Sakai T, Matsuyama T, Sano M. Comparative genomics reveals that a fish pathogenic bacterium E*dwardsiella tarda* has acquired the locus of enterocyte effacement (LEE) through horizontal gene transfer. BMC Genomics. 2013;14:642.

11. Tan YP, Zheng J, Tung SL, Rosenshine I, Leung KY. Role of type III secretion in *Edwardsiella tarda* virulence. Microbiology. 2005;151:2301–13.

12. Stevens JM, Galyov EE, Stevens MP. Actin-dependent movement of bacterial pathogens. Nat Rev Microbiol. 2006;4:91–101.

13. Yoshida S, Sasakawa C. Exploiting host microtubule dynamics: A new aspect of bacterial invasion. Trends Microbiol. 2003;11:139–43.

14. Kumar Y, Valdivia RH. Actin and intermediate filaments stabilize the *Chlamydia trachomatis* vacuole by forming dynamic structural scaffolds. Cell Host Microbe. 2008;4:159–69.

15. Hartman JJ, Mahr J, McNally K, Okawa K, Iwamatsu A, Thomas S, et al. Katanin, a microtubule-severing protein, is a novel AAA ATPase that targets to the centrosome using a WD40-containing subunit. Cell. 1998;93:277–87.

16. Sonbuchner TM, Rath U, Sharp DJ. KL1 is a novel microtubule severing enzyme that regulates mitotic spindle architecture. Cell Cycle. 2010;9:2403–11.

17. Xu T, Zhang XH. *Edwardsiella tarda*: An intriguing problem in aquaculture. Aquaculture. 2014;431:129–35.

18. Janda JM, Abbott SL, Kroske-Bystrom S, Cheung WKW, Powers C, Kokka RP, et al. Pathogenic properties of *Edwardsiella* species. J Clin Microbiol. 1991;29:1997–2001.

19. Abayneh T, Colquhoun DJ, Sørum H. Edwardsiella piscicida sp.nov., a novel species pathogenic to fish. J Appl Microbiol. 2013;114:644–54.

20. Shao S, Lai Q, Liu Q, Wu H, Xiao J, Shao Z, et al. Phylogenomics characterization of a highly virulent *Edwardsiella* strain ET080813(T) encoding two distinct T3SS and three T6SS gene clusters: Propose a novel species as *Edwardsiella anguillarum* sp. nov. Syst Appl Microbiol. 2015;38:36–47.

21. Ling SHM, Wang XH, Xie L, Lim TM, Leung KY, Ling H. M. S. Use of green fluorescent protein (GFP) to study the invasion pathways of *Edwardsiella tarda* in *in vivo* and *in vitro* fish models. Microbiology. 2000;146:7–19.

22. Ishibe K, Osatomi K, Hara K, Kanai K, Yamaguchi K, Oda T. Comparison of the responses of peritoneal macrophages from Japanese flounder (P*aralichthys olivaceus*) against high virulent and low virulent strains of *Edwardsiella tarda*. Fish Shellfish Immunol. 2008;24:243–51.

23. Ashida H, Ogawa M, Kim M, Mimuro H, Sasakawa C. Bacteria and host interactions in the gut epithelial barrier. Nat Chem Biol. 2011;8:36–45.

24. Loisel TP, Boujemaa R, Pantaloni D, Carlier MF. Reconstitution of actin-based motility of *Listeria* and *Shigella* using pure proteins. Nature. 1999;401:613–6.

25. Rottner K, Lommel S, Wehland J, Stradal TEB. Pathogen-induced actin filament rearrangement in infectious diseases. J Pathol. 2004;204:396–406.

26. Yang G, Billings G, Hubbard TP, Park JS, Leung Y, Liu Q, et al. Time resolved transposon insertion sequencing reveals 2 genome-wide fitness dynamics during infection. 2017;1–35. (Submitted).

27. Janda MJ, Abbott SL. Infections associated with the genus *Edwardsiella*: the role of Edwardsiella tarda in human disease. Clin Infect Dis. 1993;17:742–8.

28. Sharp DJ, Ross JL. Microtubule-severing enzymes at the cutting edge. J Cell Sci. 2012;125:2561–9.

29. McNally FJ, Vale RD. Identification of katanin, an ATPase that severs and disassembles stable microtubules. Cell. 1993;75:419–29.

30. Johjima A, Noi K, Nishikori S, Ogi H, Esaki M, Ogura T. Microtubule severing by katanin p60 AAA+ ATPase requires the C-terminal acidic tails of both alpha- and beta-tubulins and basic amino acid residues in the AAA plus ring pore. J Biol Chem. 2015;290:11762–70.

31. Ribet D, Cossart P. How bacterial pathogens colonize their hosts and invade deeper tissues. Microbes Infect. 2015;17:173–83.

32. McNally FJ, Okawa K, Iwamatsu a, Vale RD. Katanin, the microtubule-severing ATPase, is concentrated at centrosomes. J Cell Sci. 1996;109:561–7.

2.8. Supplements

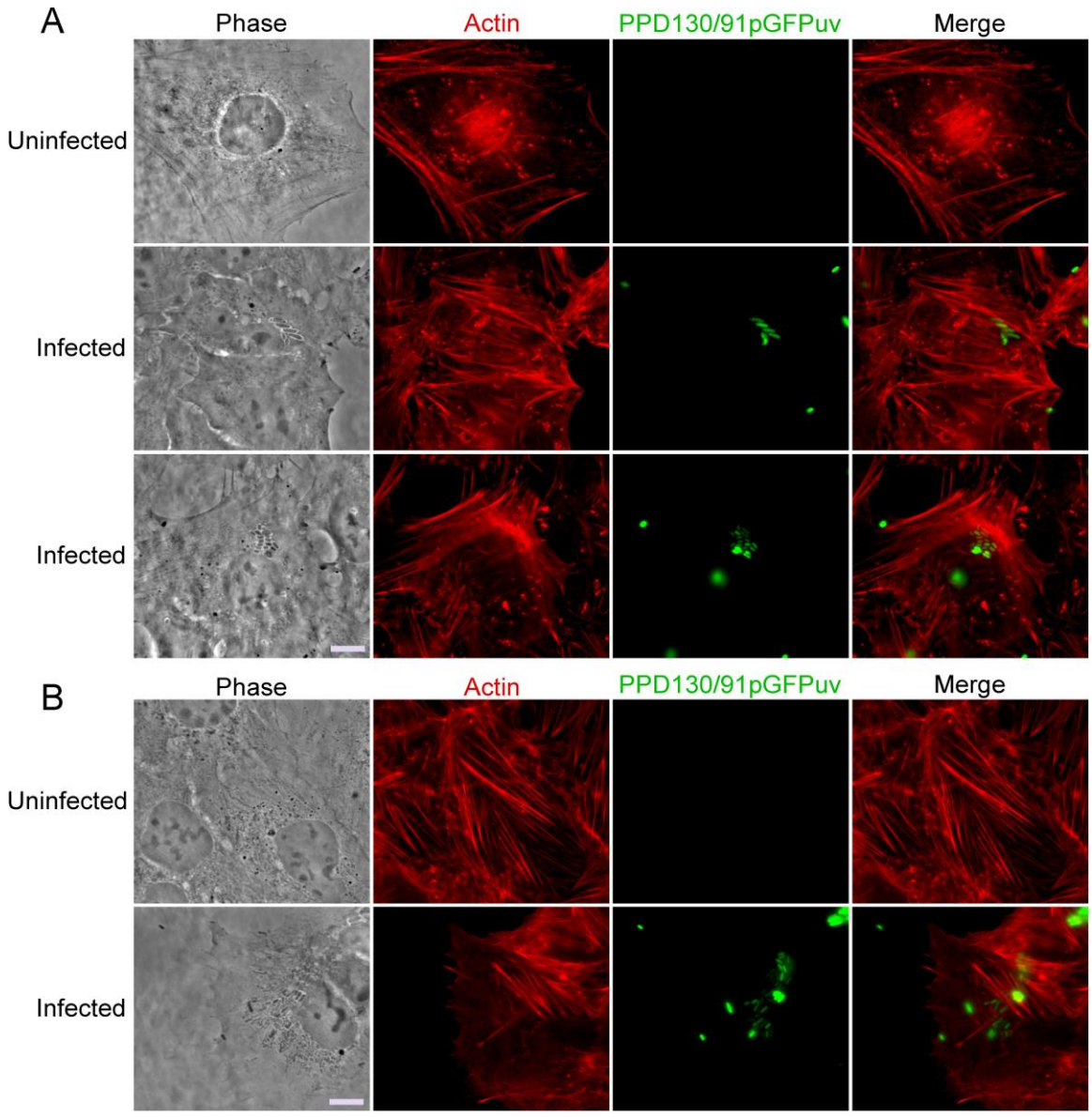

Figure S1: Actin cytoskeleton is not altered during *Edwardsiella* infections.
A) HeLa cells show no filamentous actin alterations during the 6-hour infection with *E. piscicida* PPD130/91pGFPuv. B) Caco-2 cells show no filamentous actin alterations during the 6-hour infection with *E. piscicida* PPD130/91pGFPuv. Scale bar, 10 µm.

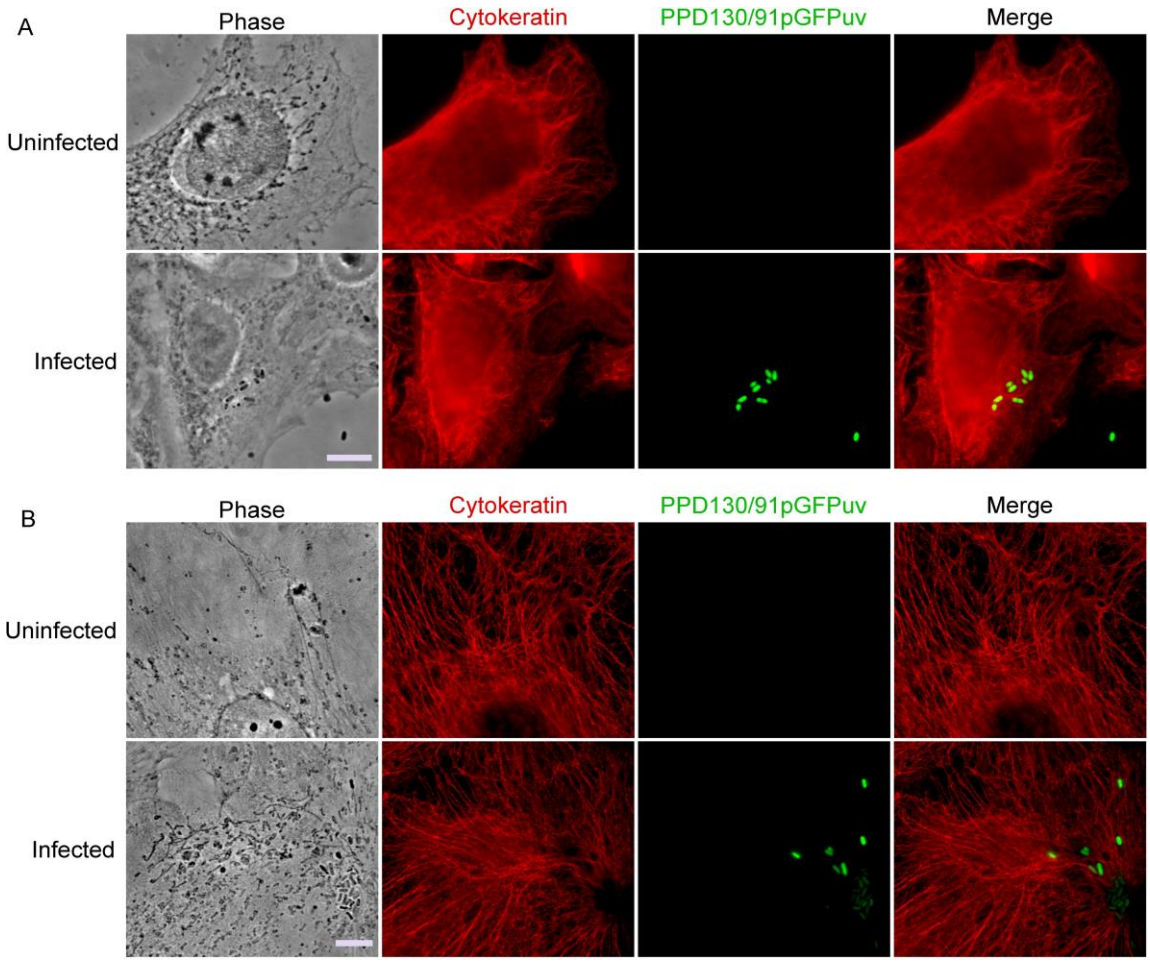

Figure S2: Intermediate filaments are not altered during *Edwardsiella* infections.

A) HeLa cells show no alterations in cytokeratin localization during the 6-hour infection with *E. piscicida* PPD130/91pGFPuv. B) Caco-2 cells show no alterations in cytokeratin localization during the 6-hour infection with *E. piscicida* PPD130/91pGFPuv. Scale bar, 10 µm.

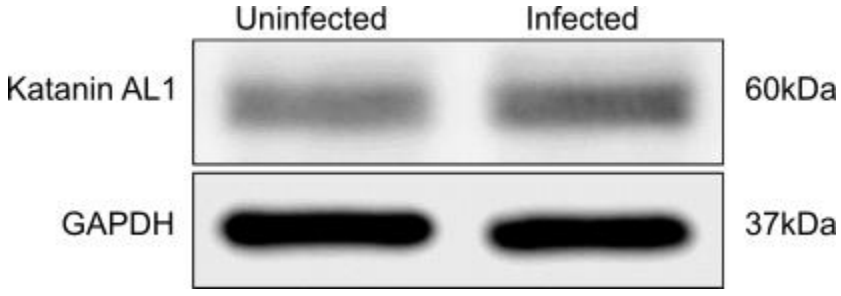

Figure S3: KATNAL1 expression levels remain unchanged during *E. piscicida* infections.

HeLa cell lysates were analyzed by Western blot, using an antibody recognizing Katanin AL1 after infection with *E. piscicida* EIB 202 for 6 hours. GAPDH was used as a loading control.

S1 Video: Host microtubules disassemble during *Edwardsiella* infections.
HeLa cells showing microtubule disassembly during *E. tarda*, ATCC 15947 infection. mkate2-EB3 expressing HeLa cells were infected for one hour and then live imaged using 2-minute intervals. The arrow heads point to microtubule cut-sites during the infection process. Scale bar, 5 μm.

Chapter 3.

Evidence that epithelial tight junctions are altered during *Edwardsiella piscicida* infections

Abstract

Bacteria from the *Edwardsiella* genus can kill all the fish in a farm within days. These infections cause an array of phenotypic alterations to fish including petechial hemorrhage in the fins and skin, rectal hernia, abscesses deep within fish musculature and most strikingly, holes within (or the liquefaction of) the fish themselves. Three main classes of intercellular junctions-tight junctions, adherens junctions, and desmosomes are crucial for maintaining tissue integrity, but are also common targets of bacterial pathogens. Because *Edwardsiella* causes alterations to various epithelia during their infectious processes, we hypothesized that intercellular junctions might be targeted by these microbes. To test this hypothesis, we immunolocalized key proteins of different types of intercellular junction in Caco-2 cells during *Edwardsiella piscicida* infections. Here we show that Claudin-3, a transmembrane tight junction protein, is extensively re-distributed during the progression of *Edwardsiella piscicida* infections, whereas adherens junction and desmosomal proteins remained at the cell periphery. This work provides the first evidence of junctional alteration during *Edwardsiella piscicida* infections, and suggests a possible molecular modification that could contribute to the observed phenotypes in diseased fish.

3.1. Introduction

Fisheries and aquaculture serve as a vital food source for billions of people (1). Consequently, ensuring the sustainable development of aquaculture is extremely important for the global economy. The intracellular Gram-negative pathogen *Edwardsiella piscicida* has the potential to destroy fish farms in a matter of days (1,2). These rod-shaped, motile, facultative anaerobes are known to be able to liquefy fish when infected (1,2). Some of the observed pathological features of these infections include petechial hemorrhaging in fins and the skin, rectal hernias, and abscesses deep within the fish musculature (2,3).

The skin, gills, and gastrointestinal tract have been reported as the primary portals of entry for *E. piscicida* into fish hosts (4,5). After proliferating on the fish host surface, these microbes ultimately invade deeper tissue layers and gain access to the bloodstream leading to a systemic infection (4). In immunocompromised humans, the gastrointestinal tract is a common zone of *E. piscicida* colonization (6,7). This opportunistic pathogen causes severe gastrointestinal and extra-intestinal diseases such as myonecrosis, septic arthritis, wound infections, and bacteremia, in immunocompromised humans (7–10). Epithelial cell-to-cell integrity and maintenance of protective barrier against bacterial invaders is normally achieved by an assortment of intercellular junctions. In the intestine, the most apically located junctions in tissues are the tight junctions (11). These junctions regulate the passage of molecules between the luminal and basolateral compartment (11). Key to the function of these junctions are the claudins, a family of at least 27 transmembrane proteins that interact cytoplasmically with the zonula occludens (ZO) proteins which ultimately link into the actin cytoskeleton. Function of occludin, another transmembrane protein at tight junctions, remains controversial as null mice survive and are for the most part unaffected by the loss of these proteins (12,13). The adherens junctions that lie immediately beneath the tight junctions associate with neighbouring cells through E-cadherin proteins as well as nectins(14). Their connection to the actin cytoskeleton functions through catenin (α and β) proteins that are also involved in signalling cascades (15). Desmosomes, mechanically bolt adjacent cells together by connecting the intermediate filament network(16). The transmembrane desmoglein and desmocollin proteins bind to their neighbours on adjacent cells forming strong attachments across the lateral membranes of the epithelial cell sheets (16).

Despite the degree of complexity in intercellular junctions, various enteric bacterial pathogens hijack these molecular structures for their benefit. This includes using junction proteins as receptors for bacterial entry and destroying the junctions as disease phenotypes progress (17–22). The complete destruction of epithelial integrity in fish suffering from edwardsiellosis (2) together with the human disease phenotypes (7) caused by *Edwardsiella* led us to develop the hypothesis that intercellular junctions are likely compromised during *E. piscicida* infections.

To test this hypothesis, we immunolocalized crucial (tight junction, adherens junction and desmosomal proteins) during *E. piscicida* infections in Caco-2 (Human

colon carcinoma) cells. This cell line was chosen because it is widely used to analyse the structure and physiology of the gut epithelium *in vitro* and because when in confluence, they express the junctional proteins at their periphery (23). We show that the tight junction transmembrane protein claudin-3 is mis-localized away from the cell borders during *E. piscicida* infections in contrast to the localization of AJ protein- E-cadherin and desmosome protein- desmoglein 2 which remained unaltered. This work provides the first evidence of junction alteration during *E. piscicida* infections and suggests that tight junction alterations may have a role in the observed disease phenotypes of edwardsiellosis.

3.2. Results

3.2.1. Rearrangement of claudin-3 during *E. piscicida* infections

Integral to the functions of tight junctions are their transmembrane proteins (11). To examine the influence *E. piscicida* had on the localization of tight junction proteins, we immunolocalized the transmembrane protein- claudin-3 (a prominent claudin of caco-2 cells) during *E. piscicida* infections. In control, untreated monolayers, claudin-3 was predominantly localized to the cell plasma membrane producing a characteristic cobblestone pattern of staining consistent with its distribution in tight junctions (Figure 3.1). More specifically, claudin 3 appeared as a distinct continuous band along the cell borders in untreated caco-2 cell monolayers. When infected, cells with bacteria present also showed clear zones devoid of claudin 3 (Figure 3.1). However, the claudin-3 localization remained unaltered in cells that did not have internalized *E. piscicida*. An unexplained nuclear localization of claudin-3 was also evident in our study, but this did not change during the infections (Figure 3.1).

3.2.2. E-cadherin and Desmoglien-2 are not altered during *E. piscicida* infections

In addition to tight junctions, adherens junctions are also major contributors to intercellular adhesion and the most well studied transmembrane protein of AJs is E-cadherin (14) which formed distinct continuous staining along the cellular boundaries in uninfected controls (Figure 3.2, panel A). This staining remained intact in cells containing internalized *E. piscicida*, suggesting that adherens junctions remained unaltered by the

microbes (Figure 3.2, panel A). The transmembrane desmogleins are integral for the proper functioning of desmosomes (24). We analysed its localization during *E. piscicida* infection in Caco-2 cells and saw the characteristic punctate staining pattern on the cell periphery (Figure 3.2, panel B). This staining was again intact during the infections which suggests that the desmosomes are intact at this stage of the infection in these cells.

3.3. Discussion

Intercellular junctions are a classic target for bacterial pathogens (17,19,21,25). Using a preliminary screen of transmembrane protein localization, we examined tight junctions, adherens junctions and desmosomes. Our finding that only claudin-3 localization was altered suggests that tight junctions are likely influenced by *E. piscicida*.

Tight junctions are integral to the barrier formation of all tissues within both fish and humans (13,26). Our evidence that a tight junction protein is no longer at the cell periphery suggests that the junctions themselves may be breached. This will have to be confirmed by electron microscopy and functional tracer assays, but none the less could be a molecular alteration that influences the diarrheal phenotypes experienced by immunocompromised individuals infected with *Edwardsiella* as well as fish that show severe tissue disintegration.

Breaching of tight junctions have been thought as major contributors to diarrheal diseases through the inflammation and potentially the “leaking” of material from the sub-tight junction area into the lumen (17). These breaches are well described during *E. coli*(17), *Salmonella*(27) and other gastrointestinal infections (28). Interestingly fish have a large assortment of claudin proteins that number into over 50 in certain species (29). Consequently, our identification of claudins as a potential target of *Edwardsiella* raises the question of whether other claudins, particularly fish claudins may also be altered by these microbes.

Our study did not identify any changes of E-Cadherin or Desmoglein 2 in caco-2 cells. This is not terribly surprizing as humans keep an intact epithelium when infected with *Edwardsiella*. An interesting study will be to examine these proteins as well as others (e.g. the nectins) in fish during *in vivo* infections as the areas where the tissue has been removed due to the infections suggests that the integrity of the cells is

compromised. None the less, our study does point to junctions being altered for the first time during *Edwardsiella piscicida* infections and sets the foundation for a deeper understanding of mechanics of intercellular junctional exploitation by these pathogens.

3.4. Materials and methods

3.4.1. Bacterial Growth Conditions

The bacterial strain used in this study is wild type *E. piscicida* strain: PPD130/91 pGFPuv. This strain was grown using standard tryptic soy broth (TSB) (BD Biosciences) supplemented with ampicillin 100 µg/mL in a standing culture overnight at 30°C.

3.4.2. Cell Culture and Infections

Human colon adenocarcinoma (Caco2) cells were cultured in DMEM/FBS [DMEM containing high-glucose (HyClone, Thermo Scientific) supplemented with 10% FBS and 1% non-essential amino acids (Gibco, Life Technologies)]. Caco-2 cells were grown at 37 °C (5% CO2). Cells were trypsinized with 0.05% Trypsin-EDTA (Gibco, Life Technologies) and seeded onto grass coverslips (Fisher Scientific) that were placed into 6-well plates (Corning, Fisher Scientific).

3.4.3. Infections

For immunofluorescence experiments, 2.0×10^5 Caco-2 cells were seeded on 22mm×22 mm square coverslips in 6 well plates three days prior to infection. Two days before infecting the cultured cells, PPD130/91 pGFPuv was streaked on TSA plate supplemented with 100 µg/mL ampicillin and grown for 24 hours at 30°C. Single colonies were picked from agar plates the following day and placed into standing culture of 2 ml TSB at 30 °C for 16 hours. Before infecting, caco-2 cells were checked for 80% confluence and the culture media was replaced with 2mL of infection media. PPD130/91 pGFPuv was used to infect host cells at a multiplicity of infection (MOI) of 25 for 6 h at 35°C prior to fixation and staining.

3.4.4. Immunofluorescence Staining

Once the infections were complete, infected cells were washed three times with phosphate-buffered saline without magnesium and calcium (PBS -/-) (Hyclone, Thermo Fisher Scientific) warmed to 37°C, fixed with 37°C 3 % paraformaldehyde for 15 min, then washed three times with PBS −/−. To permeabilize the samples, the cells were incubated with PBS−/− with 0.2 % Triton-X-100 for 5 min at room temperature (RT), then washed three times with PBS −/−. The samples were then blocked with 5 % normal goat serum (NGS) (Life Technologies) for 20 min. and stained with primary antibodies overnight at 4°C. Primary antibodies consisted of following antibodies: claudin-3 (Thermo Fisher, 2µg/mL), E-cadherin (Abcam, 2.5µg/mL), and desmoglein-2 (Cell signalling technology, 2µg/mL). After multiple washes with TPBS-0.1% BSA, Alexa-488 and Alexa-594 conjugated goat antibodies (1:1000) against mouse and rabbit IgG were added to the appropriate samples and incubated for 2 hours at RT. After the secondary antibody incubations, the samples were washed multiple times with TPBS-0.1%BSA and mounted onto glass slides using Prolong Gold containing DAPI.

3.4.5. Fluorescence Microscopy Imaging

A Leica (Wetzlar, Germany) DMI4000b inverted fluorescent microscope with a Hamamatsu Orca R2 CCD camera (Hamamatsu, Japan) and Leica ×10 HI Plan 0.25 Phase 1, ×40 HCX Plan APO 0.75 Phase 2, and ×100 HCX Plan APO 1.40 oil Phase 3 CS objectives connected to a PC with Metamorph Imaging System software (Molecular Devices, Sunnyvale, CA, USA) was used for data visualization and analysis. Images were processed using ImageJ (NIH, Bethesda, MD, USA) and Inkscape (USA).

3.5. Figures

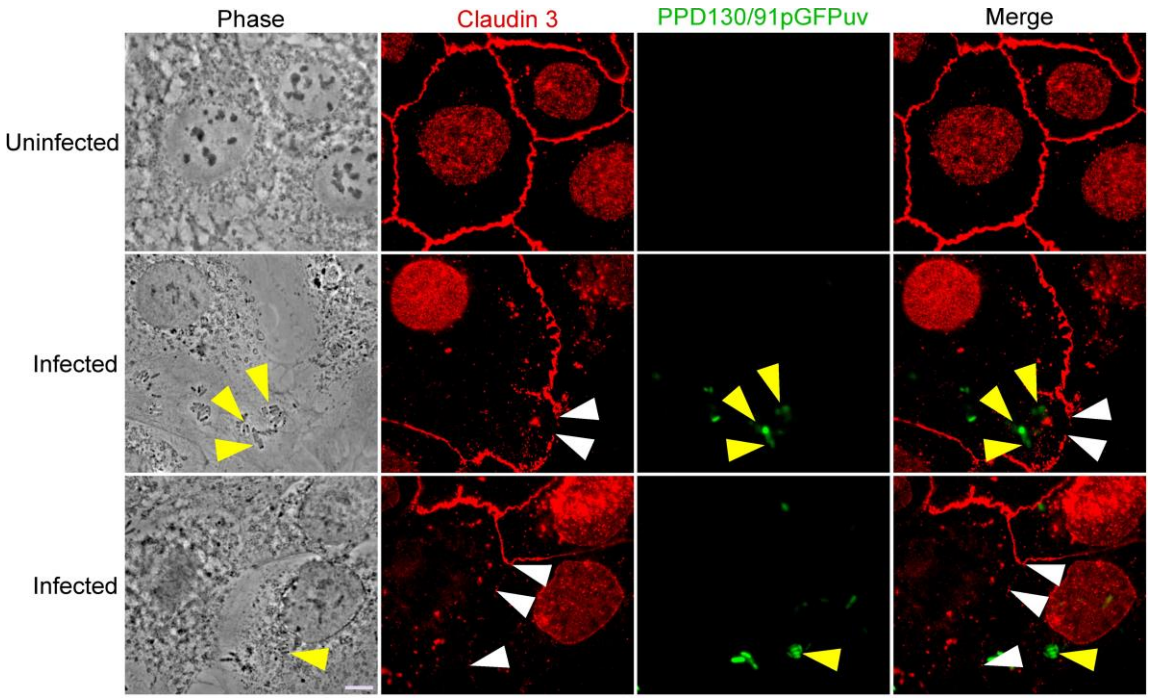

Figure 3.1: Caco-2 cells showing disruption in claudin 3 protein during *Edwardsiella piscicida* infection.

Caco-2 cells infected with *E. piscicida* PPD130/91 pGFPuv for 6 hours showing alterations in the localization of claudin 3. White arrowheads point towards disrupted localization of junctional protein – claudin 3. Yellow arrowheads depict internalized *E. piscicida*. Scale bar, 10 µm.

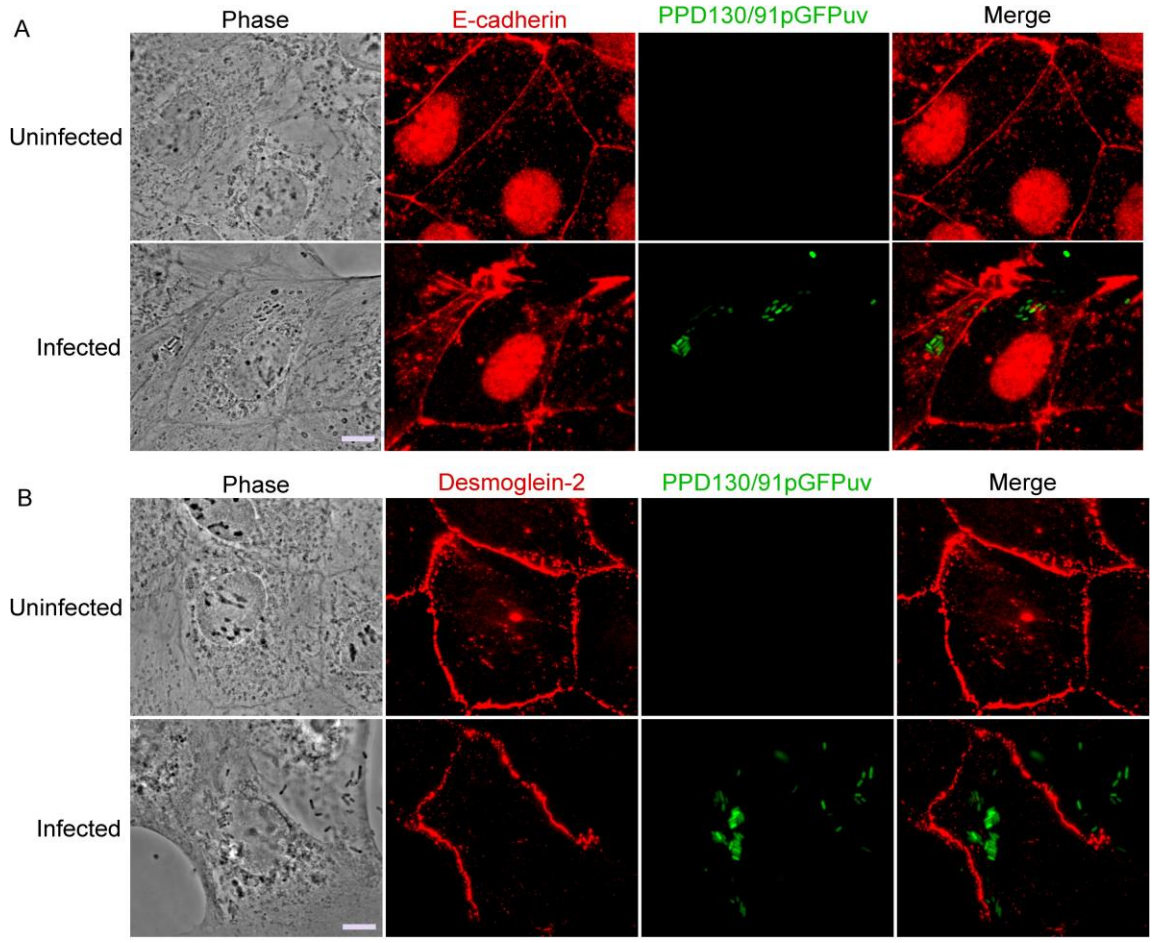

Figure 3.2: Caco-2 cells showing no alterations in E-cadherin and desmoglein-2 during *Edwardsiella piscicida* infection.

Caco-2 cells infected with *E. piscicida* PPD130/91 pGFPuv for 6 hours show no alterations in the localization of A) E-cadherin, B) desmoglein-2 proteins. Infected cell in panel A and B show missing junctions on the periphery due to missing neighbouring cell as expected. Scale bar, 10 µm.

3.6. References

1. Xu T, Zhang XH. *Edwardsiella tarda*: An intriguing problem in aquaculture. Aquaculture. 2014;431:129–35.

2. Mohanty BR, Sahoo PK. Edwardsiellosis in fish: a brief review. J Biosci. 2007;32:1–14.

3. Park S Bin, Aoki T, Jung TS. Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish. Vet Res. 2012;43:1.

4. Ling SHM, Wang XH, Xie L, Lim TM, Leung KY, Ling H. M. S. Use of green fluorescent protein (GFP) to study the invasion pathways of *Edwardsiella tarda* in *in vivo* and *in vitro* fish models. Microbiology. 2000;146:7–19.

5. Ling SHM, Wang XH, Lim TM, Leung KY. Green fluorescent protein-tagged *Edwardsiella tarda* reveals portal of entry in fish. FEMS Microbiol Lett. 2001;194:239–43.

6. Nishida K, Kato T, Yuzaki I, Suganuma T. *Edwardsiella tarda* bacteremia with metastatic gastric cancer. IDCases. 2016;5:76–7.

7. Janda MJ, Abbott SL. Infections associated with the genus *Edwardsiella*: the role of *Edwardsiella tarda* in human disease. Clin Infect Dis. 1993;17:742–8.

8. Yousuf RM, How SH, Amran M, Hla KT, Shah A, Francis A. *Edwardsiella tarda* septicemia with underlying multiple liver abscesses. Malays J Pathol. 2006;28:49–53.

9. Tamada T, Koganemaru H, Matsumoto K, Hitomi S. Urosepsis caused by *Edwardsiella tarda*. J Infect Chemother. 2009;15:191–4.

10. Hirai Y, Asahata-Tago S, Ainoda Y, Fujita T, Kikuchi K. *Edwardsiella tarda* bacteremia. A rare but fatal water- And foodborne infection: Review of the literature and clinical cases from a single centre. Can J Infect Dis Med Microbiol. 2015;26:313–8.

11. Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol. 2001;2:285–93.

12. Schulzke JD, Gitter AH, Mankertz J, Spiegel S, Seidler U, Amasheh S, et al. Epithelial transport and barrier function in occludin-deficient mice. Biochim Biophys Acta - Biomembr. 2005;1669:34–42.

13. Zihni C, Mills C, Matter K, Balda MS. Tight junctions: from simple barriers to multifunctional molecular gates. Nat Rev Mol Cell Biol . 2016;17:564–80.

14. Meng W, Takeichi M. Adherens junction: molecular architecture and regulation. Cold Spring Harb Perspect Biol. 2009;1:1–13.

15. Baum B, Georgiou M. Dynamics of adherens junctions in epithelial establishment, maintenance, and remodeling. Journal of Cell Biology. 2011;192:907–17.

16. Desai B V, Harmon RM, Green KJ. Desmosomes at a glance. J Cell Sci. 2009;122:4401–7.

17. Glotfelty LG, Hecht GA. Enteropathogenic *E. coli* effectors EspG1/G2 disrupt tight junctions: New roles and mechanisms. Ann N Y Acad Sci. 2012;1258:149–58.

18. Coyne CB, Bergelson JM. CAR: A virus receptor within the tight junction. Advanced Drug Delivery Reviews. 2005;57: 869–82.

19. Bonazzi M, Lecuit M, Cossart P. *Listeria monocytogenes* internalin and E-cadherin: From structure to pathogenesis. Cellular Microbiology. 2009;11: p.693–702.

20. Lynch T, Livingstone S, Buenaventura E, Lutter E, Fedwick J, Buret AG, et al. *Vibrio parahaemolyticus* disruption of epithelial cell tight junctions occurs independently of toxin production. Infect Immun. 2005;73:1275–83.

21. Guttman JA, Finlay BB. Tight junctions as targets of infectious agents. Biochim Biophys Acta - Biomembr. 2009;1788:832–41.

22. Fudaba Y, Nishifuji K, Andresen LO, Yamaguchi T, Komatsuzawa H, Amagai M, et al. *Staphylococcus hyicus* exfoliative toxins selectively digest porcine desmoglein 1. Microb Pathog. 2005;39:171–6.

23. Engle MJ, Goetz GS, Alpers DH. Caco-2 cells express a combination of colonocyte and enterocyte phenotypes. J Cell Physiol. 1998;174:362–9.

24. Kottke MD, Delva E, Kowalczyk AP. The desmosome: cell science lessons from human diseases. J Cell Sci. 2006;119:797–806.

25. Amagai M, Yamaguchi T, Hanakawa Y, Nishifuji K, Sugai M, Stanley JR. *Staphylococcal* exfoliative toxin B specifically cleaves desmoglein 1. J Invest Dermatol. 2002;118:845–50.

26. Duffy NM, Bui P, Bagherie-Lachidan M, Kelly SP. Epithelial remodeling and claudin mRNA abundance in the gill and kidney of puffer fish (*Tetraodon biocellatus*) acclimated to altered environmental ion levels. J Comp Physiol B Biochem Syst Environ Physiol. 2011;181:219–38.

27. Jepson MA, Schlecht HB, Collares-Buzato CB. Localization of dysfunctional tight junctions in *Salmonella enterica* serovar typhimurium-infected epithelial layers. Infect Immun. 2000;68:7202–8.

28. Guttman JA, Finlay BB. Subcellular alterations that lead to diarrhea during bacterial pathogenesis. Trends in Microbiology; 2008;16:535–42.

29. Bui P, Kelly SP. Claudins in a primary cultured puffer fish (*Tetraodon nigroviridis*) gill epithelium model alter in response to acute seawater exposure. Comp Biochem Physiol -Part A Mol Integr Physiol. 2015;189:91–101.

Chapter 4.

General discussion

Although relatively few host-pathogen labs investigate *Edwardsiella,* these microbes have decimated fish colonies and have the potential to cause national food shortages if aquaculture stocks are infected (1,2). My work examining the host cytoskeletal and intercellular junction alterations caused by these microbes marks a beginning in the examination of epithelial structural alterations that are the targets of these bacteria. By initially examining the cytoskeleton then intercellular junctions I found that the host cell microtubules were altered, but not the actin filaments or the intermediate filaments; this was a peculiar finding as most bacterial pathogens tend to target the actin filaments (3–6). This microtubule destruction strategy was likely due to activation of the Katanin-like microtubule severing proteins as their presence precisely at microtubule-cut sites was evident. Of the 15 genes identified by negative MT disassembly screen, majority were directly involved in purine biosynthesis pathway. This finding suggests a possible crosstalk between purines and katanin severing enzymes.

Interestingly, Kuo et al. reported the ability of purine-type compounds to activate katanin microtubule severing enzymes in lung cancer cell line (11). In this case, lung cancer cells undergo cell cycle arrest and apoptosis due to the purine-type compond (11). Likewise, Edwardsiella could potentially release purine-type compound(s) that trigger host katanin enzymes to be activated and cause host MT disassembly. It is also highly likely that redundant mechanisms are exploited by *Edwardsiella* to ensure microtubule destruction.

My work on intercellular junctions during *Edwardsiella* infections was intended to determine if any alterations could correspond to the observed phenotypes occurring during the infections and simultaneously follow-up on the cytoskeletal alteration study as the cytoskeleton plays a crucial role in junction integrity. Here I found that claudin 3, a key tight junction transmembrane protein, was removed from the membrane during the infections. Interestingly, the adherens junction and desmosomal proteins that were examined remained largely unchanged. That is not to say that these junctions were unchanged by *Edwardsiella* as more work will be required to solidify that conclusion. Tight junctions are arguably the most well studied junction type in relation to bacterial

infections and their alterations are thought to contribute to a range of host phenotypes including the generation of diarrhoea and inflammation (7–9). Consequently, their identification as *Edwardsiella* targets is in-line with the host phenotypes observed in infected humans. Whether this is applicable to infected fish remains to be seen.

Taken together the work I completed in this thesis begins to familiarize us with the tactics these pathogens have evolved to cause disease. By thoroughly understanding these molecular mechanisms potential therapeutic targets may be developed. Additionally, once the final bacterial genes are ultimately identified potential vaccines may be able to be developed to combat *Edwardsiella.*

4.1. Conclusion

Ultimately, complete destruction of host epithelial microtubule network and rearrangement of tight junction protein- claudin-3 command two possible strategies adopted by *Edwardsiella* to support its infectious cycle. Efficient exploitation of both structural elements of the gut epithelia—cytoskeletal proteins and intercellular junctions—makes the epithelial barrier more vulnerable. Further analysis of *Edwardsiella*-induced microtubule disassembly and alterations in the tight junctions will provide novel insights into this pathogen's biology as well as the role cellular cytoskeletal elements and intercellular junctions play in bacterial subversion of host epithelia.

4.2. Future directions

Through the *in vitro* examination of alterations in epithelial cytoskeletal proteins, I discovered a novel type III and type VI independent microtubule severing pathway utilized by *Edwardsiella*. To proceed further in understanding *Edwardsiella* pathogenesis, it would be interesting to see whether these microtubules severing events also occur *in vivo*, using previously established *in vivo* fish models such as blue gourami, zebrafish, and goldfish. Through immunolocalization of various cytoskeletal proteins-actin, microtubules, intermediate filaments, in fish epithelial tissue harboring *Edwardsiella*, molecular mechanisms involved in causing edwardsiellosis could be discovered. *In vivo* analysis of fish epithelia may also pinpoint the exact step(s) of *Edwardsiella* pathogenesis that involve host microtubule disassembly and its possible role in bacterial dissemination.

The use of *E. piscicida* EIB202 transposon insertion mutagenesis library allowed me to identify 15 key bacterial genes responsible for causing microtubule severing in host epithelial cells using a negative screening approach. Unfortunately, the precise gene that dictates microtubule disassembly was not found, possibly due to redundancies in the microtubule severing mechanism. In the future, a positive screening approach should be attempted in which clones of *Edwardsiella* genes will be inserted into non-pathogenic (lab strain) *E. coli* to be used to for microtubule disassembly screening in epithelial cells.

My identification of two host microtubule severing enzymes- KATNA1 and KATNAL1 being involved in the regulation of epithelial microtubule disassembly also warrants further study. Because overexpression of KATNA1 and KATNAL1 would likely lead to complete destruction of epithelial microtubules, knockout analysis of each of these enzymes in epithelial cells may be a good approach to conclusively determine the influence of those proteins in the microtubule severing events caused by *Edwardsiella*. To do this I would use the CRISRP /cas9 knockout system. Additionally, due to the interdependency of multiple microtubule severing enzymes on numerous host cell processes, it would be interesting to see if other microtubule enzymes including KATNAL2, FIGN, FIGN-like-1, FIGN-like-2 were also involved in *Edwardsiella*-induced microtubule severing phenotype.

Microtubules are involved in a diverse array of cellular process such as a cell division, vesicular/organelle trafficking and cell migration. Due to the complete destruction of microtubules within a eukaryotic cell, the process of mitotic cell division will likely also be hampered. I would look at the cell cycle to determine if it is halted at a certain stage using FUCCI (fluorescence ubiquitination cell cycle indicator) construct as arresting the epithelial cells at a specific stage of the cell cycle may block epithelial turnover and promote bacterial colonization and dissemination (10).

As mentioned earlier, *Edwardsiella*'s pathogenic strategy involves crossing of the epithelial barrier. Therefore, examination of the protein complexes known to connect epithelial cells together – intercellular junction complexes, allowed me to identify the involvement of tight junctions in *Edwardsiella* infectious process. More specifically, the mis-localization of claudin-3, a tight junction protein, lead me to hypothesize possible disruption of tight junctions to facilitate intercellular spread. *In vivo* visualization of this

tight junction alteration using the fish model could further strengthen the possible role of claudin-3 during *Edwardsiella* infections Additionally, functional analysis of the tight junction barrier using molecular tracers should be conducted as well as electron microscopy to look at the junctions at ultrastructural level Although no change was observed in E-cadherin and desmoglein-2, immunolocalization of other adherens junction proteins (nectins and catenins) and desmosome proteins (desmocollins, desmoglein-1,3,4) during *Edwardsiella* infections remains to be elucidated.

4.3. References

1. Xu T, Zhang XH. *Edwardsiella tarda*: An intriguing problem in aquaculture. Aquaculture. 2014;431:129–35.

2. Mohanty BR, Sahoo PK. Edwardsiellosis in fish: a brief review. J Biosci. 2007;32:1–14.

3. Ma AT, Mekalanos JJ. In vivo actin cross-linking induced by *Vibrio cholerae* type VI secretion system is associated with intestinal inflammation. Proc Natl Acad Sci USA. 2010;107:4365–70.

4. Kumar Y, Valdivia RH. Actin and intermediate filaments stabilize the *Chlamydia trachomatis* vacuole by forming dynamic structural scaffolds. Cell Host Microbe. 2008;4:159–69.

5. Cossart P. Actin-based motility of pathogens: The Arp2/3 complex is a central player. Cell Microbiol. 2000;2:195–205.

6. Colonne PM, Winchell CG, Voth DE. Hijacking host cell highways: manipulation of the host actin cytoskeleton by obligate intracellular bacterial pathogens. Front Cell Infect Microbiol. 2016;6:1-8.

7. Guttman JA, Finlay BB. Subcellular alterations that lead to diarrhea during bacterial pathogenesis. Trends in Microbiology. 2008;16:535–42.

8. Glotfelty LG, Hecht GA. Enteropathogenic *E. coli* effectors EspG1/G2 disrupt tight junctions: New roles and mechanisms. Ann N Y Acad Sci. 2012;1258:149–58.

9. Guttman JA, Finlay BB. Tight junctions as targets of infectious agents. Biochim Biophys Acta - Biomembr. 2009;1788:832–41.

10. Morikawa H, Kim M, Mimuro H, Punginelli C, Koyama T, Nagai S, et al. The bacterial effector Cif interferes with SCF ubiquitin ligase function by inhibiting deneddylation of Cullin1. Biochem Biophys Res Commun. 2010;401:268–74.

11. Kuo TC, Li LW, Pan SH, Fang JM, Liu JH, Cheng TJ, et al. Purine-type compounds induce microtubule fragmentation and lung cancer cell death through interaction with katanin. J Med Chem. 2016;59: 8521–34.