DEFINING THE ROLE OF *YERSINIA PESTIS* AIL IN HOST CELL INTERACTIONS AND PLAGUE VIRULENCE FUNCTIONS

By

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Dedication

To Mom, Dad, Francis, Erin, Madeline

and most importantly JP

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ABSTRACT

Defining the role of *Yersinia pestis* Ail in host cell interactions and plague virulence functions

by

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Chair: Eric S. Krukonis

Adhesion to host cells and delivery of cytotoxic Yops are essential steps in the infection process of the plague pathogen *Yersinia pestis*. The *Y. pestis* adhesin Ail was recently demonstrated to mediate host cell binding and be critical for Yop delivery and virulence. Thus, the goal of my thesis was to identify a host cell substrate with which Ail interacts and elucidate the mechanism by which Ail engages host cells. To identify Ail ligands, the Ail protein was purified following overexpression in *E. coli* and reconstituted in detergent micelles. Purified Ail bound specifically to fibronectin (Fn), an extracellular matrix protein that may act as a bridge between Ail and the host cell. Fibronectin is a large, complex molecule with many known binding domains. We demonstrated Ail binds to host Fn specifically and this Ail-Fn interaction is important for efficient delivery of Yops. Thus, I sought to understand this interaction in more detail. I wanted to identify the Ail binding site along fibronectin and also to identify the amino acids

of Ail that are important for host cell binding and Yop delivery. Ail binds the 120kDa fragment, but less effectively than full-length Fn. Antibody studies provided additional evidence that Ail binds the $^{8-9}$FNIII modules within the 120kDa fragment. However, inhibitor studies suggest that the Ail-binding site may include a neighboring 45kDa fragment. Together, these data suggest there may be two sites within fibronectin that are required for optimal Ail binding, the collagen/gelatin binding domain in the N-terminal half of fibronectin and the $^{8-9}$FNIII modules within the 120kDa cell binding domain more C-terminally located. To study residues that contribute to the various functions of Ail, we used a mutagenesis scheme termed SWIM (selection without isolation of mutants). We found a serine and a phenylalanine in the third exposed loop of Ail that are defective for cell binding and Yop delivery when substituted with alanine. The double S128A/F130A mutant is modestly defective for Fn binding and Yop delivery. Together, my thesis work has contributed to the understanding of how *Y. pestis* Ail uses fibronectin to engage host cells and lead to virulence functions.

CHAPTER ONE

INTRODUCTION

Hypothesis

Ail mediates an efficient interaction with host cells and facilitates *Y. pestis* Yop delivery.

Study Objectives

- Identify the host component(s) with which Ail interacts (Chapter 2)
- Identify residues of Ail that are needed for the various functions of Ail (Chapter 3)
- Understand the mechanism of binding between Ail and fibronectin and map the Ail-binding site on fibronectin (Chapter 4)
- Characterize the differences between *Y. pestis* Ail and *Y. pseudotuberculosis* Ail (Chapter 5)

Yersinia pathogenesis and disease

The three pathogenic *Yersinia* species – *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* cause very distinctive diseases. *Y. enterocolitica* and *Y. pseudotuberculosis* are enteric pathogens and cause a self-

limiting gastroenteritis disease with *Y. enterocolitica* causing more severe disease than *Y. pseudotuberculosis* [1, 2]. On the other hand, *Y. pestis* causes the more pathogenic plague disease. Interestingly, *Y. pseudotuberculosis* and *Y. pestis* are more closely related to each other based on molecular characterizations than either is related to *Y. enterocolitica*, and *Y. pestis* is believed to have evolved from *Y. pseudotuberculosis* 5,000 – 20,000 years ago [3]. *Y. pestis* is historically known for causing three large pandemics [1, 4].

Rodents are the natural reservoir of *Y. pestis* and bacteria are passed to humans via flea bites [5]. Within fleas, *Y. pestis* colonizes the midgut, replicates and creates a biofilm that blocks the flea intestine [6]. This biofilm blockage then starves the fleas. The infected, biofilm-blocked fleas aggressively feed and regurgitate bacteria into the host with each flea bite [7]. Studies have shown biofilm formation is required for *Y. pestis* persistence in the flea [8] and the *hms* genes are responsible for formation of the biofilm [5]. *Y. pestis* can cause three forms of the plague. The first, bubonic plague, is the most infamous for historical outbreaks and is now the most common form of the disease. After a flea bite, bacteria drain to in the lymphatic vessels which then drain to the regional lymph nodes. Highly replicative bacteria and inflammatory infiltrates then fill the lymph nodes and cause them to swell creating the tender buboes [1]. Bubonic plague can then progress to secondary septicemic plague if bacteria gain access to and replicate within the bloodstream [1]. Alternatively, primary septicemic plague can also occur if there is direct inoculation into the blood via flea bite or wound infection [9]. Intravenous inoculation into the blood stream in the mouse model of

infection is representative of septicemic plague. The last form of the disease is known as pneumonic plague. Similar to septicemic plague, pneumonic plague can be a secondary disease following bubonic or septicemic plague via spreading to the lungs. Also, this form can also be primary when *Y. pestis* is directly inhaled into the airways. This primary pneumonic plague has a short 1-3 day incubation period within the host and is highly deadly with 100% mortality in the absence of antibiotic treatment [10, 11].

One of the hallmarks of *Yersinia* infections is the delivery of cytotoxic proteins called *Yersinia* outer proteins (Yops) directly into the cytoplasm of the host cells via the Ysc type three secretion system (T3SS) [12, 13]. The Ysc T3SS system consists of the Ysc (Yop secretion) apparatus and an array of Yops that are secreted by this apparatus. The T3SS apparatus transverses both membranes of the gram-negative bacteria and the needle-like protrusion penetrates host cell membrane [14, 15]. Three of the Yops, YopB, YopD and LcrV , are known as structural Yops and translocate the effector Yops across the target-cell membrane by forming the translocation channel [16, 17]. The effector Yops includes YopE, YopH, YopO/YpkA, YopT, YopM, and YopJ/YopP, and these Yops exert their effects within the host cell [12, 18, 19]. YopE, YopH, YopT, and YopO are responsible for the dysregulation of actin filaments and cytoskeleton rearrangements [15]. The Yop-mediated actin rearrangements prevent bacterial engulfment by phagocytosis. YopJ/YopP inhibits transduction of host signaling that leads to the activation of proinflammatory cytokines, thus down-modulating the immune response [20-22]. Both the T3SS apparatus and

Yops are encoded on a virulence plasmid (pCD1), and without this plasmid Yersinia species are avirulent [23]. Expression of the T3SS and Yops is induced *in vitro* under low calcium conditions at 37^{o}C, but this induction is likely an artifact of *in vitro* laboratory conditions. Within a host, T3SS proteins are induced at 37^{o}C during extracellular growth and intracellular growth in macrophages [24].

Y. pestis is a facultative intracellular pathogen [1]. Bacteria are generally found intracellularly during the early stages of infection [25, 26], but at later stages more bacteria are found extracellularly in blood, lymphatics or within tissues. Upon transmission to a mammalian host via a flea bite, *Y. pestis* is phagocytosed by resident neutrophils and macrophages within tissues and blood [27]. *Y. pestis* is killed by neutrophils, but can survive within unactivated macrophages [28, 29]. Within the macrophage, *Y. pestis* proliferates and expresses anti-phagocytic structures as well as the T3SS apparatus and Yops. After expressing these factors, the bacteria escape from macrophages and are prepared for an extracellular lifestyle. Also, after a few hours of growth at 37^{o}C, F1 capsule is highly expressed and forms an anti-phagocytic envelope around the bacteria [30-32]. Together these factors make *Y. pestis* effective for thwarting host response. Once outside the host cell, *Y. pestis* disseminates to the bloodstream or lymphatic vessels of the host and causes other pathological effects characterized by plague infections.

Adhesion to host tissues facilitates colonization, an important step in infection. For Yersinia infection, physical host cell contact initiates the expression of Yops and Ysc translocation apparatus [33-35]. The

enteropathogenic Yersinia, *Y. enterocolitica* and *Y. pseudotuberculosis* both encode two well studies adhesins: Invasin (Inv) and YadA [36-39]. Invasin is known to bind to β_1 integrins on the surface of host cells [40] and initiate host cell signaling required for invasion into non-phagocytic cells [41, 42] and for the efficient delivery of Yop proteins [43]. Alternatively, YadA binds to fibronectin [44] and uses fibronectin as a bridge to engage β_1 integrins on host cells. This interaction also initiates host cell signaling for bacterial invasion [45].

However, *Y. pestis* does not express either adhesin due to an IS*1541* element insertion within *inv* [46] and a frameshift mutation in *yadA* [33, 47]. *Y. pestis* does however, have other adhesin molecules. Plasminogen activitor (Pla) [48] and pH 6 antigen (Psa) [49] are two well studied adhesins. Pla, is expressed at 26^{o}C, but further induced at 37^{o}C [50], and is known to bind several extracellular matrix components [48] including laminin [51] and glycolipids [52]. Pla is important for escape from the site of tissue inoculation following a flea bite, presumably due to its proteolytic activity and ability to facilitate digestion of fibrin clots [53]. Pla has been shown to bind to DEC-205, a member of the C-type multilectins family, on macrophages and dendritic cells [54] and this interaction was important for dissemination of *Y. pestis* in infections of mice. *In vivo* infections provide evidence that Pla is essential for *Y. pestis* to cause primary pneumonic plague, however, Pla is less important for dissemination during pneumonic plague than bubonic plague [55]. Psa is a pilus with tightly regulated expression at pH<6.7 and 37^{o}C [56-58] and is known to bind to β-linked galactosylated glycosphingolipids [59], LDL [60], and human IgG [61]. Psa is

induced within acidified vacuoles of macrophages [62], and provides protection against phagocytosis. Other chaperone/usher systems have been studied for their ability to confer adhesion when expressed in *E. coli* and the y0561-y0563 locus encodes a pilus with modest adhesion activity but did not significantly reduce adhesion to host cells when deleted from *Y. pestis* [63]. Similarly, YapC, a *Y. pestis* autotransporter, confers host cell adhesion when expressed in *E. coli*, however, in *Y. pestis,* deletion of YapC does not lead to an adhesion defect, likely due to low levels of YapC expression during conditions tested [64]. Recently, Ail from *Y. pestis* has been described as a key adhesin and virulence factor [65]. This work showed that Ail conferred adhesion to and invasion into host cells. Additionally, an *ail* mutant had a significant defect in Yop delivery *in vitro*. In an *in vivo* model of septicemic plague via intravenous inoculation, an *ail* mutant exhibited an LD_{50} more than 3,000-fold greater than parental *Y. pestis* indicating Ail is an important virulence factor. Finally, Ail may down-regulate the host immune response, as an *ail* mutant had more inflammatory infiltrate within tissues. Ail is highly expressed under many environmental conditions. Therefore, one may postulate that Ail mediates virulence functions at all stages of infection.

Without Invasin or YadA, *Y. pestis* requires another high affinity adhesin that is able to signal the efficient delivery of cytotoxic Yops. As mentioned previously, *Y. pestis* expresses a few adhesins, however, both Pla and PsaA are both regulated under different environmental conditions while Ail is highly

expressed at all conditions examined. Therefore, Ail may be the adhesin molecule responsible for an activity similar to those of Invasin or YadA.

Ail molecular characteristics

Ail is chromosomally encoded. The gene is 549bp, encoding a 182 amino acid protein. The first 26 amino acids are predicted to contain the signal sequence for placement into the outer membrane. The protein has a molecular mass of 20kDa with the signal sequence and 17kDa without the signal. Ail has eight transmembrane domains that creates four extracellular loops. Loop 1 is predicted to contain 17 amino acids, loops 2 and 3 contain 20 amino acids each, and loop 4, the shortest, loop only 10 amino acids, (Fig. 1.1).

Functions of Ail

Pathogenic bacteria must establish colonization of host tissues. Attachment can be mediated by various bacterial surface components (proteins, lipids, and/or carbohydrates). Generally, adhesion is needed for colonization of the host and adhering to non-phagocytic cells can lead to an extracellular lifestyle. One of the main functions of Ail is to mediate this close cell contact and also to deliver Yops into host cells. *Y. pestis* KIM5 (our parental strain) deletion for *ail* lead to a 50% decrease in adhesion to epithelial and macrophage-like cell types. Cell association can be measured by visually counting the number of bacteria found in close contact to host cells or colony forming unit (CFU) plating assays.

Some bacterial adhesins are able to mediate binding to host cells and also initiate intracellular uptake. While adhesion and invasion into non-phagocytic cells are related functions, there is a difference between simply adhering to the host cell and active invasion. The bacterial adhesin Inv from *Y. pseudotuberculosis* initiates high affinity contact with surface expressed β1 integrins, which then leads to invasion [66, 67]. In this example, Invasin decorates the entire bacterial surface and promotes multiple high affinity interactions to host cells. These multiple high affinity interactions initiate a zippering type effect around the entire circumference of the bacteria [68]. Inv interaction with integrins generates intracellular host cell signaling leading to actin rearrangement that facilitates this high efficiency invasion. Not all adhesive proteins mediate invasion into host cells. For example, Ail adheres to host cells to a high extent but does not mediate efficient invasion (~0.05% of the inoculum invades).

Another function of Ail includes binding to other bacteria when grown in liquid culture. This interaction with other bacteria leads to pellicle formation and these bacterial aggregates settle to the bottom when left undisturbed. When over expressed in *Y. pestis*, Ail mediates autoaggregation [69]. Pellicle formation can be measured by observing a pellet on the bottom of a culture tube, visualization of aggregates under microscopy, or the reduction in optical density of a liquid culture as the aggregates fall out of solution.

Another function of Ail related to adhesion activity is the translocation of the Yop effector proteins [65]. As mentioned before, one of the hallmarks of

Yersinia infection is the delivery of cytotoxic Yop proteins into host cells. These cytotoxic Yops are a critical part of *Y. pestis* virulence. Intimate contact with host cells is required for the engagement of the injection apparatus and secretion of effector Yops [33, 34]. Thus, Ail is an important adhesion molecule that facilitates host cell contact for Yop translocation. This function may be particularly important for Ail as other *Y. pestis* adhesins do not mediate Yop delivery as effectively [70].

Invading organisms encounter innate immune responses upon infection of a host. The complement system is one of the first lines of defense and is found within the serum component of blood. Complement is made up of a number of proteins that lead to deposition of factors for opsonization of bacteria and the membrane attack complex (MAC) that can generate holes in bacterial membrane. Some bacteria express proteins or carbohydrate capsules that bind to these complement proteins to bacterial complement-mediated lysis of the bacterium [71-73]. This resistance to complement lysis is also termed serum resistance. Many bacterial pathogens, including Yersinia species, express proteins that confer serum resistance [71, 74]. Ail and YadA both provide serum resistance to *Yersinia*, a function has been studied extensively in *Y. enterocolitica* [75]. Ail from *Y. pestis* can also provide resistance to most species of serum, although a *Y. pestis Δail* mutant is not sensitive to mouse serum [76], because mouse serum is not bactericidal for *Yersinia* species [77].

Ail from other *Yersinia* species

Ail was first described in analysis of *Y. enterocolitica* invasive proteins [78]. The *ail* gene was identified as a protein that confers attachment and invasion into cultured host cells, thus Ail stands for attachment-and invasion locus [79]. Further characterization of *Y. enterocolitica* Ail provided evidence that Ail was responsible for serum resistance but is not required to cause infection in a murine model of infection [80]. When expressed in *E. coli*, Ail confers high invasion activity into CHO (Chinese hamster ovary) cells and low to moderate levels with HEp-2 (human epithelial) and HEC-1B (human endometrial) cells [78]. Bacteria expressing Ail do not invade MDCK (canine kidney) cells. The observed invasion activity of *E. coli* expressing Ail is less than that of *E. coli* expressing Invasin with each cell line. Cell-associated bacteria were also determined and bacteria expressing Ail bound to HEp-2 cells better than CHO cells and this adhesion activity was better than Invasin. These data indicates that while Ail may not have much invasion activity, Ail can mediate host cell adhesion to a high level.

Ail was further characterized by identifying regions of Ail that are required for the adhesion, invasion and serum resistance phenotypes. A number of surface exposed residues of *Y. eneterocolitica* Ail were identified that affect adhesion, invasion and/or serum resistance [81]. Those residues required for invasion into host cells were found C-terminal half of exposed loop 2 and throughout exposed loop 3. The residues required for serum resistance were also found in exposed loops 2 and 3. This work suggested loops 2 and 3 play

important roles in the various Ail functions. Ail from *Y. pestis* and *Y. enterocolitica* are only 30-65% identical within each of the four extracellular loops when compared to *Y. pestis* Ail. Thus, identifying homologous residues between *Y. enterocolitica* and *Y. pestis* Ail that affect similar functions has been difficult.

Ail from *Y. pseudotuberculosis* was also studied for the ability to confer adhesion and invasion ability when expressed in *E. coli* [82]. An *ail* mutant shows normal cellular adhesion or invasion into HEp-2 cells, suggesting Ail from *Y. pseudotuberculosis* does not mediate cellular adhesion or invasion. *Y. pseudotuberculosis* Ail did, however, confer resistance to complement-mediated killing by human serum [82]. Ail from *Y. pseudotuberculosis* and *Y. pestis* are nearly identical, the amino acid sequences differ by only two residues – position 43 and 126. These two residues lie in the exposed loops 2 and 3, respectively, of the protein. Therefore, as I will describe in this thesis, I analyzed the two amino acid differences for contributions to the functions of Ail, including adhesion, invasion and Yop delivery activity.

Homologues of Ail

As a protein with eight transmembrane domains and four extracellular loops, Ail has a number of homologues with which to make structural and functional comparisons. Interestingly, bioinformatic comparison on sequence identity sheds some light on the structure-related function of these proteins. Most of the similarity is found within the membrane spanning sections of the protein, while the extended loops have much more variation in sequence. The

identity and similarity of Ail and its homologues range from no similarity to 80% similar. This suggests that comparisons may not be possible as they do vary greatly and these variations reflect the differences in function each protein confers to the bacterium. Many of these homologues have been studied for the same functions as Ail including adhesion, invasion, serum resistance, and autoaggregation. In the following section the functions and domains of these homologues will be compared to Ail.

One of the first described homologues of Ail was first discovered in *Enterobacter cloacae* and designated OmpX, as it was an outermembrane protein [83]. Over expression of OmpX in *E. cloacae* lead to resistance to beta-lactam antibiotics. The *ompX* gene is located on the chromosome and encodes a 172 amino acid protein of 18kDa. Biological characterization of OmpX showed it is localized to the outer membrane and an *ompX* deletion did not affect cell growth or regulation of porin proteins [84]. Interestingly, when OmpX was over expressed, other Omps were then down regulated, leading to the idea that the bacteria regulates outer membrane proteins to maintain the amount of proteins in the outer membrane.

OmpX from *E. coli* has also been characterized. *E. coli* strains deleted for *ompX* were analyzed for growth phenotypes, serum resistance, and adherence to mammalian cells. No differences in growth rates, serum resistance, or adherence to mammalian cells were observed with this *ΔompX* mutant [85]. The structure of OmpX from *E. coli* has also been solved by crystallography and NMR studies [86, 87]. A review of the structure will be covered in future sections.

Salmonella encodes a PhoP-activated gene (*pagC*), which is needed for survival within cultured macrophages [88]. PagC was characterized as a 188-amino acid protein similar to *Y. enterocolitica* Ail [89]. When expressed in *E. coli* PagC did not confer serum resistance, host cell attachment or invasion [90].

Another homologue of Ail is Rck from *Salmonella*. Rck is encoded on a virulence plasmid, and like Ail, Rck is an outer membrane 17kDa protein that prevents complement-mediated killing [90]. Rck also confers host cell adhesion and invasion [91]. In addition, experiments with *E. coli* expressing Rck from *Salmonella* and Ail from *Y. enterocolitica* resulted in Ail and Rck promoting serum resistance, attachment, and invasion to cultured cells. In order to study the molecular mechanisms of Rck activities, a random mutant screen was performed. A glycine in ascending loop 3 when mutated to aspartic acid greatly reduced serum resistance and host cell invasion. A chimeric protein constructed with Rck replaced with PagC loops 3 and 4 eliminated the Rck-mediated serum resistance and invasion phenotypes, while Rck-PagC loop 4 chimeric protein did not affect serum resistance or cell invasion. This provides evidence that the loop 3 of Rck is important for both functions. Also, this data also suggest the mechanism of serum resistance and epithelial cell invasion are similar and requires the same domain of Rck.

Bacteriophage lambda encodes Lom, another homologue of Ail [92]. Studies show Lom is an outer membrane protein that is expressed during lysogeny. Expression of Lom promotes the adhesion of *E. coli* to human cells, thus increasing the competitiveness of lambda-infected bacteria [93, 94], 1997).

Other Ail homologues are the colony opacity-associated (Opa) proteins from *Neisseria gonorrhoeae* and *N. meningitidis* strains. Opas were first described as surface-exposed proteins and also contain four extracellular loops in a structure similar to Ail and other homologues. Interestingly, numerous (10-11) genes encoding for Opas are found within one *Neisseria* bacterium [95]. These *opa* genes contain conserved coding repeat regions within the signal sequence and hypervariable regions (HV1 and HV2) located within the structural gene. HV1 and HV2 are found in the exposed loops 2 and 3 and hybridization studies with Opa proteins show the HV regions confer a number of biological functions such as adhesion and invasion into host cells that similar to Ail and other Ail homologues such as adhesion to host cells that are specific to the HV region. This suggests the importance of Opa proteins during *Neisseria* infections.

Neisseria Opa proteins are known to bind to carcinoembryonic antigen cell adhesion molecules (CEACAM) receptors on host cells [96]. CEACAM receptors are found on epithelial cells and neutrophils and the Opa binding site on CEACAM shows Opa binds to a non-glycosylated face [97] [98]. Additional studies with OpaB identified residues required for binding CEACAM1 and CEA receptors by generating alanine-substitution and hybrid mutants [99]. This study found that the conserved regions of hypervariable regions 1 and 2 were important for formation of the binding site. The important sites were found in the descending portions of loop 2 and 3.

Another Ail homologue is the enterotoxigenic invasion protein A (Tia) from enterotoxigenic *E. coli*. Tia is a 25kDa outer membrane protein that also mediates attachment to and invasion into cultured human gastrointestinal epithelial cells [100, 101]. A synthetic peptide corresponding to loop 3 of Tia inhibited invasion into cultured cells [101]. Purified Tia interacts with the cell surface heparan sulfate proteoglycans on epithelial cells [100]. A synthetic peptide consisting of residues 76 through 94 representing surface exposed loop 2 of Tia inhibited binding to heparin. Further studies suggests that Tia binds to glucoaminoglycans which then facilitates binding to other heparin binding proteins such as fibronectin and vitronectin. This provides evidence that bacterial adhesins utilize sulfated proteoglycans as a molecular bridge to many other host cell surface components [102].

Hek from another class of pathogenic, invasive *E. coli*, neonatal meningitic *E. coli* (NMEC), is also another homologue of Ail. Hek is localized in the outermembrane and mediates adhesion, invasion, autoaggregation [103]. Similar to other Ail homologues, Hek was also shown to bind heparinated proteoglygans [103]. Additionally, exposed loop 2 of Hek mediates the ability to autoaggregate, adhere to and invade host cells.

A summary of Ail homologs and regions required for various activities as defined by mutagenic or hybrid analysis is shown in Fig. 1.2.

Structure of homologues and Ail

As described previously, OmpX is an Ail homologue. The crystal structure of both OmpX of *E. coli* and another outer membrane protein, OmpA, from *E. coli* were determined at 1.9Å and 1.65Å resolutions, respectively [86, 87]. It was determined that OmpX has an eight-stranded antiparallel all-next-neighbor β barrel with hydrophilic loops on both intracellular and extracellular portions of the protein. The diameter of the barrel is ~20 Å with an overall height of 50 Å. The loops are about 28 Å (Fig. 1.3). Within the outer membrane, OmpX maintains an ellipsoidal cross-section shape (referred to as a flattened β-barrel), while OmpA maintains a more cylindrical cross-sectional shape [104].

The OmpA crystal structure transmembrane domains are very similar to OmpX, however, the extended loops 1, 2, and 4 are less dense suggesting the loops are more mobile [104]. The differences in loop mobility may correspond to the different functions of these two proteins.

Like other outer membrane homologues, OmpX and Ail contains most of the homology and similarity in the transmembrane domains with little homology within the loops. This may explain the various roles and functions these types of proteins confer to the bacterium.

Host cell binding components

Many bacteria utilize extracellular matrix components to colonize host tissues. A well-characterized host cell matrix component is fibronectin. Fibronectin plays an integral part in various aspects of host physiology and

development [105]. Fibronectin produced by the liver is found in the blood and this soluble form is known as plasma fibronectin [106, 107]. Within tissues, cells use soluble fibronectin to assemble insoluble fibrils to form the fibronectin matrix. Fibronectin along with other extracellular matrix proteins are critical for the architecture and structure of host tissues [108, 109]. Fibronectin is a very large, complex glycoprotein found as a dimer linked together with two disulfide bonds at the C-terminus of the molecule, giving it an appearance of a wishbone [110-112]. A single fibronectin molecule is about 220kDa (dimer ~440kDa). Fibronectin is made up of three types of modular domain repeats: type I, II, and III [113]. Type I = 45 amino acids while type II = 60 amino acids, and type III = 90aa [113]. These structurally similar domains are held together with proteolytically vulnerable spans, which generates a structure similar to beads on a string. All three modules are composed of two anti-parallel β-sheets; however, type I and type II are stabilized by intra-chain disulfide bonds, while type III modules do not contain any disulfide bridges. The entire fibronectin molecule consists of twelve type I (FNI), two type II (FNII), fifteen type III (FNIII) repeat, and a variable region towards the C-terminal end of the molecule (Fig. 1.4) [114].

Fibronectin performs numerous important functions within a host. First, as part of the extracellular matrix, cellular fibronectin is needed for structure of tissues and other matrix assembly [115]. Cells deposit fibronectin for cell adhesion within tissues and to crawl upon for migration. Concentrations of fibronectin within various organs of rats were examined through ELISA techniques. The heart contained 38μg/ml extractable fibronectin, liver contained

82μg/ml, lung, 123μg/ml, kidney, 60μg/ml, and intestine, 100μg/ml [116]. Plasma fibronectin plays an important role in wound healing as it is deposited at sites of injury and initiates the formation of a blood clot [117]. In addition, fibronectin is vital component for the proper development of the whole organism as a homozygous knockout is embryonic lethal at early stages [118].

As mentioned above, fibronectin plays key roles in adhesive functions within a host and binds to various host components including integrins on host cell surfaces, fibrin, and heparin presented on proteoglycans [119]. These binding substrates have specific binding sites along fibronectin (Fig. 1.4). One of the best-known fibronectin interactions is with the $\alpha_5\beta_1$ integrin via the well-studied RGD site within the 10th Fn type III repeat (^{10}FNIII) of fibronectin.

Fibronectin binds to heparin and fibrin at the N-terminal (N-^{5}FNI), then, collagen and gelatin bind to the next region (^{6}FNI-^{9}FNI). Further down the molecule the well-characterized RGD integrin-binding domain is found in the ^{10}FNIII modular repeat with a synergy region in the neighboring ^{9}FNIII [120, 121]. Finally, at the C-terminal end of fibronectin is another heparin binding site. In addition to the RGD site [122], are numerous integrin-binding sites along the entire length of fibronectin [123, 124] (Fig. 1.4).

One of the characteristic features of fibronectin is the fact the molecule can be cleaved into smaller fragments by proteolytic enzymes. Limited cleavage with cathepsin D creates the 70kDa N-terminal fragment and then this 70kDa fragment can be further digested with trypsin to generate the 30kDa heparin-binding fragment and the 45kDa gelatin-binding domain.

In order to study the various complex functions and binding sites of fibronectin in more detail, monoclonal antibodies (mAbs) have been developed to recognize specific epitopes along the length of the molecule [125, 126]. Many of the mAbs block various fibronectin substrates such as collagen, heparin, and cell surface integrin receptors and these mAbs can be very useful for functional studies. Additionally, many other groups have taken advantage of these antibodies to study other bacteria binding sites on fibronectin. These have provided great insights in fibronectin interactions.

Binding to fibronectin by bacterial proteins is a common phenomenon. Both gram-positive and gram-negative bacteria take advantage of binding to fibronectin to initiate host cell contact and, in some cases, cellular invasion [127]. One such example of a bacterial protein that binds fibronectin is protein F of *Streptococcus pyogenes* [128]. A study with protein F has mapped fibronectin binding site to two distinct domains within protein F [129]. This well characterized interaction has been studied extensively and protein F is known to bind to the N-terminal end of fibronectin in solution [130, 131]. Another *Streptococcal* species, *S. pneumoniae*, has been shown to bind to immobilized fibronectin rather than soluble fibronectin [132]. These differences in binding may be due to conformational changes of fibronectin when in solution or splayed out on an immobilized plastic surface.

Staphylococcus aureus is also known to bind to fibronectin, using FnBPA (fibronectin binding protein A) [133]. Utilizing proteolytic fragments as inhibitors of binding, it was determined that *S. aureus* binds to the 30kDa N-terminal

domain of fibronectin [127, 134, 135]. Additional studies have also shown that *S. aureus* can bind to both 30kDa and 120kDa fragments suggesting there are multiple *S. aureus* binding sites along fibronectin [136, 137].

Protein F and FnBPA fall within a larger group of fibronectin binding proteins called MSCRAMMS (microbial surface components recognizing adhesive matrix molecules). As their name describes, these microbial components binds to matrix molecules such as fibronectin, fibrinogen, collagen, and heparin-related polysaccharides [138]. Many nice reviews of MSCRAMMS describe molecular models for the interactions between fibronectin binding proteins and fibronectin [139-141].

Another bacterial adhesin with fibronectin binding activity is the *Y. pseudotuberculosis* YadA protein. YadA mediates binding to fibronectin-coated coverslips but not soluble fibronectin [44]. Further studies with YadA from *Y. pseudotuberculosis* demonstrated YadA binds to fibronectin and uses that interaction to bridge *Y. pseudotuberculosis* interaction in with β_1 integrins on the surface of host cells and initiate intracellular signaling events [45]. This YadA/Fn interaction and signal transduction was required for invasion into cultured cells. The YadA binding site on Fn is not currently known.

Implications for Ail in *Y. pestis* infection

Recent studies identified Ail of *Y. pestis* as a key virulence factor, mediator of host cell contact and a critical adhesion for Yop delivery [65, 70]. Other *Yersinia* species express Inv and YadA, which facilitate not only host cell

adhesion and invasion, but also induce host cell signaling for efficient delivery of cytotoxic Yops to host cells [36, 39, 43, 45]. *Y. pestis* does not express Inv or YadA, therefore, we hypothesize that Ail functions in a similar manner to YadA in which host cell contact is initiated first through fibronectin and then fibronectin acts as a bridging molecule to surface exposed host integrins (Fig. 1.5). This leads to the downstream signaling cascade required for Yop delivery [43].

Y. pestis expresses three adhesins – Ail, Pla, and Psa. *In vivo* studies with various plague models of infection provide evidence that each adhesin is important at different stages of infection and under different environmental conditions depending on the route of infection. Pla contains proteolytic activity, is induced at 37^{o}C, and has been demonstrated to be required for dissemination during intranasal and subcutaneous infections of mice. Therefore, it is hypothesized that the ability of Pla to proteolytically cleaves and activate plasminogen leads to plasmin-mediated degradation of fibrin clots surrounding the inoculate *Y. pestis*. This allows *Y. pestis* to disseminate from the area of inoculation. The pilus Psa is induced under acidic environments at 37^{o}C, particularly within acidic compartments of macrophages and possibly necrotic tissue and provides anti-phagocytic activity to the bacteria. Experiments with subcutaneous and intranasal inoculations in mice provide evidence that Psa is required for early stages of infections. Therefore, it appears Pla and Psa play distinct roles depending on the route of infection and environmental conditions.

Finally, Ail is highly expressed at many environmental conditions and intravenous inoculation with mice suggests Ail is needed for later stages of

infection. We propose that Ail is important for host tissue colonization and Yop delivery at later stages of infection. Understanding the mechanism by which Ail interacts with the host cell is key to our understanding of *Y. pestis* and plague pathogenesis.

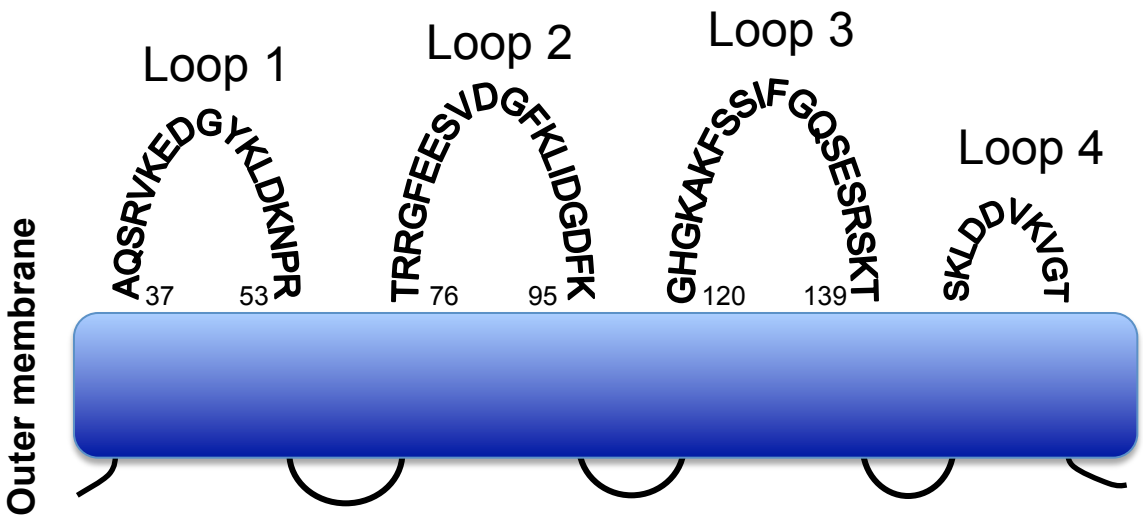
Hypothesis

Ail mediates an efficient interaction with host cells and facilitates *Y. pestis* Yop delivery.

Study Objectives

- Identify the host component(s) with which Ail interacts (Chapter 2)
- Identify residues of Ail that are needed for the various functions of Ail (Chapter 3)
- Understand the mechanism of binding between Ail and fibronectin and map the Ail-binding site on fibronectin (Chapter 4)
- Characterize the differences between *Y. pestis* Ail and *Y. pseudotuberculosis* Ail (Chapter 5)

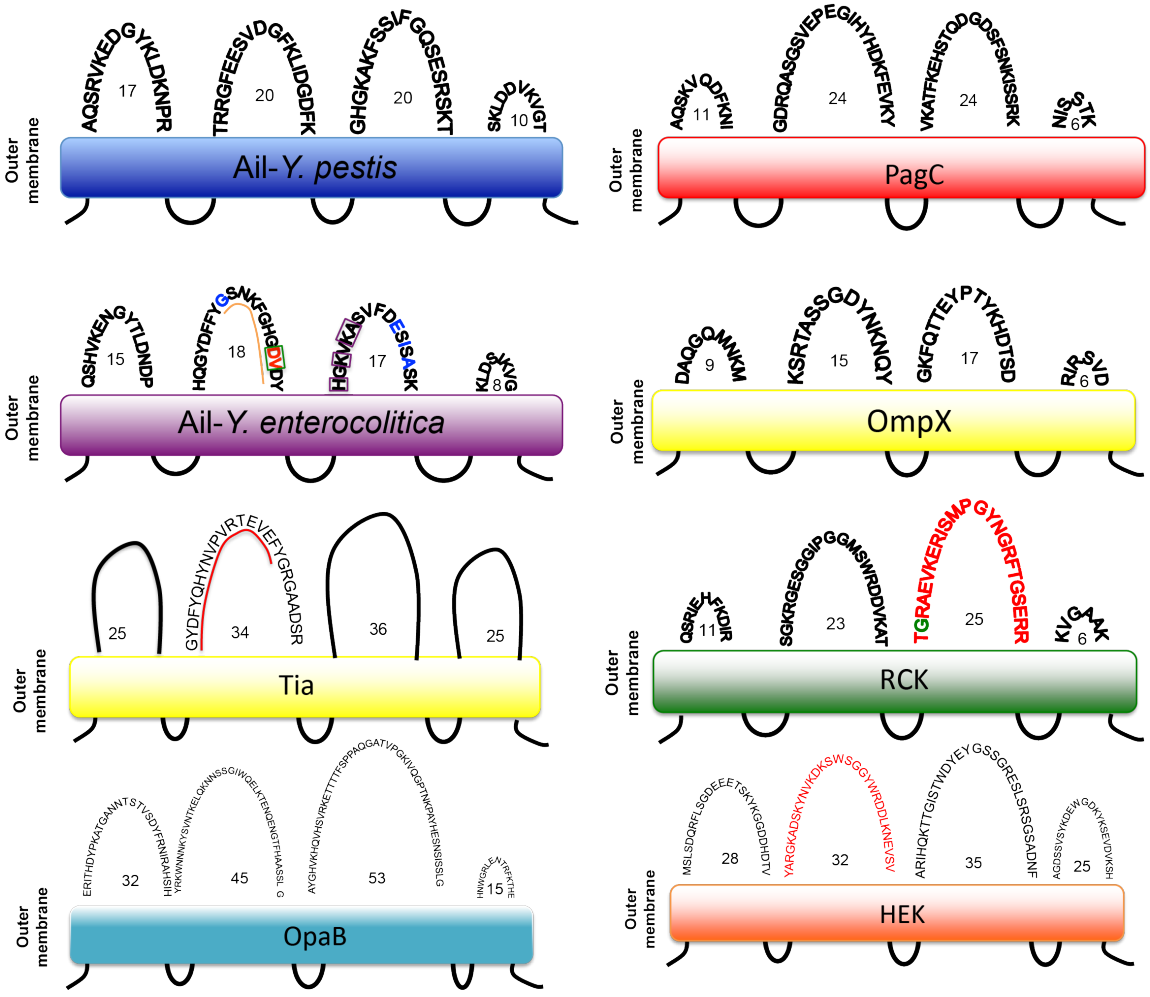
Figure 1.1



Exposed loops of Ail. A depiction of the predicted exposed extracellular loops of Ail. Modeled on Miller and Falkow, 2001. [79].

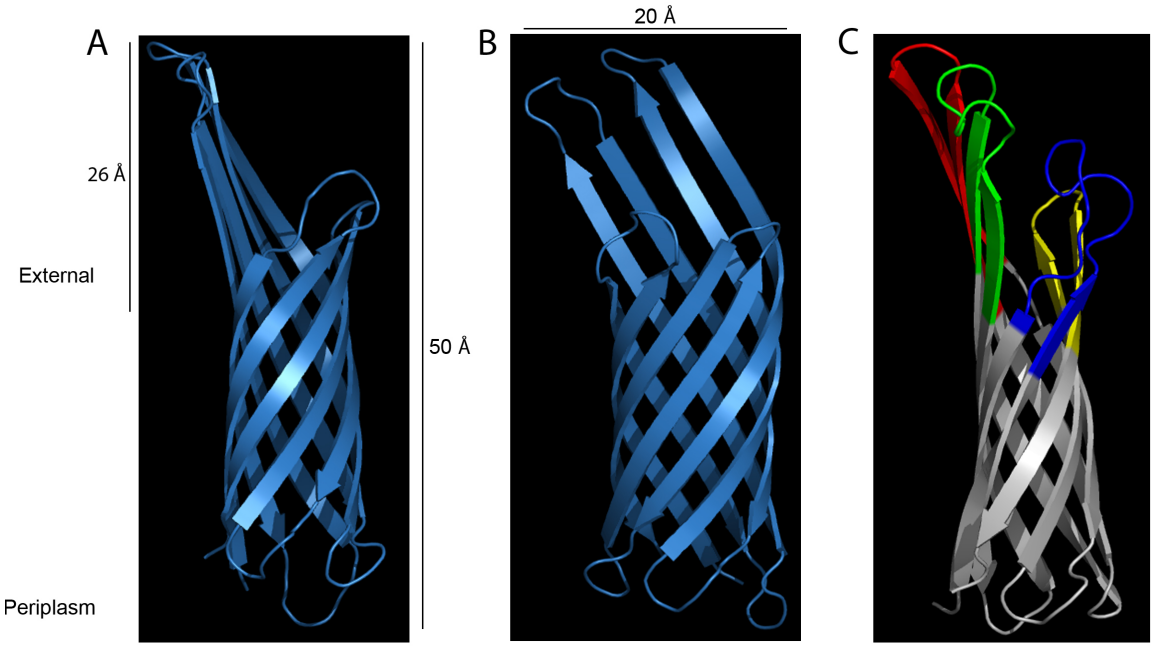
Figure 1.2

Red = non-invasive
Green = serum sensitive
Blue = intermediately invasive
Purple = intermediate serum resistance



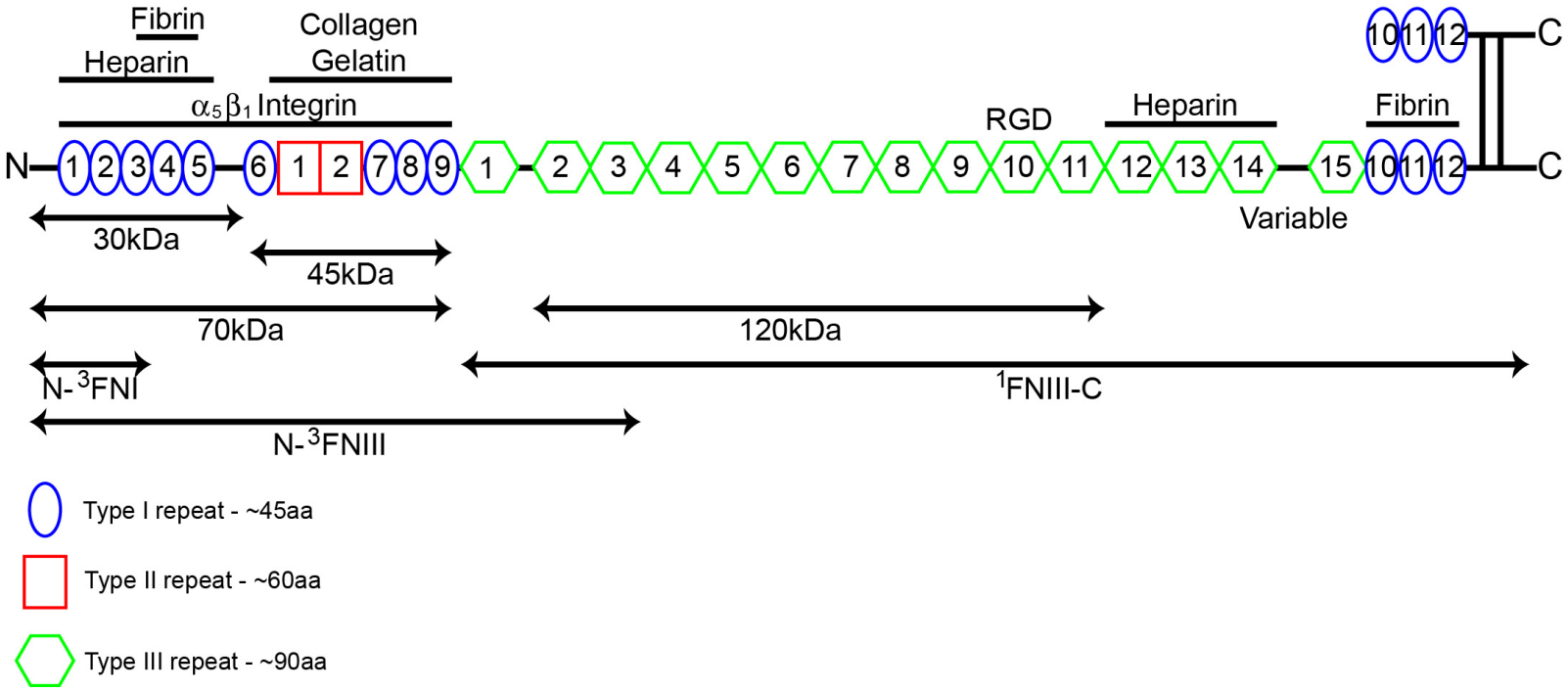
Ail and Ail-homologues loop comparisons. Depictions of Ail and structure homologues. The residues highlighted in red are non-invasive, blue indicates intermediate invasive residues, green indicates serum sensitive residues, and purple residues exhibit intermediate serum resistance. The red line indicates synthetic peptides that block invasion into host cells.

Figure 1.3



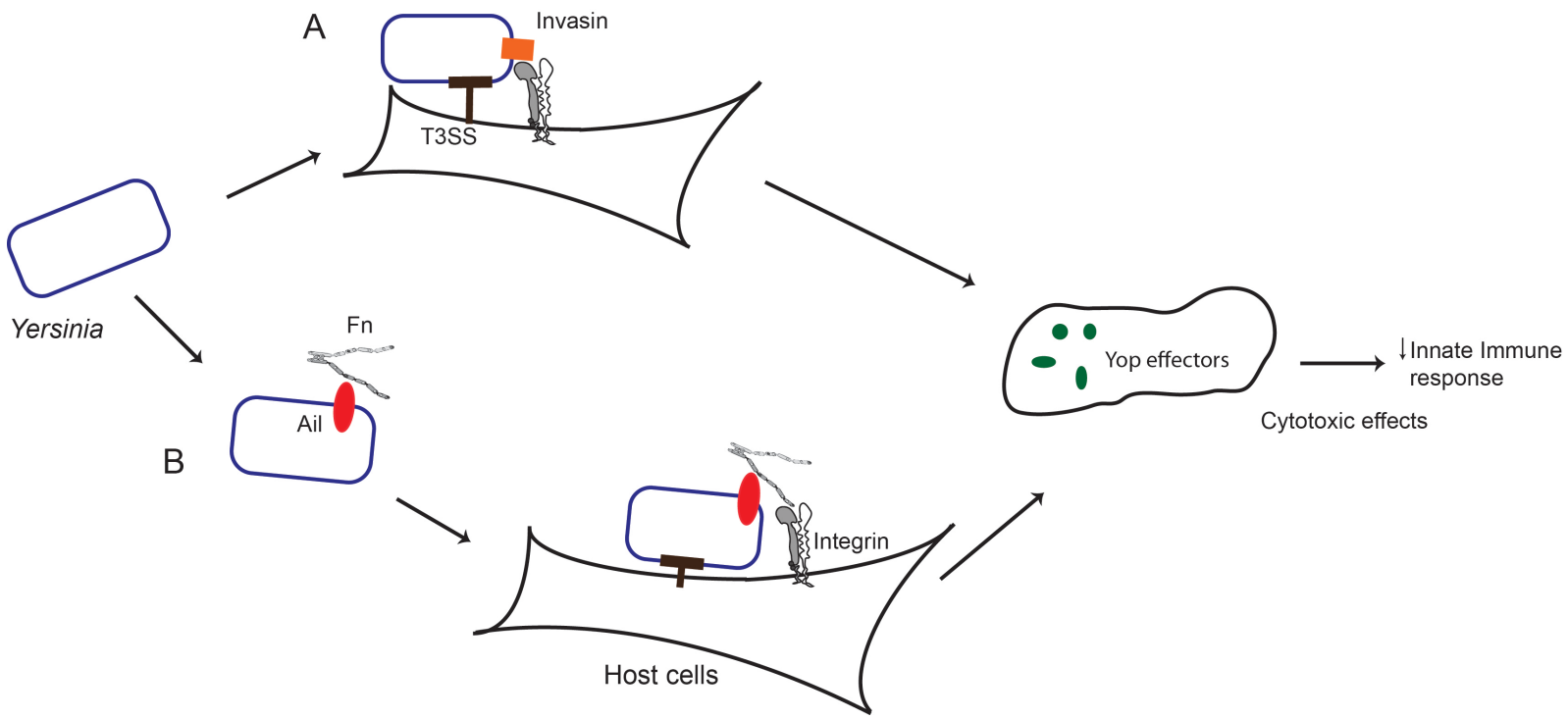
Structure of OmpX and Ail. The OmpX crystal structure is displayed in the side view (A) and the front view (B) as solved by Vogt and Schulz, 1999 [84]. Note the smaller loops, Loop 1 and Loop 4 are found in the front plane while the larger loops, Loop 2 and Loop 3 are found in the back plane. (C) Ail loops were grafted onto the known OmpX crystal structure as described by I-TASSER computational program developed by the Zhang lab [142-144]. Loop 1 is in blue, Loop 2 in green, Loop 3 in red, and Loop 4 in yellow.

Figure 1.4



Structure of fibronectin. Diagramatic depiction of fibronectin modules and important binding domains and fibronectin fragments used in this study. Based on Pankov and Yamada, 2002 [122] and Maurer et al., 2010 [129].

Figure 1.5



Importance of Ail during *Y. pestis* infection. This model depicts the outcome of *Yersinia* infections. (A) *Yersinia* species (*Y. enterocolitica* and *Y. pseudotuberculosis*) expressing YadA and Invasin (inv) can bind to β_1 integrins on the surface of host cells. Through this interaction, Invasin mediates translocation of Yops into host cells. (B) *Y. pestis* does not express YadA or Inv, but Ail can bind Fn which then interacts with β_1 integrins. We hypothesize this interaction facilitates the efficient delivery of Yops.

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CHAPTER TWO

AIL BINDING TO FIBRONECTIN FACILITATES *YERSINIA PESTIS* BINDING TO HOST CELLS AND YOP DELIVERY

Summary

Yersinia pestis, the causative agent of plague, evades host immune responses and rapidly causes disease. The *Y. pestis* adhesin Ail mediates host cell binding and is critical for Yop delivery. To identify the Ail receptor(s), Ail was purified following over-expression in *Escherichia coli*. Ail bound specifically to fibronectin, an extracellular matrix protein with the potential to act as a bridge between Ail and host cells. Ail expressed by *E. coli* also mediated binding to purified fibronectin, and Ail-mediated *E. coli* adhesion to host cells was dependent on fibronectin. Ail expressed by *Y. pestis* bound purified fibronectin, as did the *Y. pestis* adhesin plasminogen activator (Pla). However, a KIM5 *Δail* mutant had decreased binding to host cells, while a KIM5 *Δpla* mutant had no significant defect in adhesion. Furthermore, treatment with anti-fibronectin antibodies decreased Ail-mediated adhesion by KIM5 and the KIM5 *Δpla* mutant, indicating that the Ail-fibronectin interaction was important for cell binding. Finally, anti-fibronectin antibodies inhibited the KIM5-mediated cytotoxicity of host cells in an Ail-dependent fashion. These data indicate that Ail is a key adhesin that mediates binding to host cells through interaction with fibronectin on

the surface of host cells, and this interaction is important for Yop delivery by *Y. pestis*.

Introduction

The three species of *Yersinia* pathogenic for humans, *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*, cause distinctive diseases. *Y. pseudotuberculosis* causes mild self-limiting gastroenteritis, and *Y. enterocolitica* causes a more severe gastroenteritis, while *Y. pestis* is the causative agent of the plague, one of the most deadly human infectious diseases. *Y. pestis* is a close relative of *Y. pseudotuberculosis,* diverging only 5,000 to 20,000 years ago [1]. To establish an infection, all three pathogenic *Yersinia* species inject cytoxic Yersinia outer proteins (Yops) into host cells via the Ysc type III secretion system (TTSS) [2]. Host cell contact is essential for engagement of the TTSS and secretion of the Yop proteins [3, 4]. Within the host cell, Yops affects actin rearrangements, inhibit phagocytosis, and block pro-inflammatory signals [5-7]. Both *Y. enterocolitica* and *Y. pseudotuberculosis* express the well-studied adhesin molecules Invasin (Inv) and YadA, capable of mediating Yop delivery [3, 4]. However, *Y. pestis* does not express either adhesin due to an IS*1541* element insertion within *inv* [8] and a frameshift mutation in *yadA* [9, 10]. *Y. pestis* has a number of other adhesins capable of mediating host cell interaction. pH 6 antigen (Psa) [11, 12] and plasminogen activator (Pla) [13] of *Y. pestis* have both been shown to be adhesins. Psa is a tightly regulated pilus expressed at pH<6.7 and 37^{o}C [14, 15] and is known to bind to gangliosides [16], LDL [17], and IgG [18]. Pla is expressed at 37^{o}C [19] and is known to bind several

extracellular matrix components [13, 20, 21]. The putative autotransporter, YapC, is also capable of mediating cell adhesion when expressed in *E. coli* [22] as is the pilus encoded by the chaperone/usher system locus *y0561-0563* [23], but neither result in significantly decreased adhesion when deleted in *Y. pestis* [22, 23].

Recently, an additional adhesin of *Y. pestis*, Ail (adherence and invasion locus), was determined to facilitate cell binding [24, 25]. Ail is a 21.5kDa outer membrane protein of the OmpX family, predicted to have eight transmembrane domains and four extracellular loops extending above the surface of the bacterium [26, 27]. Ail homologues include OmpX of *E. coli* [28], OmpX from *E. cloaca* [29], PagC in *Salmonella* [30], and Opa protiens from *Neisseria* [31]. Ail from *Y. enterocolitica* has been studied previously and shown to have three activities, cell adhesion and invasion [32], and the ability to confer serum resistance [33] [34] by binding to complement regulatory proteins [35]. The residues for all three activities have been mapped to particular amino acids in the surface-exposed loops [36]. *Y. pseudotuberculosis* Ail also confers adhesion and invasion functions (TMT and ESK, unpublished observations) and serum resistance [37]. More recently, *Y. pestis* Ail was also shown to mediate cell adhesion [24] [25] autoaggregation [24], serum resistance [24, 35, 38], and facilitate Yop delivery to host cells [25]. Furthermore, *Y. pestis* Ail is required for virulence, as a *Y. pestis Δail* mutant has a >3,000 fold increase in LD_{50} [25]. A *Y. pestis Δail* mutant shows reduced binding to both epithelial and phagocytic cell lines, and in a mouse model of infection, a *Y. pestis* KIM5 *Δail* mutant colonizes

host tissue to much lower levels than the parental KIM5 strain. Over the course of the seven days, the *Δail* mutant is cleared from the host [25]. Together, these data demonstrate Ail is an important adhesin that contributes to colonization and virulence.

Adhesion is an important step for the establishment of a successful infection. Adhesion is also significant in *Y. pestis* pathogenesis because host cell contact is required for the production and secretion of the Yop effector proteins [39]. Therefore, host cell binding is a critical step in establishing an effective infection of the host. Bacteria can bind directly to host cell receptors [40] or use molecules like extracellular matrix (ECM) components to mediate attachment to host cells [20] [41] [42] [43]. Common components of the cellular matrix that facilitate bacterial binding include fibronectin [41] [13], collagen [21], and laminin [13] [20]. Particularly, fibronectin binding proteins on bacterial pathogens have been identified and studied. Those fibronectin binding proteins include SigB from *Staphylococcus aureus*, [44], protein F from *Streptococcus pyogenes* [45], and YadA from *Y. pseudotuberculosis* [42], and *Y. enterocolitica* [41]. Interactions between bacteria and the ECM lead to bridge-like attachments to host cells. Fibronectin is a large glycoprotein that is a key structural component in many tissues. This ~220kDa protein is commonly found as a dimer that is linked by two disulfide bonds located at the C-terminal end of the molecule. Fibronectin is a large, very complex molecule made up of three types of modular repeating units [46, 47]. Fibronectin can bind to many substrates including collagen [48], integrin receptors on host cells [49, 50] and heparin [51]. Additionally, fibronectin

contains a binding site for several bacterial pathogens at the N-terminal end of the molecule [52, 53]. YadA from *Y. pseudotuberculosis* has been shown to interact with fibronectin and binding of YadA to fibronectin allows *Y. pseudotuberculosis* to utilize β1 integrins on the surface of host cells for invasion [42]. Given the key role of *Y. pestis* Ail in cell adhesion, Yop delivery, and virulence, we sought to determine the component on host cells to which Ail binds.

Although Ail has been studied extensively in other *Yersinia* species, the substrate on the host cells to which Ail interacts is not known. In this study, we used a purified *Y. pestis* Ail to identify the extracellular matrix component, fibronectin, as a protein bound by Ail. Furthermore, Ail mediated binding to host cells through fibronectin is important for the delivery of Yop effector proteins.

Experimental Materials and Methods

Strains and culture conditions. *Y. pestis* strains were cultivated in heart infusion broth (HIB) overnight or on heart infusion agar (HIA) for 48 hours at 28°C. *Escherichia coli* strains were cultured in Luria-Bertani (LB) broth or LB agar at 28°C or 37°C. Antibiotics were used at the following concentrations: streptomycin (Sm), 100 μg/ml; chloramphenicol (Cm), 10 μg/ml; and ampicillin (Amp), 100 μg/ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used at a 100 μM concentration unless otherwise noted.

HEp-2 cells were cultured at 5% CO_2 (37°C) in modified Eagle's medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco), 1% sodium pyruvate (Gibco), and 1% non-essential amino acids (Gibco). THP-1

cells were cultured at 5% CO_2 (37°C) in RPMI medium supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco). THP-1 cells were activated by treatment with 10pg/ml phorbol myristate acetate (PMA, Sigma) for three days to stimulate attachment to 24-well plates.

Purification of Ail-His_6. The coding region of the processed form of *ail* was PCR amplified with the 5' primer: 5' - GCGCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATatggaaggc gaaagcagtatttc - 3' and 3' primer: 5' - GCGCGGTACCgaaccggtaacccgcgcc - 3', digested with *Kpn*I and *Xba*I and ligated into the C-terminal His-tag vector pET30b+ (Qiagen) using the *Kpn*I and *Xba*I sites. *E. coli* BL21(DE3) expressing the pET30b+ Ail-His_6 construct was grown in LB Kan at 37°C overnight. The next day, the culture was diluted 1:50 into LB + antibiotics and expression of Ail-His_6 was induced with 10μM IPTG. Ail-His_6 was purified under denaturing conditions (8M Urea) from whole cells (including inclusion bodies). Ail-His_6 was subjected to batch column affinity purification with Ni-NTA Superflow beads (Qiagen) by following the manufacturer's protocol (Qiagen). Cells were lysed with lysis buffer: 100mM NaH2PO4, 10mM Tris-Cl, 8M Urea, pH 8.0. Cell lysates were allowed to bind to the beads for 1 hour at room temperature and the beads were loaded onto a gravity flow column. The column was washed twice with 5ml wash buffer: 100mM NaH2PO4, 10mM Tris-Cl, 8M Urea, pH 7.3. Elution from the column was achieved with stepwise lowering of the pH of the elution solutions: 100mM NaH2PO4, 10mM Tris-Cl, 8M Urea, pH 5.9 and pH 4.5 in two

0.5ml fractions. The fraction eluates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and assessed for purity by staining with Coomassie Blue. Equal volumes of column fractions and SDS-PAGE sample buffer were mixed. The samples were boiled for 5 min, microcentrifuged for 5 min at 13.2K, and then subjected to SDS-PAGE followed by Coomassie blue staining. Fractions containing a single Ail-His_6 band with similar concentrations were pooled. Ail-His_6 was re-folded with stepwise dialysis in 20mM HEPES pH 7.0 buffer with 1% dodecyl maltoside (DDM, Anatrace) against decreasing concentrations of urea. The final dialysis step was against 20mM HEPES pH 7.0 with 0.1% DDM. The concentration of each preparation was determined by Bradford assay (Biorad) and each preparation was stored at -20^{o}C in 50µl aliquots.

While in the end, Ail-His_6 was purified under denaturing conditions, we also attempted to purify this Ail-His_6 from the outer membrane in its natural conformation. The outer membranes of *E. coli* expressing the Ail-His_6 were isolated; however, the Ail-His_6 protein was not recovered upon purification on Ni-NTA Superflow beads. Thus, the denatured form of Ail-His_6 was purified and slowly refolded in DDM detergent. We also tried other detergents, N-Dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, but the Ail-His_6 precipitated out of solution.

Extracellular Matrix Binding Assay. 96-well plates (Pro-bind, BD Falcon) were coated with appropriate concentrations of plasma fibronectin (Sigma, F2006),

cellular fibronectin (Sigma, F2518), collagen I (Sigma, C5483), collagen IV (Sigma, 27663) overnight at 4^{o}C. The wells were washed twice with PBS before blocking with Tris buffered saline (50 mM Tris 7.4, 150 mM NaCl) (TBS) + 2% bovine serum albumin (BSA) (blocking buffer) for two hours at room temperature. Purified Ail-His_6 was added to the ECM coated wells at the appropriate concentration in blocking buffer and incubated for 3 hours at 37^{o}C or overnight at 4^{o}C to allow binding. To detect Ail-His_6 binding, rabbit anti-His_6 tag polyclonal antibody (Qiagen) was added at a dilution of 1:250 and allowed to bind the wells at room temperature for 2 hours. After washing away unbound primary antibody, goat anti-rabbit antibody conjugated to alkaline phosphatase (Zymed) was added at a 1:3000 dilution and incubated for 1 hour at room temperature. Wells were washed with PBS three times and once with 100mM Tris 8.0. The alkaline phosphatase substrate, *p*-Nitrophenyl phosphate (PNPP) was added at a concentration of 4mg/ml in 100mM Tris 8.0. Absorbance was measured at A_{405}.

Bacterial binding assay and antibody blocking. 96-well plates (Pro-bind, BD Falcon) were coated with appropriate concentrations of plasma fibronectin, cellular fibronectin, collagen I, or collagen IV overnight at 4^{o}C. *E. coli* or *Y. pestis* derivatives were cultured overnight at 37^{o}C in LB or 28^{o}C in HIB respectively (IPTG and Cm was added for the strains containing pMMB207). The following day, wells were washed twice with PBS before blocking with blocking buffer (TBS + 2% BSA) for 2 hours at room temperature. The bacterial cells were normalized to an OD_{600} or OD_{620} (*E. coli* or *Y. pestis*) of 1.2 in PBS+0.4% BSA. 50µl of

bacterial suspension was added to the appropriate wells. The plate was incubated at 37°C for 2 hours. The wells were washed three times with PBS before fixing with 100µl methanol. The bacteria bound to the wells were stained with 0.01% crystal violet. After washing away excess crystal violet, the bacterial-associated crystal violet stain was solubilized with ethanol/acetone solution. The absorbance was measured at A_{595}.

Antibody blocking assays were preformed similarly to the bacterial binding assays except the polyclonal rabbit anti-human fibronectin antibody (Sigma, F3648) was diluted in 40µl of PBS + 0.4% BSA to the proper concentrations and was added to fibronectin coated wells 1 hr prior to the addition of 10µl five-fold concentrated bacteria (ie OD_{600} or OD_{620} =6.0).

Adhesion assays. HEp-2 cells were cultured in 24-well tissue culture plates until reaching 100% confluence. THP-1 cells were plated at $1X10^5$ cells/ml and activated with 10pg/ml phorbol myristate acetate (PMA, Sigma) for three days to stimulate attachment to 24-well plates. Y*. pestis* KIM5 or *E. coli* AAEC185 derivatives were cultured in HIB or LB overnight at 28°C or 37°C, respectively. Cm was added for the strains containing pMMB207 for complementation studies. Overnight *E. coli* cultures were diluted 1:10 in fresh LB with Cm and 100µM IPTG to induce plasmid-based expression. Strains were allowed to grow for an additional 3 hours at 37°C. *Y. pestis* strains were diluted 1:10 in fresh HIB with Strep and Cm and 100µM IPTG to induce expression of genes encoded on the vector pMMB207 as required. *Y. pestis* strains were allowed to grow an

additional 3 hours at 28°C or 37°C as indicated. Tissue culture cells were washed twice with 1ml of serum-free minimal essential medium (MEM) for HEp-2 cells or RPMI for THP-1 cells. A polyclonal rabbit anti-human fibronectin antibody (Sigma, F3648) was diluted at the appropriate concentrations in tissue culture media without serum with 20mM HEPES pH 7.0 and 0.4% BSA. 200μl of antibody was added to cultured cells for 1 hr prior to the addition of bacteria. For THP-1 cells, the cells were pre-treated with 5μg/ml cytochalsin D (Sigma) for 1 hour prior to infection to inhibit phagocytosis. After the 1 hr pre-incubation, 50μl of bacteria were added to each well for a final MOI of 100 or 10, for *E. coli* or *Y. pestis*, respectively. Plates were incubated with bacteria for 2 hr at 37°C in 5% CO_2. Cells were then washed two times with PBS, and cell-associated bacteria were liberated by the addition of sterile H_2O containing 0.1% Triton X-100 for 10 min. Serial dilutions of samples were plated onto HIA or LB agar for CFU analysis. In parallel wells, the entire population of bacteria was harvested and enumerated by dilution and CFU analysis to determine the total number of bacteria per well. Percent adhesion was calculated by dividing bound CFU by total bacteria in the well at the end of 2 hours of incubation and then multiplying by 100.

Cytotoxicity assay. HEp-2 cells and THP-1 cells were cultivated until they reached about 50% confluence in 24-well tissue culture plates. THP-1 cells were activated by treatment with 10pg/ml phorbol myristate acetate (PMA, Sigma) for three days to stimulate attachment to 24-well plates.

Y. pestis KIM5 derivatives strain were cultured in HIB overnight at 28°C. Overnight cultures were diluted 1:10 in fresh HIB and incubated for 3 hours at 28°C. Tissue culture plates were washed twice with PBS and 0.5ml serum-free tissue culture medium was added to each well. Bacteria were added to each well at an MOI of 10. Plates were incubated at 37°C in 5% CO_2. Cells were fixed with 0.5ml methanol and stained with 0.76mg/ml Geimsa stain at 4 hours post-inoculation. Rounding was observed and pictures were taken with a phase-contrast microscope. Cytotoxicity was enumerated by counting total cells and the number of round dark purple (shrunken cytoplasm) cells experiencing cytotoxicity in three microscopic fields (~75 cells/field). Percent cytotoxicity was calculated by dividing rounded cells by total cells.

Results

Purification of Ail using a C-terminal 6X-His tag

To study the interaction of Ail with host cells, we purified a histidine-tagged Ail derivative, Ail-His_6, using a C-terminal His-tag. A form of Ail, lacking the N-terminal signal sequence (representing the processed form) was cloned for over expression. *E. coli* BL21 (DE3) cells were induced with 10μM IPTG to express Ail-His_6. Bacterial cells were pelleted and subjected to 8M urea to denature all Ail-His_6, including proteins potentially present in inclusion bodies. The cell lysates were mixed with Ni++ beads and subjected to gravity flow chromatography column to purify the Ail-His_6 protein. Fraction eluants were analyzed by SDS-PAGE (Fig. 2.1). Purified Ail (indicated with an arrow) runs at

the predicted size of 21kDa and the identity was confirmed by MS/MS. Some pure Ail-His_6 came off in later washes at pH = 6.3, however, most of the protein eluted at pH = 5.9 and 4.5.

Purified Ail binds fibronectin specifically.

Several bacterial adhesins are known to bind components of the extracellular matrix (ECM). Since Ail-mediated binding to host cells shows many bacteria binding to regions between cells [25], therefore, we tested the binding capability of purified Ail-His_6 to the common ECM components fibronectin, collagen I, and collagen IV. Binding of Ail-His_6 to extracellular matrix was performed by coating plates with 20µg/ml of ECM and adding purified Ail-His_6 at increasing concentrations. The amount of purified Ail-His_6 bound to fibronectin increased with increasing concentrations of purified Ail-His_6 (Fig. 2.2A). By 40µg/ml, Ail-His_6 binding to fibronectin was approaching saturating levels. No binding of Ail-His_6 was detected with collagen I, collagen IV, or the negative control bovine serum albumin (BSA). Thus, binding of Ail-His_6 to fibronectin was specific. The Ail-His_6/fibronectin interaction was also studied in the converse experiment, where increasing concentrations of ECM components were coated onto a microtiter plate and the amount of Ail-His_6 was held constant. When 10µg/ml Ail-His_6 was added to increasing concentrations of fibronectin, there was increased binding which saturated at 5µg/ml fibronectin (Fig. 2.2B). This binding was specific as Ail-His_6 never bound collagen I or collagen IV at any concentration tested. Binding of Ail-His_6 to fibronectin was not due to the His_6

tag since a control His_6-tagged protein,ToxR-His_6 (a transcription factor of *Vibrio cholerae*, [54]) did not bind any ECM component tested (Fig. 2.2B-D).

Bacteria expressing Ail bind purified fibronectin.

To determine whether Ail expressed on the surface of bacteria can mediate binding to fibronectin, an IPTG-inducible construct encoding *ail* [25] was expressed in *E. coli* AAEC185, a derivative of *E. coli* lacking type-1 fimbrae [55]. *E. coli* expressing Ail were added to increasing concentrations of purified ECM components coated on microtiter plates. *E. coli* expressing Ail on its surface bound to both plasma and cellular fibronectin in a specific manner that saturated at concentrations higher than 5μg/ml (Fig. 2.3). This binding was specific for fibronectin since *E. coli* expressing Ail does not bind to Collagen I or Collagen IV (Fig. 2.3). Additionally, *E. coli* expressing the empty vector, pMMB207, does not bind to any of the extracellular matrix components tested (Fig. 2.3).

Anti-fibronectin antibody blocks Ail-mediated interaction with purified fibronectin and host cells.

Having demonstrated that *E. coli* expressing Ail can bind to purified fibronectin, we used a polyclonal antibody against fibronectin to block Ail-mediated bacterial binding to purified fibronectin. Without addition of antibody, *E. coli* expressing Ail bound purified fibronectin. Addition of both 1:50 and 1:100 dilution of anti-fibronectin (anti-Fn) polyclonal antibody resulted in a pronounced decrease in Ail-mediated *E. coli* binding to fibronectin (Fig. 2.4A). *E. coli*

expressing the empty vector control showed minimal binding to purified fibronectin irrespective of treatment with antibody.

To determine whether fibronectin is the predominant Ail substrate on the surface of host cells, the polyclonal anti-Fn antibody was used to block bacterial adhesion to cultured HEp-2 (human epithelial-like) (Fig. 2.4B) cells and THP-1 (human monocyte-like) (Fig. 2.4C) cells. The percentage of HEp-2 adhesion by *E. coli* expressing Ail was normalized to 100% to compare the adhesion across multiple experiments. Adhesion of *E. coli* expressing Ail to HEp-2 cells decreased four-fold with the anti-Fn antibody used at 1:25 dilution, while *E. coli* expressing empty vector (pMMB207) bound poorly to HEp-2 cells and was not affected by the addition of anti-Fn antibody (Fig. 2.4B). This indicates the anti-Fn antibody is specific to the Ail-fibronectin interaction. *E. coli* expressing the *Y. pestis* adhesion, pH6 antigen (Psa) encoded by *psaABC* was only weakly affected for cell binding by the anti-Fn antibody. Psa in known to interact with several cellular receptors and substrates [16, 56] (SF and ESK, unpublished results).

Ail-mediated adhesion to THP-1 cells was also affected by anti-Fn antibody although the decrease in binding was only two-fold with this cell line (Fig. 2.4C). Again, the empty vector control directed very little binding to THP-1 cells and the antibody did not affect background binding. Psa also mediated binding to THP-1 cells and this adhesion was modestly decreased in the presence of anti-Fn antibody. These data indicate that fibronectin is the main substrate for Ail-mediated adhesion on both phagocytic and non-phagocytic

human derived host cells. Taken together, anti-Fn antibody can inhibit Ail-mediated binding to purified fibronectin and host cells.

Y. pestis binding to fibronectin is mediated through Ail and Pla.

Having demonstrated that Ail expressed on the surface of *E. coli* can mediate specific and saturable binding to fibronectin, we wanted to determine whether Ail expressed in its natural context, on the surface of *Y. pestis,* can also bind fibronectin. Again, wells were coated with purified fibronectin at increasing concentrations. *Y. pestis* KIM5 binding to fibronectin increased with increasing concentrations of fibronectin, and was saturable (Fig. 2.5A). However, a KIM5 *Δail* strain also bound fibronectin in a specific and saturable manner (Fig. 2.5A), suggesting another *Y. pestis* adhesin may also bind fibronectin. Since Pla is known to bind several ECM components, [20, 21, 57] we tested a KIM5 *Δpla* mutant for fibronectin binding. The KIM5 *Δpla* mutant had decreased adhesion to fibronectin (Fig. 2.5A), and a KIM5 *Δail Δpla* double mutant displayed an even further reduction in binding to fibronectin ($p < 0.004$, relative to KIM5 *Δpla*). To determine whether Ail can restore fibronectin binding to the KIM5 *Δail Δpla* double mutant, we complemented the strain with the pMMB207-*ail* plasmid. Fibronectin binding was restored upon induction with 100μM IPTG, while the KIM5 *Δail Δpla* strain harboring pMMB207 alone bound fibronectin poorly (Fig. 2.4B). Thus, *Y. pestis* KIM5 can bind fibronectin via both Ail and Pla.

Anti-Fn antibody blocks *Y. pestis* Ail-mediated binding to host cells.

We have shown that fibronectin is the primary substrate on host cells for Ail-mediated adhesion of *E. coli*. In addition, *Y. pestis* can bind purified fibronectin via Ail, and Pla also contributes to that binding. Therefore, we wanted to address whether *Y. pestis* adhesion to cultured host cells is also dependent upon Ail/fibronectin interactions. *Y. pestis* KIM5 and various mutant derivatives were tested for their adhesive abilities to HEp-2 cells (Fig. 2.6A) and THP-1 cells (Fig. 2.6B) in the absence and presence of the polyclonal anti-Fn antibody. *Y. pestis* KIM5 adhesion without anti-Fn antibody was normalized to 100% and adhesion of the KIM5 *Δail* mutant (Pla-dependent) to HEp-2 cells (Fig. 2.6A) was 50% of parental KIM5. However, adhesion by the KIM5 *Δpla* mutant (Ail-dependent) was similar to the parental strain, KIM5. The KIM5 *Δail Δpla* double deletion mutant also showed a 50% decrease in adhesion compared to KIM5. After pre-treatment with a 1:25 dilution of anti-Fn antibody, the adhesion by the parental strain KIM5 adhesion was reduced by 40%. The anti-Fn antibody did not affect Pla-dependent adhesion (KIM5 *Δail)*, but there was a trend toward decreased Ail-mediated (KIM5 *Δpla*) adhesion in the presence of anti-Fn antibody, although this decrease did not reach statistical significance ($p = 0.085$).

Similar results were observed with adhesion to THP-1 cells. Again, when the parental *Y. pestis* strain, KIM5, was set at 100%, adhesion by KIM5 *Δail* mutant (Pla-dependent) was about 50% of parental KIM5, but adherence by the KIM5 *Δpla* mutant (Ail-dependent) was similar to the level of parental KIM5 (Fig. 2.6B). The *Δail Δpla* double deletion strain showed a further decreased level of adhesion relative to the *Δail* mutant, although this is not significant ($p = 0.38$). In

the presence of anti-Fn antibody, the parental KIM5 strain showed 50% adhesion to THP-1 cells. Again, when the fibronectin on THP-1 cells was blocked with anti-Fn antibody, the Pla-dependent adhesion (KIM5 *Δail*) was not affected, but there is a significant decrease in adhesion of Ail-mediated (KIM5 *Δpla*) adhesion with treatment by the anti-Fn antibody. The adhesion of the KIM5 *Δail Δpla* double mutant was also reduced in the presence of anti-Fn antibody, although this reduction in adhesion did not reach significance (P = 0.066). These data indicate that *Y. pestis* KIM5 expressing Ail engage host cells, primarily via Ail and this interaction is bridged by Fn since treatment with anti-Fn antibody, blocks Ail mediated interactions with host cells.

Anti-Fn antibody blocks Ail-mediated Yop delivery.

Previous studies have shown Ail to be a primary mediator of Yop delivery in *Y. pestis* KIM5 [25]. Given that Ail can mediate bacterial binding to host cells in a fibronectin-dependent manner (Figs. 2.4 and 2.6) and *Y. pestis*-expressed Ail can bind purified fibronectin (Fig. 2.5), we determined whether anti-Fn antibodies can inhibit Yop delivery by *Y. pestis*. Cytotoxicity (cell rounding) was used as a measure of Yop delivery. The anti-Fn antibody was added to HEp-2 cells one hour prior to the addition of *Y. pestis* KIM5 and derivatives. *Y. pestis* strains were incubated with host cells for four hours prior to visualization of cell rounding. 60% of HEp-2 cells showed cytotoxicity upon interaction with the parental KIM5 strain in the absence of antibody treatment (Fig. 2.7A, K). The KIM5 *Δail* mutant mediated reduced levels of Yop delivery, consistent with a

previous report and suggesting Ail is a key mediator of Yop delivery [25] (Fig. 2.7C, K). The deletion of *pla* resulted in levels of cytotoxicity similar to the KIM5 parental strain (Fig. 2.7E, K), indicating Pla is not a key mediator of Yop delivery under these conditions, even though it can mediate binding to fibronectin (Figs. 2.5). After the addition of the anti-Fn antibody, KIM5-mediated cytotoxicity was reduced to 32% (Fig. 2.7A, B, K). A KIM5 *ΔyopB* control strain (unable to deliver Yops due to lack of a component of the translocation apparatus, [2]) showed very little cytotoxicity indicating cytotoxicity being measured is Yop-dependent (Fig. 2.7I, K).

In the presence anti-Fn antibody, the KIM5 *Δail* mutant was not affected for its level of cytotoxicity for host cells (Fig. 2.7C, D, K). However, the KIM5 *Δpla* mutant showed a two-fold reduction in cytotoxicity in the presence of anti-Fn antibody, similar to the parental KIM5 strain (Fig. 2.7E, F, K). Thus, in the presence of anti-Fn antibody, both KIM5 and KIM5 *Δpla* were significantly reduced for Yop delivery, while the KIM5 *Δail* strain was not affected. Furthermore, levels of Yop-mediated cytotoxicity for KIM5 and KIM5 *Δpla* in the presence of anti-Fn antibody approached the levels of cytotoxicity elicited by the KIM5 *Δail* strain. The anti-Fn antibody does not generally decrease Yop delivery since a *Y. pestis* KIM5 *Δail Δpla ΔpsaA* mutant expressing Psa from a plasmid is not inhibited for Yop delivery (Fig. 2.7G, H, K). HEp-2 cell cytotoxicity was evaluated by counting three fields with ~100 cells/field (Fig. 2.7K).

To determine whether Yop delivery through the *Y. pestis* Ail interaction with fibronectin was specific to HEp-2 cells, we also analyzed the ability of the

anti-Fn antibody to block cytotoxic effects on THP-1 cells. Similar to the HEp-2 experiment, we pre-treated THP-1 cells with the anti-fibronectin antibody for one hour prior to the addition of *Y. pestis* KIM5 derivatives. We scored cytotoxicity by observing a shrunken cytoplasm and darker staining (data not shown). The number of cytotoxic cells and normal cells were counted and plotted (Fig. 2.7L). The percent of KIM5-infected THP-1 cells showing cytotoxicity was about 30% while the KIM5 *Δail* mutant exhibited about 10% cytotoxicity (Fig. 2.7L). The KIM5 *Δpla* mutant induced similar levels of cytotoxicity as the parental KIM5 strain (Fig. 2.7L). This data is in agreement with the data using HEp-2 cells and shows Ail is important for the cytotoxicity caused by *Y. pestis*. The KIM5 *ΔyopB* mutant caused about 10% of the THP-1 cells to show cytotoxicity indicating cytotoxicity was due to the delivery of the Yop effector proteins (Fig. 2.7L). With the treatment of anti-Fn antibody, the cytotoxicty caused by the parental KIM5 strain dropped from 30% to about 10%, while the anti-Fn antibody reduced the levels of KIM5 *Δpla* mutant from 30% to about 15% (Fig. 2.7L). This shows that the Ail-mediated cytotoxicity of the KIM5 *Δpla* mutant can be inhibited by blocking fibronectin. Finally, the anti-Fn antibody had no effect on the KIM5 *Δail* or KIM5 *ΔyopB* mutants (Fig. 2.7L). Again, Psa-mediated toxicity for THP-1 cells is not affected by addition of anti-Fn antibody (Fig. 2.7L), indicating that the effect of the anti-Fn antibody is Ail-specific.

These results suggest Ail is the key mediator of Yop delivery to HEp-2 cells and blocking the interaction between Ail and fibronectin on the surface of host cells decreases Yop delivery. Since Ail has been shown to interact with

fibronectin, we conclude that Ail interacting with fibronectin on host cells is a key event in the efficient delivery of Yops.

Discussion

A critical step in the infection process of *Y. pestis* is the adhesion to host cells, the first step in engagement of the Ysc T3SS and the delivery of cytotoxic Yop effector proteins. One *Y. pestis* adhesin, Ail, has been shown to mediate cell binding, serum resistance and Yop delivery [24, 25, 38]. However, the component on the surface of host cells to which Ail interacts as an adhesin, has not been identified. In this paper, we sought to identify the host cell receptor for *Y. pestis* Ail interaction. Using purified Ail and Ail expressed on the surface of *E. coli* and *Y. pestis*, we demonstrated that Ail interacts with fibronectin, leading to cell binding and delivery of Yop effector proteins.

While our findings demonstrated Ail binding to fibronectin and not collagen I and IV, there may be additional substrates/receptors for Ail. However, the Ail/fibronectin interaction we characterized is important for cell binding and Yop delivery to host cells.

A number of experiments employed the use of a polyclonal anti-Fn antibody to block Ail-mediated binding to host cells. To ensure the anti-fibronectin antibody was not blocking general bacterial adhesion independent of Ail or perturbing cell signaling pathways, and blocking Yop delivery [58], we expressed Psa in the *E. coli* heterologous system. Psa is known to bind to cultured cells and facilitate secretion of cytotoxic effectors (Fig. 2.4B and C) [12]

(SF and ESK in preparation). The *E. coli* expressing Psa bound to both host cell types. Treatment with the anti-fibronectin antibody resulted in only a slight decrease in bacterial adhesion. This slight decrease is likely due to the ability of Psa to mediate binding to fibronectin in addition to its other characterized receptors β-galactoxyl-linked gangliosides [16] and LDL [17].

When the *Y. pestis* parental strain, KIM5 was tested, we identified two*Y. pestis* adhesins capable of binding fibronectin, Ail and Pla (Fig. 2.5). The KIM5 *Δail* mutant still binds purified fibronectin and the KIM5 *Δpla* mutant is more defective for binding purified fibronectin. This suggests Pla is a better mediator of binding to purified fibronectin. However, complementation experiments with the KIM *Δail Δpla* double mutant confirmed that Ail can restore fibronectin binding activity. Pla has been previously reported to bind to collagen IV [21], laminin, and Matrigel [13] [20] while very low binding to both plasma and cellular fibronectin was reported [13]. This failure to bind fibronectin may be due to the concentrations tested. We show a binding curve at saturation at 5μg/ml (100ng/well) while the previous reports used a lower concentration fibronectin for their binding experiments (40ng/well). Therefore, this is the first study to report saturable Pla-mediated binding to fibronectin.

Although Pla can mediate *Y. pestis* binding to purified fibronectin, adhesion to host cells is primarily mediated through Ail since the KIM5 *Δail* mutant has only half the adhesive ability of the parental KIM5 strain, while the KIM5 *Δpla* mutant has the same level of binding as KIM5 ($p = 0.397$ for HEp-2 cells and $p = 0.517$ for THP-1 cells) (Fig. 2.6). Ail-dependent adhesion was

observed with both HEp-2 and THP-1 cells lines. These data indicate that while Pla can mediate fibronectin binding, Ail is the primary adhesin for host cell binding. This leads to the question of why is Ail better for cell adhesion and Pla is better for binding to purified fibronectin. It is possible that host cell modify the deposited fibronectin matrix such that it is a better substrate for Ail rather than Pla [59].

In order to test whether the Ail/fibronectin interaction is important for *Y. pestis* host cell adhesion, we used the polyclonal anti-fibronectin antibody to inhibit cell adhesion. Treatment with the anti-Fn antibody led to a 40-50% decrease in adhesion of the KIM5 strain, and a decrease in Ail-mediated adhesion with the KIM5 *Δpla* mutant. However, the anti-Fn antibody did not inhibit Pla-dependent adhesion in the KIM5 Δail mutant (Fig. 2.6). This indicates blocking fibronectin can inhibit adhesion of the *Y. pestis* strains expressing Ail to host cells, but not strains expressing Pla. Interestingly, the KIM5 *Δail Δpla* double deletion mutant appears to be reduced in adhesion with treatment with the anti-Fn antibody. This difference is not significant, but there is a trend toward inhibition with both HEp-2 and THP-1 cells. This experiment is done with tissue culture media at 37^{o}C at which point the other known adhesin, Psa may be expressed to low levels [15, 37], Psa can mediate some level of ECM binding. Thus binding of the KIM5 *Δail Δpla* double deletion mutant to fibronectin on host cells may be mediated through Psa. Alternatively, another uncharacterized adhesin may be responsible for this background fibronectin binding activity.

To further characterize the importance of the *Y. pestis* Ail interaction with fibronectin, we demonstrated that when the Ail/fibronectin interaction is disrupted by anti-Fn antibodies, the cytotoxicity of the HEp-2 cells is reduced (Fig. 2.7). Significant cell rounding, indicating Yop-mediated cytotoxicity, can be visualized with the parental KIM5 strain and the KIM5 *Δpla*, while the majority of host cells infected with the KIM5 *Δail* mutant appear normal. This cytotoxicity is due to the delivery of the Yop effector proteins as a YopB (a component of the Ysc translocon apparatus) mutant is defective for cell rounding and Ail-mediated adhesion is the key facilitator of Yop delivery. Upon treatment with the anti-Fn antibody, the percent of HEp-2 cells that appear cytotoxic is reduced in those strains expressing Ail (KIM5 and KIM5 *Δpla*) (Fig. 2.7A-I). This suggests that when fibronectin is no longer available for binding, the *Y. pestis* is defective for delivery of Yops, and the critical molecule for *Y. pestis* interaction with host fibronectin is Ail.

The cytotoxicity experiments were also preformed with THP-1 cells (phagocytic). As THP-1 cells have a somewhat rounded morphology and cytotoxicity is scored by observing rounded cells, we determined cytotoxicity of THP-1 cells as those that lost the cytoplasmic halo and stained a darker purple color.

Bacterial adhesion to host cells is a critical step in the infection process. Some bacteria can bind host cell surface directly by binding to cell receptors such as integrins [40], other bacteria bind ECM components found in tissues. These ECM components can serve as a bridge for binding to the host cell

surface. Likewise, Ail-mediated *Y. pestis* adhesion to host cells through fibronectin appears to use fibronectin as a bridging molecule. The adhesin, YadA, from *Y. pseudotuberculosis*, close relative to *Y. pestis*, can promote bacterial invasion into host cells and this entry was dependent upon fibronectin binding and host cell β1 integrins [42, 60].

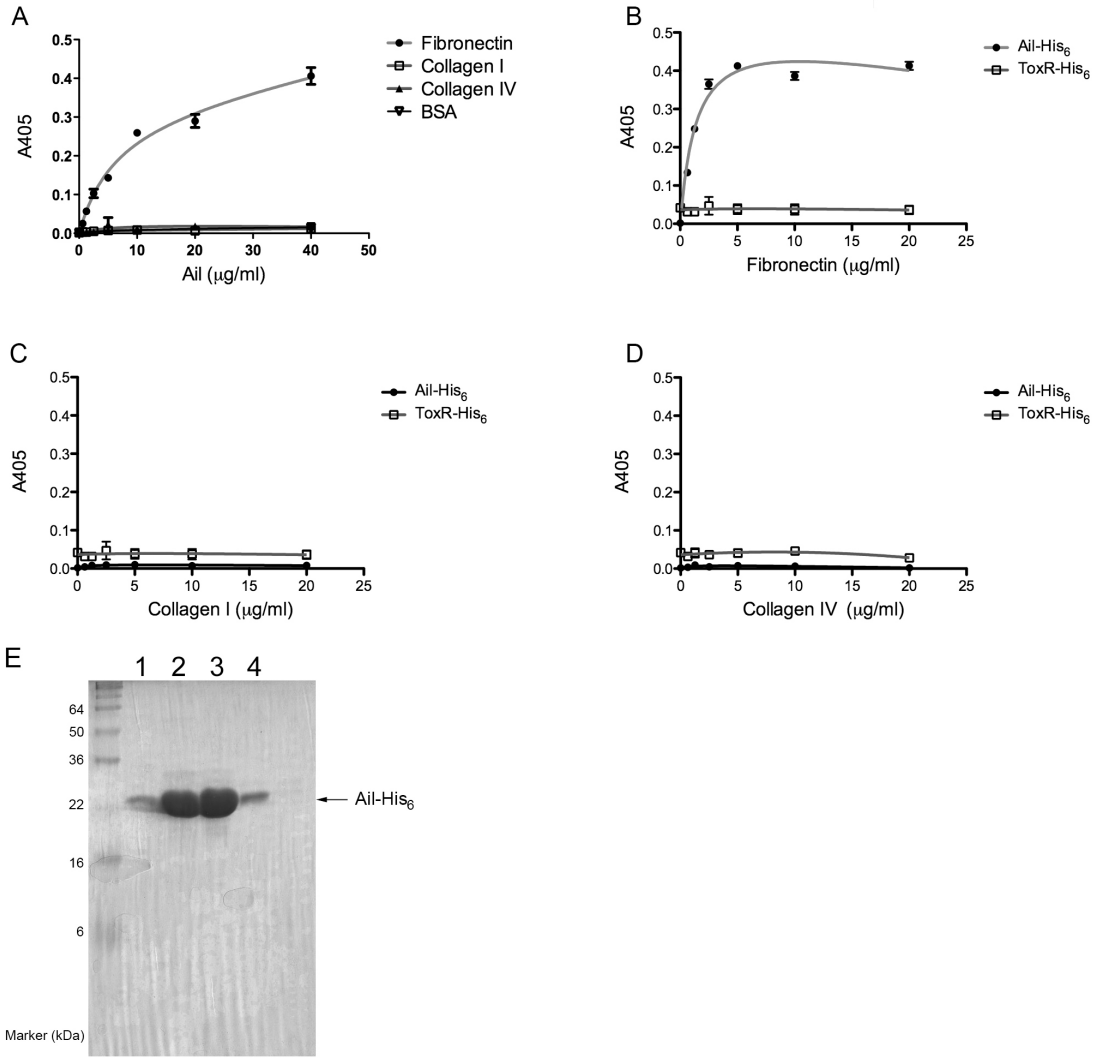
A number of bacterial pathogens bind fibronectin, often at the N-terminal end of fibronectin [52, 53]. Fibronectin is a dimer held together by a disulfide bond at the C-terminal end of the molecule. Many of the cellular binding domains along the molecule have been well studied [46]. Fibronectin is an important component of the tissues within the host. Besides the well-studied RGD cell-binding domain, there are a number of other integrin-binding sites along the length of fibronectin [46]. Therefore, bacteria can bind one region of fibronectin while allowing fibronectin to also engage host cell receptors from a different region.

We conclude that binding of Ail to fibronectin is an important event for Yop delivery to host cells. Pla on the other hand, can mediate binding to the purified fibronectin, but Pla is not a key adhesin for host cell binding and cytotoxicity of host cells is not as dependent upon Pla as Ail. We can envision a few scenarios to explain why Ail is the predominant adhesin capable of mediating the delivery of Yop effector proteins. We hypothesize Ail engages fibronectin in a unique manner that facilitates the most efficient interaction of the Ysc T3SS. This suggests Ail and Pla may have different binding domains on the fibronectin molecule. Preliminary studies suggest the Ail binding site within fibronectin is

distinct from that for Pla (TMT and ESK, unpublished data). Another possible explanation for efficient Yop delivery by Ail, may be that Ail facilitates more receptor clustering through multiple Ail molecules with the surface of host cells. We have previously reported that Ail is highly expressed in the parental KIM5 strain [25, 38], therefore, it is possible to envision that host cell binding through multiple Ail molecules leads to a more efficient cell signaling and Yop delivery.

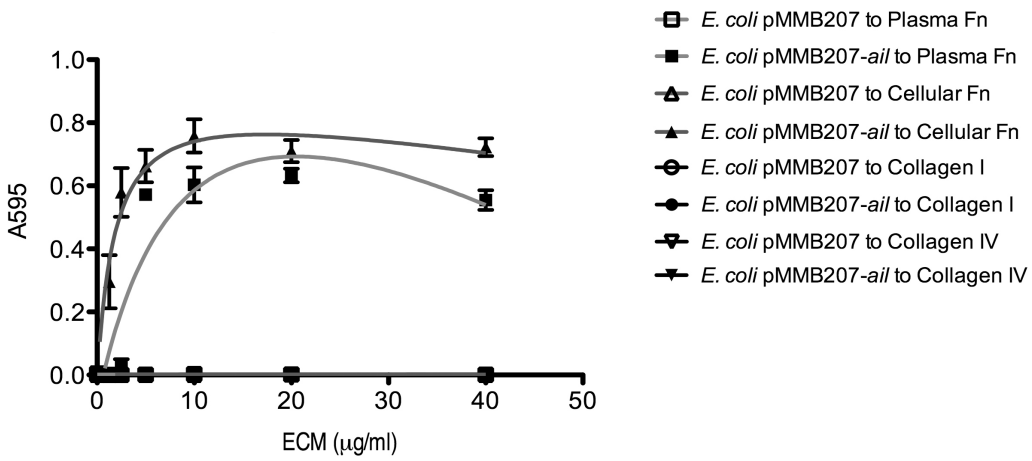
The role of efficient cell signaling in Yop delivery was demonstrated recently by Mejia et al, who showed that avid binding of invasin to β1 integrins lead to intracellular signaling and Yop delivery. Inhibitor studies showed that disrupting the cell signaling cascade leads to defective Yop delivery [58]. Thus, receptor clustering through multiple Ail molecules and fibronectin may lead to a robust signaling cascade in host cells.

Figure 2.1



Purified Ail-His$_6$ binds to fibronectin. (A) Purified Ail-His$_6$ was added at increasing concentrations to ECM and allowed to bind overnight at 4°C. Binding was detected using a mouse anti-His$_6$ antibody in an enzyme-linked immunosorbent assay. (B to D) Increasing concentrations of fibronectin (B), collagen I (C), and collagen IV (D) were preabsorbed onto a 96-well plate. Ail-His$_6$ was added at 10μg/ml and allowed to bind overnight at 4°C. The anti-His$_6$ enzyme-linked immunosorbent assay was performed again. Purified ToxR-His$_6$, an unrelated DNA binding protein, was used as a negative control to demonstrate that the His$_6$ tag played no role in binding. Shown are the results of a representative experiment (n = 3) of two trials. (E) Coomassie-stained purified Ail-His$_6$ protein used for these studies, indicating that the protein is pure. The protein concentration in lanes 1 and 4 is 117 μg/ml, and that in lanes 2 and 3 is 1.1 mg/ml.

Figure 2.2



***E. coli* expressing Ail binds to purified fibronectin.** Purified plasma fibronectin, cellular fibronectin, collagen I, and collagen IV were pre-absorbed onto 96-well plates. *E. coli* AAEC185 derivatives were added to the wells and allowed to bind at 37°C. Bound bacteria were stained with 0.01% crystal violet. The cells and crystal violet were solubilized, and the A595 of the solution was read. Shown are the results of a representative experiment done twice in triplicate.

Figure 2.3

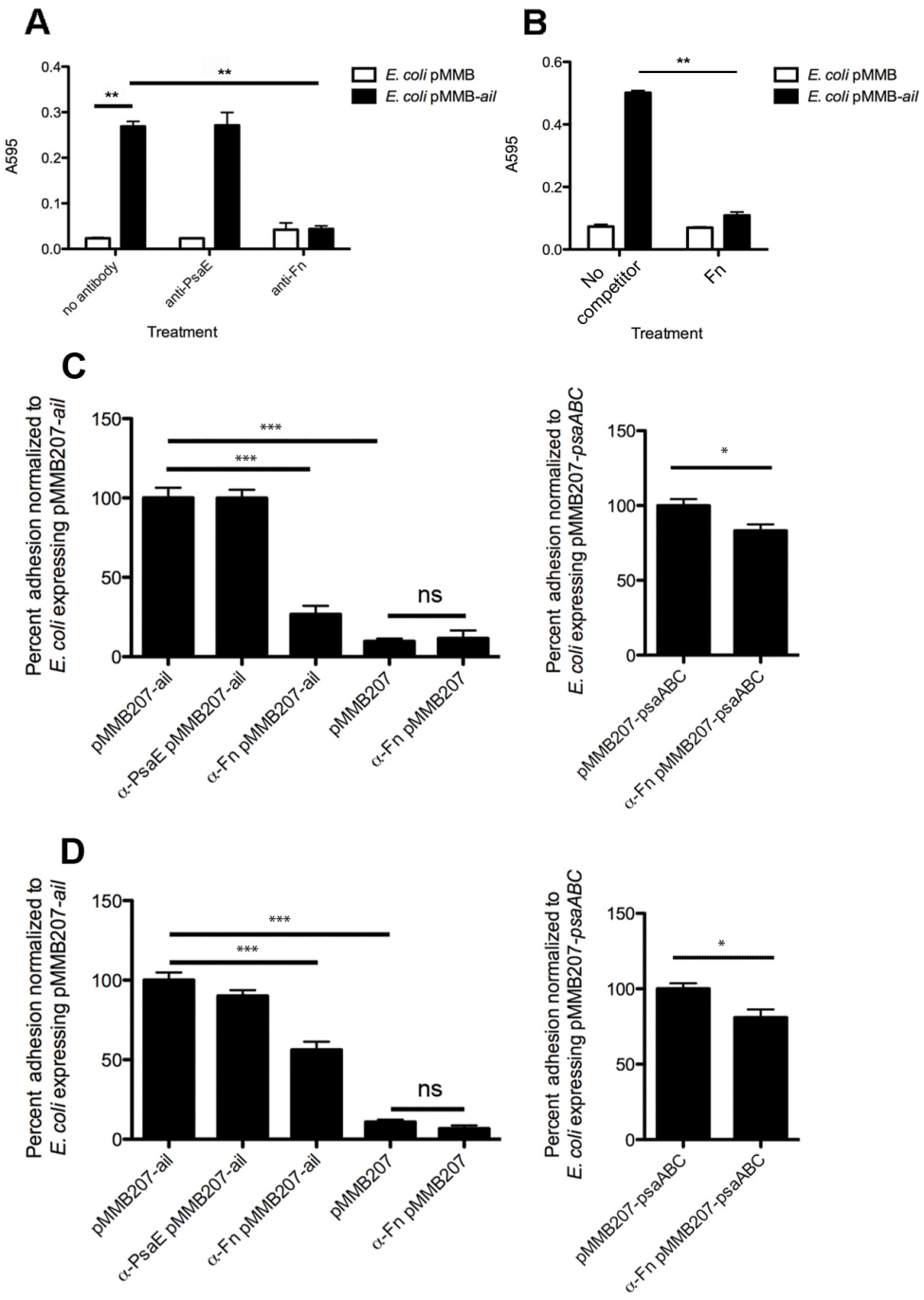
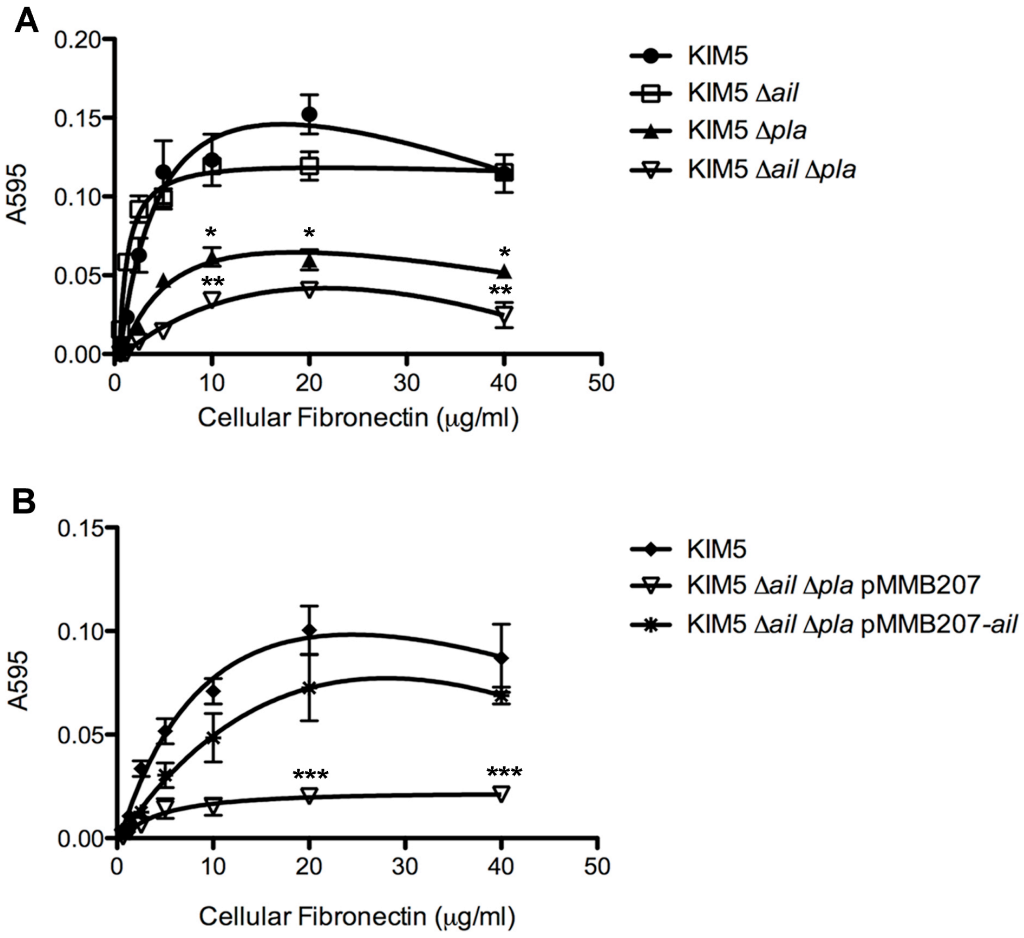

A
B
C
D
E. coli pMMB
E. coli pMMB-ail
A595
Treatment
no antibody
anti-PsaE
anti-Fn
No competitor
Fn
Percent adhesion normalized to E. coli expressing pMMB207-ail
Percent adhesion normalized to E. coli expressing pMMB207-psaABC
pMMB207-ail
α-PsaE pMMB207-ail
α-Fn pMMB207-ail
pMMB207
α-Fn pMMB207
pMMB207-psaABC
α-Fn pMMB207-psaABC
ns

Figure 2.3

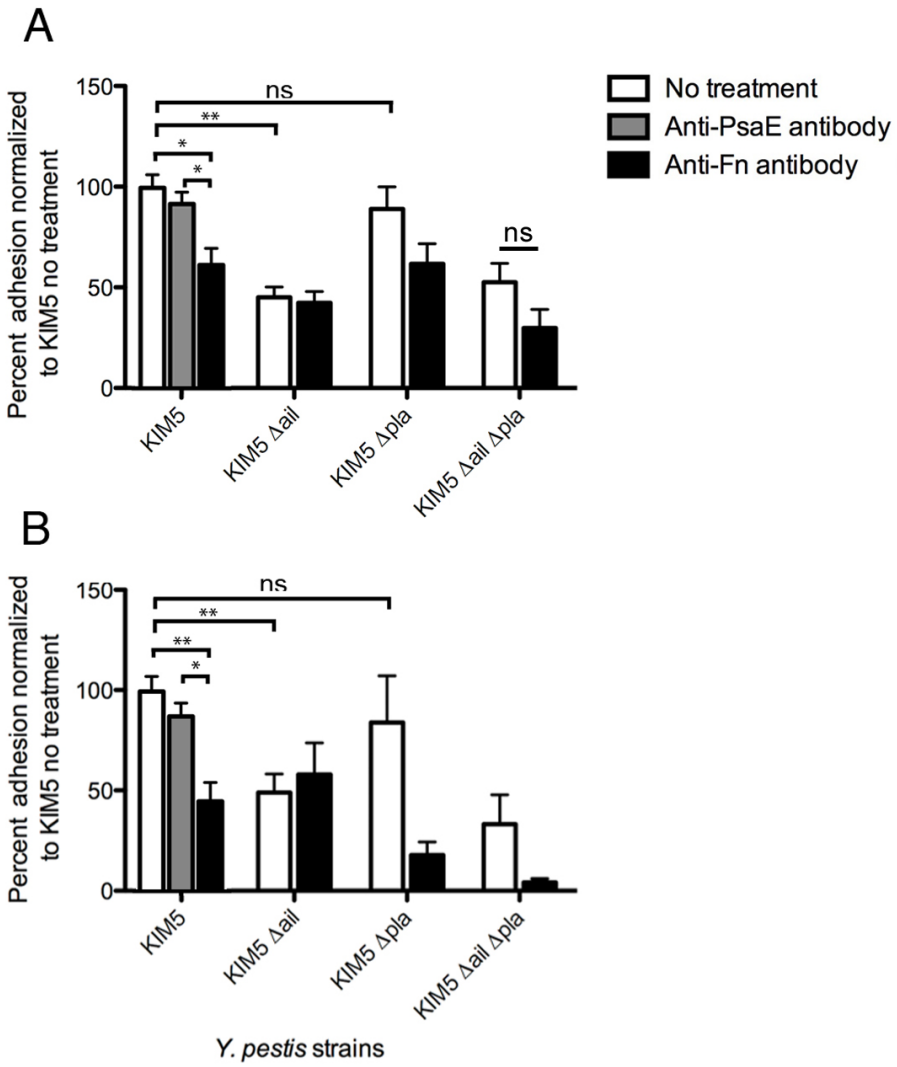
Anti-human fibronectin antibody blocks Ail-mediated bacteria binding to purified fibronectin and cultured epithelial cells. (A) Fibronectin-coated wells (10 μg/ml) were pretreated with anti-fibronectin antibody at the indicated concentrations for 1 h, and *E. coli* AAEC185 derivatives were added and allowed to bind at 37°C. Bound bacteria were stained with 0.01% crystal violet. The cells and crystal violet were solubilized, and the A595 of the solution was read. Anti-PsaE antibody was used as a negative control to show specificity of anti-Fn antibody. (B) Binding of *E. coli* expressing Ail to wells coated with 10 μg/ml plasma fibronectin could be inhibited by prebinding the bacteria with 40 μg/ml soluble cellular fibronectin. HEp-2 cells (C) and THP-1 cells (D) were infected with *E. coli* AAEC185 derivatives. Percent adhesion was calculated by dividing the number of cell-associated CFU by the total number of bacteria in the well and multiplying by 100. A 1:50 dilution (A) or 1:25 dilution (~24 μg/ml) (C and D) of a purified anti-Fn rabbit antibody was added where indicated. The adhesion of *E. coli* AAEC185 expressing Ail bound to cultured cells was set equal to 100% (HEp-2 cell average adhesion = 9.0%, THP-1 cell average adhesion = 6.8%). The adhesion of *E. coli* through Psa without antibody was set equal to 100% (HEp-2 cell average adhesion = 18.7%, THP-1 cell average adhesion = 19.0%). A control purified anti-PsaE rabbit antibody (used at the same protein concentration as the anti-Fn antibody) did not inhibit Ail-mediated binding. Data are from at least two independent experiments performed in triplicate (n = 6 to 15). *, P = 0.02; **, P = 0.0005; ***, P = 10^{-6}; ns, not significant (P = 0.05). Significance was assessed using the Student *t* test.

Figure 2.4



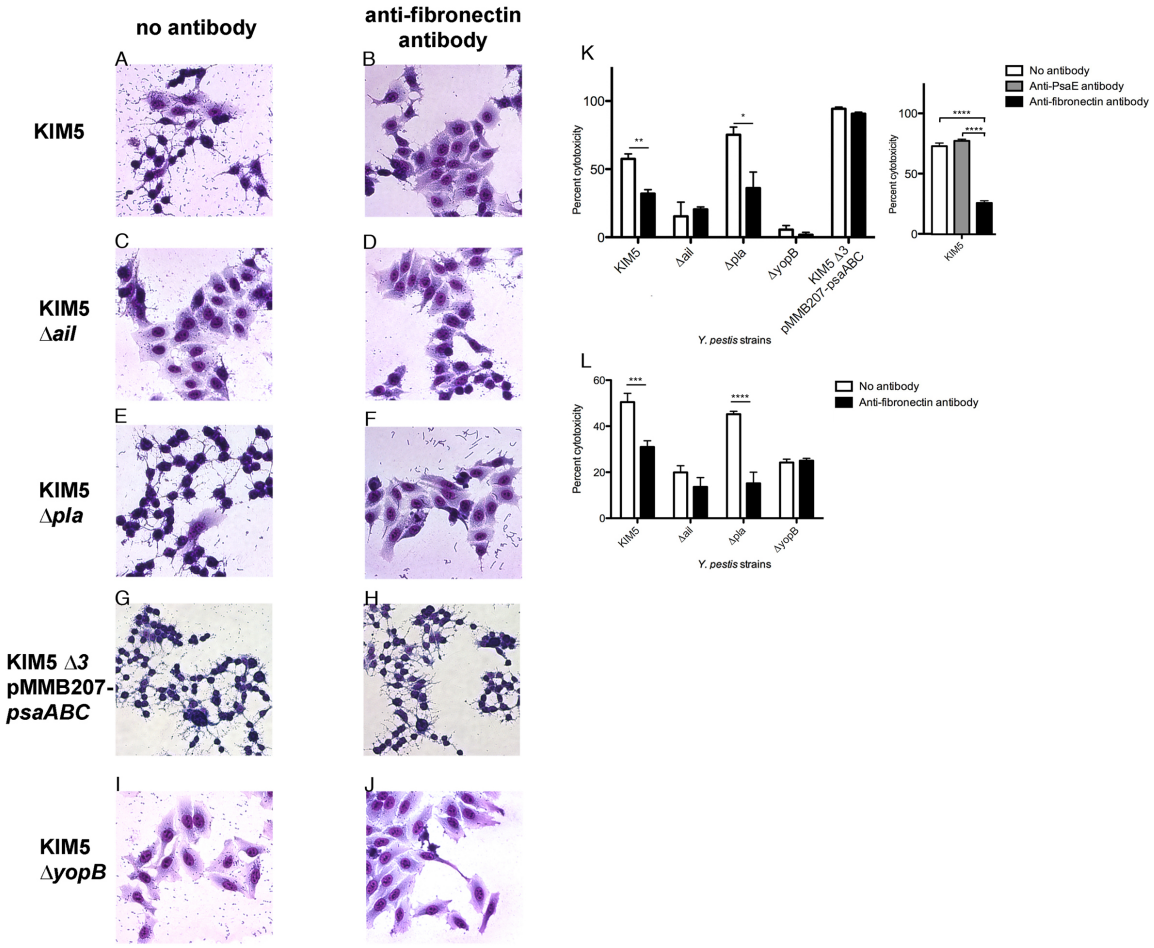
Ail and plasminogen activator (Pla) of *Y. pestis* bind to fibronectin. Purified cellular fibronectin was preabsorbed onto 96-well plates. *Y. pestis* KIM5 and derivatives were added to the wells and allowed to bind at 37°C. Bound bacteria were stained with 0.01% crystal violet. The cells and crystal violet were solubilized, and the A595 of the solution was read. *, P = 0.03 (relative to the results for KIM5); **, P = 0.04 (relative to the results for KIM5 Δpla); ***, P = 0.04 (relative to the results for KIM5 Δail Δpla with pMMB207-*ail*). Significance was assessed using the Student *t* test.

Figure 2.5



Anti-fibronectin antibody inhibits adhesion of *Y. pestis* to host cells. HEp-2 cells (A) and THP-1 cells (B) were infected with *Y. pestis* KIM5 and derivatives. Percent adhesion was calculated by dividing the numbers of cell-associated CFU by the total number of bacteria in the well (bound and unbound) and multiplying by 100. Again, the anti-PsaE antibody was used as a negative control to show specificity of the anti-Fn antibody. The adhesion of KIM5 to HEp-2 or THP-1 cells in the absence of antibody was set equal to 100% (HEp-2 cell average adhesion = 12.0%, THP-1 cell average adhesion = 9.13%). Anti-Fn and anti-PsaE antibodies were used at a 1:25 dilution (24 µg/ml). Data are from three independent experiments performed in triplicate (n = 9). *, P = 0.02; **, P = 0.0006; ns, not significant (P = 0.05). Significance was assessed using the Student *t* test.

Figure 2.6



Anti-fibronectin antibody blocks Ail-dependent Yop delivery to HEp-2 and THP-1 cells. *Y. pestis* KIM5 and derivatives were added to cells at an MOI of 10. Photos were taken at 4 h postinfection for HEp-2 cell infection. Giemsa staining reveals a shrunken cytoplasm, which is indicative of Yop-mediated cytotoxicity (A). Cytotoxicity of host cells was enumerated and plotted for HEp-2 cells (K) and THP-1 cells (L). Anti-Fn and anti-PsaE antibodies were used at a 1:25 dilution (24 μg/ml). KIM5 Δ3, = KIM5 Δail Δpla ΔpsaA (G and H). *, P = 0.05; **, P = 0.01; ***, P = 0.02; ****, P = 0.004. Significance was assessed using the Student *t* test.

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CHAPTER THREE

ISOLATION OF AIL MUTANTS DEFECTIVE IN HOST CELL ADHESION, FIBRONECTIN BINDING, AND YOP DELIVERY

Summary

The causative agent of the plague, *Y. pestis*, binds to host cells to deliver cytotoxic Yops into the host cell cytoplasm. *Y. pestis* facilitates host cell contact through an outer membrane protein, Ail, and the host extracellular matrix protein, fibronectin. This Ail-fibronectin interaction leads to efficient Yopn delivery as inhibition of this interaction with anti-Fn antibody leads to reduced level of cytotoxicity. Ail is homologous to other outermembrane β-barrel proteins such that it contains four exposed loops that may be used to contact host cells. To understand the mechanism(s) by which Ail interacts with host cells, we indentified loops and residues in each loop that are responsible for host cell contact. We first replaced each of the four extracellular loops with a mini-loop containing only glycines and alanines. These so-called loop deletion mutants of Ail were analyzed for their ability to bind to host cells. Ail-Δloop 1 was not expressed, while Ail-Δloop 2 and Ail-Δloop 3 were expressed but mediated no binding to host cells above background. Ail-Δloop 4 was expressed and mediated intermediate levels of binding. Thus, we identified loops 2 and 3 as the

most important for mediating binding to host cells. To identify individual residues that contribute to Ail binding to host cells, we utilized a unique mutagenesis screen called selection without isolation of mutants (SWIM). This codon mutagenesis scheme utilizes a functional enrichment step of adhesion to host cells to identify residues that are either needed for wild-type binding or residues that when mutated lead to a loss of binding. Using this technique we identified residues in loops 1, 2 and 3 that have binding defects to host cells, purified Fn, and a corresponding in for Yop delivery. A serine and phenylalanine at the exposed tip of loop 3 had the most severe defects in host cell binding and Yop delivery. In this chapter, we described numberous Ail mutants affecting various functions, leading to our defining key residues and contributing residues for cell binding, Fn binding, and Yop delivery.

Introduction

Ail is an outer membrane protein of *Yersinia* species that is expressed under various conditions [1]. *Y. pestis* Ail mediates adhesion to host cells, leads to autoaggregation, and confers resistance to human serum [2, 3]. Ail is also critical for delivery of cytotoxic Yop proteins to host cells [2]. In addition, an *ail* mutant is highly attenuated, with a >3000-fold increase in LD_{50} when compared to parental *Y. pestis* KIM5 strain in a mouse model of septicemic plague. Together, these data indicate Ail is a significant virulence factor for *Y. pestis* pathogenesis.

We recently demonstrated fibronectin (Fn) is a host cell ligand for Ail [4]. Fibronectin is found in the blood as soluble fibronectin and within tissues in a

fibrillar, insoluble form. In this study, both Ail and Pla (Plasminogen activator) were found to bind to fibronectin, however Ail appears to bind host cell deposited Fn more readily than Pla. Additionally, when the Ail/Fn interaction was blocked with antibodies to Fn, *Y. pestis* was defective for the delivery of cytotoxic Yop proteins. This indicates efficient delivery of Yops is dependent on the Ail-fibronectin interaction. Other *Yersinia* adhesins such as YadA bind fibronectin, which acts as a bridge to engage integrins on the host cell surface [5]. Thus, understanding this Ail-fibronectin interaction will elucidate Ail-host cell interactions.

Since Ail plays an important role in pathogenesis and we have identified Fn as a host cell substrate, we wanted to study the functional domains that contribute to the various functions of Ail. Many bacteria express outer membrane proteins that are predicted to be structurally similar to Ail (a flattened β-barrel), however, these homologues have modest similarity at the amino acid level [6-10]. Thus, elucidating amino acids required for the various functions is not possible by simple homology alignments.

Ail from *Y. enterocolitica* has been studied extensively in the past. One study identified regions of Ail required for adhesion, invasion and serum resistance [11]. In this study, key residues to examine were chosen by bioinformatic comparison with Ail homologues and natural Ail variants as well as alanine scanning of charged residues. Mutants were studied for the adhesion, invasion, and serum resistance phenotypes. Two residues in the C-terminal end of loop 2, D67 and V68 (processed form) (D90 and V91 of unprocessed form),

were identified as non-invasive into CHO cells. Ail from *Y. enterocolitica* and *Y. pestis* have only 65%, 30%, 60% and 60% identity within the four extracellular loops respectively. Thus, making analogies between the two proteins is difficult. That being said, this previous study did provide two possibly analogous residues of *Y. enterocolitica* to include in our own study of *Y. pestis* Ail, D93 and F94.

Ail is an important virulence factor of *Y. pestis* and we further defined the molecular mechanism of Ail interaction with host cells by identifying fibronectin as a host cell substrate. Thus, in order to further characterize the molecular details of this interaction, we sought to define residues/domains of Ail that are responsible for host cell attachment, binding to fibronectin and Yop delivery. Here, we utilized a unique mutagenesis technique, SWIM (selection without isolation of mutants, [12]) to generate mutant pools of *ail* that were subjected to a functional enrichment. Using this technique and site-directed mutagenesis, we have identified mutants in exposed loops 1, 2, and 3 that play a role in the various functions of Ail.

Materials and Methods

Strains and culture conditions. *Y. pestis* strains were cultivated in heart infusion broth (HIB) overnight or on heart infusion agar (HIA) for 48 hours at 28°C. *Escherichia coli* strains were cultured in Luria-Bertani (LB) broth or LB agar at 28°C or 37°C. Antibiotics were used at the following concentrations: chloramphenicol (Cm) 10 μg/ml and ampicillin (Amp) 100 μg/ml. Isopropyl-β-D-

thiogalactopyranoside (IPTG) was used at a 100 μM concentration unless otherwise noted.

HEp-2 cells were cultured at 5% CO_2 (37°C) in modified Eagle's medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco), 1% sodium pyruvate (Gibco), and 1% non-essential amino acids (Gibco).

Construction of Ail-expressing plasmids. This work has been published previously [2].

Reagents. The anti-Ail rabbit serum was kindly provided by Dr. Ralph Isberg [13].

SWIM mutagenesis. Loop mutagenesis primers were ordered from Invitrogen and utilized a degenerate sequence in order to have two nucleotides (WT and mutant) incorporated at various positions into each primer. The primer sequences for each loop mutant pool are found in Table 3.1. The pSK-*ail* construct was subjected to PCR amplification mutagenesis using the SWIM mutangenesis oligos. The resulting amplified plasmid was digested with *Dpn1* to degrade template (methylated) plasmid and the digestion product was transformed into *E. coli* DH5α. The transformed DH5α cells were allowed to grow in liquid culture for 2 hours before the addition of Ampicillin. The cultures were then grown overnight at 28°C to allow for slower growth. The next day, the liquid culture was diluted 1:100 in LB+Amp and allowed to grow another day.

The two-day grown culture is designated the input pool and 2ml were taken for isolation of the pSK-*ail* plasmid (Miniprep, Qiagen) and sent for sequencing at the University of Michigan DNA sequencing core. This culture was then diluted to an OD_{600}=0.6 and 100μl was used in adhesion enrichment assays with HEp-2 cells. After 90 min of binding to host cells, the unbound bacteria were moved to a fresh well of HEp-2 cells and incubated another 90min. After a total of four enrichments, all the bound and unbound bacteria were pooled together and cultured in fresh LB overnight to re-grow both the bound and unbound pools. The next day, the plasmids were isolated (Miniprep, Qiagen) and sent for sequencing. These pools were designated as bound and unbound mutant pools.

Generation of single point mutations. PCR-mutagenesis was performed using the enzyme Pfu (Stratagene) and primer pairs encoding the mutations. The primers used were complementary to one another and are listed in Table 3.2. Following PCR amplification using a pSK-*ail* Bluescript-derived plasmid as a template, the PCR reactions were digested with *Dpn*I to degrade the template DNA and transformed into DH5α. Potential mutant clones were sequenced to confirm that only the target site was mutated and a *Bam*HI/*Pst*I fragment containing the entire open reading frame and ribosome-binding site, was liberated, purified and ligated into the IPTG-inducible plasmid pMMB207 (CmR).

Bacterial binding assay. This assay was described previously ([4], Chapter 2). Briefly, 96-well plates were coated with appropriate concentrations of plasma

fibronectin overnight at 4°C. *E. coli* or *Y. pestis* derivatives were cultured overnight at 37°C in LB or 28°C in HIB respectively (IPTG and Cm was added for the strains containing pMMB207). The following day, wells were washed with PBS before blocking with blocking buffer. The bacterial cells were normalized to an OD_{600} or OD_{620} (*E. coli* or *Y. pestis,* respectively) of 1.0. Bacterial suspension was added to the appropriate wells. The plate was incubated at 37°C. The wells were washed with PBS before fixing with methanol and staining with crystal violet. After washing away excess crystal violet, the bacterial-associated crystal violet stain was solubilized with ethanol/acetone solution. The absorbance was measured at A_{595}.

Adhesion assays. This assay was described previously ([4], Chapter 2). Briefly, HEp-2 cells were cultured in 24-well tissue culture plates until reaching 80-90%. *Y. pestis* KIM5 or *E. coli* AAEC185 derivatives were cultured overnight and then diluted 1:50 the next day in fresh media with Cm and 100μM IPTG to induce plasmid-based expression. Strains were allowed to grow for an additional 3-3.5 hours at appropriate temperatures. Tissue culture cells were washed with PBS and serum free media with appropriate bacterial cultures were added at MOI ~100. Plates were incubated with bacteria at 37°C in 5% CO_2. Cells were then washed with PBS, and cell-associated bacteria were liberated by the addition of sterile H_2O containing Triton X-100 for 10 min. Percent adhesion was calculated by dividing bound CFU by total bacteria in the well at the end of 90 min incubation and then multiplying by 100.

Cytotoxicity assay. This assay was described previously ([4], Chapter 2). Briefly, HEp-2 cells were cultivated two days until they reached about 50% confluence in 24-well tissue culture plates. *Y. pestis* KIM5 derivatives strain were cultured overnight. Overnight cultures were diluted 1:10 in fresh media and incubated for 3.5 hours at 28°C. Tissue culture plates were washed with PBS and serum-free tissue culture medium with appropriate bacterial cultures were added to each well at an MOI of 10. Plates were incubated at 37°C in 5% CO_2. Cells were fixed and stained with Geimsa stain at 2 and 4 hours post-inoculation. Rounding was observed and pictures were taken with a phase-contrast microscope. Cytotoxicity was enumerated by counting total cells and the number of round dark purple (shrunken cytoplasm) cells experiencing cytotoxicity in three microscopic fields (~150 cells/field). Percent cytotoxicity was calculated by dividing rounded cells by total cells.

Autoaggregation assay. Bacterial cultures were grown overnight with induction with IPTG. The OD_{600} was measured the next day. The OD_{600} of unshaken cultures was then measured every 30 min for 3 hours. The OD_{600} was normalized to the time=0 and plotted against time.

Western blot assays. Normalized cultures of bacteria were resuspended in Laemmli sample buffer. Bacterial cell extracts were boiled and run on a 15% SDS-polyacrylamide gel electrophoresis (PAGE) gel. Proteins were transferred

to nitrocellulose and probed with rabbit anti-Ail serum (a kind gift from Dr. Ralph Isberg) at a 1:500 dilution.

Results

Deletions of loop 2 and 3 lead to defective host cell binding. In order to study Ail, we took a generalized approach to identify which of the four extracellular loops of Ail were required for host cell adhesion. We replaced each extended loop with a smaller loop made up of five to six glycines and alanines via PCR mutagenesis. These mutants were considered deletions of entire loops leaving just enough amino acids to allow an extracellular turn motif prior to the next transmembrane domain. This should maintain the overall structure of the protein within the outer membrane. Upon initial studies, *E. coli* AAEC185 expressing IPTG-inducible constructs of the Ail loop mutants did not express Δloop 1, and both Δloop 2 and Δloop 3 were expressed at lower levels than wild-type under the same concentration of IPTG (100μM). The Δloop 4 mutant produced similar levels of protein when compared to wild-type Ail. Thus, in host cell adhesion studies, the concentration of IPTG was adjusted to produce similar levels of protein between the Δloop 2, Δloop 3, Δloop 4 and WT Ail (Δ loop1 was not well expressed even with increased (250μM) IPTG concentrations) (Fig. 3.1B). IPTG concentrations were as follows: Wild-type Ail 50μM; Ail-Δloop 2 200μM; Ail-Δloop 3 200μM; and Ail-Δloop 4 100μM. The percentage of HEp-2 adhesion by *E. coli* expressing wild-type Ail was normalized to 100% to compare adhesion across multiple experiments (Fig. 3.1A). *E. coli* expressing vector

alone bound poorly to HEp-2 cells. *E. coli* expressing Ail-Δloop 2 and Ail-Δloop 3 does not confer adhesion to HEp-2 cells as the levels of adhesion are similar to vector alone. Ail-Δloop 4 mutant mediated about 40% of host cell adhesion when compared to wild-type Ail. Thus, it appears both loop 2 and loop 3 are required for adhesion to host cells, while Ail-Δloop 4 contributes to but is not necessary for HEp-2 cell binding.

SWIM enrichment identified mutants reduced for host cell adhesion. We had identified loop 2 and 3 as domains needed for host cell binding, but we wanted to identify individual residues in the exposed loops that contribute to the various functions of Ail. Thus, we employed a unique mutagenesis screen called SWIM (selection without isolation of mutants). This technique was previously described by Krukonis et al. [12]. This mutagenesis technique is based on PCR-generated mutagenesis via primer pools containing certain codon positions encoding 50% wild-type and 50% mutant codons. Each mutant codon was generated with a single nucleotide change to a amino acid with a smaller, uncharged side chain (Fig. 3.2A). After PCR mutagenesis, the pool of mutants are run through a functional enrichment, in this case, we used adhesion to HEp-2 cells. The input mutant pool and enriched bound and unbound pools were sequenced and the resulting chromatograms were compared for enrichment of either the wild-type codon in the bound pool or mutant codon in the unbound pool. SWIM mutagenesis and HEp-2 enrichment was performed with loop 1,

loop 2, and loop 3. To cover ~15-20 residue loops, each loop was split into two SWIM pools (an N-terminal and C-terminal half).

A sample of chromatograms generated with this mutagenesis scheme is presented in Figure 3.2B. This segment is part of the first half of Loop 3, amino acids 125-128. The input pool chromatogram shows how well the mutant codon is represented in the pool. Then, after four rounds of enrichment with adhesion to HEp-2 cells, the chromatograms show an increase in the mutant residue at positions 125 and 128 in the unbound pool relative to the bound pool. This suggests the K125I mutant and S128A mutant bind to host cells less well than wild-type Ail, and more of the mutant forms of Ail are found in the unbound pool. These two mutants were chosen for further characterization as were several others in other loop regions. Once this process identified important amino acids, the single amino acid change to alanine was generated. These point mutants were then analyzed for the various functions of Ail.

These mutagenesis techniques led us to generate many single point mutations within loop 1, loop 2, and loop 3. A summary of all the mutants generated in this study is presented in Table 3.3. Reconstituted alanine point mutants were first tested for their ability to confer host cell adhesion. Only those mutants with reduced adhesion to host cells were pursued further.

Host cell adhesion of single and double point mutations. After identifying Ail residues to mutate to alanine via SWIM, we first characterized the adhesion of alanine point mutants to host cells. In addition to the mutants identified by

SWIM, we also chose to analyze residues analogous to *Y. enterocolitica* Ail that were shown to be important for invasion and serum resistance [11]. These amino acids in *Y. pestis* are the aspartic acid (D) at position 93 and phenylalanine (F) at position 94. Thus, the single D93A and F94A mutants, and the double D93A/F94A mutant were also examined for their ability to bind host cells. *E. coli* AAEC185 expressing wild-type Ail and the Ail point-mutants in a IPTG-inducible plasmid (pMMB207) were examined for adhesion to HEp-2 cells. The mutants most defective for cell binding are shown in Figure 3.3A. The percentage of HEp-2 adhesion by *E. coli* expressing wild-type Ail was normalized to 100% to compare adhesion across multiple experiments. The loop 1 mutants, Ail-Y46A and Ail-K47A, both show a slight, but significant defect for HEp-2 adhesion as they conferred 69% and 74% adhesion (respectively). Defective Loop 2 mutants include Ail-E82A, and the *Y. enterocolitica* Ail analogous double Ail-D93A/F94A. The single Ail-D93A and Ail-F94A mutants were not as severely impaired as the double mutant. Loop 3 had the most defective mutants tested. The F130A single mutation is the most defective mutant examined with adhesion activity at the level of vector alone. We also generated the Ail-S128A/F130A double mutant and the adhesion activity is again similar to vector alone.

To assess the stability and localization of each Ail point mutant, outer membrane and inner membrane/cytoplasmic fractions were run on SDS-PAGE and probed with antibodies to Ail and RNA polymerase as a cytoplasmic control (Fig. 3.3B). All proteins are expressed similarly to wild-type Ail and are present

in the outer membrane with the exception of Ail-K47A, which is expressed to lower levels than wild-type Ail (Fig. 3.3B).

Binding of Ail mutants to Purified Fibronectin. Another Ail function that has been described is binding to purified fibronectin [4]. Thus, we tested all mutants for their ability to confer binding to purified immobilized plasma fibronectin (Fig. 3.4). The percentage of fibronectin binding by *E. coli* expressing wild-type Ail was normalized to 100% to compare binding across multiple experiments. Most single mutations defective for host cell adhesion retained some binding to purified fibronectin. Of the mutants defective for Fn binding, Ail-Y46A from Loop 1 is the most deficient while Loop 2 mutants Ail-F94A and the Ail-D93A/F94A double mutant have an intermediate phenotype. The most dramatically reduced host cell binding mutants, Ail-F130A and Ail-S128A/F130A still confer ~60% binding to fibronectin. The most defective fibronectin-binding mutant is a triple Ail-E43D/S128A/F130A mutant with 31% binding. The Ail-E43D mutant was characterized based on the presence of this substitution in *Y. pseudotuberculosis* Ail (Chapter 5). The Ail-E43D single mutant exhibited only a slight decrease in fibronectin binding that was not significant (Chapter 5, Fig. 6).

Ail mutants expressed in *Y. pestis* also exhibit a defect in host cell binding. We have characterized the wild-type Ail and mutant forms for their host cell adhesion and purified Fn binding phenotypes when expressed in *E. coli*. Some of the mutants, particularly F130A and S128A/F130A, are strongly defective for

host cell binding. We also wanted to examine their contributions to Ail functions in the natural context of *Y. pestis*. We expressed wild-type Ail and mutants forms of Ail in the KIM5 D27 *Δail* background and examined for their ability to confer adhesion to HEp-2 cells (Fig. 3.5). The level of adhesion to HEp-2 cells of the *Y. pestis* KIM5 D27 *Δail* strain complemented with wild-type Ail was normalized to 100% to compare across experiments (actual adhesion = 10.5%). Parental KIM5 D27 (also called KIM5) adhered to cells at 75% when compared to the complemented strain, likely due to the over-expression of Ail from the pMMB207 plasmid in the complimented strain. The KIM5 *Δail* bound to host cells at 20% of the complimented KIM5 D27 *Δail* pMMB207-Ail strain. The loop 1 mutants, Ail-Y46A and Ail-K47A, exhibited about 45% binding when compared to wild-type Ail complimented strain. The Ail-E82A mutant in loop 2 also had a defect for host cell binding with only 45% of the activity of wild-type Ail. This defect is more than what was observed when this mutant was expressed in *E. coli*. The other loop 2 mutants, D93A, F94A, and double D93A/F94A, also have a slight defect for host cell binding with 60%-65% adhesive ability.

Ail-K123A and Ail-K125A mutants found in exposed loop 3 were able to bind host cells at levels similar to wild-type Ail when expressed in *Y. pestis*. The other loop 3 mutants, S128A and S128A/F130, still retained a strong defect for host cell adhesion (Fig. 3.5A). The triple mutant, Ail-E43D/S128A/F130A, was also defective for HEp-2 cell binding when expressed in *Y. pestis*.

Expression levels of wild-type Ail and Ail mutants were also analyzed in *Y. pestis*. Bacteria used in adhesion assays were collected as whole cell lysates,

run on SDS-PAGE, and probed with anti-Ail rabbit serum. Surprisingly, some of the Ail mutants are not expressed at high levels. The expression levels vary between experiments. Thus, when analyzing the effects of a particular mutant, we focus on experiments where it was well expressed. The Ail-K47A is consistently poorly expressed in *Y. pestis*.

Yop delivery by Ail mutants defective for host cell binding. To assess their effect on Yop delivery, each cell-binding Ail mutant was tested for the ability to deliver Yops. We have previously reported that Ail is a key adhesin responsible for the efficient translocation of Yops into host cells [2, 4]. In addition to Ail, other *Y. pestis* adhesins, plasminogen activator (Pla) and pH6 antigen (Psa) can participate in Yop delivery [1]. Therefore, we used a KIM5 D27 *Δail Δpla ΔpsaA* (Δ3) background strain expressing the wild-type and mutant forms of Ail to explore the contribution of Ail to Yop delivery. Infection with the parental KIM5 D27 strain leads to about 60% cytotoxicity of HEp-2 cells after 2 hours (Fig. 3.6). The KIM5 D27 Δ3 lacks all three adhesins, resulting in just 10% cell cytotoxicity. The wild-type and mutant forms of Ail were expressed in the KIM5 D27 Δ3 with the IPTG inducible plasmid pMMB207. When KIM5 D27 Δ3 is complemented with wild-type Ail, the percentage cytotoxicity of HEp-2 cells is restored to about 60%. Most of the mutants examined for Yop delivery do not exhibit a defective phenotype when assayed in the D27 Δ3 background. However, the most dramatically reduced host cell adhesion mutants, Ail-F130A, the double mutant, Ail-S128A/F130A, and the Ail-E43D/S128A/F130A triple mutant, were the most

defective for cytotoxicity of host cells. The levels of cytotoxicity produced by those mutants were significantly different than wild-type Ail levels.

The Yop delivery-deficient mutant, Δ*yopB* serves as a negative control as YopB is a critical protein of the YopB/D translocation apparatus. This strain leads to only 14% cytotoxicity.

Most Ail mutants maintain Ail-mediated autoaggregration. Another function of Ail is the ability to bind to other bacteria and lead to pellicle formation in liquid culture [3]. This leads to Ail-mediated autoaggreagation of bacteria and results in the settling of bacteria at the bottom of liquid cultures. It is believed that the extended extracellular loops of Ail bind to each other to facilitate autoaggregation. Thus, cell-binding mutants identified might also play a role in autoaggregation. *E. coli* AAEC185 expressing the Ail mutants in the IPTG inducible plasmid where examined for autoaggregation. *E. coli* AAEC185 expressing wild-type Ail lead to pellicle formation and reduction in OD_{600} over a three hour period (Fig. 3.7). While most of the Ail mutants demonstrate no defect in autoaggregation, some mutants showed moderately lower levels of autoaggregation such as Ail-D93A/F94A (Fig. 3.7C) and Ail-F130A (Fig. 3.7D). The most severely defective host cell binding mutants, double Ail-S128A/F130A and triple Ail-E43D/S128A/F130A (Fig. 3.7D), failed to autoaggreagate or showed severely delayed autoaggregation similar to vector alone. Thus, it appears the same amino acids that are responsible for host cell binding are also be involved in autoaggregation of bacterial cultures.

Discussion

In this chapter, the SWIM codon mutagenesis technique and functional enrichment helped identify Ail mutants. With this technique, we generated a pool of mutants via PCR mutagenesis which was sequenced as the input pool. After one to four rounds of enrichment with HEp-2 cells, the bound and unbound pools were collected and sequenced as well. The input, bound, and unbound chromatograms were compared. Those wild-type residues that were enriched in the bound pool were considered required for host cell adhesion. Those mutant residues that were enriched in the unbound pools were also considered for their contribution to mediate host cell binding. Using this process we identified residues to create the targeted alanine substitutions. Some of the single point mutants did not exhibit a cell adhesion defect when re-examined. In fact, D49A, mediated higher than wild-type activity (Table 3.3). Additionally, we may not have identified all residues that contribute to cell binding with this SWIM technique. However, we have identified severely defective Ail mutants that we can use in future studies. In order to quantify the differences in chromatogram curves to pull out subtle differences in input versus bound or input versus unbound pools, we attempted to calculate the area under curve of chromatograms. This technique identified other mutants we may pursue further in the future. Additionally, we have identified an Ail form that contains six mutations (T76A/F80V/E81A/D85A/S128A/F130A) and appears to have a Fn

binding defect (data not shown). Thus, we would like to study this particular mutant in more detail.

From the loop deletion studies, it appears Loop 2 and 3 contribute to the host cell binding while Loop 4 plays a smaller role (Fig. 3.1A). Loop 1 could not be assessed due to expression issues - this was a common problem in loop 1. Even single alanine mutations were sometimes unstable, suggesting loop 1 is particularly sensitive to structural perturbations. Loop 4 contains only 10 amino acids and is by far the smallest of the Ail loops. Exchanging loop 4 with the glycine/alanine mini-loop led to reduced, but significant adhesion to host cells. These Ail-loop deletion mutants are in agreement with other Ail homologue mutants that have been examined. For example, Loop 2 of *E. coli* (enterotoxigenic *E. coli*) Tia was shown to be required for binding to heparin and epithelial cells [9] and loop 3 of *Salmonella* Rck is important for serum resistance [14].

Residues of Ail contributing to the cell adhesion function were identified using the innovative SWIM codon mutagenesis. Single site-directed alanine mutations of these residues were generated and were examined for various Ail functions including host cell adhesion, binding to purified Fn, facilitating Yop delivery, and mediating autoaggregation. Figure 3.8A illustrates the positions of these cell-binding defective mutants in the exposed loops of Ail. Within loop 1 of Ail, Ail-Y46A and Ail-K47A mutants were moderately defective for host cell binding (Fig. 3.3A). Interestingly, the Ail-Y46A mutant was the most defective for binding purified Fn with 45% of WT Ail activity (Fig. 3.4). Ail-E82A mutation was

the only mutant within loop 2 that was identified through SWIM mutagenesis. The others, Ail-D93A, Ail-F94A, and the double mutant, were included in this study because they are analogous to the *Y. enterocolitica* Ail mutations that were involved in host cell adhesion, invasion and serum resistance (Miller, 2002). Of these loop 2 mutations, the Ail-E82A mutant is the most defective for host cell binding in *Y. pestis*, but Ail-F94A and Ail-D93A/F94A were both significantly defective for binding purified Fn (Figs. 3.3A and 3.4). Within loop 3, two lysine to alanine mutants, Ail-K123A and Ail-K125A, had moderate effects for host cell binding when expressed in *E. coli*. This binding defect is *E. coli* specific as these mutants bind show normal host cell binding when expressed in *Y. pestis* (Fig. 3.5A). The other loop 3 mutants (Ail-S128A, Ail-F130A, and the double Ail-S128A/F130A) are more defective for binding in both *E. coli* and *Y. pestis* (Figs. 3.3A and 3.5A). Expression of some of the mutant forms of Ail in *Y. pestis* are inconsistent over different experiments and sometimes less than wild-type. Thus, we focused on experiments where bacterial cultures were expressing similar levels of Ail.

The most defective host cell binding single mutant, Ail-F130A, has a change from the wild-type bulky phenyl side chain to smaller methyl group. One may imagine the larger phenylalanine is able to establish contact with host cells via a large hydrophobic interaction. The other loop 3 mutation severely defective host cell-binding mutant is the Ail-S128A, which changes a polar serine with a hydroxyl group to the smaller alanine. Together, the Ail-S128A/F130A double mutant replaces a large hydrophobic and a small polar side chain with simpler

methyl groups near the tip of loop 3 (Fig. 3.8B). The two lysines (Ail-K123A and Ail-K125A) in loop 3 also exhibit moderate defects for host cell binding when mutated to alanine (Figs. 3.3A and 3.5A). Lysines are charged amino acids and one can imagine charge interactions may be important for host cell binding. The Ail-Y46A in Loop 1 is slightly defective for host cell binding but this mutant is the most defective mutant tested for fibronectin binding. The wild-type tyrosine also contains a large ring and a hydroxyl group. Serine and tyrosine both have hydroxyl groups, so the polar side chain my be critical for interactions with Fn or host cells. This more complex side chain may provide more interactions with Fn. In a future chapter (Chapter 4), we present evidence for charged interactions between Ail-Fn.

In respect to the structure of Ail, we attempted to gain more insights into loop orientations and positioning by changing the analogous Ail residues on the OmpX crystal structure (Fig. 3.8B). The amino acid side chains are found facing the outside of the loops, thus suggesting these side chains may interact with host cells.

Many of the mutants under investigation were found in the tips of the exposed loops. This suggests the tips of the loops engage host cell components. Also, the *Y. enterocolitica*-like mutated residues, Ail-D93A and Ail-F94A, are found on the descending, C-terminal half of loop 2, close to the membrane. Thus, membrane-proximal residues of loop 2 may make contact with host cells as well as the tip.

We have previously characterized the different functions of Ail including adhesion to host cells, binding to purified Fn, and facilitation of Yop delivery [2, 4]. In those studies we observed that interaction with Fn on host cells is different than the interaction with purified Fn. For example, Pla mediated bacterial binding to purified Fn better than Ail, however, a KIM5 *Δpla* (expressing Ail) binds to host cells better than KIM5 *Δail* (expressing Pla). In this study, we also observe similar findings. The Ail-Y46A mutants that were identified for having a moderate defect in host cell adhesion does have a more severe defect for purified fibronectin binding. Conversely, other mutants, such as Ail-S128A and Ail-F130A have a severe host cell-binding defect but are not as defective for Fn binding. It is likely that purified fibronectin adopts a different structure when purified and immobilized on plastic than when deposited and arranged by host cells.

Overall, in this study, we have used an innovative mutagenesis technique to identify residues of Ail that contribute to various functions. This provides insights into the molecular interactions of Ail with host cell.

Table 3.1. SWIM codon mutagenesis PCR primers

Primer name	Sequence (5'-3')
Loop 1.1	TCTATTGGATATGCT**caa agt cgt gtc aag gaa gat**GGGTACAAGTTGGAT – WT **t g t c t c c**
Loop 1.2	GTCAAGGAAGATGGG**tac aag ttg gat aaa aat cca aga**GGTTTCAACCTGAAG – WT **c t g c t t t t**
Loop 2.1	GGTTCATTTGCTCAG**act cgc cgg ggg ttc gag gaa tct gtt gac**GGATTCAAACTTATT – WT **g t t g c c g c c**
Loop 2.2	GAATCTGTTGACGGA**ttc aaa ctt att gat ggt gat ttt aag**TACTACTCAGTGACT – WT **g t g g c c g t**
Loop 3.1	TTATTGGGGGCAGGT**cat gga aag gct aaa ttt tcc tca ata**TTTGGTCAATCAGAG – WT **t t t g g g g**
Loop 3.2	AAAtTTTCCTCAATA **ttt ggt caa tca gag agt aga agt aaa aca**TCTCTGGCATACGG – WT **g t g c g g g t g**

PCR primers used for SWIM codon mutagenesis are displayed. Bold-lower case letters indicate the region of mutagenesis and split into codons for easy visualization. Mutant nucleotide and position is indicated on the bottom half of each primer. Each primer contains approximately 50% WT nucleotide and 50% mutant.

Table 3.2. PCR primers for the generation of point mutations within Ail

Primer Name	Sequence (5'-3')
Ail-Y46A	GTCAAGGAAGATGGG**gcc**AAGTTGGATAAAAAT
Ail-K47A	AAGGAAGATGGGTAC**gcg**TTGGATAAAAATCCA
Ail-D49A	GATGGGTACAAGTTG**gct**AAAAATCCAAGAGGT
Ail-T76A	GGTTCATTTGCTCAG**gct**CGCCGGGGGTTCGAG
Ail-E82A	CGCCGGGGGTTCGAG**gca**TCTGTTGACGGATTC
Ail-I90A	GACGGATTCAAACTT**gct**GATGGTGATTTTAAG
Ail-D93A	CAAACTTATTGATGGT**gct**TTTAAGTACTACTCAG
Ail-F94A	CTTATTGATGGTGAT**gct**AAGTACTACTCAGTGAC
Ail-D93A/F94A	CTTATTGATGGT**gctgct**AAGTACTACTCAGAC
Ail-K123A	GGGGCAGGTCATGGA**gcg**GCTAAAtTTTCCTCA
Ail-K125A	GGTCATGGAAAGGCT**gca**TTTTCCTCAATATTT
Ail-S128A	AAGGCTAAATTTTCC**gca**ATATTTGGTCAATCA
Ail-F130A	AAATTTTCCTCAATA**gct**GGTCAATCAGAGAGT
Ail-S128A/F130A	AAGGCTAAATTTTCC**gca**ATA**gct**GGTCAATCAGAGAGT
Ail-Δloop1	ATTTCTATTGGATAT**gctggaggggcgca**GGTTTCAACCTGAAG
Ail-Δloop2	GGTTCATTTGCTCAG**gcaggggaggtgct**AAGTACTACTCAGTG
Ail-Δloop3	GGATTATTGGGGGCA**ggtggagctggtggcgca**TCTCTGGCATACGGT
Ail-Δloop4	GCTTCATATGAATAC**gcaggcggaggtgct**CGCGCCAAGCATCCA

PCR primers used for site-directed mutagenesis of mutants chosen from SWIM mutagenesis technique. Bold-lower case nucleotides indicate the region of mutagenesis.

Table 3.3. All Ail mutants examined for their various functions.

Strains	Loop	Expression in *E. coli*	Cell adhesion[a]	Purified Fn[a]	Autoaggregation[a]	Cytotoxicity[b]
pMMB207			-	-	-	-
pMMB207-Ail		+	++++	++++	++	++++
Ail-Y46A	1	+	++	+	+	+++
Ail-K47A	1	+	++	++++	++	
Ail-D49A	1	+	+++++	ND	ND	+++
Ail-T76A	2	+	+++	++++	++	+++
Ail-E82A	2	+	++	++++	++	+++
Ail-I90A	2	+	+++++	+++	++	+++
Ail-D93A	2	+	++	+++	++	+++
Ail-F94A	2	+	++	++	++	+++
Ail-D93A/F94A	2	+	++	++	-/+	+++
Ail-K123A	3	+	++	++++	++	+++
Ail-K125A	3	+	+++	++++		++++
Ail-S128A	3	+	+	+++	++	++++
Ail-F130A	3	+	-	++	-/+	+++
Ail-S128A F130A	3	+	-	++	-	++
E43D/S128A/ F130A	1 and 3	+	-	+	-	++
Ail-T76A/S128A/ F130A	2 and 3	+	+	++	-	ND
Ail-E82A/S128A/ F130A	2 and 3	+	-	+++	-	ND
Ail-D85A/S128A/ F130A	2 and 3	+	-	++++	-	ND
Ail-I90A/S128A/ F130A	2 and 3	+	-	++	-	ND
Ail-F94A/S128A/ F130A	2 and 3	-	-	-	ND	ND
Ail-Δloop1		-	ND	ND	ND	ND
Ail-Δloop2		-/+	-	ND	+	ND
Ail-Δloop3		-/+	-	ND	-	
Ail-Δloop4		+	+	ND	-	

A summary of the Ail mutant functions examined in this study. [a] Assessed by expression from pMMB207 plasmid in *E. coli* AAEC185. [b] Assessed by expression from pMMB207 plasmid in *Y. pestis*. - = 0-10% activity, + = 11%-25%, ++ = 26-50%, +++ = 51-75%, ++++ = 76%-100%, +++++ = > 101%. ND = Not determined.

Figure 3.1

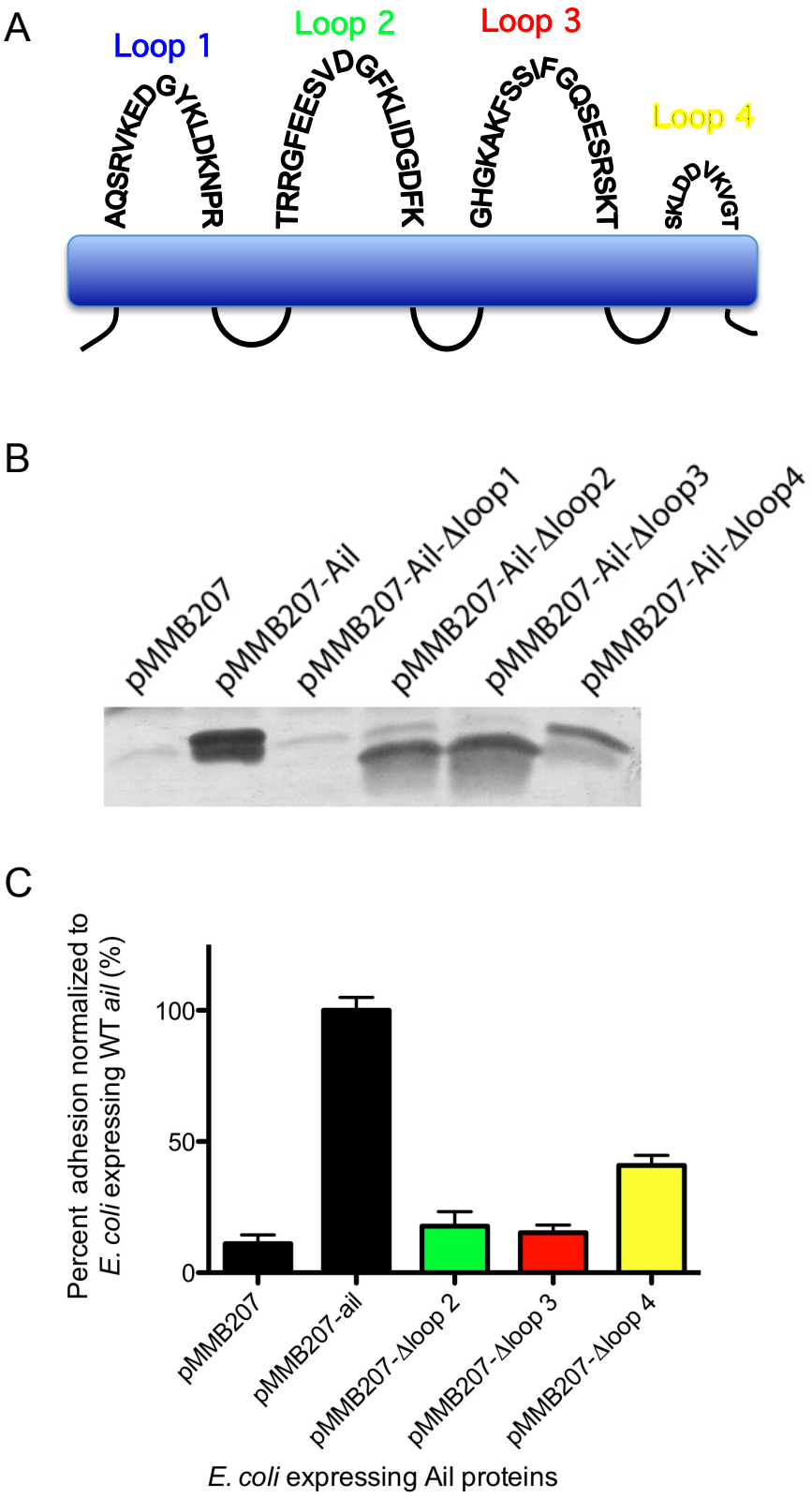

A
Loop 1
Loop 2
Loop 3
Loop 4
AQSRVKEDGYKLDKNPR
TRRGFEESVDGFKLIDGDFK
GHGKAKFSSIFGQSESRSKT
SKLDDVKVGT
B
pMMB207
pMMB207-Ail
pMMB207-Ail-Δloop1
pMMB207-Ail-Δloop2
pMMB207-Ail-Δloop3
pMMB207-Ail-Δloop4
C
Percent adhesion normalized to E. coli expressing WT ail (%)
100
50
0
pMMB207
pMMB207-ail
pMMB207-Δloop 2
pMMB207-Δloop 3
pMMB207-Δloop 4
E. coli expressing Ail proteins

Figure 3.1

Loop 2 and Loop 3 are required for host cell adhesion. (A) Illustration of Ail loops shows the amino acid sequence of the exposed loops. Note loop 1 is blue, loop 2 is green, loop 3 is red, and loop 4 is yellow. (B) The whole cell lysates of a representative experiment were run on SDS-PAGE and probed with anti-Ail rabbit serum. (C) HEp-2 cells were infected with *E. coli* AAEC185 derivatives Ail loop mutations. Percent adhesion was calculated by dividing the number of cell-associated CFU by the total number of bacteria in the well and multiplying by 100. The adhesion of *E. coli* AAEC185 expressing Ail bound to cultured cells was set equal to 100% (HEp-2 cell average adhesion = 6.0%). Data are from at least two independent experiments performed in triplicate (n = 6 to 15).

Figure 3.2

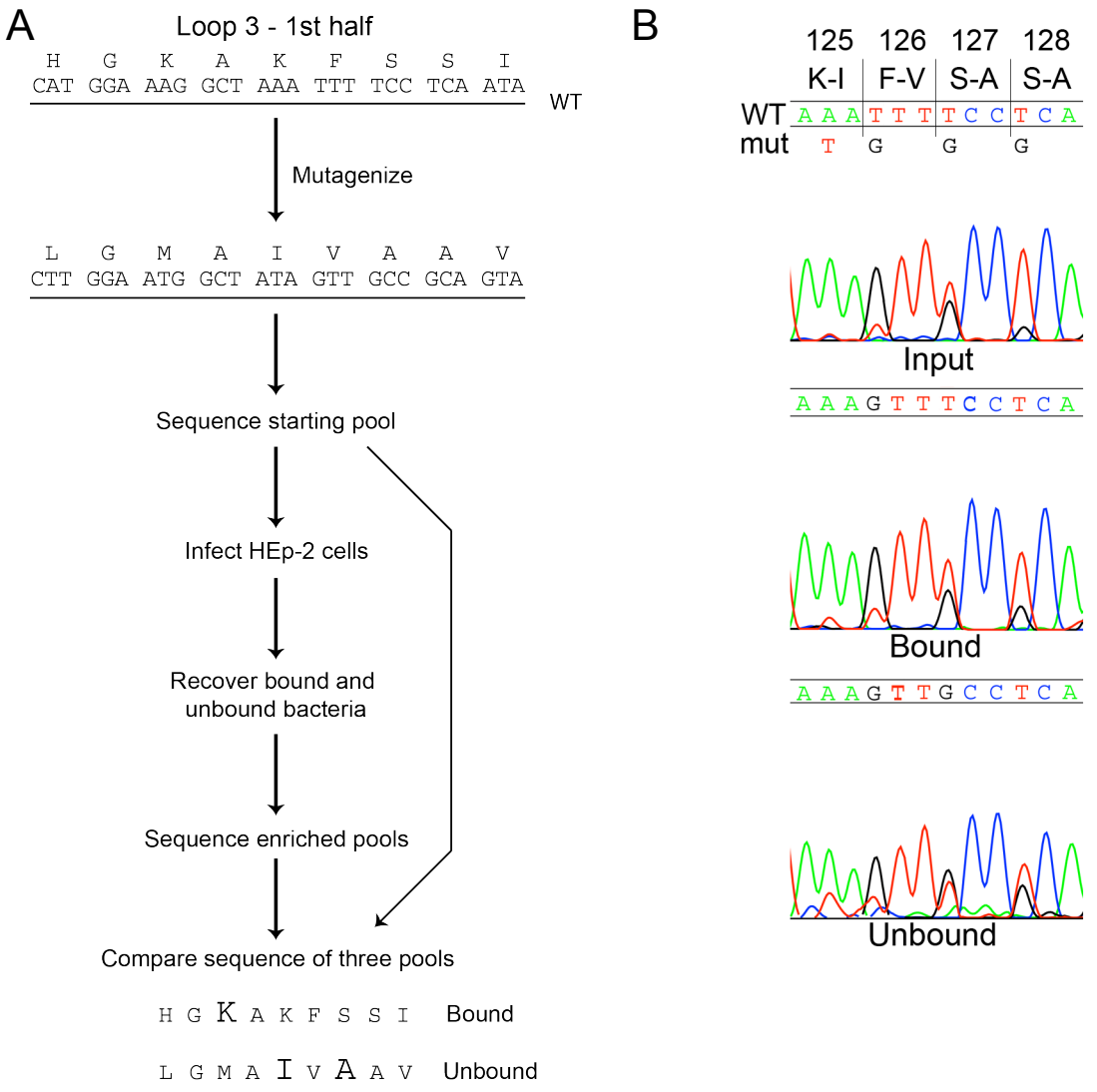
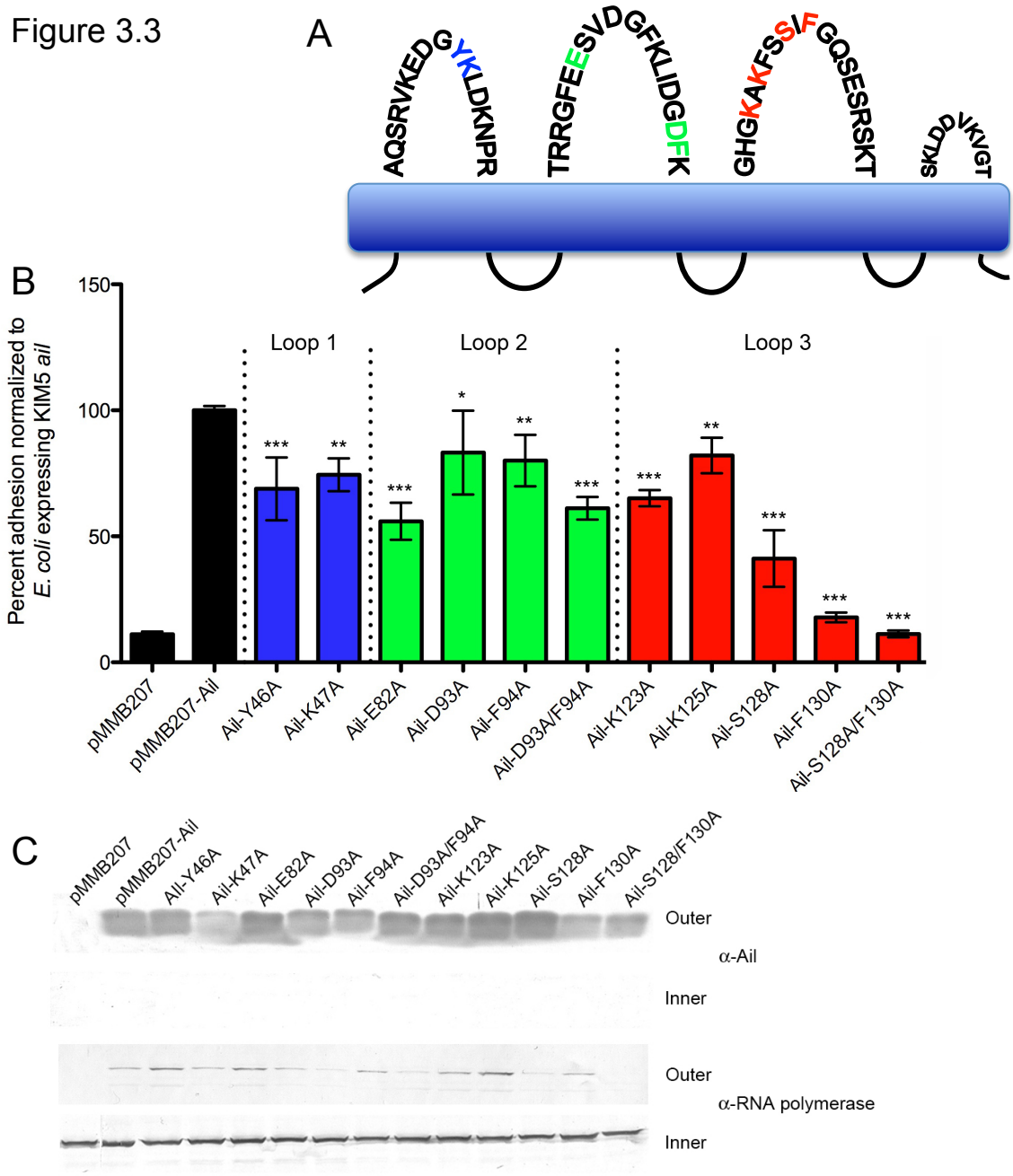


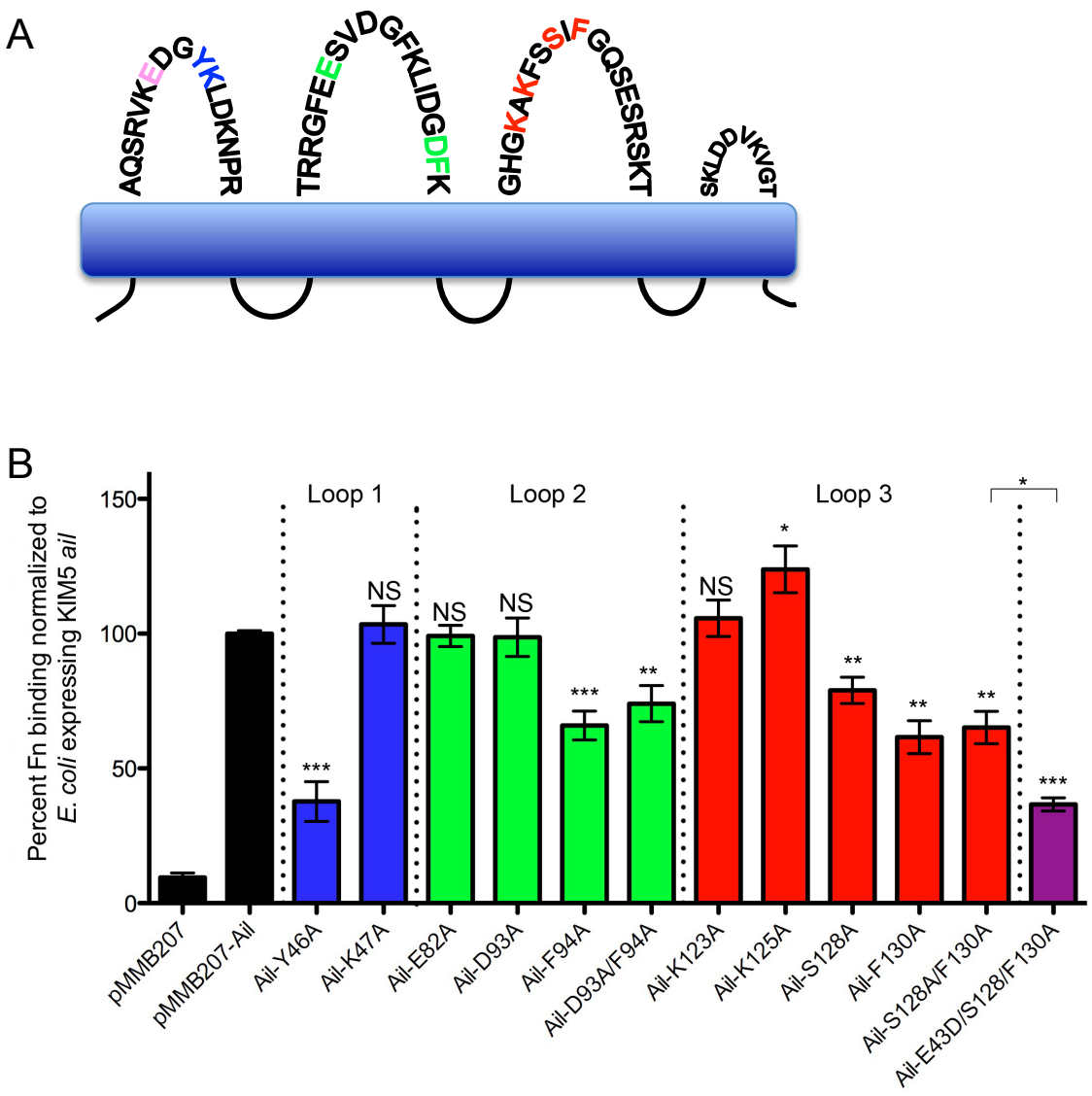
Diagram of SWIM codon mutagenesis. (A) The region representing the first half of Ail loop 3 spanning 121 to 129 was chosen for SWIM mutagenesis with all of the residues simultaneously targeted for smaller residue substitution by a single oligonucleotide. The mutagenized pool was enriched by passage through HEp-2 cells, and the starting, enriched bound, and unbound pools were compared by sequencing. (B) An example of the chromatograms analyzed after SWIM mutagenesis and enrichment sequences is shown. Residues of interest were found by noting an enrichment of wildtype residues in the bound pool and an enrichment of mutant forms in the unbound pool

Figure 3.3



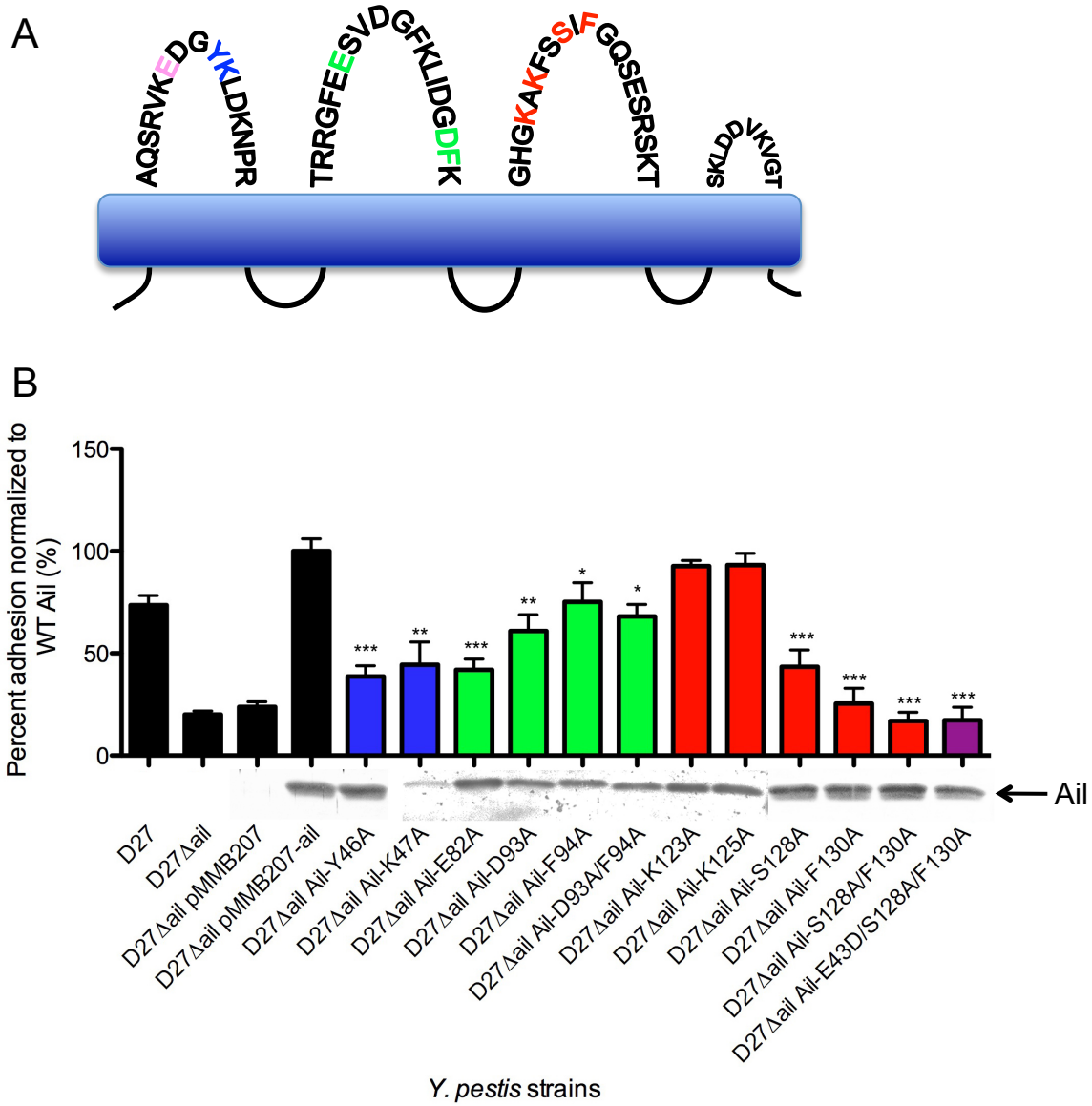
A summary of the host cell binding mutants of Ail in *E. coli*. (A) HEp-2 cells were infected with *E. coli* AAEC185 derivatives expressing mutant forms of Ail. Percent adhesion was calculated and *E. coli* AAEC185 expressing Ail bound to cultured cells was set equal to 100% (HEp-2 cell average adhesion = 5.0%). Data are from at least two independent experiments performed in triplicate (n = 6 to 15). *, P = 0.02; **, P = 0.0005; ***, P = 10^{-6}; ns, not significant (P = 0.05) when compared to *E. coli* expressing pMMB207-Ail. Significance was assessed using the Student *t* test. (B) Outer membrane fractions and inner membrane/cytoplasmic fractions were run on SDS-PAGE and probed with anti-Ail rabbit serum and anti-RNA polymerase.

Figure 3.4



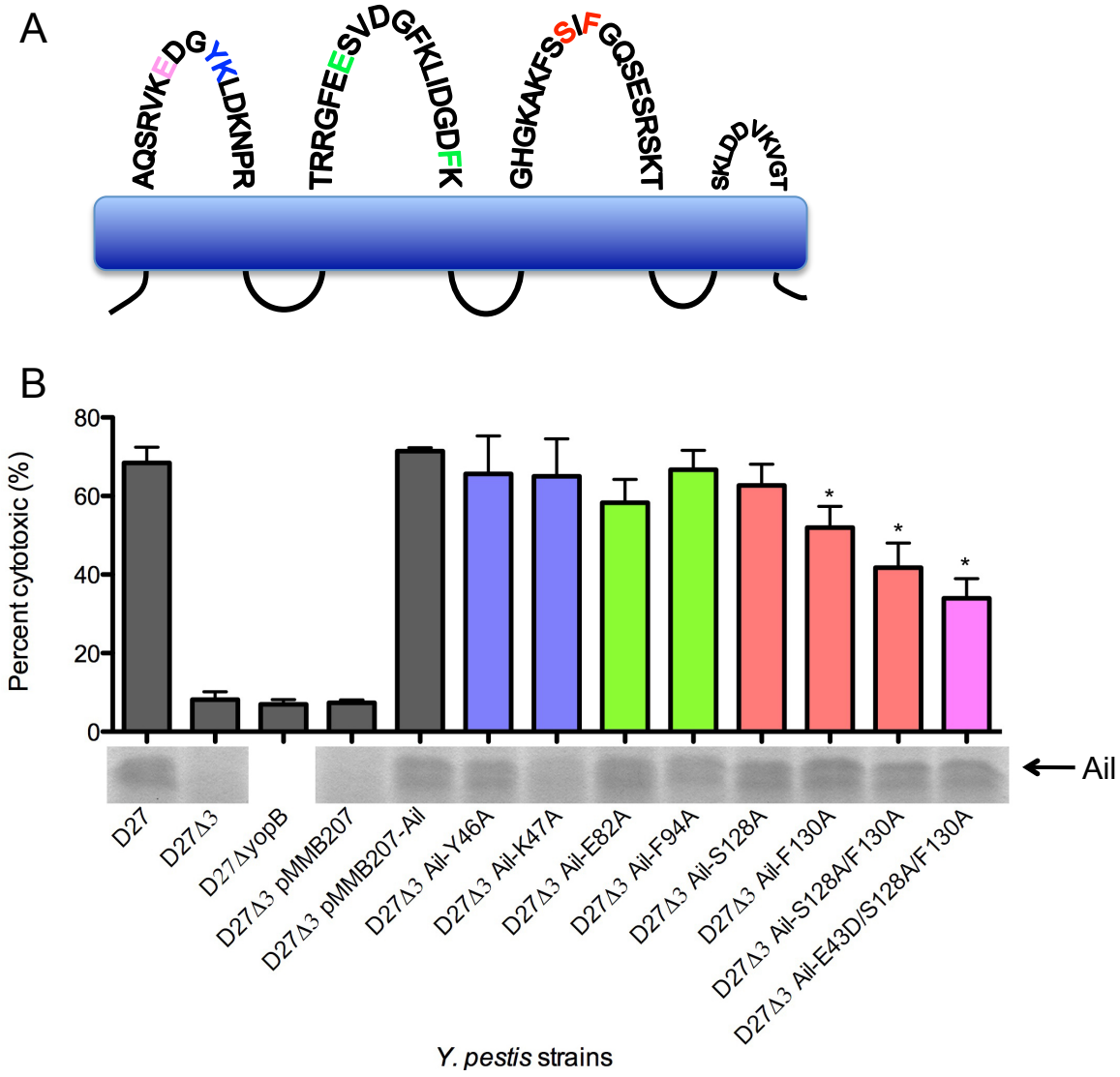
The cell-binding defective mutants do not exhibit a severe purified Fn binding. Purified plasma fibronectin was immobilized on 96-well plates. *E. coli* AAEC185 derivatives expressing the Ail mutants were added to wells and allowed to bind at 37°C. Bound bacteria were stained with 0.01% crystal violet. Stained bacterial cells were solubilized and the plates were read at ABS_{595}. Shown is representative data from two independent experiments done in triplicate (n=3). *, $P < 0.05$ compared to *E. coli* expressing pMMB207-*ail* KIM5.

Figure 3.5



HEp-2 cell binding by Ail mutants expressed in *Y. pestis*. (A) HEp-2 cells were infected with *Y. pestis* KIM5 D27 *Δail* expressing mutant forms of Ail. Percent adhesion was calculated by dividing the number of cell-associated CFU by the total number of bacteria in the well and multiplying by 100. The adhesion of *Y. pestis Δail* expressing wild-type Ail bound to cultured cells was set equal to 100% (HEp-2 cell average adhesion = 10.5%). Data are from at least two independent experiments performed in triplicate (n = 6 to 15). *, P = 0.05; **, P = 0.003; ***, P = 10^{-4} compared to KIM5 D27 *Δail* complimented with wild-type Ail. Significance was assessed using the Student *t* test. (B) Whole cell lysates of the input bacterial cultures were run on SDS-PAGE and probed with anti-Ail rabbit serum.

Figure 3.6



The cell-binding defective mutants were examined for the ability to deliver Yops. HEp-2 cells were infected with KIM D27 *Δail Δpla ΔpsaA* (Δ3) expressing wild-type and mutants forms of Ail at an MOI of 10. After two hours of infection, cells were fixed and stained with Giemsa to show shrunken, round, darker cells, which is indicative of Yop-mediated cytotoxicity. Cells were counted and percent cytotoxic cells were calculated. Whole cell lysates were run on SDS-PAGE and stained with coomassie to show Ail expression (arrow). *, $P < 0.05$ compared to KIM D27 Δ3 complemented with wild-type Ail. Significance was calculated using the Student t test.

Figure 3.7

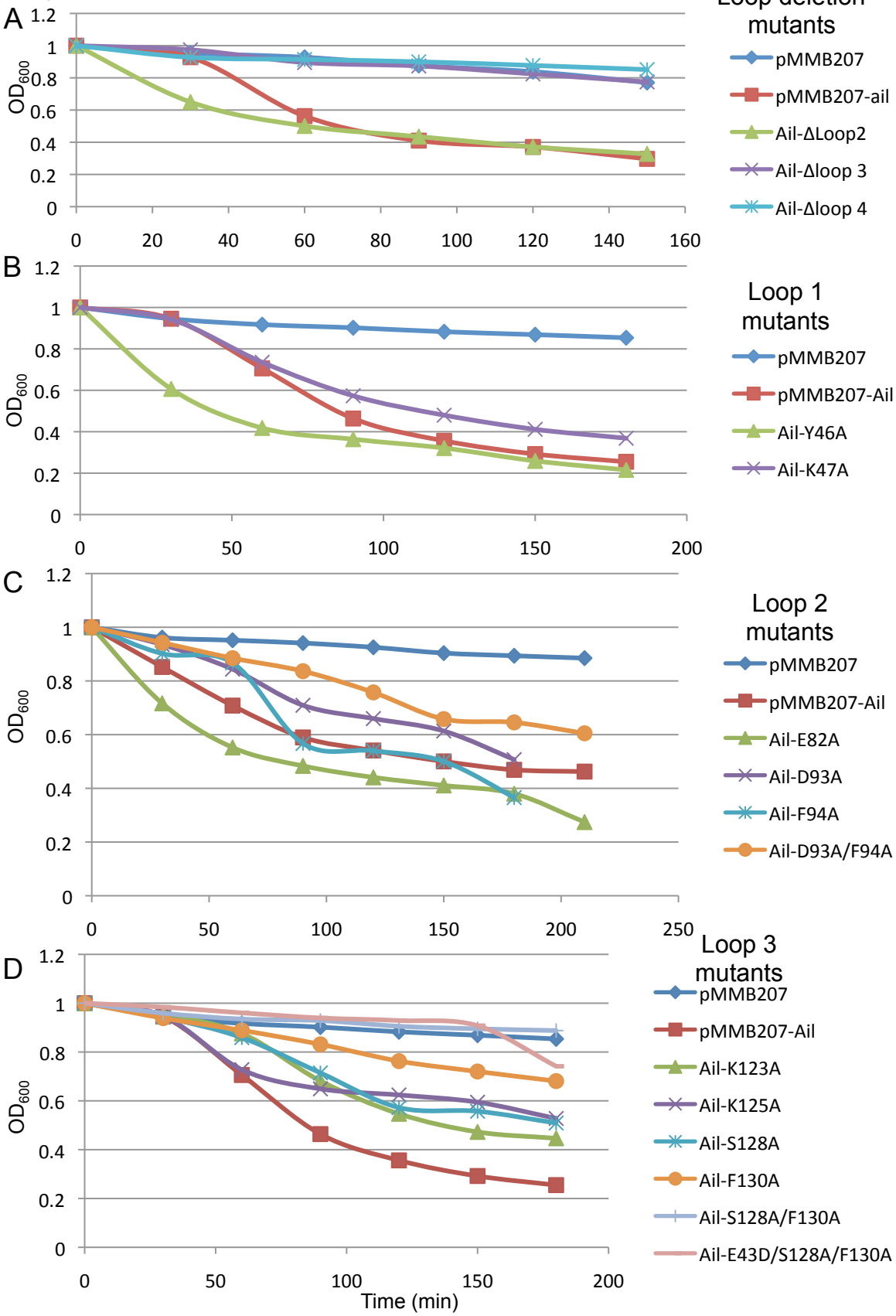
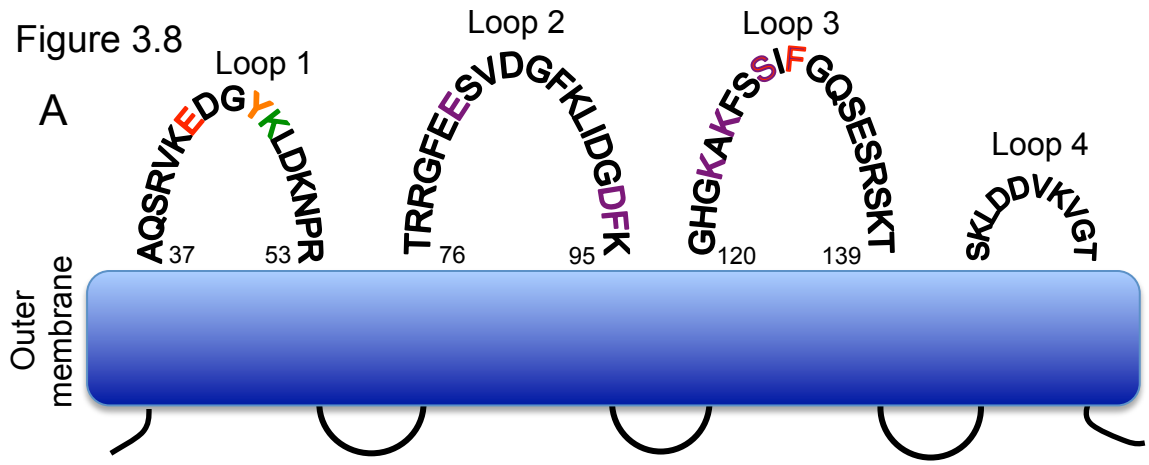
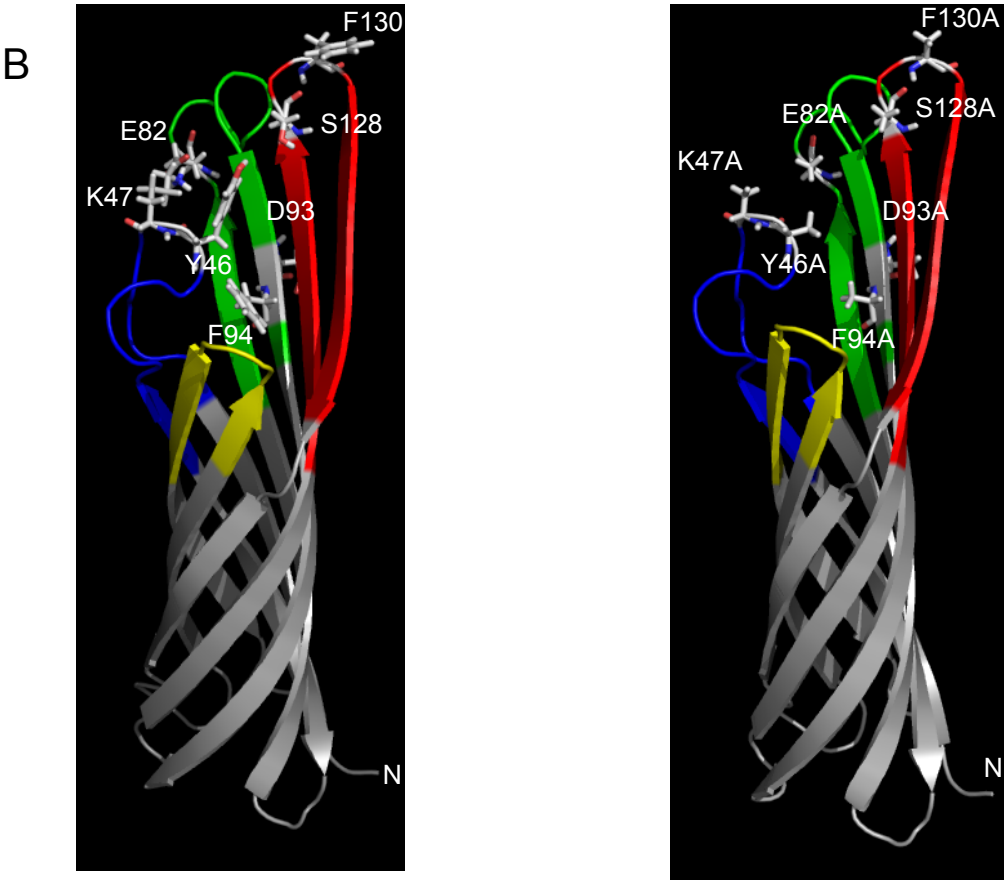

A
B
C
D
OD600
Time (min)
Loop deletion mutants
pMMB207
pMMB207-ail
Ail-ΔLoop2
Ail-Δloop 3
Ail-Δloop 4
Loop 1 mutants
pMMB207
pMMB207-Ail
Ail-Y46A
Ail-K47A
Loop 2 mutants
pMMB207
pMMB207-Ail
Ail-E82A
Ail-D93A
Ail-F94A
Ail-D93A/F94A
Loop 3 mutants
pMMB207
pMMB207-Ail
Ail-K123A
Ail-K125A
Ail-S128A
Ail-F130A
Ail-S128A/F130A
Ail-E43D/S128A/F130A

Figure 3.7

Overexpression of cell-binding mutants in *E. coli* leads to loss of autoaggregation. *E. coli* AAEC185 expressing wild-type and mutant forms of Ail were expressed of the IPTG-inducible plasmid, pMMB207. Cultures at stationary phase were allowed to settle for 3 hours. OD_{600} measurements were taken every 30 min. The OD_{600} readings over time were normalized to starting OD_{600} and plotted against time.

Figure 3.8





Illustrated positions of Ail mutants analyzed in this study. (A) The position in the exposed loops is shown for the Ail mutant residues. Purple indicates only cell-defective residues, orange indicates cell-binding and purified Fn binding defective residues, green indicates cell-binding and Yop delivery defective residues, and red indicates cell-binding, purified Fn binding, and Yop delivery defective mutants. (B) Ail residues are shown on the OmpX structure (left) and the mutated alanine residues are exchanged for wild-type residues (right). Blue = loop 1, green = loop 2, and red = loop 3.

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CHAPTER FOUR

MAPPING THE AIL BINDING DOMAIN ON FIBRONECTIN

Summary

We have previously shown that the *Y. pestis* adhesin molecule Ail interacts with the extracellular matrix protein fibronectin (Fn) on host cells. This Ail-Fn interaction is needed for the efficient delivery of the cytotoxic Yops as disruption of this interaction leads to lower cytotoxic effects on host cells upon *Y. pestis* infection. Gram-positive bacteria such as *Streptococcus pyogenes* and *Staphylococcus aureus* are known to bind to Fn at the N-terminal end of the molecule. Therefore, we wanted to examine whether Ail from *Y. pestis* interacted with Fn in a similar manner. To examine the binding site, we utilized a number of proteolytic fragments, purified fragments, and various antibodies that recognize specific epitopes along Fn. Using the proteolytic and purified fragments, we observed that Ail expressed by *E. coli* in a heterologous system bound the centrally located 120kDa fragment and not the N-terminal 30kDa or larger 70kDa fragment. Using a polyclonal antibody, which recognizes various epitopes, we found that antibodies against the 120kDa fragment were able to block bacterial binding to purified full-length immobilized Fn. Similarly, numerous monoclonal antibodies that recognize specific epitopes along Fn, monoclonal antibodies recognizing the N-terminal end and collagen-binding region did not inhibit

bacterial binding to full-length Fn, while antibodies recognizing the $^{8\text{-}9}$FNIII repeats within the 120kDa fragment were able to block *E. coli* expressing Ail binding. Finally, collagen was also able to inhibit bacterial binding through Ail, suggesting Ail may interact with the collagen-binding region of Fn as well. Together, these data presents evidence that Ail binds to primarily the $^{8\text{-}9}$FNIII repeats within the 120kDa fragment of Fn with possible contribution within the 45kDa collagen-binding site. Ail binding to these two regions presents a unique interaction with Fn that is distinct from other bacterial Fn binding proteins.

Introduction

Fibronectin is a critical molecule found throughout vertebrate host and is involved in many processes including wound healing, tissue structure, cell migration, and embryonic development. Fibronectin is a large complex glycoprotein found in blood, on cell surfaces, and within tissues [1, 2]. Fn is a dimer of 440kDa (monomer polypeptide = 220kDa), linked together with two disulfide bonds at the C-terminal end of the molecule. [3, 4]. Fibronectin is a major surface-associated glycoprotein of fibroblasts and many of the known functions of fibronectin have come from studies of cultured fibroblasts [5]. These studies found fibronectin mediates cell-cell interactions and cell-substratum contacts for tissue structure [6]. The plasma fibronectin concentration within blood is ~300 μg/ml [3, 7] and this circulating form of Fn is produced by hepatocytes [8-10]. Some of the circulating Fn is deposited into tissue matrix. In addition, cells within the tissues synthesize soluble Fn, which is arranged into insoluable fibrils that make up extracellular matrices.

Fibronectin is found throughout almost all human adult tissues [11]. Immunofluorescent studies demonstrated Fn is a major component of connective tissue matrix as it is located in basement membranes, around smooth muscle cells, and striated muscle fibers [12].

Fibronectin binds host cell integrins to facilitate cell attachment [13]. In addition, Fn binds many host cell components including collagen [14], heparin [15, 16], and fibrin [15, 17]. The binding sites of these Fn substrates are depicted in Figure 4.1. In addition to binding to the well-studied integrin $\alpha_5\beta_1$, Fn is a ligand for numerous other host cell integrins [18].

Fibronectin, like many other extracellular matrix (ECM) components, binds to various integrins on the surface of host cells and initiates a diverse set of intracellular signals to modulate host cell function such as actin rearrangement, migration, and cell cycle regulation [19]. This outside-in signaling, as the name describes, is a complex signal transduction network from the outside environment into the host cell. Focal adhesions are the structural link between ECM and the cytoskeleton during cell adhesion within tissues. Within the focal adhesion one finds local accumulation of signaling molecules that affect cytoskeleton rearrangement. This signaling occurs through the phosphorylation of specific proteins such as focal adhesion kinase (FAK), paxillin, and $p130^{cas}$ [20-22]. Thus, Fn acts as an important bridging molecule for tissue structure by linking host cells to the ECM [12] and facilitating signal transduction events [23, 24].

The N-terminal end of Fn is termed the matrix assembly site as it is responsible for the assembly of soluble Fn into insoluble fibrils. This matrix assembly site is found in repeats $^{1\text{-}5}$FNI. In addition to binding the RGD region of Fn ($^{9\text{-}10}$FNIII), $\alpha_5\beta_1$ integrins can also bind to the N-terminal matrix assembly site [25, 26]. Studies indicate $\alpha_5\beta_1$ binding to this site initiates distinct internal host cell signaling events from those observed with $\alpha_5\beta_1$ integrin interaction with the RGD domain of fibronectin [27].

The outer membrane protein Invasin (Inv) from *Y. pseudotuberculosis* mediates invasion into host cells and is a classic example of initiating contact with β_1 integrins and facilitating host cell signaling [28, 29]. Inv-integrin interaction signaling is analogous to fibronectin-integrin interaction signaling in focal adhesion [30, 31], however each trigger distinct signaling cascades. Inv binding to integrins initiates RhoA activation and the Rac-1 mediated cascade involving Arp2/3 and PIP 4, 5 [32]. The resulting actin rearrangement facilitates the high efficiency host cell uptake of *Y. pseudotuberculosis.* Thus, invasin-integrin contact mediates successful uptake of bacteria. Inv and fibronectin bind overlapping sites within the $\alpha_5\beta_1$ integrin, although Inv binds with higher affinity than Fn. Thus, Inv and Fn are competitive inhibitors of each other [33]. The high affinity interaction of Inv with integrins triggers signal transduction and actin rearrangement that leads to highly efficient cellular invasion [33] and translocation of Yop proteins [34].

YadA, another *Yersinia* adhesion and invasion protein, interacts with fibronectin [35] which then acts as a bridge to engage β_1 integrins [36]. This

interaction initiates downstream tyrosine kinase, serine/threonine kinase, and phosphatidylinositol 3-kinase (PI3K) activities. Furthermore, YadA-mediated invasion into host cells requires the PI3K-dependent signaling pathway and actin polymerization, similar to Inv-mediated invasion. Studies of YadA-induced signaling demonstrated two different downstream effectors of PI3K signaling, Akt and phospholipase Cγ1, were required for efficient host cell uptake [37]. These data provide a detailed mechanism of host cell signaling pathways generated via YadA-fibronectin-integrin interactions.

Given *Y. pestis* lacks both Inv and YadA [38-40] other surface molecules must play this role *Y. pestis*. We previously showed that Ail binds to Fn and this Ail-Fn interaction is critical step for the delivery of the Yops into host cells [41]. We hypothesize Ail from *Y. pestis* binds fibronectin and modulates host cell signaling via $\alpha_5\beta_1$,integrins, possibly in a similar fashion to YadA. This modulation may then lead to cytoskeleton rearrangements to facilitate efficient Yop delivery. Thus, we are interested in understanding how Ail binds Fn and what region of Fn is presented to integrins during the Ail-Fn interaction. The resulting Fn-integrin interaction may generate the intracellular signaling processes required to modulate cytoskeleton rearrangement for the translocation of Yop proteins. In order to understand the role of Ail in modulating host cell signaling, we first needed to map the Ail binding site on fibronectin.

To map the Ail binding site, we utilized three assays. First, we mapped Ail binding sites to specific proteolytic fragments of Fn. Ail binding to specific fragments will narrow our focus to certain regions. Second, Fn binds to various

substrates, such as heparin and collagen that can be used as competitive inhibitors for Ail binding. Finally, we can use monoclonal antibodies that recognize specific Fn regions to block Ail binding. Together, these reagents will serve as tools to characterize the Ail binding site on Fn.

Materials and Methods

Strains and culture conditions. *Escherichia coli* AAEC185 strains were cultured in Luria-Bertani (LB) broth or LB agar at 28°C or 37°C. Antibiotics were used at the following concentrations: chloramphenicol (Cm) = 25μ/ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used at a 100μM concentration unless otherwise noted.

Fibronectin and monoclonal antibodies. The following fibronectin and fibronectin fragments were used: plasma fibronectin (Sigma, F2006), cellular fibronectin (Sigma, F2518), collagen I (Sigma, C5483), collagen IV (Sigma, 27663), 30kDa of Fn (F9911), 45kDa of Fn (Sigma, F0162) 70kDa of Fn (Sigma, F0287), 120kDa of Fn (Chemicon, F1904). Dr. Deane Mosher provided the N-^{5}FNI, N-^{3}FNIII, and ^{1}FNIIII-C fragments [42]. The following antibodies were used: polyclonal rabbit anti-human fibronectin antibody (Sigma, F3648), monoclonal mouse anti-fibronectin (anti-RGD, 3E3) (Chemicon, MAB88916). Other monoclonal antibodies used in this study were kindly provided by Dr. Deane Mosher [43, 44] and Dr. Kenneth Yamada [45]

Bacterial binding assay, antibody blocking, and competitive inhibitors assays. This assay was described previously ([41], Chapter 2). Briefly, 96-well plates were coated with 10µg/ml of plasma fibronectin overnight at 4^{o}C. *E. coli* AAEC185 derivatives were cultured overnight at 28^{o}C in LB (IPTG and Cm was added for the strains containing pMMB207). The following day, wells were washed with PBS before blocking with blocking buffer. The bacterial cells were normalized to an OD_{600} of 1.0 in PBS+0.4% BSA. Bacterial suspension was added to the appropriate wells. The plate was incubated at 37^{o}C for 2 hours. The wells were washed with PBS before fixing with methanol. The bacteria bound to the wells were stained with crystal violet. After washing away excess crystal violet, the bacterial-associated crystal violet stain was solubilized with ethanol/acetone solution. The absorbance was measured at A_{595}.

Antibody blocking assays were performed similarly to the bacterial binding assays except antibodies were diluted to the proper concentrations and was added to fibronectin coated wells one hour prior to the addition of five-fold concentrated bacteria (i.e. OD_{600}=5.0).

Competitive inhibitor assays were performed similarly to the antibody blocking assays except competitive inhibitors were diluted in 40µl of PBS + 0.4% BSA to the proper concentrations and was added to fibronectin coated wells one hour prior to the addition of 10µl five-fold concentrated bacteria (i.e. OD_{600}=5.0).

Titration of Ail by addition of excess soluble fibronectin. 96-well plates (Pro-bind, BD Falcon) were coated with 10µg/ml of plasma fibronectin overnight at

4°C. *E. coli* AAEC185 derivatives were cultured overnight at 28°C in LB (IPTG and Cm was added for the strains containing pMMB207). The following day, wells were washed twice with PBS before blocking with blocking buffer (PBS + 2% BSA) for 2 hours at room temperature. The bacterial cells were normalized to an OD_{600} of 1.0 and pre-mixed with indicated concentration of soluble cellular Fn in PBS + 0.4% BSA for 1 hour at 37°C mixing. The entire mixture was added to the plasma Fn coated microtiter well and bound for 1 hour at 37°C.

Pre-adsorbed antibody blocking assay. 20µg/ml of plasma Fn, 70kDa, or 120kDa fragments were immobilized onto a plastic plate overnight at 4°C. Rabbit anti-Fn polyclonal antibody was added to each fibronectin/fragment at a concentration of 1:25. After two hours of incubation, the solution was transferred to a fresh well coated with plasma Fn, 70kDa, or 120kDa, and incubated for another two hours and the solution was again transferred to fresh fibronectin/fragment coated wells. This was done a total of four times. The resulting solution was then used in a subsequent bacterial binding assay as described above. The pre-adsorbed solutions were adjusted to the concentrations listed.

Soluble cellular and plasma Fn assay. Overnight induced *E. coli* expressing pMMB207 and pMMB207-ail cultures were normalized to OD=0.6. 200µl of 20µg/ml of cellular Fn and plasma Fn were added bacterial cultures. The

cultures were incubated at 37°C rotating for 1 hr. Bacteria were then washed and re-spun down. Pellets were resuspended in Laemmli sample buffer.

Western blot assays. Bacterial cultures incubated with appropriate Fn were resuspended in Laemmli sample buffer. Control plasma and cellular Fn were made at 10μg/ml in Laemmli sample buffer. Bacterial cell extracts were boiled and run on a 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) gel for anti-Fn blot and 15% gel for anti-Ail blot. Proteins were transferred to nitrocellulose and probed with rabbit anti-Fn polyclonal antibody at 1:1000 dilution and rabbit anti-Ail serum (a kind gift from Dr. Ralph Isberg) at a 1:500 dilution.

Results

Bacteria expressing Ail bind to the 120kDa fragment of Fn. To understand the Ail interaction with Fn in more detail, we wanted to map the Ail binding domain within Fn. As mentioned previously, fibronectin can be proteolytically cleaved into various sized small fragments (Fig. 4.2A). Others have previously shown bacteria can bind to these smaller fragments and mediate binding similar to full-length fibronectin. Thus, we used these fragments to help identify the Ail binding site. We obtained the commercially available Fn fragments: 30kDa, 45kDa, 70kDa, and 120kDa. These fragments were adsorbed to plastic plates and used in bacterial binding assays (Fig. 4.2B). *E. coli* expressing Ail from pMMB207 bound immobilized full-length Fn (plasma Fn) to high levels. Ail did not mediate binding to the N-terminal 30kDa, 45kDa, or 70kDa fragments.

However, Ail did bind to the centrally-located120kDa fragment, albeit less robust than the full length Fn binding. This indicates Ail interacts with a region contained within the 120kDa fragment, which includes the $^{2\text{-}10}$FNIII modules. Since binding to the 120kDa fragment was not as efficient as full length Fn, these this data suggest there may be regions outside the 120kDa fragment that participate in Ail interaction. Similar to previous results, *E. coli* expressing empty vector does not bind to any fragment tested.

The proteolytic fragments used in the previous study were commercially obtained and we observed contamination of other various sized fragments in each preparation (data not shown). Therefore, we sought to use fragments that were purified without contamination of other fragments. Dr. Deane Mosher provided additional recombinant Fn fragments to test Ail-mediated binding to Fn. These fragments were generated in cultured insect SF9 cell line to allow appropriate eukaryotic modifications and purified via His-tagged modification [42]. Bacteria expressing Ail bound to full-length plasma Fn to high levels similar to previous results (Fig. 4.2C). This binding was set at 100% to normalize binding across different experiments. The 30kDa and 120kDa fragments were included in these studies as some of the Mosher fragments are similar to commercially available fragments. As seen previously, Ail did not mediate bacterial binding to the 30kDa fragment, but did mediate binding to the 120kDa fragment. Bacteria expressing Ail did not bind to the most N-terminal fragment, N-^{5}FNI, nor to the larger N-terminal fragment, N-^{3}FNIII. This fragment contains sequences from the N-terminus of fibronectin to ^{3}FNIII, including modules ^{2}FnIII-

^{3}FNIII, which are present in the 120kDa fragment. Thus, this provides evidence that Ail does not bind those sites. Finally, Ail bound to the ^{1}FNIII-C fragment, and this binding was better than plasma full-length Fn. This binding supports the identification of an Ail-binding site in the 120kDa fragment of Fn and suggests there may be something beyond the 120kDa fragment that enhances Fn binding or that Ail mediated bacterial binding to dimeric Fn is more efficient than binding to monomeric Fn. These data suggest Ail binds to a region within the central and C-terminal end of the molecule.

Pre-adsorbed antibodies to the 120kDa fragment can no longer inhibit Ail binding to Fn.

The previous Fn fragment data suggests Ail binds to the 120kDa fragment. Our commercially available polyclonal anti-Fn antibody potentially contains antibodies that recognize various epitopes along the entire length of the Fn molecule. To examine the epitopes recognized by the polyclonal antibody, we performed a modified enzyme-linked immunosorbent assay (ELISA) (Fig. 4.3A). Plasma Fn and fibronectin fragments were coated onto microtiter wells and the polyclonal anti-Fn antibody was added. The polyclonal antibody recognized the full-length plasma Fn and this signal was normalized to 100 to compare across different experiments. The polyclonal antibody recognized the 120kDa fragment, but less than full length Fn. The 30kDa, 45kDa, and 70kDa fragments were recognized but to a lower level. Without any Fn coated onto well, the polyclonal antibody gave no signal. This demonstrates the polyclonal antibody does recognize the

various Fn fragments, but the epitopes present are not as numerous as the full length or 120kDa fragment. One might expect this result as the Fn fragments are smaller and contain less area for epitope recognition.

The previous ELISA data provide evidence that the polyclonal anti-Fn antibody does contain antibodies that recognize the various proteolytic Fn fragments. We then wanted to use this polyclonal antibody as a tool to identify antibodies that can inhibit Fn bacterial binding through Ail. We pre-adsorbed the polyclonal antibody to the N-terminal 70kDa and central 120kDa fibronectin fragments to remove antibodies that bind those fragments. After collecting unbound antibodies we used that solution to determine if the remaining soluble antibodies block bacterial binding to plasma fibronectin (Fig. 4.3B). Without any antibodies present, bacteria expressing Ail bound to immobilized Fn at a high level. Pre-incubation with the polyclonal anti-Fn antibody lead to inhibition of Ail-mediated binding to immobilized plasma Fn. Upon pre-incubation with antibodies pre-adsorbed to plasma Fn, Ail still was able to mediate binding to plasma Fn, due to the fact that all the antibodies were previously pre-adsorbed to plasma Fn and removed from solution. Thus, the resulting solution does not contain enough antibodies to block Ail binding to Fn. Polyclonal anti-Fn antibodies pre-absorbed to the 70kDa fragment were still able to block Ail-mediated binding. In this case, antibodies that recognize the 70kDa fragments were removed from the solution and the remaining solution contained antibodies to the rest of the Fn molecule, including the 120kDa region. When the polyclonal antibody was pre-absorbed to the 120kDa Fn fragment, the resulting solution was unable to block Ail-mediated

binding. Thus, antibodies capable of blocking Ail-mediated cell binding map to the 120kDa fragment. Together, this strategy provided further evidence that Ail binds the central to C-terminal region of Fn, which includes the 120kDa fragment, but not the N-terminal region of Fn.

Monoclonal antibodies to regions in the 120kDa fragment block Ail binding. Thus far we have evidence that Ail binds to the 120kDa region of Fn from the Fn fragment studies and pre-adsorbed antibody studies. In order to further define the Ail-binding site on Fn, we utilized monoclonal antibodies with known binding epitopes. These monoclonal antibodies have been previously described in the literature, and their recognition sites are illustrated in Fig. 4.4A. We used monoclonal antibodies kindly provided by Dr. Deane Mosher [43] and Dr. Kenneth Yamada [45]. Additionally, we used a commercially available monoclonal antibody (3E3) that recognizes the RGD integrin-binding site on Fn. Plastic wells were coated with plasma Fn and pre-incubated with the monoclonal antibodies for 1 hr before the addition of *E. coli* expressing Ail or empty vector. Without antibody addition, Ail mediated binding to plasma Fn to high levels while the polyclonal antibody blocked all Ail binding (Fig. 4.4B). None of the antibodies provided by Dr. Mosher directed against type I or early type III repeats blocked Ail-mediated binding (Fig. 4.4B). However, Ail-mediated binding to Fn was inhibited by three antibodies from Dr. Yamada (3B8, 13G12, and 12B4) (Fig. 4.4C). All three of these antibodies mapped to the Fn modules $^{8\text{-}9}$FNIII. The 3E3 and16G3 antibodies, which map to the RGD-containing ^{10}FNIII module, showed

modest or no inhibiting activity, respectively (Fig. 4.4C). Because Dr. Mosher's Lab Mab antibody, recognizing modules $^{4-8}$FNIII, did not inhibit Ail-mediated binding while the Yamada antibodies recognizing $^{8-9}$FNIII modules did inhibit, this suggests Ail binds to the $^{8-9}$FNIII modules.

Collagen, gelatin, and dextran sulfate inhibit binding to Fn, while heparin does not. Fibronectin is known to bind many substrates such as fibrin, heparin-sulfated proteoglycans, and collagen/gelatin. In order to test additional regions of FN for a role in Ail-mediated binding, we used some of these Fn substrates as competitive inhibitors for Ail binding. Plasma Fn was again immobilized onto plastic and pre-incubated with collagen I, collagen IV, gelatin (a partially hydrolyzed form of collagen), heparin (a highly sulfated form of heparan sulfate), or dextran sulfate (as a control for charge effects of heparin) (Fig. 4.5B-F). *E. coli* expressing Ail was then added to the solution. Collagen I and IV as well as gelatin inhibited Ail-mediated binding as concentrations increased (Fig. 4.6B-D). Gelatin is derived from hydrolysis of collagen so it is consistent that both collagen and gelatin inhibit binding. As presented in Chapter 2, Ail does not bind collagen I or IV so this collagen inhibition of Ail-Fn interaction is not due to Ail-collagen interaction. This blocking of bacteria expressing Ail could be by direct inhibition indicating Ail binds two regions of Fn or steric inhibition by the long fibrillar protein, trimeric collagen. Interestingly, heparin did not inhibit binding even at very high concentrations (Fig. 4.5F), while dextran sulfate was able to block Ail binding (Fig. 4.5E-F). Dextran sulfate was included as a control because it has

many sulfated negative-charged groups similar to heparin. The fact that heparin does not inhibit binding suggests Ail does not interact with fibronectin at the N-terminal 30kDa heparin binding site. The fact that dextran sulfate inhibits binding, some charge interaction may be involved in the Ail/Fn interface. Together, these data suggests Ail binds to the 45-kDa collagen/gelatin-binding fragment or binding of collagen to this region interfere with our previously defined interaction of Ail with the downstream FNIII repeats (Fig. 4.4). We have previously shown Ail does not bind the 45kDa fragment, thus this discrepancy will be addressed in the following discussion section.

Excess fibronectin enhances binding, but can block binding at high concentrations. To study the Ail-fibronectin interaction, we wanted to determine whether this interaction was specific and could be competed with excess fibronectin. Plasma fibronectin was adsorbed onto plastic plates at 10μg/ml. Then, *E. coli* expressing Ail from an IPTG-inducible plasmid was pre-incubated with soluble cellular fibronectin in order to coat the bacteria. The bacteria plus fibronectin solution was then added to plasma Fn-coated wells. Bound bacteria were fixed and stained with crystal violet to visualize bacterial binding. *E. coli* expressing the empty vector did not bind immobilized plasma Fn in any of the conditions tested (Fig. 4.6A). Without excess fibronectin, *E. coli* expressing Ail bound plasma fibronectin to a level seen previously (Chapter 2 and 3). At a lower concentration of fibronectin (1.25μg/ml), *E. coli* expressing Ail was still able to bind plasma fibronectin at levels similar to that observed without the addition of

excess Fn (Fig. 4.6B). At intermediate concentrations, addition of excess fibronectin enhanced binding to plasma Fn. We take this to indicate that cellular fibronectin binds to bacterial-expressed Ail, but at these concentrations, cellular fibronectin does not saturate all Ail. Then, free Ail is able to bind plasma Fn but since Fn is found in dimers, Ail also cross-links two or more bacteria expressing Ail to one another, thus leading to enhanced binding. At higher concentrations of cellular fibronectin (40µg/ml and above), excess Fn inhibits Ail-mediated binding. At this concentration of excess Fn, we interpret this as all the Ail is bound by cellular Fn and thus, no unbound Ail molecules are available to interact with plasma Fn on the plate or with cellular Fn bound to other bacteria. Together, these data provide evidence that Ail interacts with Fn in a specific manner and addition of excess Fn leads to a modular binding pattern depending on the concentration of excess Fn used.

Ail preferentially binds soluble cellular Fn. During *Y. pestis* infections, bacteria can be directly deposited into the bloodstream or can be taken to the bloodstream from the site of inoculation. In blood, most Fn exists a soluble globular protein, plasma Fn (unless unfolded in response to certain binding events) whereas in tissue Fn exists as extended soluble Fn (cellular Fn) or insoluble Fn matrices. To determine whether Y. pestis Ail binds plasma Fn in the blood, cellular Fn in tissues, or both, *E. coli* expressing Ail was used as a preceipation reagent where the bacteria were allowed to bind Fn in solution. The bacteria were then spun and washed and whole cell lysates were subjected to

Western blot analysis with anti-Fn to determine interaction between Ail and aspecific forms of Fn (Fig. 4.7). *E. coli* expressing either vector alone or Ail incubated with buffer alone did not contain any cross-reacting bands where probed with the anti-Fn antibody (Fig. 4.7 lanes 1 and 2). Incubating bacteria with soluble cellular Fn does lead to a small signal with *E. coli* pMMB207 and a much stronger band with *E. coli* expressing Ail, indicating Ail interacts with soluble cellular Fn (Fig. 4.7 lane 4). Finally, incubation with soluble plasma Fn leads to a very light band with *E. coli* expressing Ail, indicating Ail does not bind plasma Fn as well (Fig. 4.7 lane 6). Control lanes with purified plasma and cellular Fn (used at the same concentrations) indicate that both forms are detected with the anti-Fn antibody and that plasma Fn may be detected better than cellular Fn (Fig. 4.7 lane 7 and 8). Finally, an anti-Ail blot was performed to show *E. coli* expressing Ail did indeed have adequate and similar levels of Ail. Together, these data suggests Ail preferentially binds cellular Fn than plasma Fn and suggests the primary role for the Ail/Fn interaction occurs in tissues not blood.

Discussion

We have observed that the Ail-Fn interactions are important for Ail virulence functions such as host cell binding and Yop delivery (Chapter 2, [45]). In this study, we sought to identify the Ail binding site on the Fn molecule to understand this critical interaction in more detail. The fragment binding and antibody inhibition studies provided evidence that Ail binds to the 120kDa fragment, particularly the $^{8\text{-}9}$FNIII modules as shown with experiments using the

Yamada antibodies, 384, 13G12 and 12B4 (Fig. 4.4C) and the Mosher Lab Mab antibody. In addition, some of the fibronectin fragment experiments suggested there are additional binding sites at the most C-terminal end of Fn that enhance binding or that dimer formation by Fn enhances Ail binding (Fig. 4.2). Pre-incubating fibronectin with collagen blocked bacterial binding through Ail. Therefore, it is possible that the neighboring 45kDa fragment acts in synergy with the central 120kDa site to provide the best binding structure. Fn is a complex multi-dimensional molecule and it is also possible collagen binding to the 45kDa region affect the Ail binding site within the 120kDa region.

In our studies, we have evidence that the C-terminal end of Fn provides regions that enhance Ail binding. The two constructs that facilitate the best Ail binding, full length and Mosher fragment ^{1}FNIII-C+, include the C-termini (Fig. 4.2). In fact, the Mosher fragment containing the C-terminal end even enhanced bacterial binding. Like the full length Fn, the ^{1}FNIII-C+ construct contains the C-terminal disulfide bonds and is found as a dimer which leads to more bacterial binding to two molecules of Fn. The reason for enhanced binding to ^{1}FNIII-C+ over full-length Fn is currently not known.

To map the binding site of Ail on Fn, we utilized anti-Fn monoclonal antibodies that have been previously characterized [43, 45]. Using monoclonal antibodies provided evidence that Ail bound to the modules $^{8-9}$FNIII within the 120kDa fragment. Monoclonal antibodies in general are very useful to study molecular interactions, however, the exact recognized epitopes are not fully known and binding of antibodies to molecules can lead to changes in

intramolecular interactions. Binding of 3B8, 13G12, and 12B4 to the $^{8\text{-}9}$FNIII may distort other regions. Additionally, we have observed variable results with antibody inhibition studies with other antibodies such as 5C3, 7D5 and Lab Mab, suggesting these antibodies change the conformation of Fn and can modestly affect Ail binding. Also, intermediate inhibition with antibodies such as Lab Mab and 3E3 may indicate weaker binding affinities with their epitopes. Ail may interact with Fn more strongly than some antibodies. Thus, Ail would compete away the antibody and Ail would still appear to bind Fn at high levels.

We used known Fn binding substrates such as collagen, gelatin, and heparin to map the Ail-binding site on Fn. Dextran sulfate is a similar highly sulfated polysaccharide to heparin. Pre-incubation with collagen I, IV, or gelatin inhibited Ail-mediated bacterial binding, but this inhibition happened only at high concentrations (Fig. 4.5). It is not known whether the collagen inhibition is due to collagen directly inhibiting Ail-Fn interactions or simply by having another large neighboring molecule exerting steric hindrance the Ail-Fn interaction. Heparin does not inhibit Ail binding to fibronectin even at very high concentrations, but dextran sulfate is able to inhibit bacterial binding through Ail (Fig. 4.5E-F). This suggests general charge interactions are required for Ail binding to Fn, but such charges must be presented in a certain context such as on dextran but not heparin. In chapter 3, we identified residues of Ail that contribute to host cell binding and Fn binding. The charged residues, aspartic acid (D), glutamine (E), and lysine (K) may contribute to the Ail-Fn interactions.

On the Fn molecule, synergy sites for cell binding have also been observed in regions that support binding to the well-characterized RGD site in module ^{10}FNIII. One study generated site-directed mutants that had reduced cell spreading. Two synergy sites for cell binding were found, one at the N-terminal region of ^{8}FNIII and one in the center of the ^{9}FNIII [46]. Another study analyzing the structure of the synergy sites has shown the adjacent ^{9}FNIII synergy residues and RDG residues of ^{10}FNIII are held in linear fashion on the same face of Fn and create an easily accessible site for the integrin molecule, thus again suggesting module ^{9}FNIII acts as a synergy site for integrin binding by ^{10}FNIII [47]. The surrounding domains, $^{7-8}$FNIII, are not as ordered and there is more rotational tilt between the adjacent domains.

These two synergy regions fall within modules ^{8}FNIII and ^{9}FNIII – the same proposed Ail binding location on fibronectin. By binding to this synergy site on Fn, Ail may cause Fn to bind integrins on host cells in different manner. As mentioned in the introduction, Fn binding to the $\alpha_5\beta_1$ integrins in the two distinct sites, the N-terminal matrix assemb-ly site and the RGD ^{10}FNIII domain, leads to distinct host intracellular signaling events. Thus, Ail may be directing the Fn molecule to engage $\alpha_5\beta_1$ integrin binding in a manner that facilitates the translocation of Yops into the host cell to a site distinct from RGD region (Fig. 4.8). Since Fn is a dimer, binding by Ail may only occlude one RGD region leaving the other to freely engage the β_1 integrin. This area of study is something we would like to pursue further as it may provide important information for the role of Ail in initiating Yop delivery.

It is also possible that Ail binds a region near the synergery region in ^{9}FNIII which brings Ail in close proximity with integrins. Then Ail itself can engage the integrins. I do not present any data that Ail interacts with integrins, so it would be interesting to pursue those interactions in more detail.

Other models presented with the functional upstream domain (FUD) of Protein F1 of *Streptococcus pyogenes* suggest FUD interacts with FNI modules of the 70kDa fragment in a β-strand donation model [43]. This study calls this interaction a β-zipper mechanism in which FUD contributes another antiparallel β-strand to complete the modular structure of the FNI repeats. This β-zipper mechanism was first described with a *Streptococcus dysgalactiae* fibronectin binding protein [48]. It is possible that Ail also interacts in a similar manner with the Ail loops completing the β-sheet module of Fn.

One complication of Fn binding studies is that fibronectin is a multi-functional molecule with a complex structure that can bind to other Fn molecules, fold onto itself, and interact with numerous other molecules. These types of interactions may distort the usual epitopes for Ail binding. Another problem is that purified Fn and other fragments may not coat plastic plates in same fashion as host cells that produce fibronectin matrix. Use of the monoclonal antibodies provides an assay that relies on immobilized full length Fn. Thus, alleviating concerns about differential coating efficiencies and differing conformations of Fn fragments.

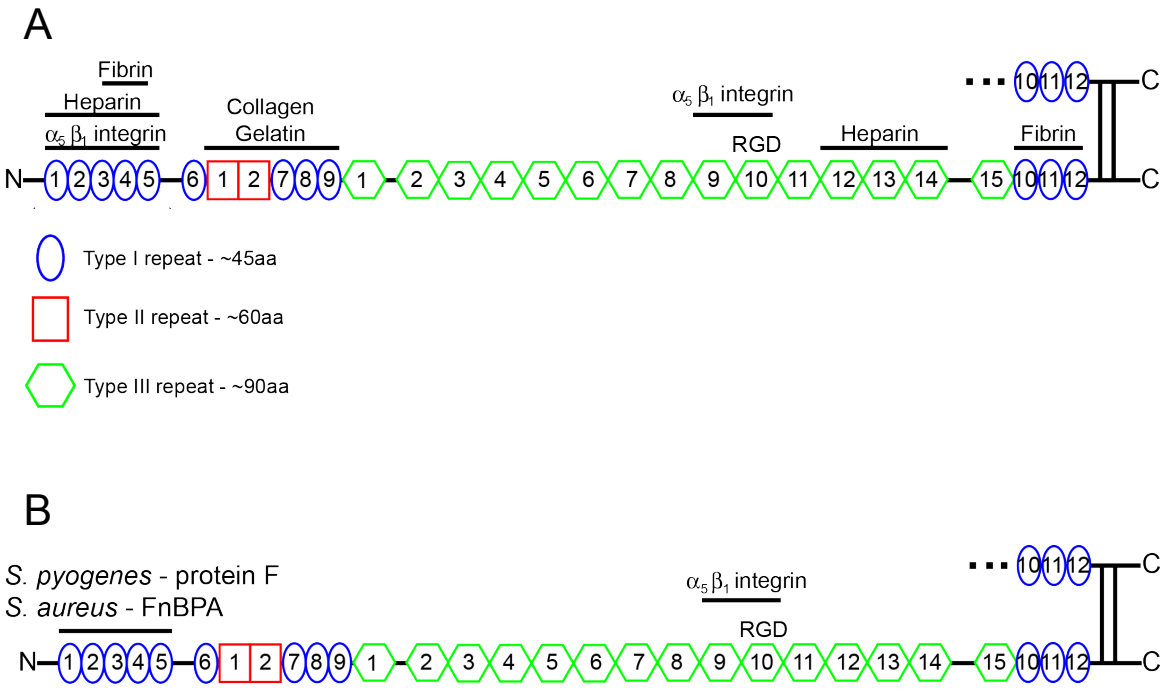
In attempts to verify Ail binding to specific Fn modules, we wanted to use known Fn binding protein controls such as *S. pyogenes* Protein F. Protein F is

known to bind to the N-terminal 30kDa fragment of Fn. We examined a *S. pyogenes* wildtype strain and isogenic *prtF* mutant for binding to immobilized Fn; unfortunately this strain only binds soluble Fn. Soluble Fn binding assays with these *S. pyogenes* strains were hard to perform and inconsistent. We currently have *Lactococcus lactis* expressing *Staphylococcus aureus* Fibronectin Binding Protein A (FnBPA, provided by Dr. Ruth Massey) which we will use as a control for bacterial binding to the 70kDa Fn fragment and antibody inhibition studies by monoclonal antibodies directed against the N-terminus of Fn.

We also want to examine whether Fn is presented to β_1 integrin via the N-terminal matrix assembly site or the RGD-containing ^{10}FNIII module. Engaging each of these sites triggers unique intracellular signaling within host cells [27]. Therefore, Ail may present a certain part of the Fn molecule to trigger the appropriate host cell-signaling cascade for the efficient delivery of Yops by *Y. pestis*. We will address this hypothesis in future studies.

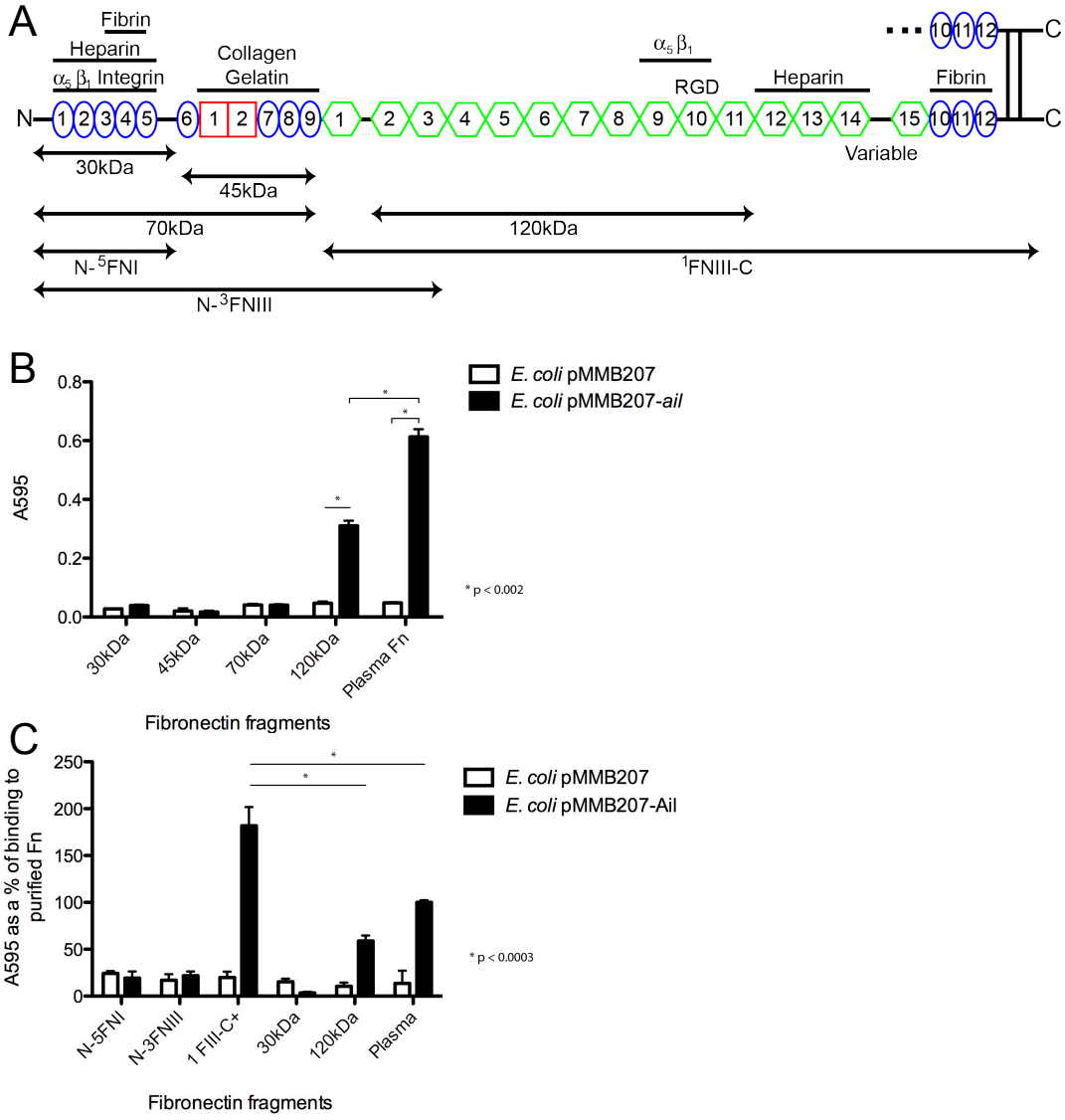
In all, we have shown Ail binds the 120kDa fragment of Fn and likely interacts with $^{8\text{-}9}$FNIII (Fig. 4.2-4.4). In addition, we know that binding of collagen presumably to the N-terminally located 45kDa Fn fragment inhibits Ail-Fn interaction. This could be by direct inhibition indicating Ail binds two regions of Fn or steric inhibition by the long fibrillar protein, trimeric collagen. Future studies will aim to determine how Fn is presented to host cells by Ail to trigger intracellular signaling and facilitate Yop delivery, a critical step in *Y. pestis* virulence.

Figure 4.1



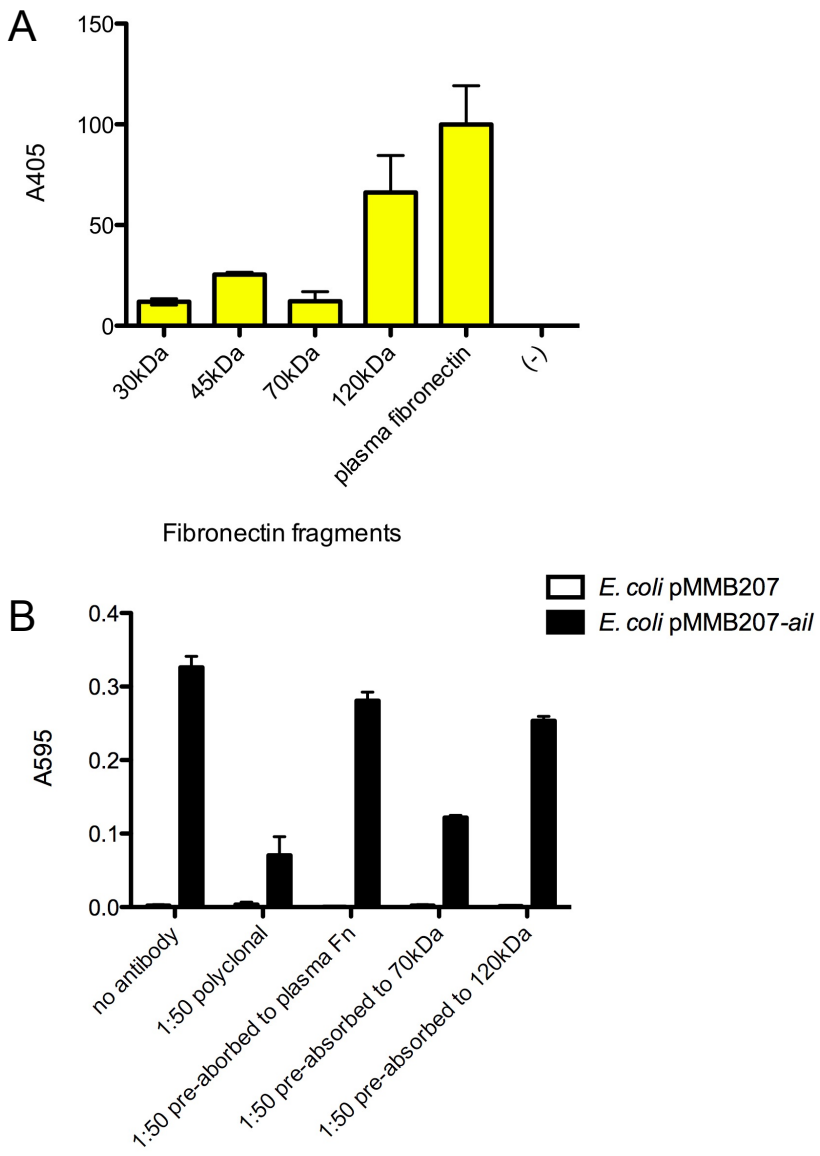
Model of fibronectin. **(**A) Fn modular repeats are shown in this illustration. The two disulfide bonds are found at the C-terminal end. The $\alpha_5\beta_1$ integrin binds to the ^{10}FNIII RGD domain and the ^{9}FNIII synergy site. Known fibronectin substrates are shown above the molecule while the fibronectin fragments used in this study are shown below. There are 12 Type I repeats (FNI), 2 Type II repeats (FNII), and 15 Type III repeats (FNIII). Type I repeats are in blue, Type II in red, and Type III in blue. Modeled on Maurer et al. [43]. (B) Diagram indicating the binding site of the other characterized Fn binding proteins of *S. pyogenes* and *S. aureus*.

Figure 4.2



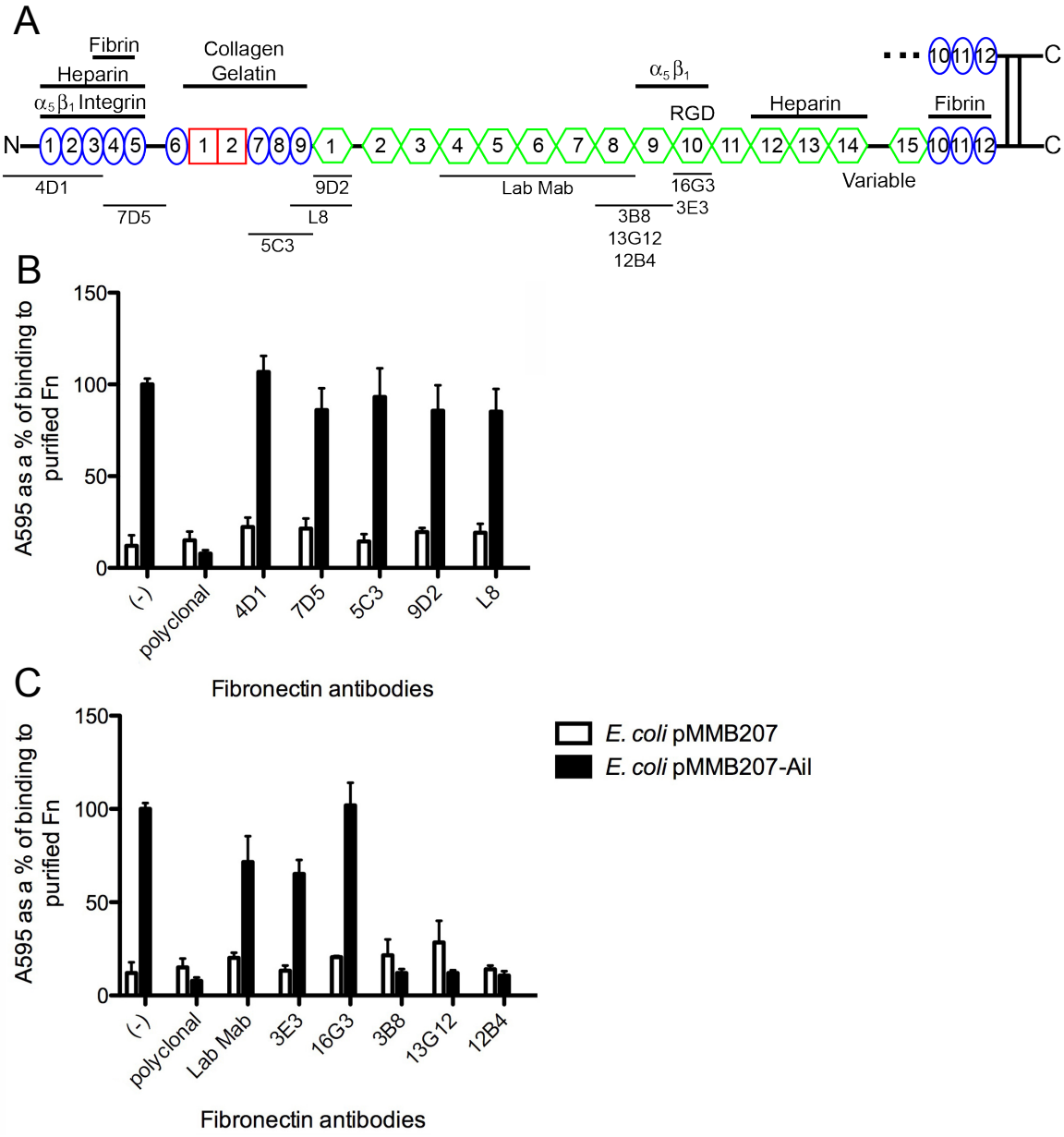
Fibronectin fragment binding. (A) This fibronectin diagram shows the fragments used in this study. (B) Fibronectin and commercially available Fn fragments were adsorbed onto plastic wells (10µg/ml). *E. coli* AAEC185 derivatives expressing empty vector and Ail were added to the wells and allowed to bind at 37°C. Bound bacteria were stained with 0.01% crystal violet. The cells and crystal violet were solubilized, and the A_{595} of the solution was read. (C) Fibronectin and fragments obtained from Dr. Mosher were adsorbed onto plastic wells (10µg/ml). *E. coli* AAEC185 derivatives were added as described above.

Figure 4.3



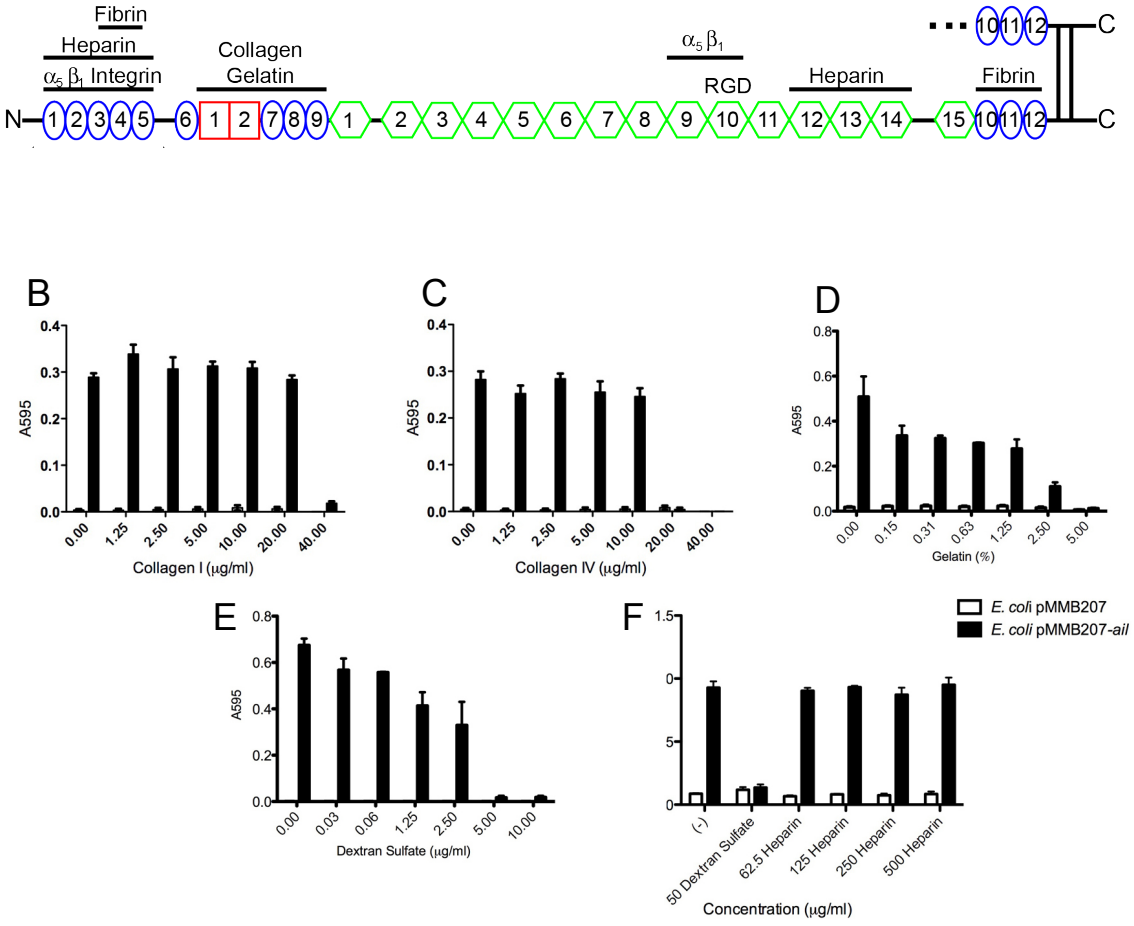
Polyclonal α-Fn antibody pre-adsorbed to 120kDa can no longer inhibit Ail binding. (A) Full-length plasma Fn and Fn fragments were immobilized onto microtiter wells. Polyclonal anti-Fn antibody was added in this modified enzyme-linked immunosorbent assay (ELISA). The signal produced with plasma was set to 100 to normalize across different experiments. (B) Microtiter wells were coated with plasma Fn (10μg/ml). The polyclonal antibody and pre-adsorbed antibodies were added at appropriate concentrations 1 hr at 37°C prior to addition of *E. coli* AAEC185 derivatives expressing empty vector and Ail. Bacteria were allowed to bind for an additional 1 hr at 37°C. Bound bacteria were stained with 0.01% crystal violet. The cells and crystal violet were solubilized, and the A_{595} of the solution was read. Shown are the results of a representative experiments (n = 2) of two trials.

Figure 4.4



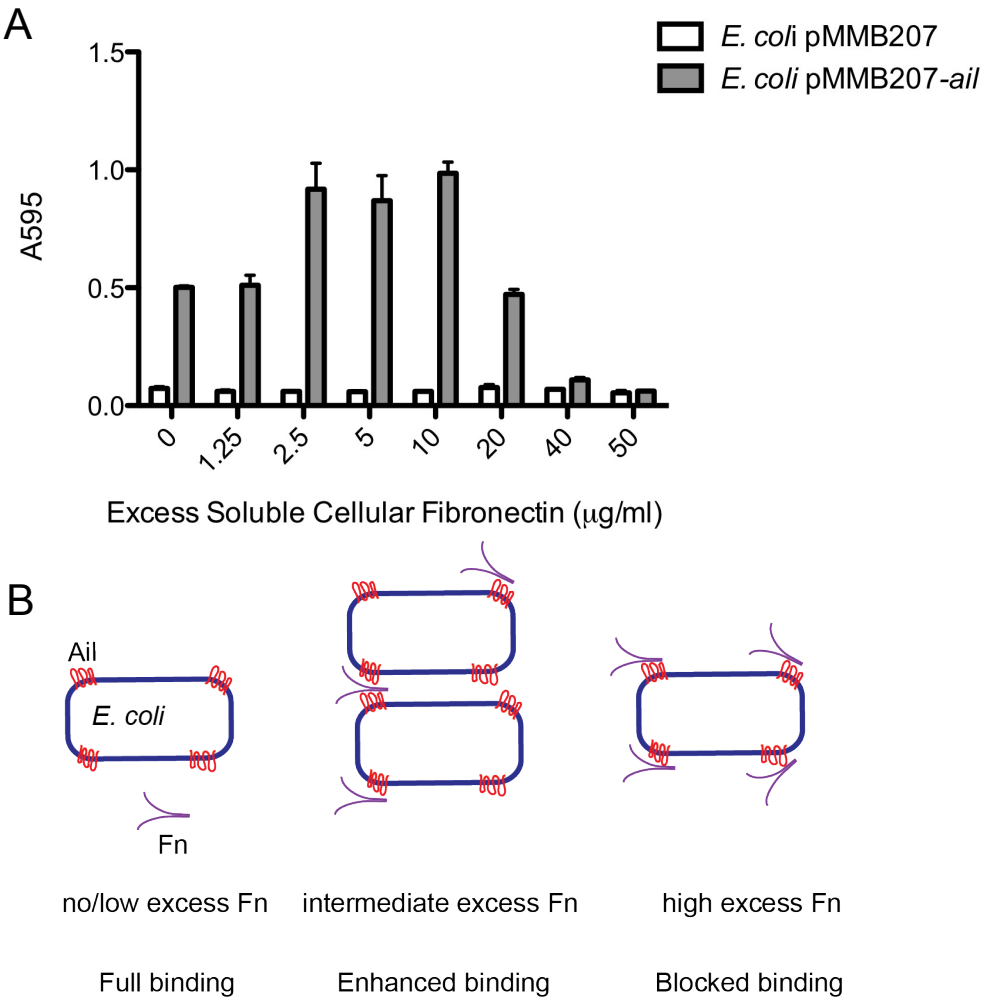
Monoclonal antibodies to regions in the 120kDa fragment block Ail binding. (A) This fibronectin diagram shows the antibodies used in this study. Plasma fibronectin was adsorbed onto plastic wells (10µg/ml). Antibodies provided by Dr. Mosher (B) and Dr. Yamada (C) was added to Fn-coated wells 1 hr prior to the addition of *E. coli* AAEC185 derivatives expressing empty vector and Ail. Bacteria were allowed to bind for additional 1 hr at 37°C. Bound bacteria were stained with 0.01% crystal violet. The cells and crystal violet were solubilized, and the A_{595} of the solution was read. *E. coli* expressing Ail without treatment with antibodies was set at 100% to normalize bacteria binding across multiple experiments (n=6 – 9).

Figure 4.5



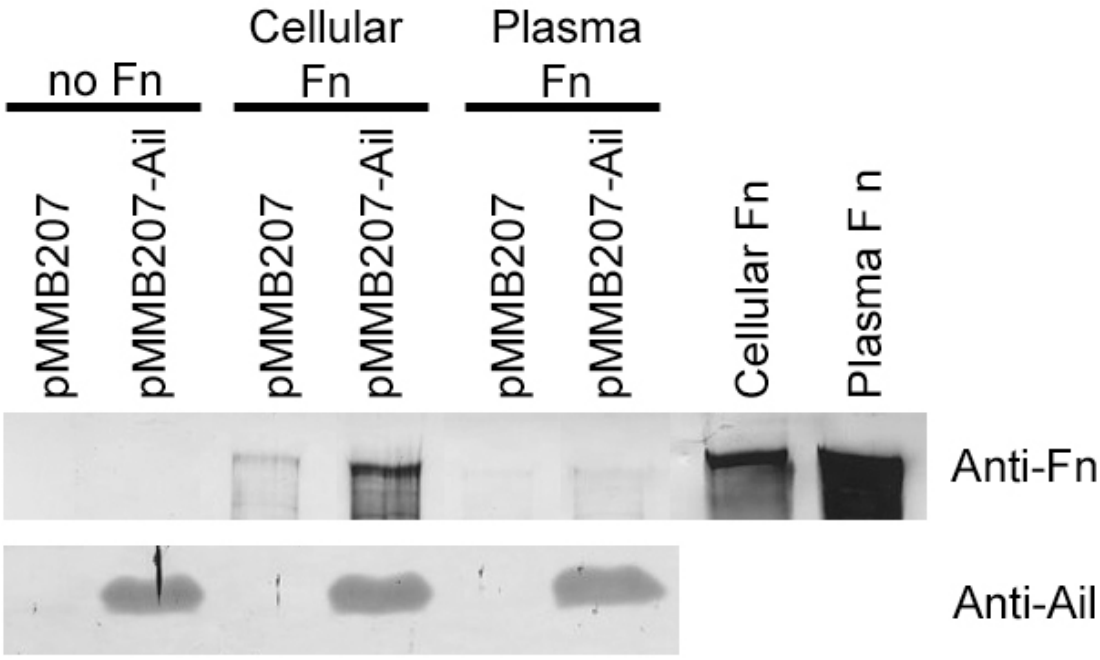
Collagen, gelatin, and dextran sulfate inhibit Ail binding to Fn, while heparin does not. (A) This fibronectin diagram shows the binding site of the Fn substrates used in this study. Plasma fibronectin was pre-adsorbed onto plastic wells (10 μg/ml). Collagen I (B), collagen IV (C), gelatin (D), dextran sulfate (E), and haparin were added at the indicated concentrations for 1 hr prior addition of *E. coli* AAEC185 derivatives. Bacteria were allowed to bind for additional 1 hr at 37°C. Bound bacteria were stained with 0.01% crystal violet. The cells and crystal violet were solubilized, and the A_{595} of the solution was read. *E. coli* expressing Ail without treatment with antibodies was set at 100% to normalize bacteria binding across multiple experiments (n=6 – 9).

Figure 4.6



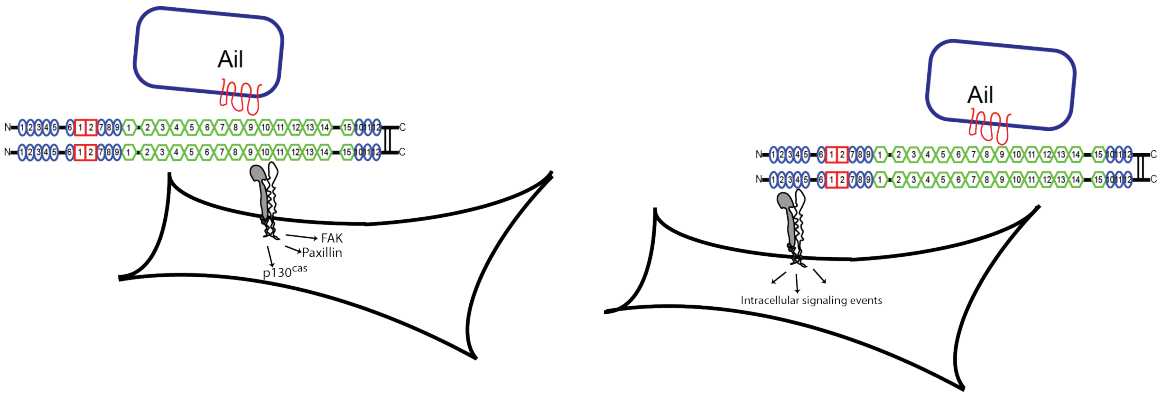
Excess soluble fibronectin can inhibit Ail binding to fibronectin. (A) Plasma fibronectin was pre-adsorbed onto plastic wells (10μg/ml). *E. coli* AAEC185 derivatives expressing empty vector or Ail was coated with excess soluble cellular Fn a the indicated concentrations for 1 hr at 37°C. The solution of Fn and bacteria was added to immobilized plasma Fn and allowed to bind another 1 hr at 37°C. Bound bacteria were stained with 0.01% crystal violet. The cells and crystal violet were solubilized, and the A_{595} of the solution was read. Shown are the results of a representative experiment (n=3) of three trials. (B) A model of the enhancement of excess FN. At no to low excess Fn, the bacteria expressing Ail binds to immobilized plasma Fn at the normal level. However, with intermediate concentration, the dimeric Fn molecule can cross-link Ail expressing bacteria which leads to enhancement of binding. At the high concentrations of excess Fn, all the Ail is bound which prevents bacterial binding to immobilized Fn on the plate.

Figure 4.7



Ail binds cellular Fn preferentially in solution. *E. coli* expressing empty vector or Ail was coated with the no Fn, cellular Fn, and plasma Fn (each 20μg/ml) in solution. After 1 hr rotating at 37^{o}C, the bacteria were spun and washed to remove excess Fn. Bacterial whole cell lysates were then subjected to SDS-PAGE and blotted with the indicated antibodies.

Figure 4.8



Model of Ail directing the binding site to either the N-terminal matrix assembly site or RDG containing ^{10}FNIII. Based on our studies presented in this chapter, we have evidence for Ail binding to the ^{9}FNIII module. With Ail binding to this ^{9}FNIII module, Fn can engage the host cell $\alpha_5\beta_1$ integrin via the N-terminal matrix assembly site or the RDG containing ^{10}FNIII module. Future studies will address these different scenarios.

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The following work was submitted for publication:

Analysis of the differences between Ail proteins of *Yersinia pestis* and *Y. pseudotuberculosis* for cell binding and invasion activities

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PLoS One, August 2010.

CHAPTER FIVE

ANALYSIS OF THE DIFFERENCES BETWEEN AIL PROTEINS OF *YERSINIA PESTIS* AND *Y. PSEUDOTUBERCULOSIS* FOR CELL BINDING AND INVASION ACTIVITIES

Summary

The *Yersinia pestis* adhesin Ail mediates host cell binding and facilitates delivery of cytotoxic Yop proteins. Ail from *Y. pestis* and *Y. pseudotuberculosis* are identical except for two amino acids at positions 43 and 126. Ail from *Y. pseudotuberculosis* has been reported to lack host cell binding ability, thus we sought to determine which amino acid difference(s) are responsible for the difference in cell adhesion. We found *Y. pseudotuberculosis* Ail binds host cells, albeit at ~50% the capacity of *Y. pestis* Ail. *Y. pestis* Ail single mutants, Ail-E43D and Ail-F126V, both have decreased adhesion and invasion when compared to wild-type *Y. pestis* Ail. *Y. pseudotuberculosis* Ail also had decreased binding to the Ail substrate fibronectin, relative to *Y. pestis* Ail. The modest adhesion defect of *Y. pseudotuberculosis* Ail relative to *Y. pestis* Ail had no effect on the efficiency of Yop delivery.

Introduction

There are three pathogenic *Yersinia* species. While the enteric pathogens *Y. enterocolitica* and *Y. pseudotuberculosis* cause primarily self-limiting gastroenteritis, and in some cases mesenteric lymphadenitis, *Y. pestis* causes the rapidly fatal disease plague [1, 2]. While *Y. enterocolitica* and *Y. pseudotuberculosis* are similar in the disease they cause via an oral route of infection, *Y. pseudotuberculosis* and *Y. pestis* are more closely related genetically, estimated to have evolved from one another between 5,000 and 20,000 years ago [3].

All three pathogenic *Yersinia* species harbor a virulence plasmid that encodes cytotoxic Yop proteins and the Type III Secretion System (T3SS) required for their delivery to host cells [2]. This process requires adhesion of *Yersinia* to host cells [4-7]. Adhesins can bind host cells directly or via bridging molecules such as extracellular matrix components [8-12]. Pathogenic *Yersinia* species produces many adhesins including Invasin (Inv) [13], YadA [14], plasminogen activator (Pla) [15], pH 6 antigen (Psa) [16], and Ail [6, 17, 18]. Ail from *Y. enterocolitica* has been well characterized and many functions have been elucidated, including serum resistance and adhesion to and invasion into host cells [19-21]. The crystal structure of OmpX from *E. coli* (an Ail homologue) has been determined [22, 23] and this family of proteins is described as a flattened β-barrel with four extracellular loops extending above the surface of the bacterium. The four proposed loops of Ail contain 8-20 amino acids.

Ail from *Y. enterocolitica* binds cultured cells in a species-specific manner with adhesion to CHO and HEp-2 cells but not MDCK cells [19]. Further studies identified Ail point mutants with intermediate and severe serum sensitivity that also affected invasion [24]. In particular, an aspartic acid (D67) and valine (V68) at the C-terminal end of loop 2 were required for both Ail functions.

Ail from *Y. pseudotuberculosis* is reported to lack adhesion and invasion activities when expressed in *E. coli*, although *E. coli* expressing Ail was still able to confer serum resistance [25]. Thus, *Y. pseudotuberculosis* Ail has long been believed to have no adhesion capacity.

Recently, Ail from *Y. pestis* has been shown to mediate serum resistance [18, 26], auto-aggregation [18], and cell adhesion [6, 18]. Additionally, Ail is a key *Y. pestis* adhesin for Yop delivery and virulence [6, 27]. The extracellular matrix protein fibronectin is a substrate for *Y. pestis* Ail and this Ail-fibronectin interaction is important for adhesion to host cells and Yop delivery [12].

Since Ail is a critical molecule for *Y. pestis* adhesion, Yop delivery, and virulence, we wanted to identify residues of Ail required for adhesion and Yop delivery. Although the previous studies of OmpX and *Y. enterocolitica* Ail are useful, the functions of these proteins cannot be translated to *Y. pestis* Ail, as even *Y. enterocolitica* Ail (the protein more similar to *Y. pestis* Ail) is only 30%-65% identical to *Y. pestis* Ail within the four extracellular loops. *Y. pseudotuberculosis* Ail is 98.9% identical to *Y. pestis* Ail with only two amino acid changes, E43D and F126V (*Y. pestis* relative to *Y. pseudotuberculosis*).

Therefore, we hypothesized these two amino acids should be important for the adhesive activity of Ail. In this study, we revisited the adhesion capacity of *Y. pseudotuberculosis* Ail and observed reduced but significant binding to cultured host cells. Single mutations based on *Y. pseudotuberculosis* Ail introduced into the *Y. pestis* Ail molecule were analyzed for their cell adhesion and Yop delivery functions. While *Y. pseudotuberculosis* Ail and single mutations were defective for host cell binding by up to 65%, this level of adhesion defect did not disrupt the ability of Ail to deliver Yop proteins.

Experimental Materials and Methods

Strains and culture conditions. Bacterial strains and plasmids used in this study are listed in Table S1. *Y. pestis* strains were cultivated in heart infusion broth (HIB) overnight or on heart infusion agar (HIA) for 48 hours at 28°C. *Escherichia coli* strains were cultured in Luria-Bertani (LB) broth or LB agar at 28°C or 37°C. Antibiotics were used at the following concentrations: streptomycin (Sm), 100 μg/ml; chloramphenicol (Cm), 10 μg/ml; and ampicillin (Amp), 100 μg/ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used at a 100 μM concentration unless otherwise noted. YPIII was obtained from Dr. Ralph Isberg and YPIII and IP2666 from Dr. James Bliska.

HEp-2 cells were cultured at 5% CO_2 (37°C) in modified Eagle's medium (MEM) (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco), 1% sodium pyruvate (Gibco), and 1% non-essential amino acids (Gibco).

Construction of Ail-expressing plasmids. pSK-*ail* Bluescript encoding wild-type *Y. pestis* Ail was constructed by PCR amplification of the *ail* locus from *Y. pestis* strain KIM5 [6] using primers 5'-GCGCGGATCCTTGGCTGGCCACTTTAGTCT-3' and 5'-GCGCCTGCAGGGTTAGGAGGACGTTAGAAC-3'. The *ail* PCR product was digested with *Bam*HI and *Pst*I and ligated into *Bam*HI/*Pst*I-cut pSK Bluescript (Stratagene). *ail* from the Y. pseudotuberculosis strains YPIII and IP2666 were similarly PCR amplified and ligated into pSK Bluescript. All clones were also moved into the IPTG-inducible vectors pMMB207 [28] and pMMB66EH [29] for cell binding studies.

Generation of mutations. PCR-mutagenesis was performed using the enzyme Pfu (Stratagene) and primer pairs encoding the mutations Ail-E43D and Ail-F126V. The primers used were complementary to one another and are listed here: ail-E43D top strand 5' – caaagtcgtgtcaagGACgatgggtacaagttgg and bottom strand 5' – ccaacttgtacccatcGTCcttgacacgactttg; ail-F126V top strand 5' – catggaaaggctaaaGTTtcctcaatatttggtc and bottom strand 5' – gaccaaatattgaggaAACtttagcctttccatg. Following PCR amplification using a pSK-*ail* Bluescript-derived plasmid as a template, the PCR reactions were digested with *Dpn*I to cut the template DNA and transformed into DH5α. Potential mutant clones were sequenced to confirm that only the target site was mutated and a *Bam*HI/*Pst*I fragment containing the entire open reading frame and ribosome-

binding site, was liberated, purified and ligated into the IPTG-inducible plasmid pMMB207 (CmR) or pMMB66EH (AmpR).

Adhesion and invasion assays. Adhesion assays were preformed as described previously [12]. HEp-2 cells were cultured in 24-well tissue culture plates until reaching 100% confluence. Bacteria at the proper dilution were added to cultured HEp-2 cells at an MOI~100. After 1 hour 45 minutes incubation at 37°C in 5% CO_2, cell-associated bacteria were liberated by the addition of sterile H_2O containing Triton X-100. Percent adhesion was calculated by dividing bound CFU by total bacteria in the well and then multiplying by 100.

Invasion assays were performed similarly except that at the end of 1 hour 45 min of bacterial binding, cells were washed once with phosphate-buffered saline (PBS) to remove unbound bacteria and minimal essential medium containing gentamicin was added for 1 hour at 37°C in 5% CO_2 to kill extracellular bacteria. Cells were then washed twice with PBS and lysed and plated as described for the adhesion assay.

Bacterial binding to fibronectin. Bacterial binding assays were performed as described previously [12]. Briefly, bacterial cells were diluted and added to immobilized fibronectin (Sigma, F2006) and allowed to bind at 37^{o}C. Bacteria bound to the wells were stained with crystal violet. After washing away excess

crystal violet, the bacterial-associated crystal violet stain was solubilized with a methanol/acetone solution. The absorbance was measured at ABS_{595}.

Cytotoxicity assay. Cytotoxicity assays were preformed as described previously [12]. Briefly, *Y. pestis* KIM5 derivatives were added to HEp-2 cells at an MOI of 10. After 2 hours of incubation at 37°C in 5% CO_2, cells were fixed with 0.5ml methanol and stained with Geimsa stain. Rounding was observed and pictures were taken under a phase-contrast microscope. Cytotoxicity was enumerated by counting total cells and the number of round dark purple (shrunken cytoplasm) cells experiencing cytotoxicity in three microscopic fields (~150 cells/field). Percent cytotoxicity was calculated by dividing rounded cells by total cells. This experiment was performed twice in triplicate, n=6. Statistical significance was assessed using the student t test.

Western blot assays. Normalized cultures of bacteria were resuspended in Laemmli sample buffer. Bacterial cell extracts were boiled and run on a 15% SDS-polyacrylamide gel electrophoresis (PAGE) gel. Proteins were transferred to nitrocellulose and probed with rabbit anti-Ail serum (a kind gift from Dr. Ralph Isberg) at a 1:500 dilution.

Results

Amino acid sequence of two *Y. pseudotuberculosis* strains. Prior to comparing relative binding capacities of *Y. pestis* and *Y. pseudotuberculosis* Ail,

we sequenced Ail from both *Yersinia* species. For *Y. pestis*, strain KIM5 was used and for *Y. pseudotuberculosis* strains IP2666 and YPIII were used. The sequence alignment (Fig. 5.1) shows *Y. pseudotuberculosis* Ail has an aspartic acid, at position 43, instead of a glutamic acid (E43D) and a valine at position 126 instead of a phenylalanine (F126V). The E43D substitution is predicted to lie in exposed loop 1 while F126V is predicted to lie in loop 3. At these exposed positions, these two residues may directly contact cell components such as fibronectin. Unlike a previously published YPIII Ail sequence [25], our *Y. pseudotuberculosis* YPIII Ail sequence did not contain an additional amino acid change at position 7, T7I.

Ail from *Y. pestis* and *Y. pseudotuberculosis* exhibit adhesion to host cells. To determine the relative binding efficiencies of Ail proteins from different *Yersinia* strains to host cells, Ail from the *Y. pestis* strain KIM5 [6], *Y. pseudotuberculosis* IP2666 and *Y. pseudotuberculosis* YPIII were expressed from an IPTG-inducible construct in *E. coli* AAEC185 [30], and adhesion to cultured HEp-2 cells was measured. Adhesion of *E. coli* expressing KIM5 Ail to HEp-2 cells was set to 100% (actual adhesion was 4.0%). In contrast to a previous report [25], we observed significant adhesion activity from the two *Y. pseudotuberculosis* alleles (both encode identical ORFs). Ail from the two *Y. pseudotuberculosis* strains exhibited ~60% adhesion to HEp-2 cells while *E. coli* harboring the pMMB207 empty vector had ~2% adhesion (Fig. 5.2A). Whole cell lysates demonstrated *E. coli* expressing the various Ail constructs made similar

amounts of Ail protein (Fig. 5.2B). These data indicate that while *Y. pseudotuberculosis* Ail has reduced adhesion ability relative to *Y. pestis* Ail, it does mediate adhesion to cultured cells *in vitro*.

The various Ail proteins were also tested for invasion of HEp-2 cells, an activity previously reported for *Y. enterocolitica* [19, 31], and *Y. pestis* Ail [18]. While KIM5 Ail protein mediates low levels of invasion in this system (0.01%), this level is 50-fold above background *E. coli* invasion. Relative to KIM5 Ail-mediated invasion (set at 100%), Ail from *Y. pseudotuberculosis* had 25% invasion activity. *E. coli* expressing vector alone invaded with only 2% of the efficiency of KIM5 Ail (Fig. 5.2C). These results indicate that like adhesion function, invasion activity of *Y. pseudotuberculosis* Ail was significantly lower than *Y. pestis* Ail when expressed in *E. coli*.

Single *Y. pseudotuberculosis*-like mutations within *Y. pestis* Ail also confer reduced adhesion and invasion function. We demonstrated that Ail from *Y. pseudotuberculosis* has reduced adhesive activity relative to *Y. pestis* Ail (Fig. 2A). Thus, we wanted to determine which of the two amino acid differences contributed to the reduced adhesion ability. We generated each of the single amino acid mutant derivatives, Ail-E43D and Ail-F126V, and tested their ability to bind HEp-2 cells. Adhesion to HEp-2 cells was normalized to the wild-type *Y. pestis* (KIM5) Ail construct. The Ail-E43D mutant exhibited about 32% adhesion when compared to KIM5 Ail, while the Ail-F126V mutant gave about 71% adhesion (Fig. 5.3A). Like previous results (Fig. 5.2A), *Y. pseudotuberculosis* Ail

mediated 50% adhesion relative to *Y. pestis* Ail. Again, the expression of various Ail derivatives was analyzed by Western blotting and the levels of protein were comparable across all strains (Fig. 5.3B).

Invasion function of the single amino acid mutants in the KIM5 Ail protein was also assessed. The invasion capacity of the two single mutants were similarly reduced as the Ail-E43D and Ail-F126V mutations gave 45% and 55% invasion, respectively. For both adhesion and invasion, the Ail-E43D mutant had significantly less activity than the Ail-F126V mutant. We presume the slightly increased level of *Y. pseudotuberculosis* Ail-mediated invasion (45%) as compared to previous experiments (Fig. 5.2C) is due to experimental variation.

A T7I mutation in *Y. pseudotuberculosis* Ail renders the protein unstable. To address differences between our results and a previous report on *Y. pseudotuberculosis* Ail, we also generated the T7I mutation in the *Y. pseudotuberculosis* background. This Ail derivative has three amino acid changes relative to *Y. pestis* Ail – T7I, E43D, and F126V and is the reported YPIII sequence from the previous study [25]. This version of Ail was expressed in *E. coli* and tested for adhesion to HEp-2 cells. *Y. pseudotuberculosis* Ail harboring the T7I mutation was unstable and conferred no adhesive function (Fig. 5.4). Therefore, the T7I mutation likely explains why a previous report found no adhesion activity for *Y. pseudotuberculosis* Ail [25]. We did not find the T7I substitution in two *Y. pseudotuberculosis* strains from which we sequenced

ail (Fig. 5.1), one of which is the same strain (YPIII) reported previously to contain the T7I substitution.

KIM5 Ail and *Y. pseudotuberculosis* Ail binds to purified fibronectin with different efficiencies. We have recently shown that the host cell substrate for *Y. pestis* Ail is fibronectin [12]. Therefore, we determined whether the binding of *Y. pseudotuberculosis* Ail to purified fibronectin could account for the difference in host cell binding. *E. coli* expressing various Ail derivatives were added to increasing concentrations of purified plasma fibronectin coated on microtiter plates. *E. coli* expressing KIM5 Ail on its surface bound to fibronectin in a manner that neared saturation as concentrations approached 20μg/ml (Fig. 5.5). *E. coli* expressing the Ail-E43D mutant, Ail-F126V mutant, and the *Y. pseudotuberculosis* Ail bound to fibronectin revealed no defect in binding to 2.5 and 5μg/ml, however, at 10μg/ml and 20μg/ml there was decreased fibronectin binding by the bacteria expressing the Ail-E43D and Ail-F126V and *Y. pseudotuberculosis* Ail. These decreases were significant for Ail-F126V ($P = 0.02$) and *Y. pseudotuberculosis* IP2666 Ail ($P = 0.03$). Ail-E43D showed a trend toward reduced binding but did not reach statistical significant ($P = 0.08$). Thus, the various Ail proteins have similar binding patterns to purified fibronectin, but KIM5 Ail appears to bind fibronectin slightly more efficiently.

***Y. pseudotuberculosis* Ail and single mutants confer host cell adhesion and invasion when expressed in *Y. pestis*.** We have observed that *Y. pestis*

Ail binds to cultured cells better than *Y. pseudotuberculosis* Ail when expressed in *E. coli* (Fig. 5.2A). Also, the point mutants Ail-E43D and Ail-F126V have severe to moderate cell-binding defects (respectively) (Fig. 5.3A). We also wanted to test the various forms of Ail for their ability to facilitate adhesion to host cells in the natural context in *Y. pestis*. The KIM5 Ail, Ail-E43D, Ail-F126V, and *Y. pseudotuberculosis* Ail were expressed in the KIM D27*Δail* background and examined for their ability to bind host cells (Fig. 5.6A). We set *Y. pestis* KIM5 D27 (also known as KIM5) adhesion to HEp-2 cells to 100% to compare across experiments (actual adhesion = 8.6%). KIM5 *Δail* strain bound to host cells 42% when compared to parental KIM5. KIM5-Ail was expressed from the IPTG-inducible plasmid pMMB66EH was not able to fully complement KIM5 *Δail* as the adhesion was only 75% when compared to parental KIM5. This may be due to expression levels from the pMMB66EH plasmid. The other Ail derivatives, Ail-E43D, Ail-F126V, and *Y. pseudotuberculosis* Ail, bound to host cells at the same level of KIM5-Ail. The KIM5 *Δail* pMMB66EH empty vector control bound at ~50% of the activity of parental KIM5 Ail. Together these results suggest *Y. pseudotuberculosis* Ail and the two *Y. pestis* single mutants are able to mediate host cell binding at similar levels to KIM5 Ail.

We also examined the Ail-E43D and Ail-F126V *Y. pestis* Ail mutants and the *Y. pseudotuberculosis* Ail for the ability to invade host cells when expressed in *Y. pestis* (Fig. 5.6B). Using the same parental KIM5 and KIM5 *Δail* strains as the adhesion assay, we examined the invasion into HEp-2 cells. Again, the KIM5 parental strain was set at 100% activity to normalize across experiments (actual

invasion = 0.24%). The KIM5 *Δail* did not confer any invasion activity into host cells, while the KIM5 Ail was able to complement the deletion strain back to parental levels. The Ail-E43D, Ail-F126V, *and Y. pseudotuberculos*is Ail also exhibited invasion levels similar to KIM5 Ail. Again, the empty vector control lacked any invasion activity. Together, these data suggest these other Ail molecules mediate full invasion activity.

The adhesion and invasion activity of the two *Y. pestis* Ail mutants and *Y. pseudotuberculosis* Ail in *Y. pestis* are different than the activity when expressed in *E. coli*. These results are a bit surprising as *E. coli* heterologous expressing system was used as a common surrogate in many previous studies. Reasons for this difference will be addressed in the discussion section.

***Y. pestis* and *Y. pseudotuberculosis* Ail lead to similar levels of Yop-mediated cytotoxicity.** We have provided evidence that Ail from *Y. pseudotuberculosis* and the single mutants do confer binding to host cells when expressed in *Y. pestis*. Therefore, we wanted to test the various forms of Ail for their ability to facilitate delivery of cytotoxic Yop proteins to cultured cells, a process known to be facilitated by Ail [6, 12]. To assess Yop delivery, we visualized cytotoxicity by cell rounding and darkening of the perinuclear region following Geimsa staining of cultured HEp-2 cells [6, 12]. To compare *Y. pseudotuberculosis* Ail and *Y. pestis* Ail single mutants for their ability to elicit cytotoxicity we expressed each Ail derivative in the KIM D27*Δail* background. *Y. pestis* KIM D27 (also known as KIM5) caused about 60% cytotoxicity of HEp-2

cells after two hours of infection (Fig. 5.7). The KIM D27 *Δail* derivative lead to only 8% cytotoxicity, comparable to the *ΔyopB* derivative. YopB is part of the YopB/D translocon complex of the T3SS, and is required for Yop delivery [7]. *Y. pestis* Ail complemented the KIM D27 *Δail* strain back to wild-type levels of cytotoxicity. Similar to *Y. pestis* Ail complemented strains, the Ail-E43D, Ail-F126V, and *Y. pseudotuberculosis* (Ail-E43D/F126V) Ail-complemented strains also exhibited wild-type levels cytotoxicity (Fig. 5.7). When protein expressed from these constructs was analyzed by Western blot analysis, the *Y. pestis*, *Y. pseudotuberculosis*, and two single mutations complemented expression of Ail to similar levels, albeit somewhat less then endogenous levels (Fig. 5.7). Thus, the intermediate defects in adhesion by *Y. pseudotuberculosis* Ail, *Y. pestis* Ail-E43D and Ail-F126V were not sufficient to reduce Yop delivery by *Y. pestis*.

Discussion

To identify residues important for the interaction of *Y. pestis* Ail with host cells, we analyzed the differences between *Y. pestis* and *Y. pseudotuberculosis* Ail. There are two amino acid changes between these two Ail derivatives. Two mutants in the *Y. pestis* background, Ail-E43D and Ail-F126V, were analyzed for cell binding, cell invasion and Yop delivery capacity. When expressed in *E. coli*, we found the Ail-E43D mutation is more defective than Ail-F126V for host cell binding and invasion, although both mutations affected these activities (Fig. 5.3). As the differences between Ail-E43D and Ail-F126V for adhesion and invasion were significant, it appears the glutamic acid at position 43 is more critical for cell

binding than the phenylalanine at position 126 within *E. coli*. To determine whether elimination of most of the side chain of residue 43 would completely abolish cell-binding activity, we made an Ail-E43A substitution. Unfortunately, this mutation was unstable when expressed in *E. coli* (data not shown). Interestingly, these derivatives of Ail no longer had a defect when expressed in *Y. pestis*. The Ail-E43D, Ail-F126V, and *Y. pseudotuberculosis* Ail exhibited the same levels of adhesion and invasion activity as the KIM5 Ail.

We previously reported that one host cell substrate for *Y. pestis* Ail is the extracellular matrix component, fibronectin [12]. We hypothesized the defect observed in host cell binding by the single mutants as well as *Y. pseudotuberculosis* Ail, was due a lower affinity for purified fibronectin. Upon binding to immobilized fibronectin Ail-E43D, Ail-F126V, and *Y. pseudotuberculosis* Ail had slightly reduced binding to fibronectin as compared to *Y. pestis* Ail at 10μg/ml and 20μg/ml fibronectin (Fig. 5.5; although the defect for the E43D mutant did not reach statistical significance; P = 0.13 at 10μg/ml and P = 0.08 at 20μg/ml.) It is unclear why defects in binding to purified fibronectin do not precisely reflect defects in host cell binding, although we have noted previously that Ail recognizes cell deposited fibronectin somewhat differently than immobilized purified fibronectin [12].

Close bacterial-host cell interactions are required for the efficient delivery of Yop cytotoxic proteins [4, 32]. Therefore, we wanted to examine the various forms of Ail, KIM5 Ail, Ail-E43D, Ail-F126V, and *Y. pseudotuberculosis* Ail, for their ability to deliver Yops into host cells. When the various Ail proteins were

expressed in KIM *Δail* they mediate adhesion activity to host cells at levels similar to the KIM5 Ail (Fig. 5.6). This full adhesion activity leads to proficient Yop delivery as all Ail forms tested lead to similar levels of cytoxocity of host cells (Fig. 5.7). This provides evidence that there is no functional difference between *Y. pestis* Ail and *Y. pseudotuberculosis* Ail in Yop delivery.

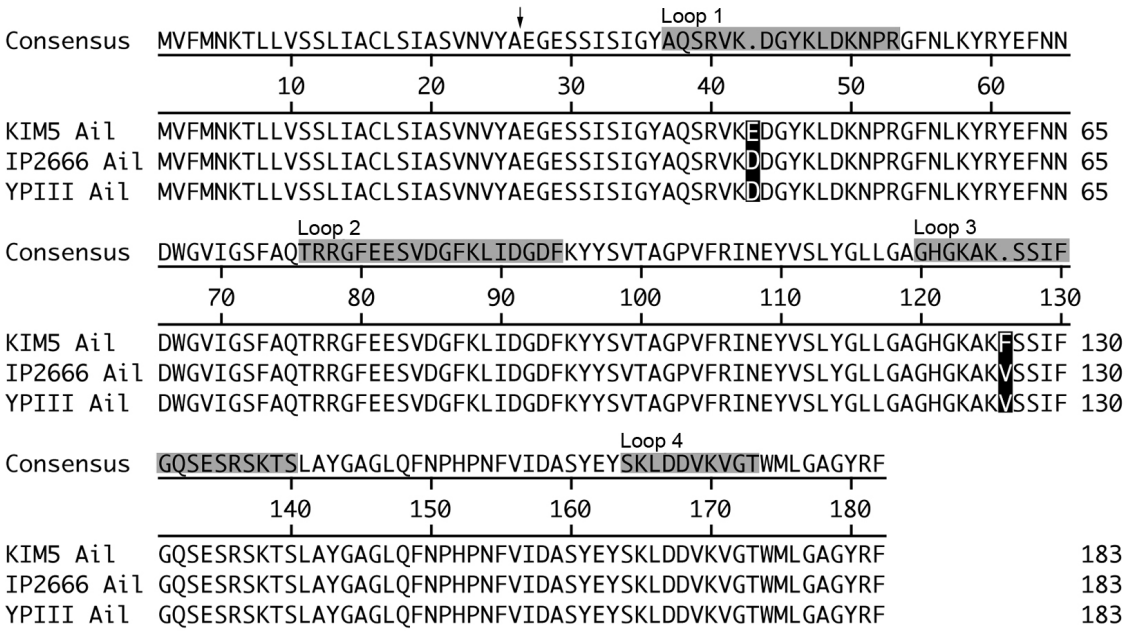
In order to test the adhesion ability of the *Y. pseudotuberculosis* Ail and single Ail mutants, in the future we will express these various Ail proteins in *Y. pseudotuberculosis* as well. These Ail forms should be expressed in a *Y. pseudotuberculosis Δail Δinv ΔyadA* to examine the contribution of Ail activity alone.

The difference of host cell adhesion and invasion activity between *E. coli* and *Y. pestis* is curious. One possible explanation is observation is that the structure of *E. coli* LPS is different than *Y. pestis* LPS. Future studies will be designed to analyze whether LPS structure is masking other proteins presented on the surface of the bacterium.

Y. pseudotuberculosis Ail was previously reported to lack adhesion and invasion ability to HEp-2 cells [25]. The previous experiments were done similarly to our experiments in a heterologous *E. coli* expression system. The previously published YPIII *Y. pseudotuberculosis* Ail sequence from that study contained an additional T7I substitution that we did not observe in either of our *Y. pseudotuberculosis* strains, YPIII or IP2666. We also generated the *Y. pseudotuberculosis* Ail-T7I substitution, but found this particular protein was unstable when expressed in *E. coli*.

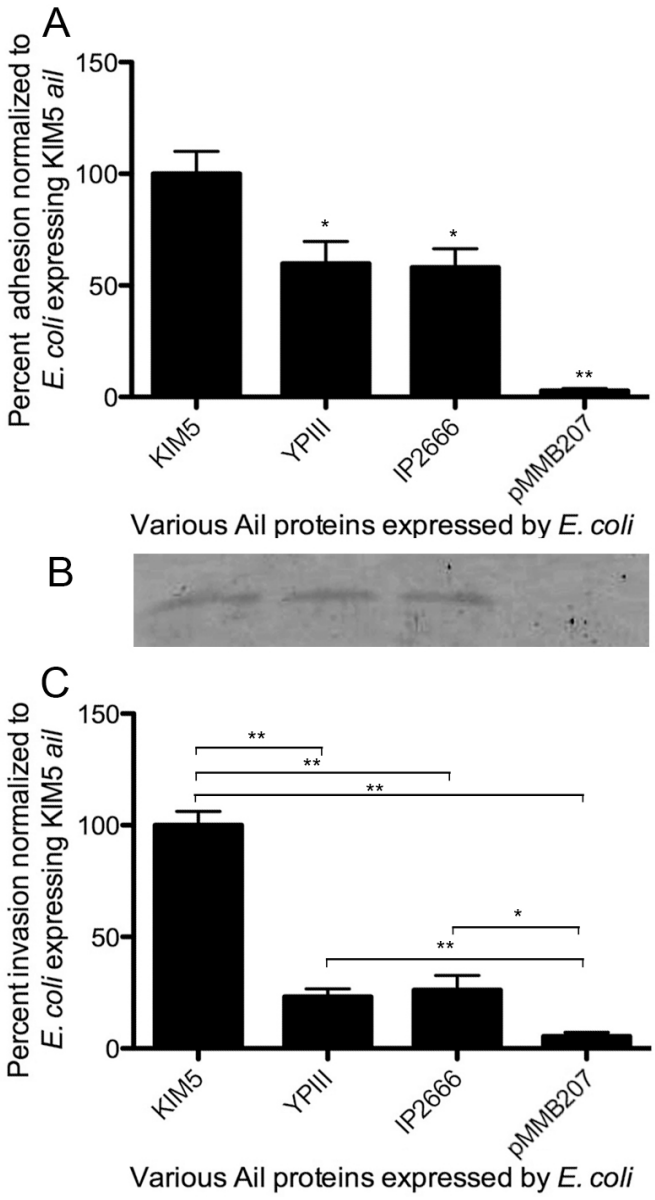
Studies presented here indicate that *Y. pseudotuberculosis* Ail does confer adhesion similar to *Y. pestis* Ail. This work is different than what has been published in the past and contributes to the knowledge of the residues of Ail that are important for the various functions.

Figure 5.1



Protein alignment of the various Ail proteins. Ail from *Y. pestis* strain KIM5 and two *Y. pseudotuberculosis* isolates, IP2666 and YPIII, are shown. The two variant amino acids; positions 43 and 126 are highlighted. The arrow indicates the predicted processed cleavage site. The putative extracellular loop regions are indicted with grey boxes. The alignment was performed with the MegAlign program from Lasergene.

Figure 5.2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Percent adhesion normalized to E. coli expressing KIM5 ail
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50
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KIM5
YPIII
IP2666
pMMB207
Various Ail proteins expressed by E. coli
B
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Percent invasion normalized to E. coli expressing KIM5 ail
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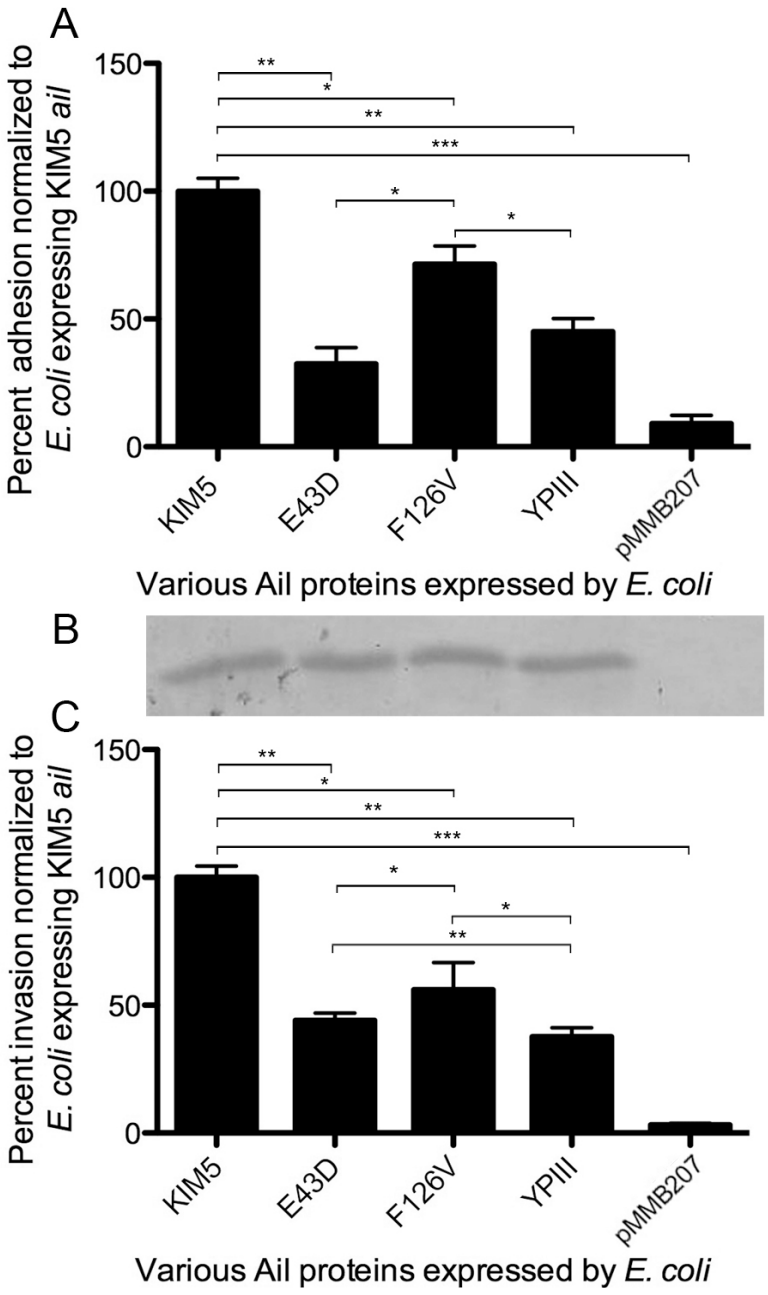
KIM5
YPIII
IP2666
pMMB207
Various Ail proteins expressed by E. coli

Figure 5.2

***Y. pseudotuberculosis* Ail exhibits reduced adhesion and invasion function.** (A) Cultured HEp-2 cells were infected with *E. coli* AAEC185 expressing various forms of Ail from the indicated *Yersinia* strains. The empty vector (pMMB207) serves as a negative control. Percent adhesion was calculated by dividing the number of cell-associated CFU by the total number of bacteria in the well and multiplying by 100. The adhesion of *E. coli* AAEC185 expressing KIM5 Ail was set to 100% (HEp-2 cell adhesion average = 4.0%). (B) Ail expression levels were determined in whole cell extracts were separated by SDS-PAGE followed by anti-Ail Western blotting. (C) Invasion assays were performed similarly to adhesion assays except infected cells were treated with gentamicin to kill external bacteria. The level of KIM5 Ail-mediated invasion was 0.05% prior to normalization. Data are from two independent experiments performed in triplicate (n=6). *, $P < 0.02$; **, $P < 0.00002$. Significance was calculated using the Student t test.

Figure 5.3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Various Ail proteins expressed by E. coli
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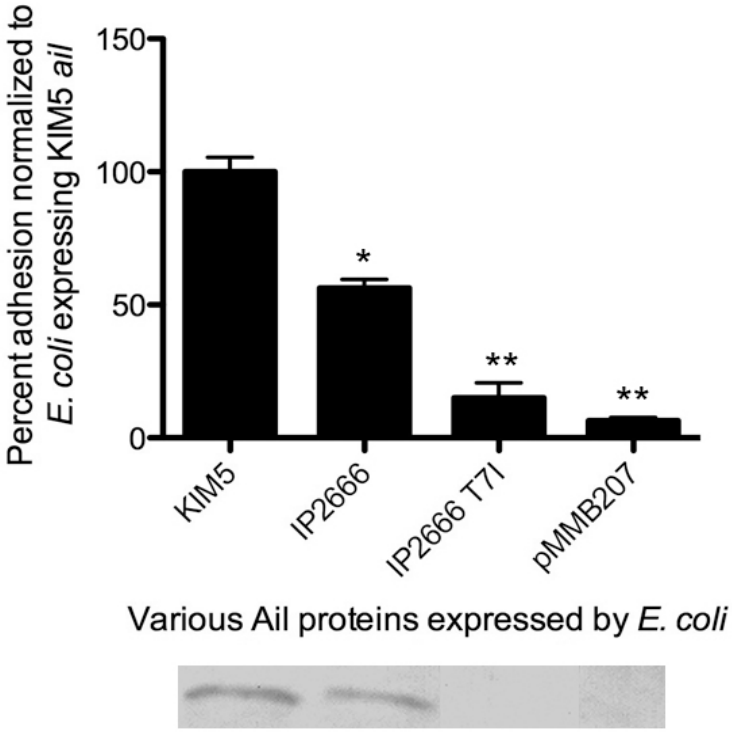
KIM5
E43D
F126V
YPIII
pMMB207
Various Ail proteins expressed by E. coli

Figure 5.3

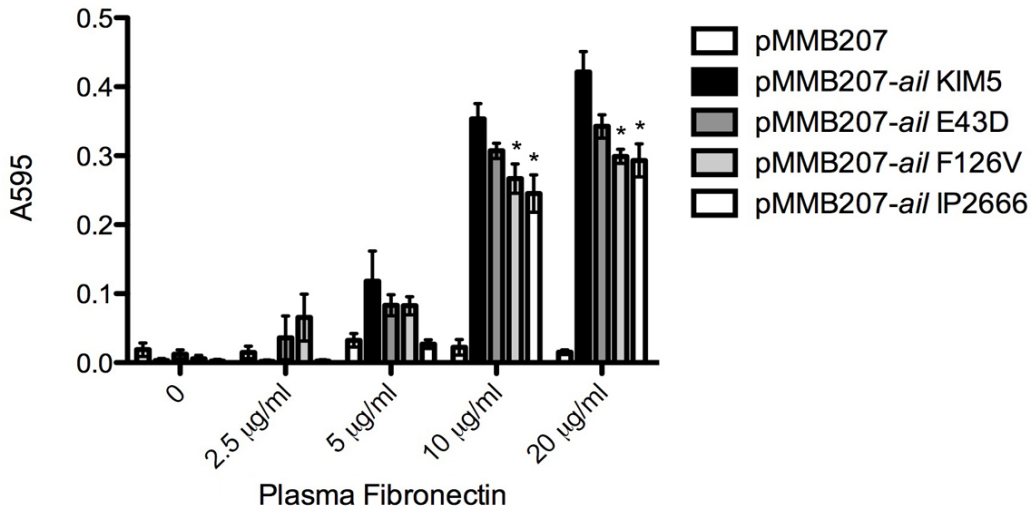
***Y. pestis* Ail single mutants, E43D and F126V, have decreased adhesion and invasion ability.** (A) HEp-2 cells were infected with *E. coli* AAEC185 expressing the *Y. pestis* KIM5 Ail, single mutants of Ail, *Y. pseudotuberculosis* Ail (double mutant), and vector alone. Percent adhesion was calculated by dividing the number of cell-associated CFU by the total number of bacteria in the well and multiplying by 100. HEp-2 adhesion average = 4.7%. (B) Ail expression levels were determined in whole cell extracts separated by SDS-PAGE followed by anti-Ail Western blotting. (C) Invasion assay were performed similar to adhesion assay except infected cells were treated with gentamicin to kill external bacteria. The level of KIM5 Ail-mediated invasion was 0.05% prior to normalization. Data are from two independent experiments performed in triplicate (n=6). *, $P < 0.03$; **, $P < 10^{-6}$; ***, $P < 10^{-9}$. Significance was calculated using the Student t test.

Figure 5.4



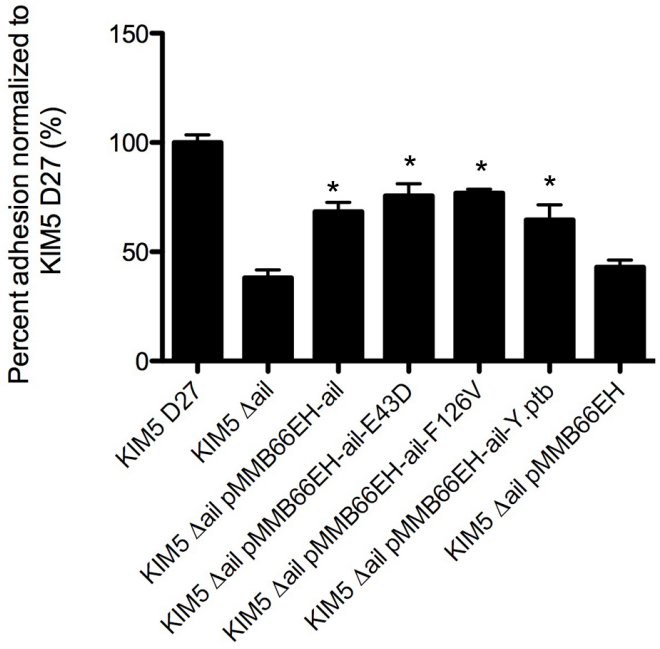
***Y. pseudotuberculosis* Ail containing the T7I mutation is unstable.** HEp-2 cells were infected with *E. coli* AAEC185 expressing the *Y. pestis* KIM5 Ail, *Y. pseudotuberculosis* Ail, and *Y. pseudotuberculosis* Ail harboring a T7I substitution. Percent adhesion was calculated by dividing the number of cell-associated CFU by the total number of bacteria in the well and multiplying by 100. HEp-2 adhesion average = 4.7%. (B) Ail expression levels were determined in whole cell extracts separated by SDS-PAGE followed by anti-Ail Western blotting. Data are from two independent experiments performed in triplicate (n=6).

Figure 5.5



***Y. pseudotuberculosis* Ail is attenuated for binding to purified fibronectin relative to *Y. pestis* Ail.** Purified plasma fibronectin was immobilized on 96-well plates. *E. coli* AAEC185 derivatives expressing the indicated Ail derivatives were added to wells and allowed to bind at 37°C. Bound bacteria were stained with 0.01% crystal violet. Stained bacterial cells were solubilized and the plates were read at ABS_{595}. Shown is representative data from two independent experiments done in triplicate (n=3). *, $P < 0.05$ compared to *E. coli* expressing pMMB207-*ail* KIM5.

Figure 5.6


Percent adhesion normalized to KIM5 D27 (%)
150
100
50
0
*
*
*
*
KIM5 D27
KIM5 Δail
KIM5 Δail pMMB66EH-ail
KIM5 Δail pMMB66EH-ail-E43D
KIM5 Δail pMMB66EH-ail-F126V
KIM5 Δail pMMB66EH-ail-Y.ptb
KIM5 Δail pMMB66EH

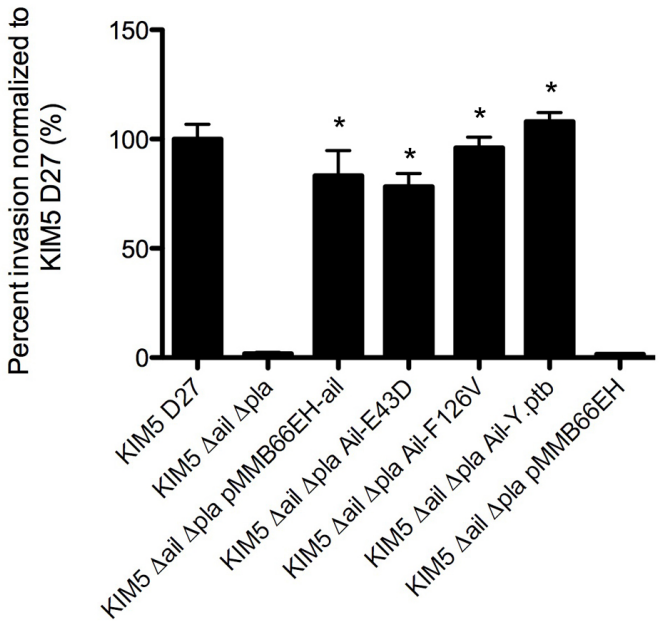
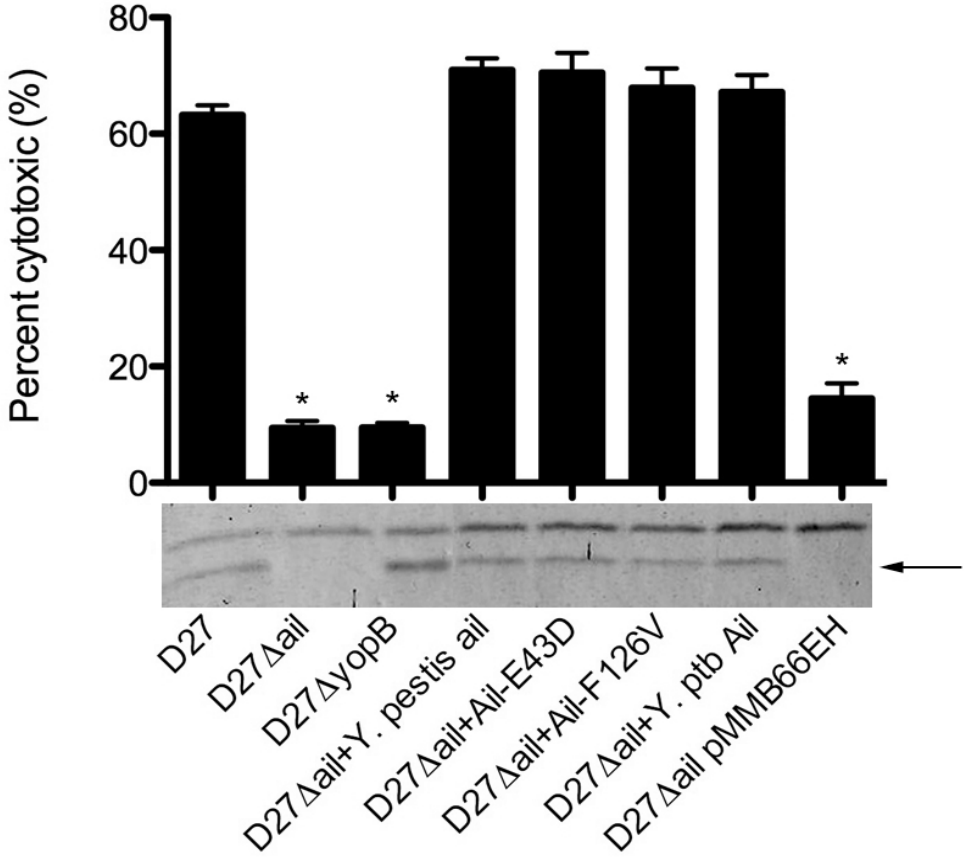

Percent invasion normalized to KIM5 D27 (%)
150
100
50
0
*
*
*
*
KIM5 D27
KIM5 Δail Δpla
KIM5 Δail Δpla pMMB66EH-ail
KIM5 Δail Δpla Ail-E43D
KIM5 Δail Δpla Ail-F126V
KIM5 Δail Δpla Ail-Y.ptb
KIM5 Δail Δpla pMMB66EH

Figure 5.6

***Y. pseudotuberculosis* Ail exhibits reduced adhesion and invasion function.** (A) Cultured HEp-2 cells were infected with *Y. pestis* expressing various forms of Ail from the indicated *Yersinia* strains. The empty vector (pMMB66EH) serves as a negative control. Percent adhesion was calculated by dividing the number of cell-associated CFU by the total number of bacteria in the well and multiplying by 100. The adhesion of KIM5 D27 was set to 100% (HEp-2 cell adhesion average = 8.6%). (B) Invasion assays were performed similarly to adhesion assays except infected cells were treated with gentamicin to kill external bacteria. The invasion of KIM5 D27 was set to 100% (HEp-2 cell invasion average = 0.2%). Data are from two independent experiments performed in triplicate (n=6). *, $P < 0.02$ when compared to Δail for adhesion assay or Δail Δpla for invasion assay. Significance was calculated using the Student t test.

Figure 5.7



***Y. pseudotuberculosis* and the *Y. pestis* Ail single mutants cause levels of HEp-2 cell cytotoxicity similar to *Y. pestis* Ail.** HEp-2 cells were infected with KIM D27 derivatives at an MOI of 10. After two hours of infection cells were fixed and stained with Giemsa to show shrunken, round, darker cells, which is indicative of Yop-mediated cytotoxicity. Cells were counted and percent cytotoxic cells were calculated. Anti-Ail Western blots were performed to assess Ail expression (arrow). *, $P < 0.0002$ compared to KIM D27. Significance was calculated using the Student t test.

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CHAPTER SIX

DISCUSSION

Summary of work presented

In this thesis, I have shown that the *Y. pestis* adhesin, Ail, binds to the host cell extracellular matrix component, fibronectin (Chapter 2) [1]. Ail bound both purified Fn and Fn deposited by host cells in culture. This Ail-Fn interaction was blocked with anti-Fn polyclonal antibody. We also provided evidence that both *Y. pestis* adhesins, Pla and Ail, can bind purified Fn. However, Ail is the main adhesion when binding to host cells as demonstrated by the *Y. pestis* Δ*pla* mutant adhering to host cells at levels similar to parental *Y. pestis*, while a *Y. pestis* Δ*ail* provided only 50% of parental adhesion. Additionally, treatment of cultured cells with the anti-Fn antibody lead to reduced Ail-mediated Yop delivery (Fig. 2.6). Together, this suggests Ail is a critical adhesin and the interaction between Ail and Fn generates a key event for delivery of Yops.

Given the critical nature of the Ail/Fn interaction and the growing body of literature on the mechanism of Fn binding by bacterial Fn-binding proteins [2, 3], we characterized the residues of Ail that contribute to host cell and Fn binding (Chapter 3). SWIM codon mutagenesis used with a functional enrichment identified key residues required for cell adhesion function. Cell-binding mutants

were identified in each exposed loop of Ail that were examined through SWIM mutagenesis. The most host cell defective binding mutants were found in the tip of exposed loop 3, residues S128A, F130A, and the double S128A/F130A mutant. Some of the mutants that exhibited reduced host cells attachment were also defective for binding purified Fn, although none of the mutants isolated were completely defective for binding purified Fn. Most importantly however, the most defective cell binding mutants, Ail-S128A/F130A double and Ail-E43D/S128A/F130A triple mutant, did facilitate Yop-mediated cytotoxicity of host cells at a reduced level compared to wild-type Ail. The most defective mutant, Ail-E43D/S128A/F130A, still exhitbited half of the cytotoxicity ability of the wild-type Ail, so it may take multiple mutants to obtain an Ail molecule that leads to background levels of cytotoxicity. Together, these data provide molecular details about the residues of Ail required for a critical virulence function.

Several bacterial proteins bind to extracellular matrix components such as Fn to colonize host tissues. Ail binds to Fn in a specific manner and as shown previously, this interaction mediates the translocation of Yops (Chapter 2) [4]. Therefore, we sought to understand the region of Fn bound by Ail (Chapter 4). We utilized Fn fragments, antibodies that recognize epitopes on Fn, and Fn substrates to map the Ail binding domain. We showed Ail bound to the 120kDa fragment, particularly modules $^{8\text{-}9}$FNIII within the 120kDa fragment since antibodies mapped to that region can block Ail mediated bacterial binding. However, we also provided additional evidence that synergy sites may be working with the 120kDa fragment to enhance Ail binding. For example,

collagen, which binds to the 45kDa fragment N-terminal to the 120kDa fragment, inhibited Ail-mediated bacteria binding, suggesting the 45kDa fragment along with the 120kDa fragment may provide optimal Ail-Fn binding (Fig. 4.5). Also, fragments containing the most C-terminal end of Fn appear to enhance binding through Ail (Figure 4.3).

Finally, in our studies to examine residues of Ail that contribute to various functions, we also addressed the published observation that Ail from *Y. pseudotuberculosis* does not mediate attachment to host cells (Chapter 5). In some strain isolates, the difference between Ail from *Y. pestis* and *Y. pseudotuberculosis* is only one amino acid, F126V, but, in other isolates, two amino acids are different between the two proteins, E43D and F126V. Both of the amino acid differences are found in exposed loops of Ail. In an *E. coli* heterologous system, Ail from *Y. pseudotuberculosis* mediated host cell adhesion, although this adhesion activity is less than *Y. pestis*-Ail adhesion activity. Additionally, Ail from *Y. pseudotuberculosis* exhibited invasion activity, however, similar to adhesion, the invasion was reduced when compared to Ail from *Y. pestis*. Therefore, Ail from *Y. pseudotuberculosis* confers adhesion to and invasion into host cells. These observations are different from what was previously reported [5]. We made the additional T7I single substitution cited in the previous work, but that particular mutant was unstable when expressed in *E. coli*. Interestingly, when these various Ail constructs are expressed in *Y. pestis*, Ail from *Y. pseudotuberculosis* conferred the same adhesive and invasive ability as Ail from *Y. pestis*. This suggests that expressing *Yersinia* proteins *in E. coli*

may not always reflect the natural context, and Ail from *Y. pseudotuberculosis* does confer adhesion and invasion into host cells (Chapter 5).

In this thesis I have shown that Ail binding to Fn facilitates Yop delivery, a critical virulence function of *Y. pestis*. Additionally, I have provided mechanistic details at the amino acid level for the interaction between Ail and Fn. A model of the Ail interaction with Fn is found in Figure 6.1. The Ail side chains found to be important in binding Fn are shown and modeled next to the structure of Fn modules $^{8\text{-}9}$FNIII. From the model, residues found in the tips of loop 3 are available to contact host Fn and one can imagine how the side chains of those particular residues facilitate the critical Ail/Fn interaction.

Implication of Ail during plague disease development

Y. pestis is infamous for causing millions of deaths world wide during three large plague pandemics throughout history. Today, *Y. pestis* infections are not as common, but each year, about 2000 cases are reported in Asia and areas such as the Four-corners region of the United States. *Y. pestis* is considered a re-emerging Category A biological agent and a potential bioterrorism threat. Thus, it is essential to define mechanisms of virulence in order to develop targeted vaccine strategies and therapeutics.

Through a transposon mutagenesis screen, Ail from *Y. pestis* was identified as a critical adhesin and an important virulence factor during infections [4]. In addition to identifying Ail through a mutagenesis screen, we were the first laboratory to show Ail facilitates delivery of the cytotoxic Yops into host cells.

Therefore, we sought to define the host cell interaction in more detail to understand the importance of Ail during infections. Taken together, my thesis work has strong implications for *Y. pestis* virulence and infections and makes a significant impact in the field of *Y. pestis* pathogenesis.

Additionally, understanding the mechanism in which Ail interacts with host cells would help develop vaccines or therapeutics that may be used against the development of the plague. Because Ail is an exposed, highly expressed *Y. pestis* protein, it is possible Ail could be used for vaccine development as a protective antigen against the plague in a preventative manner. Also, small molecule inhibitors can be developed that can be used as therapeutic treatment of those individuals already infected with *Y. pestis*.

During the development of the plague diseases, *Y. pestis* progresses through different environments at different points of the infection process. *Y. pestis* is deposited either into the skin or directly into the bloodstream from a flea-bite [6]. Alternatively, *Y. pestis* can be directly deposited into the lungs if infectious droplets are inhaled. Bacteria can be taken up by resident macrophages in the areas where the bacteria are initially deposited. *Y. pestis* can survive within unactivated macrophages and under this acidic intracellular environment, *Y. pestis* induces the expression of the anti-phagocytic component Psa. *Y. pestis* grow and are eventually released into the extracellular environment [7]. As the infection spreads, bacteria remain extracellular in the tissues and can gain access to the lymphatic vessels, which drain into the

regional lymph nodes. This is a characteristic of the bubonic plague leading to painful swollen lymph nodes (bubos). *Y. pestis* in the lymphatic system can gain access to the blood stream via the thoracic duct and replicate rapidly leading to septicemic plague. During many stages of infection delivery of cytotoxic Yop proteins are delivered upon contact with host cells.

Y. pestis expresses two other adhesins, Pla and Psa, in addition to Ail. Our lab has demonstrated that all three adhesins can mediate Yop delivery to tissue culture cells [8]. Because of their regulation, expression patterns, and possibly cell tropism, each adhesin plays a role in Yop delivery under specific environmental conditions. Pla is induced at 37^{o}C [9], and may not play a major role at the earliest stages septicemic infections. With subcutaneous and intranasal inoculation, reflecting bubonic and pneumonic plague, respectively, Pla plays a more important role as a Δ*pla* mutant is highly attenuated for virulence in bubonic plague, and the proteolytic activity was required for the development of fulminant pneumonic plague [10]. However, infection via intravenous inoculation the *Y. pestis* Δ*pla* mutant did not dramatically impact the LD_{50} (~35 fold increase LD_{50}) [8]. It is believed that during infections the protease activity may be the dominant function of Pla for dissemination from the site of inoculation.

In studies with the *in vivo* septicemic plague model, mimicked through intravenous inoculation, a *Y. pestis* Δ*ail* mutant was attenuated with a >3,000-fold increase in LD_{50} when compared to parental KIM5. In this mouse model, a Δ*psaA* mutant was only 75-200-fold increase in LD_{50} [8, 11]. Various *in vivo*

studies suggest distinct temporal roles for these two adhesins. Although tested via subcutaneous and intranasal routes, a *Y. pestis* Δ*psaA* mutant has a colonization defect early while a Δ*ail* mutant has a defect at later stages of infection. Furthermore, when tested via intravenous route, Psa plays a role in infections, but Psa expression is down-regulated by day four [8]. This suggests Psa is required for early stages of infection and Ail is important for later stages. Psa is induced at 37^{o}C under acidic conditions. Thus, it is expressed after initial intracellular growth within macrophages [11]. We provided evidence that Ail binds soluble cellular Fn but not plasma Fn (Fig. 4.7). This preferential binding suggests that *Y. pestis* may not bind plasma Fn in the blood during septicemic plague, but once *Y. pestis* arrives in tissues, such as lymph nodes, Ail binds cellular Fn. This supports the importance of Ail/Fn interaction within the tissues at the later stages of infection to bind host cells, deliver cytotoxic Yops, and thus cause tissue damage in the host. Additional studies with intranasal inoculation with *Y. pestis* Δ*ail*, suggest Ail is required for dissemination from the lung. Thus, Ail may be a critical adhesion for many routes of inoculation based on its universal expression pattern [8], and other adhesins play unique roles depending on the inoculation route, environmental condition and stage of the infection process (Figure 6.2)

Ail has been studied for about twenty years, however, it was only recently demonstrated that Ail was critical for infections. Until now, the host cell substrate for Ail was not known. Thus, it was worth pursuing the identification of a host cell substrate for Ail. We now have more insights into how Ail is facilitating adhesion

to host cells, as our data establishes direct Ail-Fn binding [1]. Although we have not fully addressed how Ail mediates Yop delivery, we have generated useful mutants of Ail that will help us identify the host signaling events that generate the efficient pore formation for the translocation of Yops.

Finally, we took innovative strategies in order to analyze Ail in more detail. Others have identified Ail mutants previously [12], but residues of interest were pulled out of homologue comparisons and alanine scanning. In our study, we used the SWIM mutagenesis and biologically based functional enrichment to identify residues contributing to adhesive function.

Future directions

- *Determine whether the N-terminus or C-terminal-RGD domain of Fn is presented to host cell integrins upon Ail binding.* As mentioned in previous chapters, Fn can bind β1 integrins through either the N-terminal matrix assembly site or the well-characterized RGD domain in the central region of Fn [13, 14]. We provided evidence that Ail binds to the $^{8\text{-}9}$FNIII modules. In this manner, Ail may present Fn to host cell intregrins through either the N-terminus or RGD-domain. Therefore, determining how Fn is used as a bridging molecule may help understand the role of Ail plays in adhesion and Yop delivery.

- *Characterize the host cell signaling events generated by the Ail-mediated Yop delivery.* Invasin triggers specific host cell signaling pathways to

rearrange actin cytoskeleton to open the pore for efficient delivery of Yop delivery. We will examine some of the same signaling molecules such as Rho and Rac GTPases. Additionally, YadA mediates binding in a manner similar to Ail, in that it binds fibronectin as a bridge to engage β1 integrins. The interaction results in a PI3K-dependent, Akt and phospholipase Cγ1 signaling cascade [15]. So, examining the generation of YadA-like signals is another line of investigation. Many commercially available inhibitors are known and easily accessible for further studies. In a complementary approach, RNAi targeting may knockdown specific small GTPases, kinases, or phosphatases. Thus, we can demonstrate the dependence of Yop delivery upon certain signaling pathways.

- *Determine whether the defective host cell-binding mutants generate a different host cell signal.* In understanding the signaling cascade generated by Inv, Mejia *et al*., [16] used a low affinity Inv mutant in their studies. This single point mutation, D911E, was unable to trigger critical host cell signaling required for opening of the T3SS apparatus and Yop delivery. Thus, in a similar manner, we can compare intracellular host signaling of wild-type Ail and host cell binding mutants (generated in Chapter 3). Additionally, we have recently obtained the reagents for a more quantitative, sensitive assay for Yop delivery that may detect subtle and temporal differences [17]. This technique uses a YopM-Bla protein fusion that when translocated into host cells can cleave a fluorescent

molecule CCF2-AM. The CCF2-AM molecule participates in FRET and emits green fluorescence upon excitation of a blue fluorophore, but upon cleavage by YopM-Bla, the two CCF2-AM fluorophores will be separated and upon excitation of the blue fluorophore and blue fluorescence will be emitted. The fluorescence can be measured over time, providing kinetic data on the extent of Yop delivery by wild-type Ail and cell-binding defective mutants.

- *Generate deletions and point mutations of Fn.* Over the course of my studies, we obtained a clone of cDNA encoding human Fn [18]. This clone has been expressed within SF9 insect cells for proper eukaryotic glycosylation of Fn [19]. We can generate Fn constructs of various lengths including a Fn fragment that contains both the 45kDa collagen-binding fragment and the 120kDa central fragment and examine the ability of Ail to bind these domains together. Additionally, we can generate just the $^{8\text{-}9}$FnIII modules alone to test whether there are synergy sites for the optimal Ail binding. If the two modules alone are sufficient for binding, one can conclude no synergy sites exist for Ail bidning. Also, we can generate smaller Fn peptides to test if these can modulate binding or inhibit Ail binding to full-length Fn. All these approaches are various ways to map the Ail binding domain to the molecular level. In all of these cases, the Fn construct can be expressed and purified for analysis of Ail-binding.

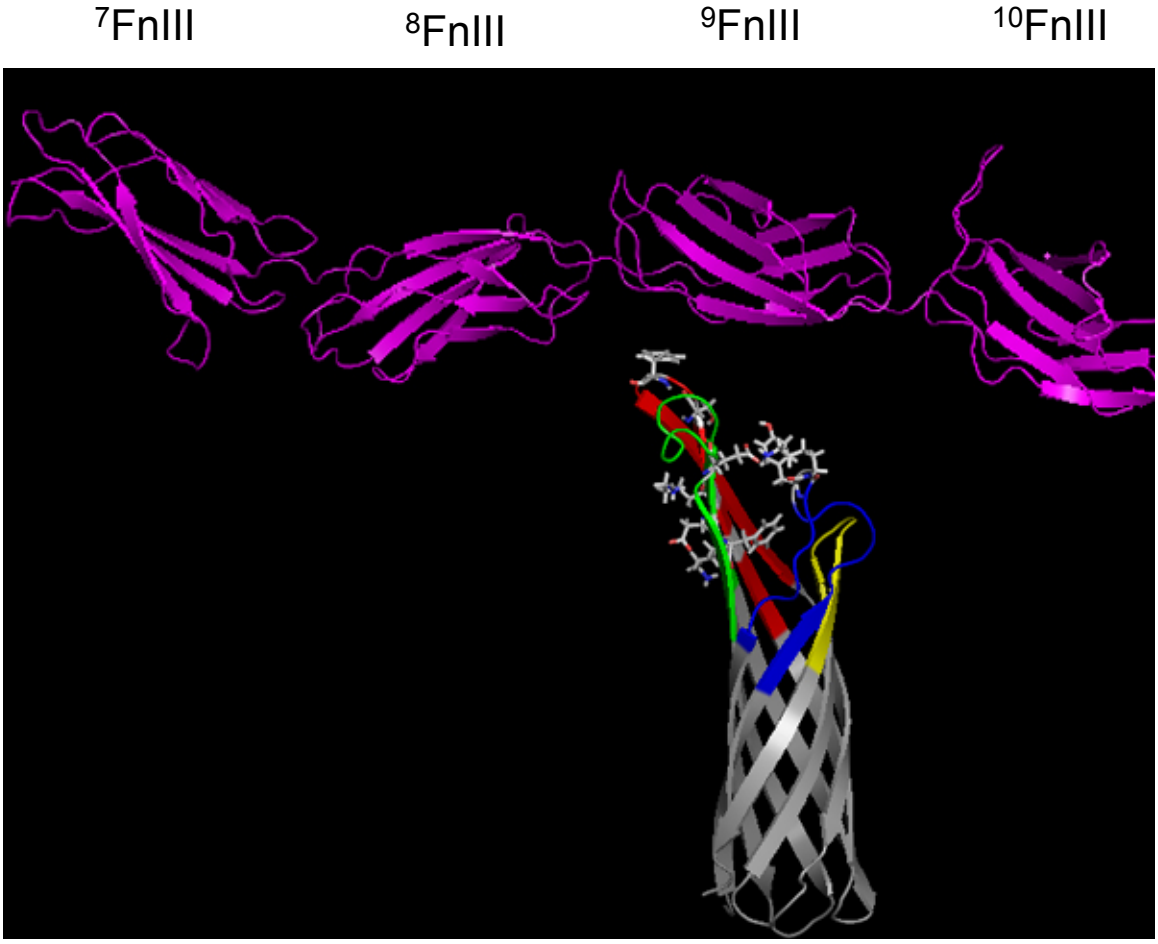
- *Expressing Fn constructs in the Fn-/- cell lines.* One of the drawbacks of using purified Fn and Fn fragments is not knowing the conformation or structure of Fn when it is immobilized onto plastic. We have evidence that *Y. pestis* Ail binds to purified Fn differently than Fn that is deposited by host cells in tissue culture. Thus, to study the Ail-binding domain in a natural context, we could express the various Fn constructs in the Fn-/- cell lines [20]. Such a cell line has been generated by our collaborator Dr. Deane Mosher.

- *Identify additional receptors for Ail.* Upon purification of Ail, we observed binding to purified Fn, but not to collagen I or IV. We only tested those ECM components, and there is a possibility that Ail binds to other receptors or ligands on the surface of host cells.

- *Mouse model of infection with Ail mutations.* Previous *in vivo* studies with Ail have been done in an intravenous route of infection with the attenuated KIM5 strain, which lacks the *pgm* locus. Thus, we want to examine the contribution to infection of Ail in the fully virulent KIM6+ (pCD1Ap) via the subcutaneous and intranasal route of infection in the new University of Michigan ABSL3 laboratory. In addition, we can express the cyototoxic-defective mutants, F130A and S128A/F130A, (and possibly E43D/S128A/F130A) from the chromosome in the KIM6+ (pCD1Ap), and test the role these Ail mutants play during plague infections.

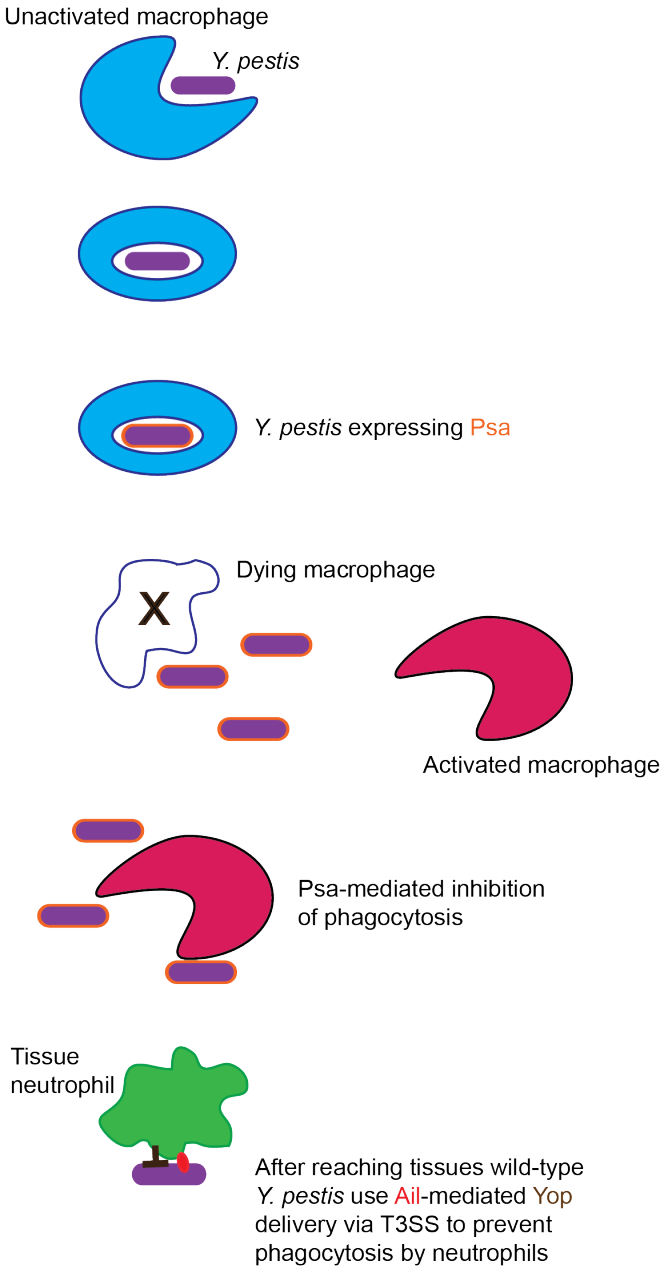
- *Role of serum resistance via Ail in vivo.* As mentioned previously, mouse serum is not bactericidal for *Yersinia* species [21], thus the previous *in vivo* models with mice do not study the role of serum resistance of Ail. Ail does confer resistance to complement-mediated lysis by guinea pig serum. Therefore, the role of Ail-mediated serum resistance can be studied in a guinea pig model of infection.

Figure 6.1



Ail interactions with Fn. The structure of Ail is modeled next to the structure of modules $^{7\text{-}10}$FNIII of the central region of Fn. The size of Ail is shown in proportion to the size of Fn modules.

Figure 6.2



Model of infection. A model of *Y. pestis* Ail during infection. The model hypothesizes that Psa is required at early stages of infection while Ail-mediated Yop delivery is required at later stages.

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