Defining the Ail Ligand-Binding Surface: Hydrophobic Residues in Two Extracellular Loops Mediate Cell and Extracellular Matrix Binding To Facilitate Yop Delivery

Tiffany M. Tsang,^a Jeffrey S. Wiese,^b Jamal A. Alhabeil,^b Lisa D. Usselman,^c Joshua J. Thomson,^b Rafla Matti,^c Malte Kronshage,^c Natalie Maricic,^a Shanedah Williams,^c Naama H. Sleiman,^b Suleyman Felek,^c Eric S. Krukoni^{a,b,d}

Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan, USA^a; Department of Biomedical and Diagnostic Sciences, University of Detroit Mercy School of Dentistry, Detroit, Michigan, USA^b; Department of Biologic and Materials Sciences, University of Michigan School of Dentistry, Ann Arbor, Michigan, USA^c; Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, Michigan, USA^d

ABSTRACT *Yersinia pestis*, the causative agent of plague, binds host cells to deliver cytotoxic Yop proteins into the cytoplasm that prevent phagocytosis and generation of proinflammatory cytokines. Ail is an eight-stranded β -barrel outer membrane protein with four extracellular loops that mediates cell binding and resistance to human serum. Following the deletion of each of the four extracellular loops that potentially interact with host cells, the Ail- Δ loop 2 and Ail- Δ loop 3 mutant proteins had no cell-binding activity while Ail- Δ loop 4 maintained cell binding (the Ail- Δ loop 1 protein was unstable). Using the codon mutagenesis scheme SWIM (selection without isolation of mutants), we identified individual residues in loops 1, 2, and 3 that contribute to host cell binding. While several residues contributed to the binding of host cells and purified fibronectin and laminin, as well as Yop delivery, three mutations, F80A (loop 2), S128A (loop 3), and F130A (loop 3), produced particularly severe defects in cell binding. Combining these mutations led to an even greater reduction in cell binding and severely impaired Yop delivery with only a slight defect in serum resistance. These findings demonstrate that *Y. pestis* Ail uses multiple extracellular loops to interact with substrates important for adhesion via polyvalent hydrophobic interactions.

KEYWORDS plague, type III secretion, serum resistance

Ail is a multifunctional outer membrane protein of *Yersinia* species that is expressed under various environmental conditions (1–7). Specifically, *Yersinia pestis* Ail mediates adhesion to host cells, leads to autoaggregation, confers resistance to human serum, and facilitates the delivery of cytotoxic Yop proteins to host cells (8–10). As a consequence of these various activities, an *ail* mutant is highly attenuated, with a >3,000-fold increase in its 50% lethal dose (LD₅₀) compared to that of the parental *Y. pestis* KIM5 strain in a mouse model of septicemic plague (8), and shows a similar defect in a mouse model of bubonic plague using a fully virulent KIM5⁺ strain (11). In a rat model of pneumonic plague, where serum-mediated killing of *Y. pestis* is more robust, an *ail* mutant is >10⁵-fold attenuated in terms of its LD₅₀ (10, 12). An *ail* mutant is also highly attenuated (>3,000-fold) in rats in a bubonic plague model, although the LD₅₀ has not been precisely determined (11). Thus, Ail is a significant virulence factor for *Y. pestis* pathogenesis.

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Address correspondence to Eric S. Krukoni, krukones@udmercy.edu.

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A number of ligands have been identified for Ail, including fibronectin (Fn) (13), laminin (Ln), and heparan sulfate proteoglycans (HSPGs) (14). The binding site for Ail on Fn has been determined to be within Fn type III repeats 9 and 10 (15), while on Ln, Ail binds the C-terminal 40-kDa fragment LG4-5 (14). Additionally, when the Ail-Fn and/or Ail-Ln interaction was blocked with antibodies to Fn or both Fn and Ln, *Y. pestis* was defective for the delivery of cytotoxic Yop proteins (13, 14). This indicates that efficient delivery of Yops is dependent on Ail-ECM (extracellular matrix) interactions, presumably by using ECM as a bridge to host cell receptors, similar to the well-studied YadA-Fn interaction that bridges enteropathogenic *Yersinia* binding to host cell integrins (16). Thus, understanding Ail-ECM interactions will elucidate how Ail facilitates the efficient delivery of critical Yop proteins to host cells.

Since Ail plays a critical role in pathogenesis, we sought to determine domains that contribute to the various functions of Ail. Many bacteria express outer membrane proteins that are predicted to be structurally similar to Ail (an eight-stranded flattened β -barrel); however, these homologues have modest similarity at the amino acid level (17–21). Thus, defining the amino acids required for the various functions is not possible by simple homology alignments.

Ail from *Y. enterocolitica* has been studied extensively. One study identified regions of Ail required for invasion (a process dependent on adhesion) and serum resistance (4). In this study, key residues to examine were chosen by comparison with Ail homologues and natural Ail variants, as well as alanine substitution of charged residues. Two residues in the C-terminal end of extracellular loop 2, D67 and V68 (numbered according to the proteolytically processed form following secretion; D90 and V91 of the unprocessed form), were required for the invasion of CHO cells (4). The Ail proteins of *Y. enterocolitica* and *Y. pestis* have only 65, 30, 60, and 60% identity within the four extracellular loops, respectively, making analogies between the two proteins difficult. However, we also included two possible analogous residues of *Y. enterocolitica* in our study of *Y. pestis* Ail, D93 and F94 (analogous to *Y. enterocolitica* Ail D90 and V91). Two other important findings from the studies with *Y. enterocolitica* Ail were that often multiple mutations were required to acquire a strong cell invasion phenotype (suggesting a polyvalent interaction) and a peptide corresponding to the C-terminal 12 amino acids of loop 2 inhibited Ail-mediated invasion of CHO cells (4).

To specifically define the molecular details of *Y. pestis* Ail interaction with the host, we determined Ail residues responsible for host cell attachment, Fn binding, Ln binding, and Yop delivery. We utilized the mutagenesis technique SWIM (selection without isolation of mutants [22]) to generate mutant pools of *ail* that were subjected to a functional enrichment. Using this technique and site-directed mutagenesis, we have identified residues in loops 1, 2, and 3 that play a role in the various functions of Ail.

RESULTS

Deletion of loop 2 or 3 leads to defective host cell binding. To identify which of the four extracellular loops of Ail (Fig. 1A) is required for host cell adhesion, we replaced each loop with a smaller loop made up of five or six glycines and alanines to allow an extracellular turn motif and measured the percentage of HEp-2 adhesion. *Escherichia coli* expressing Ail- Δ loop 2 and Ail- Δ loop 3 conferred levels of adhesion similar to that conferred by the empty vector pMMB207, while the Ail- Δ loop 4 mutant protein mediated about 40% of the HEp-2 cell adhesion mediated by wild-type (WT) Ail (Fig. 1B; adhesion conferred by WT Ail was set to 100%). This level of adhesion was achieved by the Δ loop 4 mutant protein, even though it was not as highly expressed as the Δ loop 2 or Δ loop 3 mutant protein (Fig. 1C). The Ail- Δ loop 1 mutant protein was unstable in *E. coli*, and the Ail- Δ loop 2, Ail- Δ loop 3, and Ail- Δ loop 4 mutant proteins were not as highly expressed as WT Ail, as demonstrated by Western blotting (Fig. 1C) and Coomassie-stained gels (data not shown). Thus, residues in loops 2 and 3 (and possibly loop 1) are required for adhesion to host cells, while Ail loop 4 is not required for cell binding. Adhesion studies with the loop deletion mutants of *Y. pestis* confirmed the importance of loops 2 and 3 in the natural bacterial host (see Table S4 in the

FIG 1 Loops 2 and 3 of Ail are required for host cell adhesion. (A) An illustration of Ail loops showing the amino acid sequence of the exposed loops. (B) HEP-2 cells were infected with *E. coli* AAEC185 derivatives carrying specific Ail loop deletions. Percent adhesion was calculated by dividing the number of cell-associated CFU by the total number of bacteria in parallel wells and multiplying by 100. The adhesion of *E. coli* AAEC185 expressing WT Ail bound to cultured cells was set to 100% (HEP-2 cell average adhesion of 6.0%). (C) Whole-cell lysates of various Ail derivatives were subjected to SDS-PAGE and probed with anti-Ail rabbit serum. Since the loop mutant proteins were expressed at lower levels in *E. coli* AAEC185 (*fim* mutant) than WT Ail at the same concentration of IPTG (100 μ M, because of instability of the mutant proteins), the concentration of IPTG was adjusted to approach similar levels of protein among Δ loop 2, Δ loop 3, Δ loop 4, and WT Ail (Ail- Δ loop1 was not well expressed, even at increased IPTG concentrations). Coomassie gels also confirmed the decreased stability of loop mutant proteins (data not shown). The IPTG concentrations used were as follows: WT Ail, 50 μ M; Ail- Δ loop 1, 500 μ M; Ail- Δ loop 2, 200 μ M; Ail- Δ loop 3, 200 μ M; Ail- Δ loop 4, 100 μ M. Data are from three independent experiments performed in triplicate ($n = 9$). Error bars indicate the standard deviations. *, $P < 0.001$. Significance was assessed with the Student *t* test.

supplemental material). In *Y. pestis*, adhesion conferred by the Ail- Δ loop4 mutant protein was not statistically significantly different from that conferred by WT Ail (see Table S4), indicating that the 60% cell-binding defect in *E. coli* is likely due to poor expression levels relative to WT Ail.

SWIM enrichment identifies mutants with reduced host cell adhesion. To identify individual residues in loops 1, 2, and 3 that contribute to the cell-binding activity of Ail, we employed a previously described mutagenesis screening method called SWIM (22). This mutagenesis technique is based on PCR-generated mutagenesis

via primer pools containing certain codon positions with 50% WT and 50% mutant codons. Each mutant codon was generated with a single nucleotide change to an amino acid with a smaller, uncharged side chain (Fig. S1A; not all amino acids could be changed to alanine with a single nucleotide change). After PCR mutagenesis, the mutant pools in *E. coli* were run through a HEp-2 cell-binding enrichment. Following four rounds of enrichment, the input mutant pool and enriched bound and unbound pools were sequenced. The resulting chromatograms were compared for enrichment of either the WT codon in the bound pool or a mutant codon in the unbound pool. SWIM mutagenesis and HEp-2 enrichment were performed with loops 1, 2, and 3. To cover the 17- to 20-residue loops, each loop was split into two SWIM pools (an N-terminal and a C-terminal half; see Table S2).

A sample chromatogram generated with this mutagenesis scheme is presented in Fig. S1B. The region shown is from the N-terminal half of loop 3, amino acids 125 to 128. The input pool chromatogram shows how well the mutant codon is represented in the pool (some are represented better than others). Then, after four rounds of enrichment with adhesion to HEp-2 cells, the chromatograms show an increase in the mutant residue at positions K125 and S128 in the unbound pool relative to the bound pool (see Fig. S1B). This suggests that the K125I and S128A mutant proteins bind to host cells less well than WT Ail does. These two mutant proteins were chosen for further characterization, as were several others with changes in the loop regions. Once this process identified important amino acids, single site-directed amino acid changes to alanine were generated since each clone in the SWIM mutant pool has an average total of four mutations. These point mutant proteins were then analyzed for cell-binding activity. A summary of the *Y. pestis* mutants generated in this study that expressed stable protein and their activities is presented in Table S4.

Cell adhesion of Ail mutant proteins. Next we characterized the adhesion of alanine point mutant proteins to host cells. In addition to mutant proteins identified by SWIM, we chose to analyze residues analogous to *Y. enterocolitica* Ail that were shown to be important for adhesion, invasion, and serum resistance (D90 and V91 [4]). In *Y. pestis*, these correspond to D93 and F94. When the various Ail mutant proteins were expressed from a plasmid in a *Y. pestis* KIM5 Δ ail background, the Ail-F80A, Ail-S128A, and Ail-F130A single mutant proteins had about 30% adhesion relative to WT Ail, while Ail-D93A, Ail-F94A, and Ail-D93A/F94A had no effect on Ail-mediated cell adhesion (Fig. 2B). Combining mutations in both loops 2 and 3 led to an even greater decrease in cell adhesion, with Ail-F80A/F130A and Ail-F80A/S128A/F130A showing the least binding activity (Fig. 2B). This suggests that the interaction of Ail with host cells is polyvalent and that both loops 2 and 3 can establish interactions with host cells. The expression of each protein was assessed by Coomassie staining (Fig. 2B).

Residues in Ail loops 2 and 3 contribute to Fn binding. By binding the central region of Fn, Ail can facilitate cell adhesion and Yop delivery to host cells (13, 15). Thus, we tested the various Ail mutant proteins for the ability to bind Fn immobilized on 96-well plates. The percentage of Fn binding by *Y. pestis* expressing WT Ail was normalized to 100% after the level of binding by WT Ail to the control protein bovine serum albumin (BSA) was subtracted. *Y. pestis* strain KIM5 Δ ail Δ pil was used for Ail expression since both Ail and plasminogen activator (Pla) can bind Fn (13). The single mutant proteins Ail-F94A, Ail-S128A, and Ail-F130A all mediated low levels of Fn binding, approaching background levels seen with the empty vector pMMB207 (Fig. 3A). The Ail-F80A single mutant protein had no defect in Fn binding; in fact, it tended to have a higher level of Fn binding than WT Ail (although it did not reach the cutoff for statistical significance at $P = 0.07$). However, when combined with the F130A mutation, the double mutant protein Ail-F80A/F130A had no detectable Fn binding activity (Fig. 3A). Together, these data indicate a role for loops 2 and 3 in Fn binding.

It is unclear why the Ail-S128A/F130A mutant protein did not show a defect in Fn binding. One possibility is that once Fn is completely disengaged from loop 3, it is able to make more efficient contacts with residues in loop 2 (like F94). The fact that Ail-F80A

FIG 2 Mutations in F80, S128, and F130 of Ail drastically reduce cell binding by *Y. pestis*. (A) A diagram of Ail highlighting residues of interest. (B) HEp-2 cells were infected with KIM5 Δ ail strains expressing various mutant forms of Ail. Percent adhesion was calculated with cells expressing WT Ail set to 100% (HEp-2 cell average adhesion of 10.5%). Whole-cell lysates of the input bacterial cultures were subjected to SDS-PAGE, and Ail was identified as the predominant 15-kDa protein by Coomassie staining (note that Ail-D93A and Ail-F94A levels were assessed on a different gel). Data are from at least two independent experiments performed in triplicate ($n \geq 6$). Error bars indicate the standard deviations. *, $P < 0.05$; **, $P < 0.001$ (compared to strains expressing pMMB207-Ail). Significance was assessed with the Student *t* test. The white lines in the gel in panel B indicate lanes that were cropped and rearranged to match the histogram. The gel for D93A and F94A mutant proteins was run on a different day, but the presence of additional bands above Ail serves as a loading control.

tends to have increased interaction may also be due to some altered interactions with F94 (loop 2) and S128 and F130 (loop 3) in the context of the Ail-F80A mutant protein. F94 lies at the bottom of loop 2 oriented toward the center of the barrel (14); thus, its substrate accessibility could be influenced by other Ail-substrate interactions. Further studies are required to explain these observations.

Residues in Ail loops 2 and 3, especially F80, contribute to Ln binding. Ln is another defined ECM substrate for Ail. To determine whether the nature of Fn binding and that of Ln binding are distinct, Ail derivatives altered in various residues in loops 2 and 3 were assessed for Ln binding in the *Y. pestis* KIM5 Δ ail Δ pla background. Alteration of F80 had the most dramatic impact on Ln binding of the single mutant proteins, with the Ail-F80A mutant protein having only background levels of binding. Unlike Fn binding, mutations in loop 3 (S128A and F130A) had only intermediate defects on Ln binding and these were significantly greater than binding by the Ail-F80A mutant protein ($P < 0.05$). Thus, interaction between Ln and Ail and that of Fn and Ail are distinct, with F80 being critical for Ail-Ln interaction but dispensable for Ail-Fn interaction.

Multiple Ail mutations are required to strongly affect Yop delivery. To assess their effect on Yop delivery, the loop 2 and 3 cell-binding Ail mutant proteins were

FIG 3 Residues in loops 2 and 3 of Ail contribute to Fn and Ln binding by *Y. pestis*. (A) KIM5 Δ ail Δ p_{ail} strains expressing various forms of Ail were assessed for binding to purified Fn immobilized on microtiter wells. Binding by plasmid-expressed WT Ail was set to 100% after subtracting the binding of WT Ail to BSA alone. Levels of Ail expression were assessed by Coomassie staining as indicated by the arrow. Fn binding was measured twice in triplicate ($n = 6$). (B) KIM5 Δ ail Δ p_{ail} strains expressing various forms of Ail were assessed for binding to purified Ln immobilized on glass coverslips. Binding by plasmid-expressed WT Ail was set to 100%. Levels of Ail expression were assessed by Coomassie staining as indicated by the arrow. Ln binding was measured three times in duplicate ($n = 6$). Error bars indicate the standard deviations. *, $P < 0.05$; **, $P < 0.001$ (compared to strains expressing pMMB207-Ail). Significance was assessed with the Student *t* test. White lines in the gel in panel A indicate lanes that were cropped and rearranged to match the histogram. Two different gels were used to assemble all of the mutant proteins, but the presence of additional bands above Ail serves as a loading control.

assessed for the ability to confer cytotoxicity. We have previously reported that Ail is a key adhesin responsible for the efficient translocation of Yops into host cells (1, 8, 13). To ensure endogenous expression levels for each *ail* allele, we integrated each mutation of interest into the *Y. pestis* KIM5 genome at the *ail* locus. This resulted in similar amounts of all of the mutant proteins (Fig. 4). Chromosomally expressed WT Ail (either from the parental KIM5 strain or from a reconstructed WT *ail* strain, Δ ail::*ail* WT) mediated 90% cytotoxicity by 3 h, as measured by cell rounding due to delivery of Yop proteins. An Δ ail mutant strain showed only 5% rounding, similar to a Δ yopB mutant lacking a functional type III secretion (T3S) translocation apparatus (Fig. 4; see Table S5).

Some single mutations in Ail that had intermediate defects in cell adhesion had modest effects on Yop delivery (Ail-S128A and Ail-F130A). However, combination of loop 2 and 3 mutations in Ail-F80A/F130A and F80A/S128A/F130A resulted in dramatic defects in Yop delivery with ~20% cell rounding (Fig. 4). This indicates that both loops 2 and 3 of Ail can establish contact with host cells to facilitate Yop delivery and both

FIG 4 Loops 2 and 3 of Ail contribute to Ail-mediated Yop delivery. Strains expressing various mutant forms of Ail from the chromosome were used to infect HEP-2 cells at an MOI of 10. After 3 h of infection, cells were fixed and stained with Giemsa to visualize shrunken, round, darker cells indicative of Yop-mediated cytotoxicity. Cells were counted by a researcher blinded to the source of the image, and the percentage of cells showing cytotoxicity was calculated. Whole-cell lysates were subjected to SDS-PAGE and stained with Coomassie to assess Ail expression (arrow). Experiments were performed twice in duplicate, and two fields of ~ 100 cells/field were counted per infected well (eight measurements, $n = 4$). Black bars denote WT *ail* or the Δ ail mutant, the dark gray bar indicates a mutation in loop 2, white bars denote mutations in loop 3, and light gray bars indicate mutations in both loops 2 and 3. *, $P < 0.05$; **, $P < 0.001$ (compared to the KIM5 Δ ail::ail reconstructed WT strain). Error bars indicate the standard deviations. Significance was assessed with the Student *t* test.

interactions must be disrupted to strongly impair T3S of Yops. This reflects our findings with cell and ECM binding (Fig. 2 and 3), although single mutant proteins had stronger defects in those two activities than in Yop delivery. This suggests that Fn and/or Ln may serve as bridging molecules to host cells for Ail-mediated Yop delivery (14). As with cell binding and Fn binding, the F80A/F130A double mutation produced the strongest defect in Yop delivery, highlighting the critical nature of these two bulky hydrophobic residues. We hypothesize that the Ail-F130A mutant has a strong cell-binding defect yet maintains most of its Yop delivery activity because even with reduced cell adhesion relative to that of WT Ail, the chromosomal Ail-F130A mutant still has several cell-bound bacteria capable of Yop delivery. Thus, by 3 h postinoculation, no readily observable defect in Yop delivery is observed.

Plasmid-based Ail-mediated Yop delivery experiments using a KIM5 Δ ail Δ pla Δ psaA (Δ 3) background also confirmed that mutations in both loops 2 and 3 are required to detect a strong Yop delivery defect (see Table S4). For plasmid-based studies, we used the KIM5 Δ 3 strain since the *Y. pestis* adhesins plasminogen activator (Pla) and pH 6 antigen (Psa) can also participate in Yop delivery (1) and Ail-mediated Yop delivery from a plasmid was less efficient than delivery from chromosomally expressed Ail. Thus, Pla and Psa expression led to higher relative background levels when Ail was expressed from a plasmid (data not shown).

Residues required for Ail-mediated cell adhesion and Yop delivery are not critical for serum resistance. In addition to serving as an adhesin, Ail functions as a

FIG 5 Ail cell adhesion mutants maintain strong serum resistance in human serum. *Y. pestis* strains carrying chromosomally encoded alleles of *ail* were mixed into 80% NHS or HIS and incubated for 1 h at 37°C. Bacteria were then diluted and plated for CFU counting. Percent serum resistance was determined by the number of colonies surviving in NHS/HIS $\times$ 100. Strains were measured twice in duplicate ($n = 4$). Error bars indicate the standard deviations. *, $P < 0.05$; **, $P < 0.001$ (compared to the KIM5 Δ ail::ail [WT] strain). Significance was assessed with the Student *t* test.

mediator of serum resistance in various pathogenic *Yersinia* species (3–5, 9, 10, 23–25). To determine whether the same residues that are critical for adhesion and Yop delivery are required for Ail-mediated serum resistance, we measured the survival of *Y. pestis* KIM5 expressing various forms of chromosomally expressed Ail in 80% human serum for 1 h at 37°C. While an Δ ail mutant strain decreased >10,000-fold in viable cells in normal human serum (NHS) relative to heat-inactivated serum (HIS), a reconstructed WT *ail* strain survived at levels indistinguishable from those of the parental strain in NHS (Fig. 5). Even strains expressing the mutant proteins most defective in cell adhesion, Ail-F80A/F130A and Ail-F80A/S128A/F130A, had only a modest 3- to 4-fold defect in serum resistance (Fig. 5; see Table S5). Thus, these strains maintain a significant level of serum resistance (10,000-fold above that of an Δ ail mutant), despite losing almost all of their adhesion and Yop delivery activities. This indicates that there are other residues critical for Ail-mediated serum resistance still to be defined and that they are likely distinct from the residues required for cell adhesion and Yop delivery.

Residue F130 of loop 3 strongly promotes autoaggregation. Another function of Ail is the ability to cause bacterial autoaggregation (9). We hypothesized that the extracellular loops of Ail interact with each other to facilitate autoaggregation. Thus, we analyzed several cell-binding mutant proteins to determine whether similar residues are required for autoaggregation. *Y. pestis* KIM5 Δ ail expressing various mutant Ail proteins from plasmid pMMB207 was examined for autoaggregation. Ail expression was induced overnight at 28°C with 100 μ M isopropyl- β -D-thiogalactopyranoside (IPTG). Following overnight growth, the optical density at 620 nm (OD_{620}) of each culture was measured. The cultures were then allowed to settle without shaking for 60 min at room temperature, and the OD_{620} was measured again. The change in OD_{620} over 60 min was calculated as the percent autoaggregation. While Ail-F80A had a modest (<50%) defect in autoaggregation, Ail-F130 had a strong defect in autoaggregation, indicating that loop 3 plays a more dominant role in this activity (Fig. 6). Combination of the F80A and F130A mutations led to a further reduction in autoaggregation compared to results with the Ail-F130A single mutant protein ($P = 0.03$), indicating a supporting role for loop 2 in autoaggregation (Fig. 6).

To determine whether the phenomenon of Ail-mediated autoaggregation of *Y. pestis* relies on Ail being present on both cells or is simply due to the general promiscuous adhesiveness of Ail for other cell surfaces, we labeled *Y. pestis* strains with green fluorescent protein (GFP) or red fluorescent protein (RFP) and determined

FIG 6 The cell-binding Ail-F130A mutant of *Y. pestis* is severely defective for autoaggregation. *Y. pestis* KIM5 Δ ail expressed WT Ail or mutant forms of Ail from IPTG-inducible plasmid pMMB207. After overnight growth with 100 μ M IPTG, stationary-phase cultures were allowed to settle for 60 min. OD₆₂₀ measurements were taken at time zero and 60 min. The OD₆₂₀ at 60 min was subtracted from the starting OD₆₂₀ and expressed as a percentage of the starting OD₆₂₀ to quantify the amount of autoaggregation. Autoaggregation was measured three times in duplicate and once independently ($n = 7$). Error bars indicate the standard deviations. *, $P < 0.05$ relative to WT Ail; #, $P < 0.05$ relative to Ail-F130A. Significance was assessed with the Student t test.

whether red cells expressing Ail can aggregate with green cells lacking Ail (KIM5 Δ ail plus the pMMB207 vector alone) or *vice versa*. Mixed populations of cells induced overnight with 100 μ M IPTG were assessed for coaggregation by visualizing bacterial aggregates after 30 min of settling (Fig. 7) or by measuring the settling of red or green fluorescent cells from solution after 60 min of settling at room temperature with a fluorescence plate reader (see Fig. S2). These studies demonstrated that aggregation required that Ail be present on both cells (Fig. 7; see Fig. S2), indicating an Ail-Ail interaction. Furthermore, if Ail-F80A/F130A or Ail-F80A/S128A/F130A was expressed on the surface of the red strain, it aggregated poorly with green cells expressing WT Ail (Fig. 7; see Fig. S2) and *vice versa*. Thus, the same aggregation defects observed in single-strain cultures (Fig. 6) hold true in mixed cultures (Fig. 7; see Fig. S2). By fluorescent imaging of aggregated clumps, strains expressing Ail-F130A are partially (although inefficiently) recruited to clumps of bacteria expressing WT Ail (Fig. 7E and F), compared to strains expressing the multiple-mutant protein Ail-F80A/F130A or Ail-F80A/S128A/F130A being excluded from the bacterial aggregates (Fig. 7G to J). Assessment of the levels of coaggregation by fluorescence spectrophotometry gave a more quantitative measure of coaggregation (see Fig. S2).

DISCUSSION

Using an Ail loop deletion strategy, followed by SWIM mutagenesis and functional enrichment for cell adhesion mutant proteins, we identified several residues required for Ail-mediated binding to host cells. Specifically, residues in extracellular loops 2 and 3 play a key role in host cell binding, ECM binding, and Yop delivery (Fig. 1 to 4).

On the basis of our initial findings by SWIM analysis, specific alanine mutant proteins were constructed and assessed for various Ail functions, including host cell adhesion, binding to purified Fn and Ln, facilitating Yop delivery, conferring serum resistance, and mediating autoaggregation. Figure 8 illustrates the positions of the three most influential residues for cell binding and Yop delivery (F80, S128, and F130), residing in loops

FIG 7 Mutant forms of Ail are incorporated poorly into aggregates of cells expressing WT Ail. Strains were grown overnight at 28°C to saturation. The following day, 25 μ l of each of two strains (one red, one green) was inoculated into 3 ml of fresh HIB with 100 μ M IPTG to induce *ail* expression and grown overnight again at 28°C. The following day, bacteria were allowed to settle for ~30 min, the supernatant was discarded, and 50 μ l of the settled bacterial pellet was placed on a glass coverslip for visualization of *Y. pestis* aggregated cells. Imaging was performed with a 60 $\times$ magnifying objective on an Olympus BX63 microscope.

2 and 3. These residues constitute a polyvalent contact surface that Ail utilizes to engage ligands (Fig. 8B). The concept of a bivalent (two-loop) interaction between Ail and its substrates is supported by the fact that, in many cases, both F80 and F130 must be altered to produce the most severe phenotype (Fig. 2 to 4). One exception was Ln binding, which was completely disrupted in the Ail-F80A mutation affecting loop 2 alone (Fig. 3B). It is noteworthy that F130 was unstructured in the solved crystal structure of *Y. pestis* Ail (14), suggesting that it may seek to bury itself when another hydrophobic surface is available. The minimal fragment of human Fn bound by Ail (FnIII⁹⁻¹⁰) has 14 large hydrophobic residues (phenylalanine, tryptophan, or tyrosine), suggesting candidates for Ail-Fn interaction residues (15, 26). Binding to Fn was strongly dependent on two hydrophobic residues, F94 and F130, again suggesting that

FIG 8 Positions of Ail residues critical for cell binding and Yop delivery. (A) The positions of the Ail mutant residues in the exposed loops are shown. (B) Key Ail residues F80, S128, and F130 are shown on the Ail structure with a side view and a top view. The precise position of F130 is estimated because of flexibility in the solved Ail structure (14).

key hydrophobic residues are critical for Ail-Fn interaction. A similar hypothesis can be made for Ail binding to Ln, which was exquisitely dependent on F80, a residue that may make hydrophobic interactions with the ~375-amino-acid C-terminal Ail-binding domain of Ln, LG4-5 (14), which contains 25 hydrophobic Phe, Trp, and Tyr residues (27).

In addition to the strong effects on cell binding, Fn binding, Ln binding, and Yop delivery by hydrophobic residues of loops 2 and 3, several other residues appear to contribute significantly to Ail activity. Within loop 1, mutant protein Ail-Y46A (another hydrophobic residue) was ~60% defective for host cell binding but showed no defect in Yop delivery (see Table S4). Unfortunately, many other loop 1 mutant proteins were not stable upon the introduction of alanine substitutions (data not shown). The *Y. pestis* Ail-E82A loop 2 mutant protein was also 60% defective for host cell binding but again showed no defect in Yop delivery (see Table S4). Within loop 3, two lysine-to-alanine Ail mutations, K123A and K125A, had moderate effects on host cell binding, suggesting that charge interactions may also contribute to Ail-mediated cell adhesion (see Table S4; the *P* value for the Ail-K125A adhesion defect was 0.06). Thus, while F80, S128, and F130 are key contributors to the phenotypes we assessed, other residues of Ail can also influence these functions.

It is clear that many residues found to contribute to the binding activities of Ail lie in or near the tips of exposed loops (Y46, F80, E82, S128, and F130). This suggests that the tips of the loops engage host cell components. This is in contrast to major cell invasion residues of *Y. enterocolitica* Ail, Ail_{Y.ent}-D90 and Ail_{Y.ent}-V91, which are found on the descending, C-terminal half of loop 2, close to the membrane (4). In that *Y. enterocolitica* Ail study, hydrophobic residues at the tip of Ail were not investigated for their contributions to cell adhesion/invasion as the study focused on residues conserved in other Ail homologues and charged, surface-exposed residues (4).

While specific amino acids vary in each homolog of the Ail family, our finding that loops 2 and 3 of Ail are key players in cell adhesion and Yop delivery agrees well with studies of other related proteins. The *Salmonella* protein Rck is known to confer cell adhesion/invasion and serum resistance on strains expressing Rck. By a protein fusion technique where portions of the homologous *Salmonella* protein Pac (which lacks these

activities) were spliced at different positions to Rck, it was determined that loop 3 of Rck (and/or potentially the two subsequent transmembrane domains) is critical for both cell invasion and serum resistance activities (28). A later study demonstrated that a peptide derived from loop 2 of Rck can also mediate binding to host cells (29). Similarly, loop 2 of the *E. coli* K1 Ail-like adhesin Hek is required for Hek-mediated cell adhesion, invasion, and autoagglutination, while loop 3 also participates in autoagglutination (18). Furthermore, a peptide from loop 2 of the Ail-like protein Tia of enterotoxigenic *E. coli* (ETEC) inhibited ETEC invasion of host cells, whereas a scrambled peptide did not (30), and a similar Tia loop 2 peptide can bind HSPGs, a common component of host cell surfaces (20). Finally, in a recent study where Ail was embedded into nanodiscs for nuclear magnetic resonance analysis that preserves adhesive function, antibodies directed against loop 2 of Ail inhibited Fn binding by Ail (31). In all, these studies demonstrate that loops 2 and 3 of Ail-like proteins are often involved in ligand binding and important pathogenic functions. Our studies presented here define the critical residues within these loops in *Y. pestis* Ail for several Ail-dependent activities.

We have previously characterized the different functions of *Y. pestis* Ail, including adhesion to host cells, binding to purified Fn and Ln, and facilitation of Yop delivery (1, 8, 13–15, 32). These previous studies led us to identify the minimal binding site for Ail on Fn, a stretch of just 180 amino acids in the 250-kDa Fn protein molecule. The studies presented here inform the other side of this interaction by identifying specific residues of Ail that contribute to host cell and Fn binding. As anticipated, those residues most defective for cell binding are also the most defective for Yop delivery, a key process in plague pathogenesis.

The specific contributions of Ail-mediated cell adhesion, ECM binding, and complement regulation to virulence *in vivo* are still an open question. While recent studies have indicated that Ail influences *in vivo* target cell selection for Yop delivery during *Yersinia* infections (33–35), the mechanism(s) by which Ail directs cell targeting of Yop secretion is unclear. Ail could recruit ECM components to direct Yop delivery to specific immune cells with receptors for those components to disarm the host immune response. Alternatively, Ail could affect cell adhesion/Yop delivery by directing the degradation of opsonized complement components such as C3b through recruitment of serum regulatory proteins factor H and C4bp (5, 24), thus steering interactions toward cells capable of recognizing processed C3b fragments such as C3d (36).

This study focused on the identification of Ail residues required for host cell binding and Yop delivery. While some of these residues also contribute modestly to serum resistance in *Y. pestis*, mutation of three critical adhesion residues in a single protein (Ail-F80A/S128A/F130A) resulted in a protein that maintains a level of serum resistance 10,000-fold above that of an Δ ail mutant strain and that is only 3- to 4-fold less resistant than WT Ail (Fig. 5; see Table S5). Thus, additional residues participate in the ability of Ail to protect *Y. pestis* from the bactericidal effects of serum, a critical activity in plague disease. Analysis of serum resistance conferred by *Y. enterocolitica* Ail indicated residues in loops 1, 2, and 3 that contributed to serum resistance, and often multiple mutations were required to produce a serum-sensitive phenotype (4). Future studies will focus on identifying residues critical for the serum resistance activity of *Y. pestis* Ail with the goal of developing vaccination strategies and/or postexposure therapeutics for preventing plague disease.

MATERIALS AND METHODS

Strains and culture conditions. *Y. pestis* strains used in this study were cultivated in heart infusion broth (HIB) overnight or on heart infusion agar (HIA) for 48 h at 28°C. *E. 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), 25 µg/ml; ampicillin (Amp), 100 µg/ml. IPTG was used at 100 µM unless otherwise noted. The strains and plasmids used in this study are listed in Table S1.

HEp-2 cells were cultured at 5% CO₂ (37°C) in modified Eagle's medium (MEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 1% sodium pyruvate (Life Technologies), and 1% nonessential amino acids (Life Technologies).

SWIM mutagenesis. SWIM mutagenesis was performed in a manner similar to that previously described (22). Loop mutagenesis primers were ordered from Invitrogen and utilized a sequence in which

two nucleotides (WT and mutant proteins) could be incorporated at various positions to encode WT and mutant Ail proteins. The primer sequences for each loop mutant protein pool are in Table S2 with the forward primer listed (the reverse primer is exactly complementary to the forward primer). A pSK-Bluescript-derived pSK-*ail* construct was subjected to PCR amplification mutagenesis with the SWIM mutagenesis oligonucleotides. The resulting amplified plasmid was digested with DpnI to degrade the template plasmid, and the digestion product was transformed into *E. coli* DH5 α . DH5 α transformants were allowed to grow in liquid culture for 2 h at 28°C before the addition of Amp. The cultures were then grown overnight at 28°C. The next day, the liquid culture was diluted 1:100 in LB plus Amp and allowed to grow for another day at 28°C. The 2-day grown culture is designated the input pool, and 2 ml was taken for isolation of the pSK-*ail* plasmid (Miniprep; Qiagen) and sequenced. This culture was then diluted to an OD₆₀₀ of 0.6, and 100 μ l 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 for another 90 min. After a total of four enrichments, pools of bound and unbound bacteria were collected and cultured in fresh LB plus Amp overnight to regrow both the bound and unbound pools. The next day, the plasmids were isolated (Qiagen Miniprep kit) and sent for sequencing. These pools were designated the bound and unbound mutant protein pools.

Generation of single point mutations. PCR mutagenesis was performed with the enzyme *Pfu* (Stratagene) and primer pairs encoding the mutations. The primers used were complementary to one another, and the forward primers are listed in Table S3. Following PCR amplification with the pSK-*ail* plasmid as a template, PCR products were digested with DpnI to degrade the template DNA and transformed into DH5 α . Potential mutant protein clones were sequenced to confirm that only the target site was mutated and a BamHI/PstI fragment containing the entire open reading frame and ribosome-binding site was liberated, purified, and ligated into IPTG-inducible plasmid pMMB207 (Cm^r) (37).

Introduction of *ail* alleles onto the *Y. pestis* chromosome. To facilitate the introduction of point mutations into the *ail* locus of *Y. pestis* KIM5, we first used λ -RED recombination to mark the normal *ail* locus with a kanamycin resistance/sucrose sensitivity cassette (*neo-sacB*) from IVET plasmid pRES (38). With primers Ail-sacBf and Ail-sacBr (see Table S3), the *neo-sacB* cassette was amplified by PCR and recombined at the *ail* locus by standard recombineering in *Y. pestis* as previously described (8). The *ail::neo-sacB* strain was selected on HIA plus 30 μ g/ml kanamycin, and replacement of the *ail* allele was confirmed by PCR. *ail* alleles of interest were then PCR amplified from plasmid pMMB207-*ail* with primers Ailf2 and Ailr2, and the PCR product was used to replace the *neo-sacB*-marked *ail* allele by selection on HIA plus 5% sucrose. Proper insertions were confirmed initially by PCR product size and restoration of kanamycin sensitivity. The *ail* allele from each candidate strain was then PCR amplified and sequenced to confirm the presence of the desired mutation.

Fn-binding assay. The Fn-binding assay was described previously (13). Briefly, 96-well plates were coated with 40 μ g/ml plasma Fn (Sigma) in phosphate-buffered saline (PBS) overnight at 4°C. *Y. pestis* KIM5 Δ ail Δ pla derivatives expressing various forms of Ail from inducible plasmid pMMB207-*ail* were cultured overnight at 28°C in HIB with 20 μ M IPTG and Cm. The following day, wells were washed with PBS before being blocked with PBS plus 10 mg/ml BSA (blocking buffer). Bacterial cells were pelleted and resuspended at an OD₆₂₀ of 1.0, and 50 μ l of the bacterial suspension was added to triplicate wells. The plate was then incubated for 2 h at 37°C. The wells were washed three times with PBS before being fixed with 100 μ l of methanol and stained with 0.01% crystal violet for 20 min. After excess crystal violet was washed away three times with PBS, the bacterium-associated crystal violet stain was solubilized with 100 μ l of an 80% ethanol–20% acetone solution. The A₅₉₅ was measured to quantify the binding of bacteria to Fn.

Ln-binding assay. Glass coverslips were added to 24-well tissue culture plates (Costar) and UV irradiated for 2 h. Each coverslip was then coated with 40 μ g/ml human Ln (Sigma catalog no. L6274) in 300 μ l of PBS. Coverslips were coated for 2 h at room temperature and washed once with 1 ml of PBS. Coverslips were then blocked with 500 μ l of PBS containing 10 mg/ml BSA overnight at 4°C. *Y. pestis* strains expressing various forms of Ail from plasmid pMMB207 were then grown overnight at 28°C in HIB plus 20 μ M IPTG and Cm. Bacteria were pelleted and resuspended at an OD₆₂₀ of 5 in PBS plus 0.4% BSA, and 300 μ l of bacterial cells was added to each well and incubated for 2 h at 28°C. Unbound bacteria were then removed by three washes with 1 ml of PBS, and bacteria were fixed with 300 μ l of methanol for 20 min at room temperature. The methanol was then removed, and the plates were air dried for about 2 h. The wells were then stained with 400 μ l of 0.01% crystal violet for 20 min at room temperature and washed twice with 500 μ l of water. The stained bacteria were then lysed with 500 μ l of a 20% acetone–80% ethanol mixture, and the A₅₉₅ of 100 μ l of the lysate was read. Levels of binding relative to the binding of a strain expressing WT Ail are presented.

Cell adhesion assay. The cell adhesion assay used was described previously (8, 13). Briefly, HEP-2 cells were cultured in MEM plus 10% FBS in 24-well tissue culture plates until they reached 80 to 90% confluence. *Y. pestis* KIM5 or *E. coli* AAEC185 (*fim* mutant) derivatives were cultured overnight at 28°C in HIB plus Cm (*Y. pestis*) or at 37°C in LB plus Cm (*E. coli*) and then diluted 1:50 the next day in fresh medium with Cm and 100 μ M IPTG to induce plasmid-based Ail expression. Strains were allowed to grow for an additional 3 h at 28°C (*Y. pestis*) or 37°C (*E. coli*). Tissue culture cells were washed once with 1 ml of PBS, and then 400 μ l of serum-free MEM was added, followed by 100 μ l of bacteria resuspended in serum-free MEM at an OD₆₂₀ (*Y. pestis*) or OD₆₀₀ (*E. coli*) of 0.6 for a multiplicity of infection (MOI) of ~100. Plates were incubated with bacteria at 37°C in 5% CO₂ for 90 min. Cells were then washed three times with PBS, and cell-associated bacteria were liberated by the addition of sterile H₂O containing 0.1% Triton X-100 for 10 min. Percent adhesion was calculated by dividing the number of bound CFU by the total number of bacteria in parallel wells and multiplying by 100.

Cytotoxicity assay. The cytotoxicity assay used was described previously (8, 13). Briefly, HEp-2 cells were cultivated in MEM plus 10% FBS until they reached about 50 to 80% confluence in 24-well tissue culture plates. *Y. pestis* KIM5 derivative strains were cultured overnight in HIB (with Cm if required). Overnight cultures were diluted 1:10 in fresh medium and incubated for 3.5 h at 28°C (with Cm and 100 μ M IPTG if required for plasmid-based expression). Tissue culture wells were washed once with 1 ml of PBS, and 490 μ l of serum-free MEM was added, followed by 10 μ l of bacteria resuspended in serum-free MEM at an OD₆₂₀ of 0.6 to obtain an MOI of $\sim$ 10. The plates were incubated at 37°C in 5% CO₂ for 3 h. The cells were washed twice with 1 ml of PBS, fixed with methanol, dried, stained with 0.076% Giemsa stain for 20 min, and then washed four times with H₂O. Rounding was observed, and photographs were taken with a phase-contrast microscope at $\times$ 20 magnification. Cytotoxicity was enumerated by determining the total number of cells and the number of round dark purple (shrunken cytoplasm) cells experiencing cytotoxicity in three microscopic fields ($\sim$ 150 cells/field). Percent cytotoxicity was calculated by dividing the number of rounded cells by the total number of cells and multiplying by 100.

Autoaggregation assay. *Y. pestis* cultures were induced for Ail expression overnight in HIB plus Cm and 100 μ M IPTG. After overnight induction, stationary-phase cultures were allowed to settle for 60 min. OD₆₂₀ measurements were taken at time zero (before settling) and at 60 min. The OD₆₂₀ at 60 min was subtracted from the starting OD₆₂₀ and expressed as a percentage of the starting OD₆₂₀ to quantify the amount of autoaggregation. Assessment of Ail-mediated coaggregation with fluorescently labeled strains was performed by transforming KIM5 Δ ail mutant strain SF777 with either GFP-encoding plasmid pFVP25.1 (39) or RFP-encoding plasmid pGEN-Pem7-DsRedT3-MUT (40). Each fluorescent strain was then transformed with plasmid vector pMMB207 encoding one of several alleles of *ail* (or with the empty vector). Strains were grown overnight at 28°C to saturation. The following day, 25 μ l each of two strains (one red, one green) was inoculated into 5 ml of fresh HIB plus Amp (selecting for the fluorescence-encoding plasmids) plus Cm (selecting for pMMB207) and 100 μ M IPTG to induce *ail* expression and grown overnight again at 28°C. The following day, cultures were removed from the incubator and resuspended by gentle vortexing and 1 ml was gently pelleted for 5 min at 7,000 rpm and resuspended in PBS to prevent autofluorescence from the medium and 100 μ l was moved to a black 96-well plate (USA Scientific) to measure initial levels of both the green and the red fluorescent strains before clumping with a SpectraMax M3 spectrophotometer (Molecular Devices) and excitation and emission at 488 and 511 nm for green and 557 and 592 nm for red, respectively. The remaining 5-ml cultures were left to settle at room temperature for 60 min. At the end of 60 min, 100 μ l of the supernatant was moved to the black 96-well plate for fluorescence measurements to assess the autoaggregation of both red- and green-labeled cells in the mixture. For fluorescence microscopy, red- and green-labeled cells were grown similarly, but after overnight growth at 28°C, bacteria were allowed to settle for $\sim$ 30 min, the supernatant was discarded, and 50 μ l of the settled bacterial pellet was placed on a glass coverslip for visualization of aggregated *Y. pestis* cells. Imaging was performed with a 60 $\times$ magnifying objective on an Olympus BX63 microscope.

Protein expression analysis. Bacterial cultures were resuspended in Laemmli sample buffer normalizing for OD₆₀₀ (*E. coli*) or OD₆₂₀ (*Y. pestis*). Bacterial cell extracts were boiled and subjected to 15% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were stained with Coomassie blue dye, which makes Ail visible as a unique protein band at about 15 kDa (8). Other protein bands in the cell lysate served as protein loading controls. For studies with the Ail loop deletions expressed in *E. coli*, a previously described anti-Ail antibody (41) and Coomassie blue staining were employed to detect Ail.

Serum resistance assay. *Y. pestis* strains expressing chromosomally encoded mutant forms of Ail were grown overnight at 28°C in HIB. Bacteria were diluted 1:50 the next day and grown in HIB for 3 to 4 h. After the OD₆₂₀ was read, 1 ml of each strain was pelleted and resuspended in PBS at an OD₆₂₀ of 0.5. The strains were then diluted 1:10 in PBS to a concentration of $\sim$ 1.5 $\times$ 10⁷/ml. A 50- μ l volume of diluted bacteria ($\sim$ 7.5 $\times$ 10⁵ bacteria) was mixed with 200 μ l of NHS (Sigma) or 200 μ l of HIS, resulting in a concentration of 80% serum. Bacteria and serum were incubated with rolling for 1 h at 37°C. Bacteria were then plated at various dilutions on HIB plates and grown at 28°C, and viable colony counts were enumerated at 48 h. Percent serum resistance is expressed as the number of colonies surviving in NHS/HIS $\times$ 100.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.01047-15>.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.6 MB.

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