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The *Yersinia pseudotuberculosis* Outer Membrane Protein Ail Recruits the Human Complement Regulatory Protein Factor H

Derek K. Ho,* Rauna Riva,* Mikael Skurnik,*,† and Seppo Meri*,†

Previous investigations characterizing the mechanism(s) of complement resistance in *Yersinia pseudotuberculosis* showed that the outer membrane protein Ail can functionally recruit the regulator of the classical and lectin pathways of complement, C4b-binding protein. In this study, we extend these observations and show that Ail can also recruit the regulator of the alternative pathway (AP), factor H (fH). Binding to fH was dependent on Ail expression and observed in the context of full-length LPS. Inactivation of *ail* resulted in loss of fH binding. Ail expression conferred resistance to AP-mediated killing. Bound fH was functional as a cofactor for factor I-mediated cleavage and inactivation of C3b. Ail alone is sufficient to mediate fH binding and resistance to AP-mediated killing, because Ail expression in a laboratory *Escherichia coli* strain conferred both of these phenotypes. Binding was specific and inhibited by increasing heparin and NaCl concentrations. Using a panel of fH recombinant fragments, we observed that both short consensus repeats 5–7 and 19–20 regions are responsible for mediating the interaction with Ail. Collectively, these results suggest that fH recruitment is an additional mechanism of complement resistance of Ail. Recruitment of both fH and C4BP by Ail may confer *Y. pseudotuberculosis* with the ability to resist all pathways of complement activation. *The Journal of Immunology*, 2012, 189: 3593–3599.

Within the Gram-negative genus *Yersinia*, three species are of medical importance for humans: *Y. pestis*, the causative agent of plague, and the two enteropathogens *Y. enterocolitica* and *Y. pseudotuberculosis*. Although *Y. pestis* is usually transmitted by the bite of an infected flea or inhalation of aerosolized droplets from a person with pneumonic plague, the enteropathogenic Yersiniae are ubiquitous in the environment and are transmitted by ingestion of contaminated food or water. Although *Y. pseudotuberculosis* is genetically closely related to *Y. pestis*, infections with the enteropathogenic Yersiniae are usually self-limiting. However, *Y. pseudotuberculosis* has been implicated as the causative agent of Far East Scarlet-like fever, a serious infection mimicking scarlet fever caused by group A streptococci (1).

Any microorganism that survives contact with human blood must possess mechanisms for evading complement, a key first-line innate immune defense against infection. Upon encountering an invader, complement is activated via one of three routes: the classical pathway, the lectin pathway, or the alternative pathway (AP). All three pathways converge at the C3 step, leading to covalent deposition of the opsonin C3b, generation of inflammatory anaphylatoxins C3a and C5a, and formation of the membrane attack complex (2).

All three pathogenic *Yersinia* species express a chromosomally encoded outer membrane protein, Ail, which was shown to mediate resistance to serum or complement-mediated killing (3–5). Ail belongs to a family of highly conserved 17–19-kDa outer membrane proteins found in the Enterobacteriaceae, including Rck (*Salmonella typhimurium* and *S. enteritidis*), PagC (typhoid and nontyphoid *Salmonella*, *Escherichia coli* O157:H7), and OmpX (*E. coli* and *Enterobacter cloacae*) (6, 7). The solved crystal structures of OmpX (8) and *Y. pestis* Ail (9) suggest that these proteins exhibit a common topology consisting of eight transmembrane amphipathic β-strands and four surface-exposed loops. The regions of greatest similarity are the transmembrane domains, whereas the greatest sequence diversity is found in the surface-exposed loops, which are likely responsible for interactions with the host.

The molecular mechanisms of Ail-mediated serum resistance have been extensively characterized in *Y. enterocolitica*. Biedzka-Sarek and colleagues (10, 11) demonstrated that Ail recruits C4BP and factor H (fH), which are fluid-phase complement-regulatory proteins of the classical/lectin and alternative pathways, respectively. These proteins normally act to prevent complement-mediated damage to host cells and tissues. However, this recruitment was observed only in the absence of LPS *O*-Ag or outer core. In contrast, the trimeric autotransporter protein YadA was shown to bind both C4BP and fH, regardless of LPS state. Therefore, YadA is the primary ligand for these proteins on *Y. enterocolitica*.

fH is a single-chain soluble glycoprotein ~150 kDa consisting of 20 short consensus repeats (SCRs). It is found in serum at a concentration of ~500 μg/ml. fH is the primary fluid-phase regulator of the AP, and its mechanism of action is three-fold: to accelerate the decay of the AP C3 convertase, C3bBb, to compete with factor B binding to C3b, and to act as a cofactor for factor I (fI)-mediated cleavage and inactivation of C3b (12). The end result is reduced C3b deposition on the target surface, ultimately blocking the opsonic, lytic, and proinflammatory actions of complement. Not surprisingly, a wide range of diverse bacterial pathogens possess the ability to recruit and exploit fH, facilitating resistance to

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Abbreviations used in this article: AP, alternative pathway; fH, factor H; fI, factor I; GVB, Veronal-buffered saline supplemented with 0.1% gelatin; HIS, heat-inactivated serum; LB, Luria–Bertani; NHS, normal human serum; SCR, short consensus repeat.

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complement attack (13). Disrupting a pathogen's ability to recruit fH is an attractive preventive measure, as demonstrated by the meningococcal vaccines currently in clinical trials, which specifically target fH binding (14).

Although a high degree of conservation is observed in the transmembrane regions of the Ail proteins from *Y. enterocolitica* and *Y. pseudotuberculosis*, considerable diversity can be found in the surface-exposed loops. Despite this, in previous investigations we observed that *Y. pseudotuberculosis* Ail, similar to its homolog in *Y. enterocolitica*, can bind C4BP in a functional manner (15). However, dissimilar to *Y. enterocolitica*, C4BP binding to *Y. pseudotuberculosis* Ail was unaffected by increasing heparin or NaCl concentrations (11, 15).

In this study, we extended our previous observations and demonstrate functional fH binding to *Y. pseudotuberculosis* Ail. Binding was observed in the presence of full-length LPS. Ail expression is associated with resistance to AP-mediated killing. fH bound to Ail can mediate C3b cleavage in the presence of fI. Ail expression in a laboratory *E. coli* strain conferred fH binding and resistance to AP-mediated killing. fH binding is specific, inhibited by increasing NaCl and heparin concentrations, and appears to be mediated by SCRs 5–7 and 19–20. Taken together, these results suggest that *Y. pseudotuberculosis* Ail can recruit fH as an additional complement-resistance mechanism along with C4BP recruitment, thus conferring this pathogen with resistance to all pathways of complement activation.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids are shown in Table I. For all experiments involving *Y. pseudotuberculosis*, bacteria were streaked from −70°C frozen stocks onto Luria–Bertani (LB) (Becton-Dickinson, Sparks, MD) plates. After ~48 h of growth at room temperature, a single colony was picked, inoculated into 5 ml LB broth, and grown overnight at 37°C with shaking. The following day, bacteria were subcultured into LB broth and grown for an additional 3–4 h at 37°C with shaking. *E. coli* was grown on LB plates or in broth at 37°C. Where appropriate, bacterial growth media were supplemented with ampicillin (100 μg/ml) or kanamycin (100 μg/ml). Plasmid pAY43 contains the *ail* gene from *Y. pseudotuberculosis* strain YPIII/pIB1 cloned into pBR322. pAY43 was a kind gift from Dr. Ralph Isberg (Tufts University, Boston, MA) and was described previously (4).

Sera, proteins, and Abs

Normal human serum (NHS) was pooled from blood samples collected from 7 to 10 healthy adult laboratory personnel. All persons who donated blood for this study provided a written informed consent. The study protocol was approved by the Section for Research of the Helsinki University Central Hospital Laboratory (project TYH7214). The blood was allowed to clot, and the sera were subsequently harvested, pooled, aliquoted, and stored at −70°C until used. Heat-inactivated serum (HIS) was generated by incubating NHS for 1 h at 56°C. Serum functional only in the AP (AP-only serum) was generated by supplementing NHS with EGTA and $MgCl_2$ (final concentrations 5 and 10 mM, respectively). Purified human C3b and fI were purchased from Complement Technology (Tyler, TX) and Calbiochem (San Diego, CA), respectively. Human fH was purified according to the protocol described earlier (16). BSA and heparin were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant fH constructs containing SCRs 1–5, 5–7, 8–11, 8–20, 11–15, 15–20, and 19–20 were produced as described previously (17–19). Monoclonal mouse anti-human fH 196X recognizing SCR domain 1 (20) was used as a primary Ab in FACS analysis. The appropriate Alexa Fluor-labeled secondary Abs were acquired from Invitrogen (Carlsbad, CA). Polyclonal goat anti-human fH antiserum (Calbiochem) was used in FACS analysis experiments, measurement of dissociation constants, and immunoblotting. HRP-conjugated secondary Abs were acquired from Jackson ImmunoResearch (West Grove, PA).

Measurement of dissociation constants

Y. pseudotuberculosis YPIII/pIB1, grown as described above, was resuspended to ~1×10^8 CFU/ml in PBS. Approximately 1×10^7 CFU was allowed to adhere to individual wells of a 96-well Nunc Maxisorp ELISA plate (Nunc, Roskilde, Denmark) overnight at 37°C. After washing, 50 μl purified human fH (in PBS) was added in doubling dilutions (highest concentration used: 500 nM fH) to the adhered bacteria for 30 min at room temperature. The wells were washed, and bound fH was detected with goat anti-fH antiserum, followed by washing and the addition of anti-goat HRP Abs. Blank wells and bacteria-only wells (no fH added) served as controls. All washing steps were performed with PBS/0.05% Tween 20. Wells were developed with OPD reagent (Dako), and the color reaction was measured at 492 nm in a Labsystems iEMS Reader MF plate reader (Labsystems, Helsinki, Finland). The amount of bound fH was quantified in accordance with a standard curve of bound fH. Data analysis and K_d determination were performed with GraphPad Prism (GraphPad Software, La Jolla, CA).

Serum bactericidal assay

Bacteria grown as described above were washed and resuspended in PBS to a final concentration ~1×10^5 CFU/ml. Bacterial suspensions were added to HIS or AP-only serum to a final serum concentration of 40% in a 100-μl reaction volume. Following a 30-min incubation at 37°C, the samples were placed on ice to stop further bacteriolysis. Serial dilutions of the samples in PBS were plated on LB agar plates and incubated overnight at 37°C in 5% CO_2 (*Y. pseudotuberculosis*) or room air (*E. coli*). Survival was determined by counting bacterial colonies the following day.

Flow cytometry

Bacteria grown as described above were centrifuged at 10,000 × *g* for 3 min and resuspended in PBS to a final concentration ~1×10^9 CFU/ml. Following an additional wash in PBS, an aliquot of the bacterial suspension was added to HIS or AP-only serum or mixed with purified human fH (final concentration specified in each experiment) to a final volume of 50 μl. Samples were incubated at 37°C for the indicated times, centrifuged, and washed three times in 50 μl PBS supplemented with 1% BSA. After the final wash, bacteria were resuspended in 50 μl PBS/1% BSA. Twenty microliters of a 1:100 dilution of the appropriate primary Ab (diluted in PBS) was added to the bacteria (final volume 70 μl) and incubated at room temperature for 20 min. After washing in PBS, bacteria were resuspended in 50 μl PBS, to which 20 μl 1:200 dilution of the appropriate Alexa Fluor 488-conjugated secondary Ab was added, followed by incubation at room temperature in the dark for 20 min. The cells were washed twice as above and resuspended in 0.5 ml filtered PBS containing 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). Flow cytometric analysis of 10,000 gated events was performed on a FACScan cytometer (BD Biosciences, San Jose, CA).

Cofactor activity for C3b cleavage

Y. pseudotuberculosis bacteria, grown as described above, were resuspended to a final concentration ~1×10^9 CFU/ml in PBS/1% BSA. The bacteria were then incubated with purified human fH at a final concentration of 10 μg/ml. Following a 30 min incubation at 37°C, the bacteria were washed five times in 150 μl PBS. After the last wash, bacteria were resuspended in PBS and incubated with ~50,000 cpm [^{125}I]C3b and 5 μg/ml fI (final reaction volume 25 μl). After a 1-h incubation at 37°C, the samples were centrifuged, and the supernatants were analyzed by SDS-PAGE under reducing conditions. The gels were subsequently dried, and the results were visualized by autoradiography from a phosphoimager plate.

Binding of fH fragments

E. coli BL21(DE3)/pAY43, grown as described above, were resuspended to a final OD_{600} of 1.0 in PBS. Equimolar amounts (67 nM) of purified fH or fH recombinant constructs were added to the bacteria (final volume 50 μl). The samples were incubated for 30 min at 37°C with agitation and washed thoroughly with PBS. Surface-bound proteins were eluted by resuspending the pellet in 20 μl 0.1 M glycine-HCl (pH 2.5), followed by incubation at 37°C for 20 min. Postcentrifugation, the supernatants were saved and neutralized by the addition of 3 μl 1 M Tris-HCl (pH 9.5). The neutralized supernatants were run on SDS-PAGE, followed by immunoblotting with a polyclonal goat anti-human fH antiserum (described above), which detects each fH construct.

Direct fH-binding assays

Direct fH-binding assays were performed, as described previously (21). Briefly, *E. coli* BL21(DE3)/pBR322 or *E. coli* BL21(DE3)/pAY43 were grown in LB medium with ampicillin, as described above. Thereafter, they were washed and resuspended in Veronal-buffered saline (142 mM NaCl, 1.8 mM sodium barbital, 3.3 mM barbituric acid [pH 7.4–7.6]) supplemented with 0.1% gelatin (GVB) to a final concentration of 1×10^9 CFU/ml. Twenty microliters of this solution was then incubated with 20 μl [^{125}I]

Table I. Bacterial strains and plasmids used in this study

Bacterial Strain	Description	Reference
Y. pseudotuberculosis		
YPIII/pIB1	Serotype O:3, wild type	(31)
YPIII/pIB1 *ail*	*ail::kan*, Km^R, derivative of YPIII/pIB1	(15)
YPIII	pYV-cured derivative of YPIII/pIB1	(31)
YPIII *ail*	*ail::kan*, Km^R, pYV-cured derivative of YPIII/pIB1	(15)
E. coli BL21(DE3)	Standard laboratory strain	Invitrogen, Carlsbad, CA
Plasmids		
pBR322	Standard cloning vector	Promega, Madison, WI
pAY43	pBR322 *ail*$^+$ from strain YPIII	(4)

fH (~20,000 cpm/sample) for 30 min at 37°C with agitation. After incubation, the samples were centrifuged through 250 μl 20% sucrose/GVB at 10,000 × *g* to separate free protein from protein bound to the bacteria. The supernatants and pellets were separated, and radioactivities were measured in a Wallac Wizard 3″ gamma counter (GMI, Minneapolis, MN). The ratio of bound to total radioactivity was then determined. Competition assays were performed by determining the percentage binding of [^{125}I]fH in the presence of increasing amounts of unlabeled fH, heparin, or NaCl. BSA was excluded from these assays as an irrelevant protein competitor to fH binding, because BSA was used as a blocking agent in the primary Ab-incubation step for detection of fH binding by flow cytometry (see above).

Results

Ail expression mediates resistance to AP-mediated killing

Previous work by Yang et al. (4) demonstrated that Ail can confer serum resistance to a pYV-cured (*yadA*$^-$) serotype O:3 strain of *Y. pseudotuberculosis*, YPIII, and that complementation with plasmid-expressed Ail in the *ail* knockout strain restored serum resistance (strains described in Table I). These assays were performed in NHS, where all pathways of complement are active. To test the possibility that Ail can mediate resistance to serum killing in the presence of the AP only, we incubated mid-log phase bacteria in serum treated with EGTA and Mg^{2+}, which inactivates the classical and lectin pathways but leaves the AP intact. As shown in Fig. 1, wild-type strain YPIII/pIB1 survived in 40% EGTA/Mg^{2+} serum (AP only), whereas >1 log of killing was observed in the absence of Ail. Bacteria incubated in HIS supplemented with EGTA/Mg^{2+} displayed equivalent survival as bacteria incubated in HIS alone (data not shown). To test the possible contribution of YadA to resistance under these conditions, the same assays were performed using a pYV-cured strain. The absence of YadA had no effect on serum resistance, whereas the *yadA*$^-$/*ail*$^-$ strain exhibited killing similar to the *ail*$^-$ (YadA$^+$) strain (Fig. 1). The modest killing observed in the absence of Ail is likely due to the relatively inefficient ability of the AP alone to kill *Y. pseudotuberculosis* without contribution from the other pathways. Taken together, these results suggest that Ail can mediate resistance to AP-mediated killing, regardless of the presence of YadA.

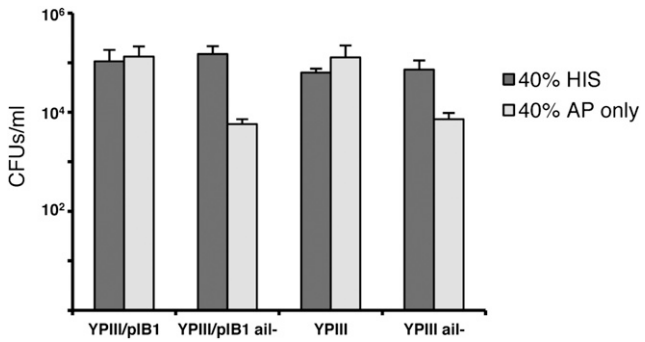

FIGURE 1. Resistance of *Y. pseudotuberculosis* to AP-mediated serum bactericidal activity. Approximately 1 × 10^5 CFU/ml of a mid-log phase broth culture of *Y. pseudotuberculosis* was incubated with 40% HIS or 40% AP-only serum (serum supplemented with 5 mM EGTA and 10 mM $MgCl_2$) in PBS for 30 min at 37°C. The reaction tubes were placed on ice, serially diluted, and plated on LB agar. Following an overnight incubation at 37°C/5% CO_2, bacterial survival was enumerated by colony counting. Data are shown as means of three experiments ± SD. YPIII, Ail$^+$ *yadA*$^-$ strain; YPIII ail$^-$, *ail*$^-$ *yadA*$^-$ strain YPIII/pIB1, wild-type strain; YPIII/pIB1 ail$^-$, *ail*$^-$ YadA$^+$ strain.

Ail expression confers factor H binding

The ability of Ail to mediate resistance to AP-mediated killing suggests the possibility that Ail can recruit fH, the key fluid-phase regulator of the AP. As shown in Fig. 2A, by FACS analysis we observed binding of fH to wild-type bacteria following a 30 min incubation in 40% HIS. No binding was observed when bacteria were incubated in PBS alone or in the absence of Ail expression. Binding of fH was dose dependent, because the addition of increasing amounts of purified fH led to increased binding (Fig. 2B). No binding to fH was observed in the absence of Ail, even at the highest fH concentration tested. Further characterization of this interaction was performed by testing the binding of purified fH dilutions to whole cells of immobilized bacteria. Based on a standard curve of bound fH, the calculated apparent K_d was 2.26 × 10^{-7} M (Fig. 2C). Ail-dependent binding of purified fH was also observed in strains PB1 and 2812/79 (serotypes O:1a and O:1b, respectively; data not shown). These results suggest that Ail can bind fH and that binding is dose dependent. Furthermore, the loss of fH binding in the presence of YadA but the absence of Ail suggests that YadA is not a fH receptor on *Y. pseudotuberculosis*, in contrast to the results observed with *Y. enterocolitica* (10).

Ail-bound fH promotes C3b cleavage

One of the main functions of fH is to act as a cofactor for fI-mediated cleavage and inactivation of C3b. Thus, we tested the possibility that fH bound to Ail is functional in this manner. As shown in Fig. 3, the incubation of purified, ^{125}I-labeled C3b with both fH and fI led to the generation of the α′-chain cleavage fragments of 67 and 41 kDa. These cleavage products were also observed with strain YPIII/pIB1 after preincubation with fH, washing, and addition of fI and C3b. Minimal cleavage was observed in the *ail*$^-$ strain after a similar treatment. Omission of fI prevented the cleavage of C3b, suggesting that the bacteria do not possess intrinsic protease activity against C3b. These results suggest that fH bound to Ail is functional in fI-mediated cleavage of C3b.

Ail expression in E. coli *confers resistance to AP-mediated killing and fH binding*

We next tested the possibility that fH binding and resistance to AP-mediated killing are independent of any other *Y. pseudotuberculosis*-specific factors. *E. coli* BL21(DE3) expressing Ail (pAY43)

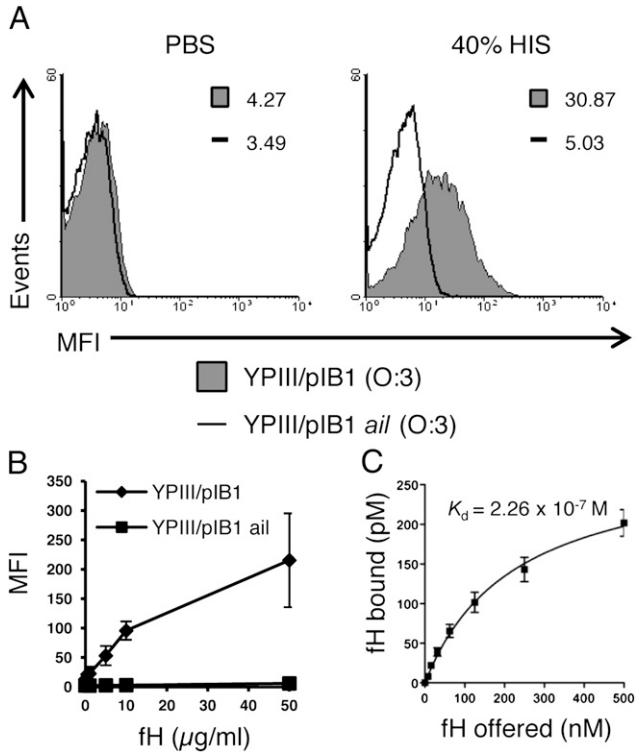

FIGURE 2. *Y. pseudotuberculosis* Ail binds fH. (**A**) Binding of fH to Ail from HIS. Approximately 1×10^8 CFU of bacteria grown to mid-log phase were washed in PBS and subsequently mock treated (PBS only) or incubated with 40% HIS for 30 min at 37°C. Bound fH was detected with monoclonal anti-human fH Ab 196X, followed by FACS analysis. Mean fluorescence intensity (MFI) values are indicated in each panel. Representative data are shown. (**B**) fH binding to Ail is dose dependent. Same experimental protocol as in (A), except that increasing amounts of purified human fH were used instead of HIS. Bound fH was detected with a goat anti-fH antiserum, followed by FACS analysis. Data are means ± SD of three experiments. (**C**) K_d of fH binding to Ail. Bacteria were immobilized on microtiter wells and incubated with varying amounts of purified fH, followed by detection with goat anti-fH antiserum and anti-goat HRP Abs. Bound fH was quantified in accordance with a standard curve of fH. Nonlinear fitting analysis and K_d determination were performed with GraphPad Prism. Data are mean ± SD of two experiments performed in triplicate wells.

was incubated for 30 min in HIS or EGTA/Mg^{2+} serum, whereas bacteria harboring the empty vector (pBR322) served as a control. As shown in Fig. 4A, bacteria expressing Ail exhibited complete survival, whereas bacteria expressing empty vector exhibited

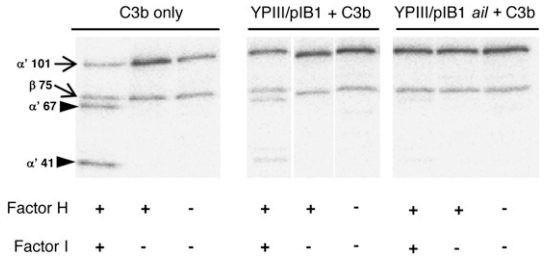

FIGURE 3. Cofactor assay for C3b cleavage. Bacteria grown as in Fig. 2 were incubated with purified fH (10 μg/ml) for 30 min at 37°C and washed. After the final wash, fI (5 μg/ml) and [^{125}I]C3b (50,000 cpm) were added. The presence or absence of fH and fI for each sample is indicated. After a 1-h incubation at 37°C, samples were analyzed with reducing SDS-PAGE and visualized by autoradiography. The α′ − and β-chains of C3b and the C3b α′ − chain cleavage products are indicated by arrows and arrowheads, respectively. Representative data of an experiment performed three times are shown.

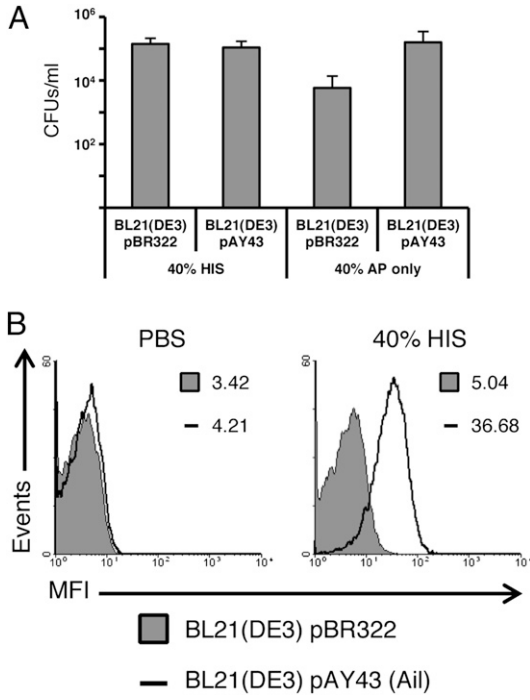

FIGURE 4. Expression of Ail in *E. coli* BL21(DE3) is associated with fH binding and resistance to AP-mediated killing. (**A**) Ail expression in *E. coli* BL21(DE3) is associated with resistance to AP-mediated killing. Approximately 1×10^5 CFU of BL21(DE3)/pBR322 and BL21(DE3)/pAY43 (Ail-expressing) from an overnight LB culture were washed in PBS, followed by incubation with either 40% HIS or AP-only serum for 30 min at 37°C. Bacterial survival was enumerated by plating on LB agar and counting colonies the following day. Data (mean ± SD) are from an experiment performed three times. (**B**) Ail expression confers fH binding. Bacteria grown as in (A) were either mock treated (PBS only) or incubated with 40% HIS for 30 min at 37°C. Bound fH was detected with mAb 196X, followed by FACS analysis.

a loss of viability ~1 log, similar to the results observed with *Y. pseudotuberculosis* (Fig. 1). By FACS analysis, we observed that Ail expression also conferred fH binding from HIS, whereas no binding was observed in PBS or in the presence of empty vector (Fig. 4B). Taken together, these results suggest that Ail expression can confer fH binding and resistance to AP-mediated killing independently of other *Y. pseudotuberculosis* factors.

fH binding to Ail is mediated by SCRs 5–7 and 19–20

Recombinant constructs of fH were used to determine which region (s) of fH was responsible for the interaction with Ail. Equimolar concentrations (67 nM) of fH constructs spanning SCRs 1–5, 5–7, 8–11, 8–20, 11–15, 15–20, and 19–20 were incubated with *E. coli* BL21 (DE3) expressing Ail, followed by elution at low pH. The eluates were neutralized and analyzed by SDS-PAGE, followed by immunoblotting with a goat anti-human fH antiserum. As shown in Fig. 5, we observed binding between Ail and fH constructs containing SCRs 1–5, 5–7, and 19–20. Consistent with these observations, binding to constructs containing SCRs 15–20 and 8–20 were also observed. As controls, bacteria were incubated in PBS alone or with purified fH. These results suggest that SCRs 5–7 and 19–20 are mediating the binding to Ail, which is consistent with observations from several other fH-binding pathogens (22).

fH-binding competition assays

To further characterize the interaction between fH and Ail, we tested the effects of increasing concentrations of unlabeled fH, heparin, and NaCl on binding of ^{125}I-labeled fH. We first con-

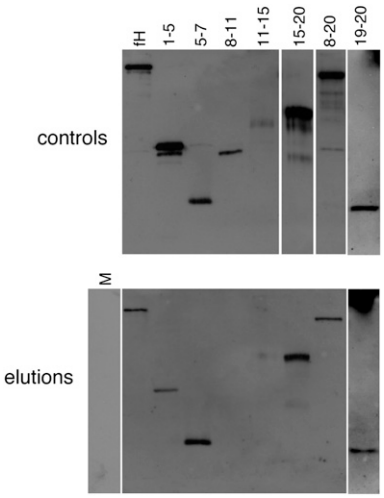

FIGURE 5. fH SCRs 5–7 and 19–20 mediate the interaction with Ail. Mapping of the Ail binding site on fH. Bacteria grown as in Fig. 4 were incubated with equimolar (67 nM) amounts of purified fH or recombinant fH constructs for 30 min at 37°C. Bacteria were washed, and bound protein was eluted under acid conditions. Samples were neutralized and analyzed by SDS-PAGE, followed by immunoblotting with a goat polyclonal anti-human fH antiserum. The *upper panel* is an immunoblot of equimolar amounts of purified fH and fH recombinant constructs. The labels above the blot in the *upper panel* correspond with the blot in the *lower panel*. Representative data are shown. M, PBS-only–treated bacteria.

firmed that binding of [^{125}I]fH was dependent on the presence of Ail. As shown in Fig. 6A, *E. coli* BL21(DE3) expressing Ail bound [^{125}I]fH, whereas minimal binding was observed by bacteria carrying an empty vector. In Fig. 6B–D, increasing amounts of unlabeled fH, heparin, and NaCl were able to compete with Ail binding to [^{125}I]fH. These results suggest that fH binding to Ail is specific, sensitive to increasing heparin concentrations (consistent with the SCR-mapping experiments, because heparin binds fH via SCRs 7 and 20), and mediated by ionic interactions.

Discussion

In this study, we showed that the outer membrane protein Ail of *Y. pseudotuberculosis* can functionally recruit the principal fluid-phase regulator of the AP, fH. Binding to fH was observed in the context of full-length LPS and was independent of the presence of YadA. Ail expression was associated with resistance to AP-mediated killing and C3b deposition. Bound fH was functional as a cofactor for fI-mediated cleavage and inactivation of C3b. Expression of Ail in *E. coli* conferred both fH binding and resistance to AP-mediated killing. Binding is specific, sensitive to increasing heparin and NaCl concentrations, and appears to be mediated by SCRs 5–7 and 19–20. These results suggest that fH binding is an additional serum-resistance mechanism in *Y. pseudotuberculosis*.

The molecular mechanisms of serum resistance in *Y. enterocolitica* have been characterized extensively. Both Ail and the trimeric autotransporter protein YadA possess functional C4BP- and fH-binding ability. However, YadA is the primary receptor for these regulatory proteins, because Ail cannot bind in the presence of full-length LPS (10, 11). Although the Ail proteins of *Y. enterocolitica* and *Y. pseudotuberculosis* are homologs, considerable diversity is observed in the surface-exposed loops, which are likely responsible for interactions with host components. Although both proteins can mediate serum resistance, it was somewhat surprising to observe conserved mechanisms between species. Nevertheless, it is important to note several differences between *Y. enterocolitica* and *Y. pseudotuberculosis*. In *Y. enterocolitica*, YadA is able to bind both C4BP and fH. In contrast, in *Y. pseudotuberculosis*, Ail appears to be solely responsible for binding of these regulatory proteins, because the absence of Ail expression results in the loss of both C4BP (15) and fH binding (Fig. 2). Differences in the nature of binding were also observed. C4BP binding to *Y. enterocolitica* Ail is sensitive to both increasing NaCl and heparin concentrations and appears to be mediated by SCRs 1–3 (11), whereas the C4BP interaction in *Y. pseudotuberculosis* is unaffected by increasing NaCl and heparin concentrations and appears to be mediated by SCRs 6–8 (15). In both *Y. enterocolitica* and *Y. pseudotuberculosis*, fH binding is sensitive to increasing NaCl and heparin concentrations, whereas SCRs 6–7 appear to

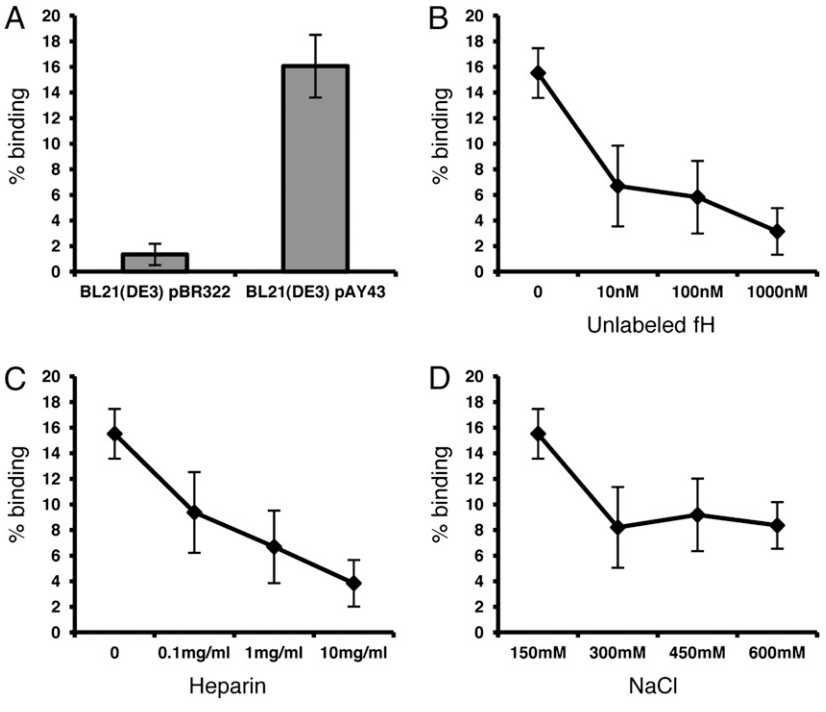

FIGURE 6. fH binding to Ail is specific and sensitive to increasing heparin or salt concentrations. Direct binding of [^{125}I]fH to Ail was performed by incubating ~1 × 10^8 CFU of BL21(DE3)/pBR322 or BL21(DE3)/pAY43 in GVB with [^{125}I]fH (~20,000 cpm/sample). The ratio of bound to total radioactivity was then determined (percentage binding). Data are mean ± SD of four independent experiments. (**A**) Expression of Ail correlates with binding to [^{125}I]fH. (**B**) Binding of fH to Ail is specific, because increasing amounts of unlabeled fH can compete with binding to [^{125}I]fH. Increasing amounts of heparin (**C**) and NaCl (**D**) reduce binding of fH to Ail.

mediate fH binding in *Y. enterocolitica*, and SCRs 5–7 and 19–20 mediate binding in *Y. pseudotuberculosis*.

The relative affinity of C4BP for Ail appears to be greater than that of fH, because the K_d value for the Ail–C4BP interaction is 4.5×10^{-9} M (15), whereas it is 2.26×10^{-7} M for the Ail–fH interaction. This increased affinity may be due to multivalent binding of C4BP via its multiple α-chains. Resistance to the AP also appears to be less critical for survival compared with all pathways combined, because ∼1 log of killing was observed with strain YPIII/pIB1 *ail*$^-$ in AP-only serum (Fig. 1) compared with the complete killing (∼7 logs) observed with the same strain in NHS (15). This suggests that, despite possessing a poorer ability to bind fH compared with C4BP, under AP-only conditions there are fewer C3 convertases and C3b molecules to inactivate. Nevertheless, it is likely that, in the host environment, both C4BP and fH binding act synergistically and that fH binding by Ail inhibits the ability of the AP to amplify the remaining C3b generated by the classical pathway.

Initial observations on the Ail family of outer membrane proteins suggested that these proteins mediated dissimilar functions, based on the sequence diversity found in the surface-exposed loops. However, as the molecular mechanisms of these proteins become characterized, it is apparent that conserved functions can be assigned to several members of this family. Rck, PagC (*Salmonella choleraesuis*), and Ail were shown to mediate serum resistance (3–5, 23, 24), whereas adhesion and/or invasion properties have been observed in all of the aforementioned proteins, with the exception of *Y. pseudotuberculosis* Ail (25–29). Furthermore, functional fH and C4BP binding have now been described in Rck (21, 30) and *Y. enterocolitica* and *Y. pseudotuberculosis* Ail (10, 11, 15). Future characterization of PagC from other species (*Salmonella typhi*, *E. coli* O157:H7) will be necessary to determine whether these proteins can also mediate serum resistance, and if so, by the mechanisms described above. If this is the case, it suggests the possibility that conserved structural epitopes exist in these proteins independent of amino acid sequence. Furthermore, therapeutic or preventive measures effective against one protein may be effective against homologous proteins.

The end result of fH regulation of complement activation is reduced C3b deposition on the target surface. While attempting to measure C3b deposition on the surface of YPIII/pIB1, we observed that the bacteria were positive for C3 after incubation in HIS (data not shown). This suggests that the bacteria are able to bind C3 in the absence of complement activation. The absence of Ail expression resulted in the loss of C3 binding. Based on these results, it is possible that direct binding to C3 is yet another complement-resistance mechanism mediated by Ail. These observations preclude our ability to accurately measure fH-mediated inhibition of C3b deposition and warrant further investigation beyond the scope of this study.

The results presented in this article indicate that the outer membrane protein Ail from *Y. pseudotuberculosis* can functionally recruit the key fluid-phase regulator of the AP, fH. In concert with C4BP recruitment (15), fH recruitment confers Ail with the ability to mediate resistance to all pathways of complement activation, thus increasing the ability of this pathogen to establish a successful infection.

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Disclosures

The authors have no financial conflicts of interest.

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