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Editor's Choice

Yersinia pestis uses the Ail outer membrane protein to recruit vitronectin

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Yersinia pestis, the agent of plague, requires the Ail (attachment invasion locus) outer membrane protein to survive in the blood and tissues of its mammalian hosts. Ail is important for both attachment to host cells and for resistance to complement-dependent bacteriolysis. Previous studies have shown that Ail interacts with components of the extracellular matrix, including fibronectin, laminin and heparan sulfate proteoglycans, and with the complement inhibitor C4b-binding protein. Here, we demonstrate that Ail-expressing *Y. pestis* strains bind vitronectin – a host protein with functions in cell attachment, fibrinolysis and inhibition of the complement system. The Ail-dependent recruitment of vitronectin resulted in efficient cleavage of vitronectin by the outer membrane Pla (plasminogen activator protease). *Escherichia coli* DH5α expressing *Y. pestis* Ail bound vitronectin, but not heat-treated vitronectin. The ability of Ail to directly bind vitronectin was demonstrated by ELISA using purified refolded Ail in nanodiscs.

INTRODUCTION

Yersiniae are Gram-negative bacteria of the family *Enterobacteriaceae* (Putzker *et al.*, 2001). The genus *Yersinia* includes three species associated with human disease. The enteropathogenic yersiniae (*Yersinia enterocolitica* and *Yersinia pseudotuberculosis*) are associated with gastrointestinal disease and are spread via the faecal–oral route. In contrast, *Yersinia pestis* is the agent of plague and is transmitted via the bite of infected fleas (bubonic plague or septicaemic plague) or via aerosols (pneumonic plague) (Perry & Fetherston, 1997). The three human pathogenic yersiniae share a number of critical virulence attributes including a virulence plasmid pCD1-encoded type III secretion system (T3SS) (Forsberg *et al.*, 1994) and an Ail (attachment invasion locus) outer membrane protein (Miller *et al.*, 1990). The T3SS mediates the cell-contact-dependent injection of Yop (*Yersinia* outer protein) effectors into targeted host cells. The injected Yop effectors function to block bacterial phagocytosis and to suppress the production of pro-inflammatory cytokines (Viboud & Bliska, 2004). Importantly, the specificity of the *Yersinia*–host cell interaction is largely determined by bacterial adhesins that recognize defined host cell receptors (Simonet *et al.*, 1996). The enteropathogenic yersiniae express two dominant adhesins/invasins (YadA and Invasin) that are required for efficient cell attachment and subsequent Yop injection (Grosdent *et al.*, 2002). *Y. pestis* does not express YadA or Invasin (Rosqvist *et al.*, 1988; Simonet *et al.*, 1996; Skurnik & Wolf-Watz, 1989), but expresses other adhesins, including Ail, Pla (plasminogen activator protease) and pH6 antigen (Kolodziejek *et al.*, 2007; Lindler & Tall, 1993; Sodeinde *et al.*, 1992). Ail is expressed by all three human pathogenic yersiniae, but appears to play a more significant role in the virulence of *Y. pestis*, in part due to the lack of functional YadA and Invasin (Felek *et al.*, 2010; Hinnebusch *et al.*, 2011; Wachtel & Miller, 1995). Importantly, *Y. pestis* Ail has established roles in cell invasion, cell attachment, Yop injection, serum (complement) resistance and virulence (Bartra *et al.*, 2008; Felek *et al.*, 2010; Hinnebusch *et al.*, 2011; Kolodziejek *et al.*, 2010).

Structural studies have demonstrated that *Y. pestis* Ail (Marassi *et al.*, 2015; Yamashita *et al.*, 2011) forms an eight-stranded antiparallel β-barrel with four extracellular loops that closely resembles the structure of *Escherichia coli* OmpX (Vogt & Schulz, 1999). The extracellular loops mediate interactions with components of the extracellular

Abbreviations: C4BP, C4b-binding protein; ECM, extracellular matrix; HBS, heparin-binding site; HIS, heat-inactivated sera; MAC, membrane attack complex; NHS, normal human sera; PAI, plasminogen activator inhibitor; T3SS, type III secretion system.

matrix (ECM) (fibronectin, laminin and heparan sulfate proteoglycans) and complement system [C4b-binding protein (C4BP)] that contribute to bacterial attachment/invasion and serum resistance, respectively (Ding *et al.*, 2015; Ho *et al.*, 2014; Tsang *et al.*, 2010; Yamashita *et al.*, 2011). Co-crystallization studies with a heparin analogue in conjunction with site-directed mutagenesis identified two heparin-binding sites (HBS) on Ail formed by residues from extracellular loops 2 and 3 (HBS-1) and extracellular loop 1 (HBS-2) (Yamashita *et al.*, 2011). Mutagenesis studies performed on *Y. enterocolitica* Ail indicate that residues critical for both cell attachment and serum resistance map to extracellular loops 2 and 3, and appear to cluster around a hydrophobic cleft on the extracellular surface of Ail – a potential site for binding of ECM proteins and/or C4BP (Miller *et al.*, 2001; Yamashita *et al.*, 2011).

Previous studies have demonstrated that *Y. pestis* Ail directly binds the ECM proteins fibronectin and laminin, as well as the complement regulatory component C4BP. Another study suggested that *Y. pestis* can recruit vitronectin to its cell surface (Duensing *et al.*, 1999). Bacterial pathogens that recruit ECM proteins as well as C4BP and/or Factor H to their cell surface, such as *Haemophilus influenzae* (Hallström *et al.*, 2007), *Neisseria gonorrhoeae* (Ram *et al.*, 1999), *Neisseria meningitidis* (Lewis & Ram, 2014) and *Moraxella catarrhalis* (Bernhard *et al.*, 2014; Nordström *et al.*, 2004), have also been shown to recruit vitronectin (Attia *et al.*, 2006; Duensing & van Putten, 1997; Hallström *et al.*, 2006, 2011; Sa E Cunha *et al.*, 2010), indicating that acquisition of vitronectin can be advantageous to pathogens even in the presence of other ECM proteins and complement regulatory factors. Vitronectin is a multifunctional glycoprotein found abundantly in serum and the ECM (Singh *et al.*, 2010). Circulating vitronectin is normally a monomer, whereas cell-bound vitronectin is typically multimeric. Vitronectin plays a critical role in many biological processes, including cell adhesion, fibrinolysis, cell migration and regulation of membrane attack complex (MAC) formation. Bacterial pathogens bind vitronectin to their surface in order to enhance adhesion to host cells and tissues as well as to protect them from MAC-mediated lysis (Singh *et al.*, 2010). In this study, we demonstrate that *Y. pestis* actively recruits vitronectin to its surface. Furthermore, we demonstrate that the acquisition of vitronectin is dependent upon the Ail outer membrane protein.

METHODS

Bacterial strains and growth conditions. All *Y. pestis* strains used in this study, including KIM5 (pPCP1$^+$; pCD1$^+$), KIM5 Δ*ail* (Bartra *et al.*, 2008), KIM8-E (pPCP1$^-$; pCD1$^+$ Δ*yopE–sycE*::*dhfr*) (Bartra *et al.*, 2006) and KIM8-E Δ*ail* (Bartra *et al.*, 2008) are Pgm$^-$ and avirulent from peripheral routes of infection (Une & Brubaker, 1984). These strains and their derivatives were routinely grown in heart infusion broth (HIB) supplemented with 2.5 mM $CaCl_2$ or on Tryptose Blood Agar Base plates (Difco) at 27 or 37 °C. In Yop secretion assays, *Y. pestis* strains were grown in TMH media as described previously (Bartra *et al.*, 2006). *E. coli* DH5α (Cambau *et al.*, 1993) and derivatives were grown at 37 °C in LB or HIB. When appropriate, media was supplemented with kanamycin (25 μg ml^{-1}) or ampicillin (50 μg ml^{-1}).

Construction of plasmids pFLAG-Ail and pFLAG-OmpX. DNA fragments encoding the mature portion of *Y. pestis* Ail (y1324; residues 24–194) and OmpX (y1682; residues 25–176) were amplified with oligonucleotides Ail-*Hin*dIII-F, Ail-*Bgl*II-R, OmpX-*Hin*dIII-F and OmpX-*Bgl*II-R. The resulting DNA fragments were digested with *Hin*dIII and *Bgl*II, and inserted into plasmid pFLAG-ATS (Sigma), generating plasmids pFLAG-Ail and pFLAG-OmpX, which direct the expression of N-terminal FLAG-tagged Ail and OmpX.

Bacterial binding of vitronectin. Bacteria were grown in HIB containing 2.5 mM $CaCl_2$ for 5 h at 37 °C. HIB-grown bacteria were washed in ice-cold PBS and suspended in PBS to OD_{620} 5.0. Washed bacteria (250 μl) were added to an equal volume of normal human sera (NHS), heat-inactivated sera (HIS) or purified vitronectin (50 μg ml^{-1}) (Sigma). Binding reactions were incubated for 2 h at 4 °C or 1 h at 37 °C. Bacteria and co-sedimenting proteins were pelleted by centrifugation at 10 000 ***g*** for 5 min at 4 °C, washed three times with 1 ml ice-cold PBS containing 0.05 % Tween-20 and lysed by boiling in 100 μl SDS-PAGE sample buffer (50 mM Tris/HCl, 2 % SDS, 5 % glycerol, 1 % β-mercaptoethanol, pH 6.8). Bacterial lysates and co-sedimenting proteins were subjected to SDS-PAGE and immunoblot analysis with mouse monoclonal anti-vitronectin (VIT-2; Sigma), rabbit polyclonal anti-vitronectin (Sigma), anti-Ail (Ding *et al.*, 2015) or anti-FLAG M2 (Sigma) antibodies.

Proteolysis of vitronectin by Pla-expressing *Y. pestis*. Bacteria were grown in HIB containing 2.5 mM $CaCl_2$ for 5 h at 37 °C. HIB-grown bacteria were washed in ice-cold PBS and suspended in PBS to OD_{620} 10.0. Washed bacteria (300 μl) were added to an equal volume of purified vitronectin (50 μg ml^{-1}). Reactions were incubated for 4 h at 37 °C. Samples (50 μl) were removed at 0, 0.5, 1, 2, 3 and 4 h and placed on ice. Samples were subjected to SDS-PAGE and immunoblot analysis with a mouse monoclonal anti-vitronectin antibody.

Flow cytometric analysis of vitronectin binding. Flow cytometry to detect complement component binding to bacteria was performed as described previously (Ram *et al.*, 2001). Briefly, 3×10^7 bacteria (*Y. pestis* KIM5 or KIM5 Δ*ail*) were incubated with purified human monomeric vitronectin (Innovative Research) or C4BP (both from Complement Technologies) at a final concentration of 5 μg ml^{-1} for 1 h at 37 °C. After three washes, antibodies against each of the proteins (anti-vitronectin mAb) (Quidel) and anti-C4BP mAb 104 (Härdig *et al.*, 1997) were added (1 : 100 dilution in Hank's balanced salt solution) followed by FITC-conjugated anti-mouse IgG. Data were analysed using FlowJo 7.2.5 data analysis software (TreeStar).

Binding of purified Ail-containing nanodiscs to vitronectin. The preparation of recombinant C-terminal His-tagged Ail (Ail-His) and its incorporation in lipid bilayer nanodiscs was as described previously (Ding *et al.*, 2015). ELISAs were performed with antibody-conjugated horseradish peroxidase and its substrate *o*-phenylenediamine (Pierce) added at a concentration of 0.5 mg ml^{-1} in stable peroxide substrate buffer (Thermo Scientific). Human plasma fibronectin (Sigma; F2006) or vitronectin (Sigma) was coated on 96-well plates (Nunc) at a concentration of 20 μM in PBS, which corresponds to 5 μg fibronectin ml^{-1} and 0.6 μg vitronectin ml^{-1}. After coating overnight at 4 °C, the wells were washed three times with PBS, then blocked with Tris-buffered saline (TBS) containing 3 % milk for 2 h at room temperature, and finally washed with TBST (TBS with 0.05 % Tween-20 included to prevent non-specific binding). Incremental concentrations of nanodisc-incorporated Ail-His in TBST were added to the coated wells and the plates were incubated at 37 °C for 3 h and then at 4 °C overnight. Bound Ail-His was probed by adding mouse

anti-His mAb (Qiagen; 1 : 100 dilution in TBST-milk) to the wells and incubating for 2 h at room temperature. Unbound primary antibody and Ail were removed by washing three times with TBST-milk. Then, secondary goat anti-mouse antibody conjugated to horseradish peroxidase (Sigma; 1 : 10 000 dilution) was added, the plates were then incubated for 1 h at room temperature, and finally washed three times with PBS and once with TBS, before adding fresh *o*-phenylenediamine to develop A_{490}.

RESULTS

Binding of vitronectin to Ail-expressing *Y. pestis*

Y. pestis Ail has been shown to interact with fibronectin, laminin and C4BP; however, the interaction of Ail with vitronectin has not been examined. To determine whether *Y. pestis* interacts with serum vitronectin, *Y. pestis* KIM8-E (Pla– Ail+) and *Y. pestis* KIM8-E Δ*ail* (Pla– Ail–) were incubated with NHS or HIS at 4 °C and evaluated for co-sedimentation of vitronectin with the bacteria by immunoblotting with a monoclonal anti-vitronectin antibody (Fig. 1a). Immunoreactive bands corresponding in size to the characteristic 75 and 65 kDa bands of vitronectin were observed in samples containing the Ail-expressing bacteria (KIM8-E) incubated with NHS; however, no vitronectin bands co-sedimented with the *ail* deletion mutant (KIM8-E Δ*ail*) or with bacteria incubated with HIS. Complementation of the *ail* deletion mutant with plasmid pFLAG-Ail (Bartra *et al.*, 2008), which expresses functional N-terminal FLAG-tagged Ail, restored binding of vitronectin (Fig. 1a).

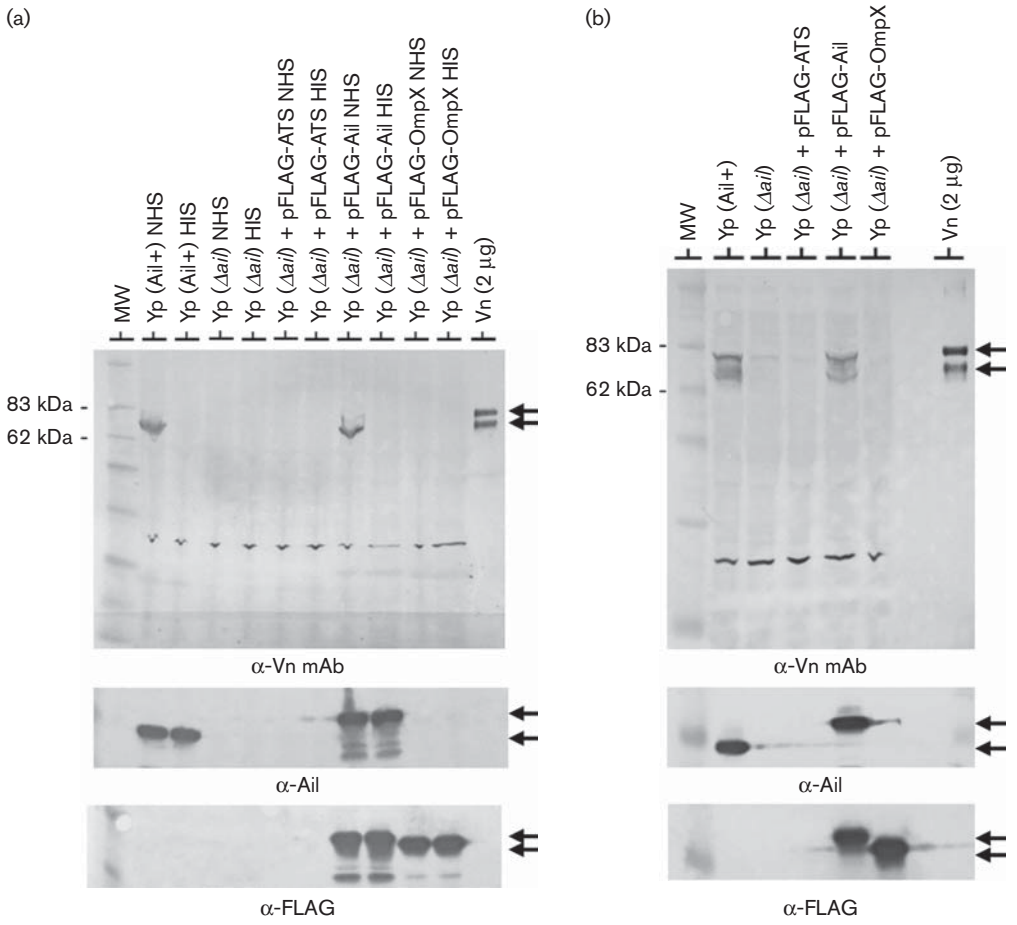

Fig. 1. *Y. pestis* expressing Ail binds vitronectin. (a) Co-sedimentation of vitronectin from NHS or HIS with *Y. pestis* KIM8-E (Yp Ail+) and *Y. pestis* KIM8-E Δ*ail* (Yp Δ*ail*). Bacteria were incubated with 50 % NHS or HIS at 4 °C for 2 h. Washed cells and co-sedimenting proteins were subjected to SDS-PAGE and immunoblotting with VIT-2 anti-vitronectin mAb (α-Vn mAb), anti-FLAG M2 mAb (α-FLAG) or anti-Ail (α-Ail) antisera. The 65 and 75 kDa vitronectin bands are marked by arrows. Complementation of *Y. pestis* KIM8-E Δ*ail* with plasmid pFLAG-Ail restores expression of Ail and binding of vitronectin. In contrast, expression of the pFLAG-ATS vector or pFLAG-OmpX does not restore vitronectin binding. (b) Co-sedimentation of purified monomeric vitronectin with *Y. pestis* KIM8-E (Yp Ail+) and *Y. pestis* KIM8-E Δ*ail* (Yp Δ*ail*). Bacteria were incubated with pure vitronectin (50 μg ml^{-1}) at 37 °C for 1 h and washed cells subjected to immunoblot analysis with anti-vitronectin mAb (α-Vn mAb), FLAG M2 mAb (α-FLAG) or anti-Ail (α-Ail) antisera. MW, molecular mass standard.

Providing the pFLAG-ATS vector or pFLAG-OmpX, which expresses FLAG-tagged *Y. pestis* OmpX (y1682), did not re-establish vitronectin binding. These results suggested that *Y. pestis* recruits vitronectin to its surface in an Ail-dependent manner and that heat inactivation of NHS denatures or alters the structure of vitronectin in a manner that interferes with its binding to Ail.

Binding of purified vitronectin to Ail-expressing *Y. pestis*

Vitronectin is known to interact with other serum components such as plasminogen activator inhibitor (PAI)-1 (Salonen *et al.*, 1989) as well as complement components C5b–7 and C9 (Milis *et al.*, 1993). To verify that the interaction of vitronectin with Ail occurs independent of these and other serum components, we examined the binding of purified vitronectin to *Y. pestis*. Purified vitronectin co-sedimented with *Y. pestis* expressing native Ail (KIM8-E) or FLAG-tagged Ail (Fig. 1b), but did not interact with KIM8-E Δ*ail* or KIM8-E Δ*ail* carrying pFLAG-ATS or pFLAG-OmpX, indicating that Ail-expressing *Y. pestis* recognize and directly bind vitronectin.

Binding of vitronectin to Ail-expressing *E. coli*

To confirm that the binding of vitronectin to *Y. pestis* Ail is direct and is not dependent upon other *Y. pestis* surface components, we examined the binding of purified vitronectin to *E. coli* DH5α as well as to *E. coli* DH5α expressing FLAG-tagged Ail (Fig. 2a). Vitronectin did not bind to *E. coli* DH5α or DH5α carrying pFLAG-ATS or pFLAG-OmpX, but did co-sediment with *E. coli* DH5α expressing FLAG-tagged Ail, confirming that Ail binds vitronectin independent of other *Y. pestis*-specific surface components or structures. Heat-treated (56 °C for 30 min) purified vitronectin did not interact with *E. coli* DH5α or DH5α expressing native Ail or FLAG-tagged Ail (Fig. 2b), confirming that heat treatment alters the conformation of vitronectin in a manner that prevents recognition by Ail.

Binding of vitronectin to Pla-expressing *Y. pestis*

Previous studies by Haiko *et al.* (2010) demonstrated that *Y. pestis* Pla degrades the serum PAI-1/vitronectin complex. To investigate the role of Pla in the interaction of Ail with vitronectin, the NHS vitronectin-binding profile of (Pla+ Ail+) *Y. pestis* KIM5 was compared with those of *N. gonorrhoeae* and *N. meningitidis* – pathogens that also recruit vitronectin, either directly, through the protein Opc in the case of *N. meningitidis* (Sa E Cunha *et al.*, 2010), or indirectly, through bridging by select glycosaminoglycans in the case of *N. gonorrhoeae* (Duensing *et al.*, 1999) (Fig. 3a). *Y. pestis* appeared to preferentially recruit the 65 kDa form of serum vitronectin (found in serum as part of a disulfide-linked dimer of the 65 kDa chain and 10 kDa chain that form after endogenous cleavage of 75 kDa vitronectin) rather than the 75 kDa monomeric protein, whereas *N. gonorrhoeae* and *N. meningitidis* appeared to recognize both forms of vitronectin approximately equally. The 75 kDa form of vitronectin also appeared to be degraded in the presence of Pla, resulting in several new anti-vitronectin antibody-reactive bands, a process that likely contributes to the apparent preferential binding of the 65 kDa form of serum vitronectin by Ail+ Pla+ *Y. pestis* KIM5.

Proteolytic degradation of vitronectin by Pla is facilitated by Ail

The co-sedimentation experiments with Pla-expressing *Y. pestis* KIM5 revealed that several new anti-vitronectin antibody-reactive bands appeared following incubation of *Y. pestis* with NHS at 37 °C, suggesting that the outer membrane Pla protease may cleave serum vitronectin. To determine whether Pla degrades isolated vitronectin and whether Ail contributes to this process, Ail+ Pla+ (KIM5), Ail– Pla+ (KIM5 Δ*ail*) and Ail+ Pla– (KIM8-E) *Y. pestis* were incubated with purified vitronectin at 37 °C and degradation of vitronectin measured by SDS-PAGE and immunoblot analysis over a 4 h time course (Fig. 3b). *Y. pestis* KIM5 (Ail+ Pla+) rapidly degraded the purified vitronectin; in contrast, *Y. pestis* KIM8-E (Ail+ Pla–) did not degrade vitronectin, confirming that vitronectin is a Pla substrate. *Y. pestis* KIM5 Δ*ail* (Ail– Pla+) also degraded vitronectin, but at a much slower rate than the Ail+ Pla+ strain. These results suggested that Ail plays a critical role in facilitating the degradation of vitronectin, possibly by recruiting vitronectin to the bacterial surface. Alternatively, the decreased degradation of vitronectin observed in the Ail– Pla+ strain could be due a decrease in Pla activity associated with the absence of Ail. To examine this possibility, the degradation of the secreted Pla substrate YopN was measured as an independent means of assessing Pla activity (Fig. 3c). The secreted YopN protein was stable in both Pla– strains. In contrast, typical Pla-dependent YopN degradation products were observed in both Pla+ strains; however, the degradation of YopN was reduced in the Ail– Pla+ strain, resulting in 1.8-fold more full-length YopN in the Ail– Pla+ strain than in the Ail+ Pla+ strain. These results suggest that Pla activity is increased in the presence of Ail or that Ail recruits both YopN and vitronectin to the bacterial surface, thus facilitating their degradation.

Y. pestis Ail binds the Pla-cleaved fragments of vitronectin

To determine whether Ail binds the vitronectin peptide fragments generated by Pla, *Y. pestis* KIM8-E (Pla– Ail+), KIM8-E Δ*ail* (Pla– Ail–), KIM5 (Ail+ Pla+) and KIM5 Δ*ail* (Pla+ Ail–) were incubated with purified vitronectin at 37 °C for 1 h to allow Pla-dependent degradation of vitronectin, and evaluated for co-sedimentation of vitronectin and/or vitronectin peptides with the bacteria by immunoblotting with a monoclonal anti-vitronectin antibody (Fig. 3d). As expected, no vitronectin peptides co-sedimented with the Ail– bacteria. In contrast, immunoreactive

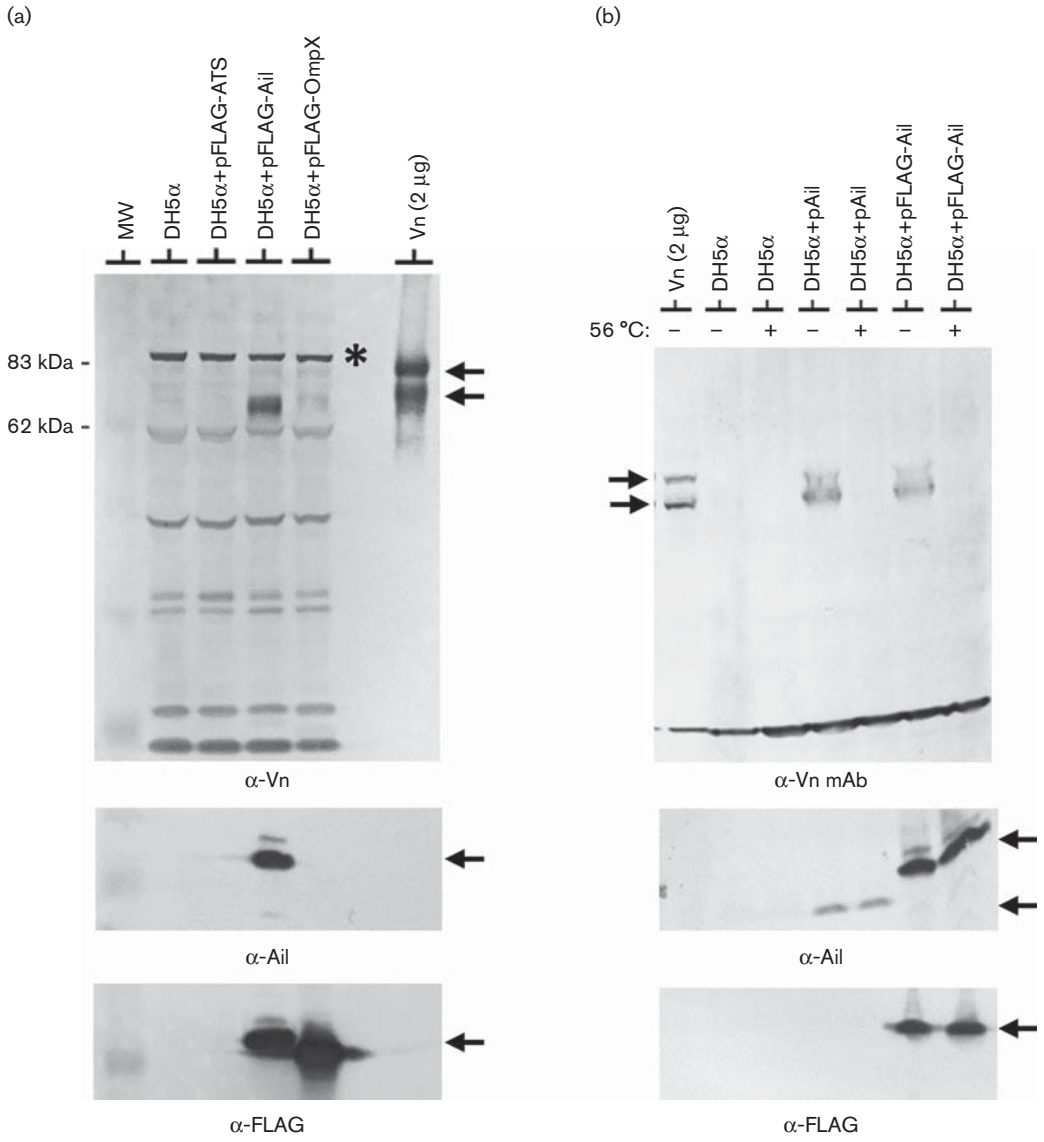

Fig. 2. *E. coli* expressing *Y. pestis* Ail binds vitronectin. (a) Co-sedimentation of purified monomeric vitronectin with *E. coli* DH5α expressing FLAG-tagged Ail. Bacteria were incubated with pure vitronectin (50 μg ml^{-1}) at 37 °C for 1 h and washed cells subjected to immunoblot analysis with anti-vitronectin antisera (α-Vn), FLAG M2 mAb (α-FLAG) or anti-Ail (α-Ail) antisera. The 65 and 75 kDa vitronectin bands are marked by arrows. *Y. pestis* KIM8-E Δ*ail* carrying plasmid pFLAG-Ail expressed FLAG-tagged Ail and bound vitronectin. In contrast, providing the pFLAG-ATS vector or pFLAG-OmpX did not result in vitronectin binding. A cross-reactive protein that is not vitronectin is indicated with an asterisk. MW, molecular mass standard. (b) Co-sedimentation of purified monomeric vitronectin with *E. coli* DH5α expressing *Y. pestis* Ail or FLAG-tagged Ail. Bacteria were incubated with purified vitronectin (50 μg ml^{-1}) or heat-treated vitronectin (56 °C 30 min; 50 μg ml^{-1}) at 37 °C for 1 h and washed cells subjected to immunoblot analysis with anti-vitronectin mAb (α-Vn mAb), FLAG M2 mAb (α-FLAG) or anti-Ail (α-Ail) antisera.

bands corresponding in size to the characteristic 75 and 65 kDa forms of vitronectin co-sedimented with Ail+ Pla– bacteria. Surprisingly, essentially all of the vitronectin peptides generated by Pla appeared to co-sediment with the Ail+ Pla+ bacteria, indicating that the degradation of vitronectin by Pla does not disrupt the Ail–vitronectin interaction.

Binding of vitronectin to Ail-expressing *Y. pestis* measured by flow cytometry

To independently confirm a role for Ail in recruiting vitronectin to the *Y. pestis* bacterial surface, the capacity of a Pla+ *Y. pestis ail* deletion mutant (KIM5 Δ*ail*) and its Pla+ parent strain (KIM5) to bind vitronectin was analysed

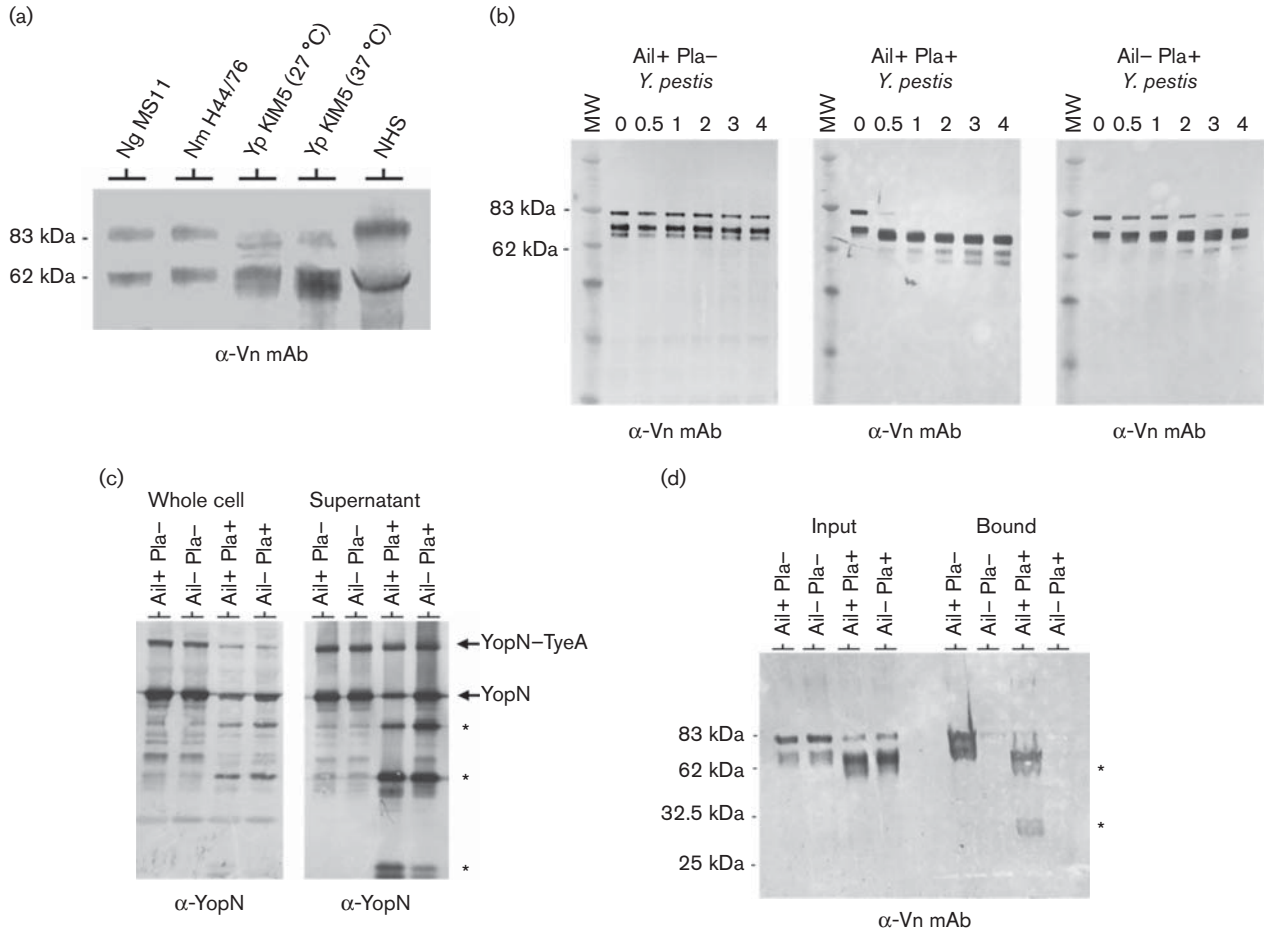

Fig. 3. Binding and degradation of vitronectin by *Y. pestis*. (a) Co-sedimentation of vitronectin from NHS with *N. gonorrhoeae* (Ng), *N. meningitidis* (Nm) and *Y. pestis* (Yp) KIM5 (strain JG150; grown at 27 or 37 °C). Bacteria were incubated with 50 % NHS at 37 °C for 1 h and washed cells were subjected to immunoblot analysis with anti-vitronectin mAb (α-Vn mAb). (b) Degradation of vitronectin by Ail+ Pla+ (*Y. pestis* KIM5), Ail– Pla+ (KIM5 Δ*ail*) and Ail+ Pla– (KIM8-E) was determined by SDS-PAGE and immunoblot analysis with anti-vitronectin mAb (α-Vn mAb) over a 4 h time course. MW, molecular mass standard. (c) Degradation of secreted YopN by Pla-expressing *Y. pestis*. Ail+ Pla– (KIM8-E), Ail– Pla– (KIM8-E Δ*ail*), Ail+ Pla+ (KIM5) and Ail– Pla+ (KIM5 Δ*ail*) *Y. pestis* were grown in TMH media for 5 h at 37 °C. Whole bacterial cells (Whole cell) and culture supernatants (Supernatant) were separated by centrifugation and subjected to immunoblot analysis with anti-sera specific for YopN (α-YopN). Pla-generated YopN degradation products are indicated with an asterisk. YopN–TyeA is a functional secreted YopN–TyeA hybrid protein that is the result of a +1 translational frameshift event (Ferracci *et al.*, 2004). (d) Binding of intact and degraded vitronectin to Ail+ Pla– (KIM8-E), Ail– Pla– (KIM8-E Δ*ail*), Ail+ Pla+ (KIM5) and Ail– Pla+ (KIM5 Δ*ail*) *Y. pestis*. Bacteria were incubated with vitronectin (25 μg ml^{-1}) for 1 h at 37 °C and an aliquot of each sample removed (Input). The input material as well as washed cells with co-sedimenting proteins (Bound) were subjected to immunoblot analysis with anti-vitronectin mAb (α-Vn mAb). Pla-generated vitronectin degradation products are indicated with an asterisk.

by flow cytometry. Bacteria were incubated with purified human C4BP or vitronectin and processed for flow cytometry (Fig. 4). Both C4BP and vitronectin bound to the parent strain, but not to the *ail* deletion mutant. A portion of the KIM5 bacteria did not bind vitronectin in this assay, which may reflect cleavage of vitronectin by Pla and subsequent loss of antibody binding. Overall, these studies confirmed that *Y. pestis* recruits the multifunctional vitronectin glycoprotein to its surface via an Ail-dependent mechanism.

Binding of vitronectin by purified recombinant Ail

To further confirm the presence of a direct interaction between Ail and vitronectin, we examined the binding of purified recombinant Ail and vitronectin by ELISA. Previously, we showed that the structure and fibronectin binding activity of *Y. pestis* Ail can be reconstituted in phospholipid bilayer nanodiscs enabling structural and activity studies to be performed in identical samples (Ding *et al.*, 2015). Nanodiscs are detergent-free, and

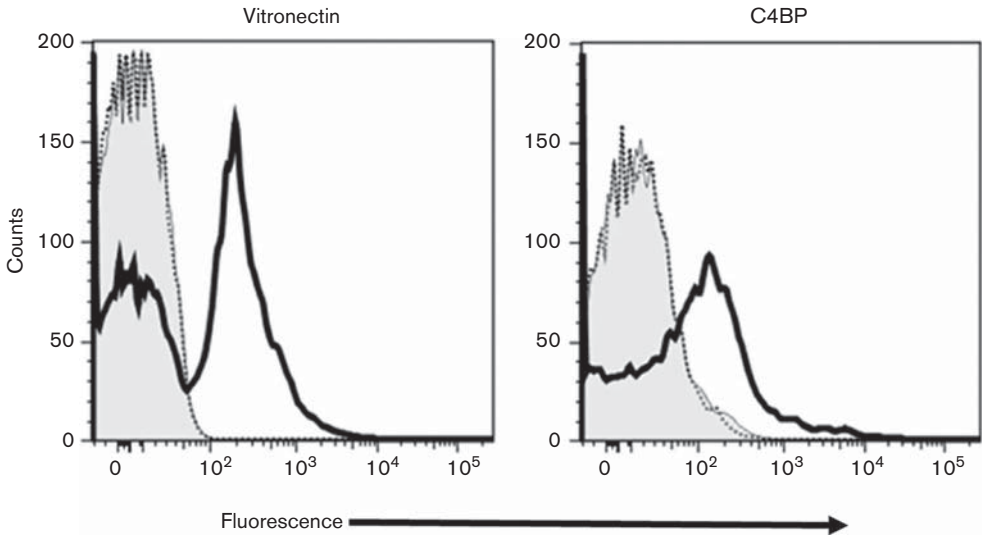

Fig. 4. Binding of vitronectin and C4BP to *Y. pestis* KIM5 and KIM5 Δ*ail*. Binding of purified human C4BP and vitronectin to *Y. pestis* KIM5 (solid black line) and KIM5 Δ*ail* (dashed line) was evaluated by flow cytometry. The concentration of each of the proteins in the reaction mixture was 5 μg ml^{-1}. Binding of primary and secondary antibodies (no added complement component) to the bacteria represents the background (grey histogram). The *x*-axis represents fluorescence on a five-decade log_{10} scale and the *y*-axis represents the number of events. One representative experiment of two reproducibly repeated experiments is shown.

provide a phospholipid bilayer environment that recapitulates the key anisotropic physical and chemical properties of biological membranes (Nath *et al.*, 2007; Ritchie *et al.*, 2009). Consistent with the pull-down assays performed with whole bacterial cells, Ail exhibits concentration-dependent binding to vitronectin-coated plates, with an ELISA profile similar to that observed for binding to fibronectin-coated plates (Ding *et al.*, 2015) (Fig. 5). In contrast, assays performed with empty nanodiscs yielded no ELISA signal, confirming that the binding signals are due to Ail in these experiments.

DISCUSSION

Y. pestis is a pathogen whose goal is to produce high-level septicaemia in its host in order to facilitate transmission via its flea vector (Lorange *et al.*, 2005). To survive in the blood and tissues of its host, *Y. pestis* must avoid engulfment by host phagocytic cells and destruction via the complement system. We have previously shown that the Ail outer membrane protein is essential for *Y. pestis* to avoid complement-dependent bacteriolysis (Bartra *et al.*, 2008). In addition, Ail has been demonstrated to play a critical role in the cell adhesion-mediated selection of cells for injection by the T3SS (Felek & Krukonis, 2009; Felek *et al.*, 2010). However, the mechanisms by which Ail facilitates adhesion to host cells and mediates complement resistance are not well understood. Ail has been shown to bind the ECM proteins fibronectin and laminin, which contribute to bacterial

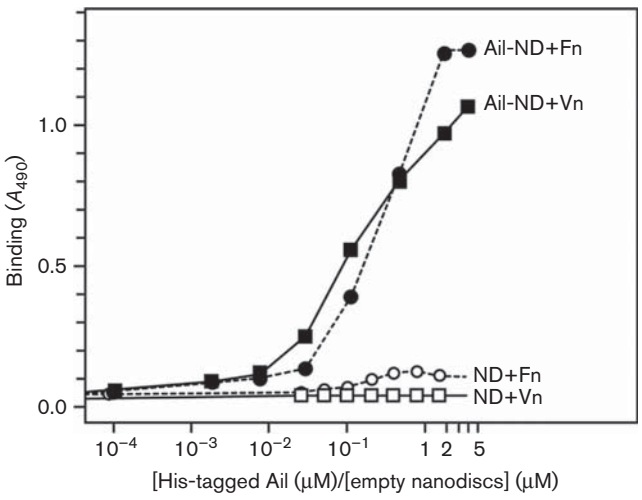

Fig. 5. Vitronectin-binding activity of purified Ail detected by ELISA. Purified refolded Ail-His in nanodiscs) (at 0.06 μM Ail concentration) binds fibronectin (Ail-ND + Fn) and vitronectin (Ail-ND + Vn). In contrast, empty nanodiscs bind neither fibronectin (ND-Fn) nor vitronectin (ND-Vn). Ail-His nanodiscs or empty nanodiscs were added at increasing concentrations to vitronectin- and fibronectin-coated plates and incubated overnight. Binding was detected by ELISA using a mouse anti-His antibody. Each point in each dataset represents the mean of three experiments.

adhesion, and to the complement inhibitor C4BP, which contributes to complement resistance (Ho *et al.*, 2014; Tsang *et al.*, 2010; Yamashita *et al.*, 2011). Here, we demonstrate that *Y. pestis* Ail also actively recruits the multifunctional glycoprotein vitronectin to the bacterial surface. Numerous other bacterial pathogens recruit vitronectin to both increase adherence to eukaryotic cells and/or evade complement-dependent killing (Attia *et al.*, 2006; Duensing & van Putten, 1997; Hallström *et al.*, 2009, 2011; Sa E Cunha *et al.*, 2010). Vitronectin contains an Arg–Gly–Asp (RGD) sequence for interaction with host $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins (Lössner *et al.*, 2009). Thus, bacterial-bound vitronectin can function as a bridge that links bacteria to select integrin-expressing cells. Vitronectin also binds the C5b–7 complex of complement and prevents its membrane insertion as well as complement component C9 to further inhibit MAC assembly (Milis *et al.*, 1993). Serum vitronectin is also a critical component of the coagulation pathway, since its interaction with PAI-1 is essential for stabilizing the enzyme's function as a key regulatory brake in the conversion of plasminogen to plasmin during fibrinolysis. Thus, the recruitment of vitronectin by *Y. pestis* could play a key role in bacterial attachment to host cells, complement resistance and inhibiting coagulation by enhancing fibrinolysis. Defining the biological role of the Ail–vitronectin interaction in bacterial attachment and/or complement resistance is complicated by the multitude of substrates recognized by Ail and the lack of defined Ail mutants defective in binding to individual substrates. Determination of the precise fragments or domains of its host ligands involved in binding to Ail could provide important information in this regard.

In several experiments (Figs 1a, 2a, b and 3a), Ail-expressing bacteria appeared to preferentially bind the 65 kDa form of serum vitronectin, whereas other pathogens such as *N. gonorrhoeae* and *N. meningitidis* bound both forms of vitronectin approximately equally (Fig. 3a). The 65 kDa form of vitronectin is normally generated by proteolytic processing of the Arg379–Ala380 bond in the C-terminal region of the vitronectin (Chain *et al.*, 1991). The resulting disulfide-linked, two-chain (65 and 10 kDa) isoform of vitronectin is a conformationally distinct form of the glycoprotein that has been shown to preferentially bind heparin (Sane *et al.*, 1990). However, the addition of high levels of exogenous heparin or pretreatment of *Y. pestis* with heparin had no effect on the interaction of Ail and vitronectin (data not shown). The processing of vitronectin by Pla (Fig. 3a, b) resulted in progressive degradation of the 75 kDa form of vitronectin and generated several degradation products similar in size to the 65 kDa form of vitronectin (Fig. 3b). However, preferential binding to the 65 kDa form of vitronectin was also observed with Pla– strains (Figs 1a and 2a, b), suggesting that there are likely multiple mechanisms that lead to preferential binding of the 65 kDa isoform of vitronectin to Ail. Finally, in several experiments where purified vitronectin was used in conjunction with Pla– *Y. pestis* strains (Figs 1b and 3d), no preferential binding to the 65 kDa form of vitronectin was observed.

Heat treatment of NHS or purified vitronectin at 56 °C for 30 min prevented recognition of vitronectin by Ail (Figs 1a and 2b). This was unexpected as previous studies had reported that heat inactivation of NHS or heat treatment (56 °C for 30 min) of purified vitronectin increased binding of vitronectin to *N. meningitidis* Opc and Msf (meningococcal surface fibril) (Griffiths *et al.*, 2011; Sa E Cunha *et al.*, 2010). Heating of vitronectin has been shown to modify its native structure by increasing the amount of a partially unfolded form termed 'activated vitronectin'. Opc and Msf are reported to specifically recognize and bind activated vitronectin to mediate complement resistance in *N. meningitidis*. The present study, however, suggests that *Y. pestis* Ail is unlikely to recognize the same form of vitronectin recognized by Opc and Msf as heat treatment of vitronectin decreased, not increased, binding to Ail. It has also been reported that proteolytic processing of the vitronectin C terminus that forms the 65 kDa fragment also results in a partial unfolding of vitronectin (Sane *et al.*, 1990). Thus, a partial unfolding of serum vitronectin, distinct from the partial unfolding associated with heat treatment, may be involved in its recognition by Ail.

Y. pestis Pla is a plasmid pPCP1-encoded outer membrane protein with aspartic protease activity (Sodeinde *et al.*, 1992; Vandeputte-Rutten *et al.*, 2001). Pla activates plasminogen to the serine protease plasmin, which subsequently cleaves fibrin. Pla also inactivates α_2-antiplasmin and PAI-1. Together, these activities enhance fibrinolysis, which promotes *Y. pestis* survival and dissemination in its host (Sebbane *et al.*, 2006; Sodeinde *et al.*, 1992). PAI-1 is a serine protease that functions as the primary inhibitor of host plasminogen activators (Wiman, 1996). It exists in both active and inactive forms. The active form of PAI-1 binds to vitronectin, thus establishing a stable active reservoir of PAI-1 (Wiman *et al.*, 1988). A previous study demonstrated that *Y. pestis* Pla and *Salmonella enterica* PgtE both degrade the vitronectin/PAI-1 complex (Haiko *et al.*, 2010), facilitating fibrinolysis. Here, we show that the presence of Ail greatly facilitates the targeting and degradation of vitronectin, indicating that Ail may function hand-in-hand with Pla to rapidly target vitronectin likely facilitating the inactivation of PAI-1. The partial degradation of vitronectin and the binding of the cleaved vitronectin peptides may also facilitate or disrupt other vitronectin-dependent activities. Further studies will be required to determine the function, if any, of the degraded and bound fragments of vitronectin.

The finding that *Y. pestis* Ail binds C4BP, the primary fluid-phase regulator of the classical and lectin pathways, in addition to vitronectin is consistent with previous findings (Ho *et al.*, 2014; Ngampasutadol *et al.*, 2005). Importantly, many other pathogens bind both C4BP and vitronectin (Blom *et al.*, 2009), suggesting that there may be a distinct advantage to recruiting both inhibitors. Indeed, the *M. catarrhalis* UspA2 protein has also been shown to bind both C4BP and vitronectin (Attia *et al.*,

2006). In this case, the binding of vitronectin and not C4BP appeared to correlate with complement resistance. Overall, the ability to interfere with both the early and late stages of the complement cascade likely provides a more complete resistance to complement-mediated attacks. Further studies will be required to sort out the roles of C4BP, vitronectin and other serum factors in the pathogenesis of *Y. pestis*.

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