Yersinia adhesins: an arsenal for infection

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Abbreviations used: Ail, attachment and invasion locus; AT, autotransporter; C3, complement component 3; C4bp, complement component 4-binding protein; Caf, cluster fraction 1 antigen; C-U, chaperone-usher; ECM, extracellular matrix; FasL, Fas ligand; FH, complement factor H; Flp, fimbiral low-molecular-weight protein; H-NS, histone-like nucleoid structuring protein; Ifp, intimin family protein; Ilp, intimin/invasin-like protein; InvA, invasin; LPS, lipopolysaccharide; M cell, microfold cell; MAM7, multivalent adhesion molecule 7; Myf, mucoid factor; NET, neutrophil extracellular trap; PGA, poly-*β*-1,6-*N*-acetyl-D-glucosamine; Pla, plasminogen activator; Psa, pH 6 antigen; TAA, trimeric autotransporter adhesin; Yap, *Yersinia* autotransporter protein; Yad, *Yersinia* adhesin; Yop, *Yersinia* outer protein

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Abstract

The *Yersiniae* are a group of Gram-negative coccobacilli inhabiting a wide range of habitats. The genus harbours three recognised human pathogens: *Y. enterocolitica* and *Y. pseudotuberculosis*, which both cause gastrointestinal disease, and *Y. pestis*, the causative agent of plague. These three organisms have served as models for a number of aspects of infection biology, including adhesion, immune evasion, evolution of pathogenic traits, and retracing the course of ancient pandemics. The virulence of the pathogenic Y*ersiniae* is heavily dependent on a number of adhesin molecules. Some of these, such as the *Yersinia* adhesin A and invasin of the enteropathogenic species, and the pH 6 antigen of *Y. pestis*, have been extensively studied. However, genomic sequencing has uncovered a host of other adhesins present in these organisms, the functions of which are only starting to be investigated. Here, we review the current state of knowledge on the adhesin molecules present in the *Yersiniae*, their functions and putative roles in the infection process.

1. Introduction

The *Yersiniae* are a large group of Gram-negative bacteria comprising 18 recognised species [1,2]. Among these, two species, *Y. enterocolitica* and *Y. pseudotuberculosis*, are causes of gastrointestinal disease in humans. A third species of medical relevance is *Y. pestis*, the causative agent of plague, which has been a scourge of humanity for at least 5000 years [3]. The virulence of all three species is reliant on adhesive properties of the bacteria, and the adhesin molecules mediating adherence to host tissues have been a focus of research for several decades. Important adhesins of *Yersiniae* were identified in the 1980s, with the *Yersinia* adhesin A (YadA) and Invasin (InvA) being the first ones [4-6], followed by others soon after. More recently, the availability of whole bacterial genome sequences have uncovered a number of potential adhesin-encoding genes. In addition, the production of adhesin fragments by recombinant methods combined with structural biology have been utilised to gain significant insights into the molecular mechanisms of bacterial adhesion. Adhesins fall into several different classes based on their structures, assembly pathways and surface export mechanisms, and most of these classes are represented in *Yersiniae*. Below, we review the current state of knowledge on the different types of adhesin molecules present in the human pathogenic *Yersiniae*, their functions and putative roles in the infection process.

2. Autotransporter adhesins

Autotransporters (ATs), or type V secretion systems, constitute the largest group of secreted proteins in Gram-negative bacteria. There are five recognised classes of ATs, type Va through Ve [7]. The pathogenic *Yersiniae* contain adhesins belonging to types Va, Vc and Ve (Table 1). Type Va-secreted proteins are classical ATs consisting of an N-terminal signal peptide, an extracellular passenger and C-terminal membrane anchor domain. The signal peptide mediates transport of the protein to the periplasm, where chaperones such as Skp, SurA and DegP protect the protein against proteases and keep them in an unfolded state until they are inserted into the outer membrane by the β-barrel assembly machinery [7]. The C-terminal β-barrel transmembrane domain forms the transport channel through which the passenger is secreted across the outer membrane. Type Vc systems or trimeric autotransporter adhesins (TAAs) are similar in architecture to classical autotransporters, but are obligate homotrimers [8]. The passengers of TAAs typically consist of a globular head domain followed by a coiled coil stalk (Figure 1). Type Ve ATs or "inverse autotransporters" have a similar overall architecture to classical ATs, but their domain order is reversed, i.e. the β-barrel translocator domain is N-terminal to the passenger [9].

2.1 Type Va adhesins in Yersinia

A number of classical ATs have been discovered in *Y. pestis* and *Y. pseudotuberculosis*, collectively known as *Yersinia* AT proteins or Yaps (Table 1). In *Y. pestis*, 13 loci code for presumably functional ATs. Among these genes, *yapK*, *yapJ* and *yapV* are close paralogues; the latter gene is present in *Y. pestis* KIM but lacking in CO92 [10]. In addition, *Y. pseudotuberculosis* encodes an AT paralogous to *yapKJV* designated *yapX*, but this is a pseudogene in all *Y. pestis* strains [11].

yapB is another probable pseudogene in *Y. pestis* due to truncation of the translocator domain; however, *Y. pseudotuberculosis* has two intact, chromosomally adjacent *yapB* paralogues [12]. *yapA* might be nonfuctional in *Y. pestis* biovar Orientalis strains due to a point mutation in the signal

sequence [12], but it is expressed in KIM strains [13]. *yapE* is the only *yap* also found in *Y. enterocolitica* [12].

The transcription profile of the *yap*s shows that they are expressed at low levels during *in vitro* growth conditions but are upregulated in a mammalian infection model [12]. A part of the passengers of YapA, YapE and YapG is cleaved by plasminogen activator (Pla; see section 3.2) and released into the culture medium; the rest of the Yaps remain intact and associated with the outer membrane [12,14]. The other Yaps are surface-localized in *Y. pestis* as shown by protease accessibility and immunofluorescence microscopy [13].

YapC plays a role in mediating autoaggregation, binding to macrophages, binding to human-derived epithelial cell lines, and biofilm formation [15]. YapG does not play a role in virulence in bubonic or pneumonic plague, and its function remains to be deciphered [16]. YapJ and YapK are upregulated during bubonic and pneumonic infections [12], though their exact functions are not yet clear [17]. YapV, a paralogue of YapJ and YapK, is similar to the *Shigella* autotransporter IcsA and, like IcsA, YapV is able to interact with N-WASP, which is involved in actin polymerization [10]. YapV, YapJ and YapK bind to a variety of extracellular matrix (ECM) molecules, and in addition YapV and, to a lesser extent, YapK interact with alveolar epithelial cells [11]. Deletion of *yapE* from *Y. pestis* effects the colonization of tissues during bubonic plague and plays a role in binding of bacteria to host cells and autoaggregation [18]. However, *Y. enterocolitica* YapE lacks the autoaggregation activity and is not proteolytically processed [14].

2.2 Type Vc adhesins in Yersinia

2.2.1 YadA

YadA is the prototypical TAA, present in all the three human pathogenic species of *Yersinia*. However, in *Y. pestis*, *yadA* is a pseudogene due to a single base pair deletion causing a frame shift [19,20]. YadA is an essential virulence factor of *Y. enterocolitica* and its absence renders the bacteria avirulent in a mouse model [21]. *yadA* mutants are able to penetrate the mouse intestinal mucosa but are not

able to persist for more than two days [22]. In contrast, YadA is not essential for virulence in *Y. pseudotuberculosis*. Introduction of a functional copy of *yadA* into *Y. pestis* causes a modest reduction in virulence [19]. This is particularly interesting because the same protein can cause different effects in different species of *Yersinia*.

YadA is encoded on the 70-kb virulence plasmid, pYV, and is induced upon a shift of temperature to 37 °C [23]. The expression of *yadA* is regulated by the temperature-sensitive *lcrF* gene [24]. *lcrF* is transcribed at comparable levels at both 26 °C and 37 °C in *Y. pestis* and *E.coli*, but translation is efficient only at 37 °C and not at 26 °C [25]. The activator of the plasmid-encoded virulence genes, including *yadA*, in *Y. enterocolitica* is known as VirF, which is a homologue of LcrF [26]. VirF is synthesized at high temperatures but its artificial expression at 30 °C does not lead to expression of virulence factors [27], which indicates that factors other than VirF are also required. YmoA is a chromosomally encoded histone-like protein which thermoregulates the induction of virulence genes in *Y. enterocolitica*. The deletion of this gene allows expression of the virulence factors below 30 °C [28]. Intergenic RNA thermosensors are also involved in regulating *lcrF*/*virF* translation. Combined action of both YmoA and RNA thermosensors seems to effectively regulate the infection efficiency of *Yersinia* [29]. A recent study showed that *yadA* expression is also modulated by the transcriptional regulator OmpR, which represses YadA by directly binding to the *yadA* promoter. OmpR-mediated control of *yadA* expression is independent from the thermoregulatory mechanism mentioned above [30].

YadA varies in size from strain to stain and ranges from 422 to 455 residues. It has a lollipop-like appearance and covers the entire surface of the bacteria [31] (Figure 1). A trimeric β-barrel domain anchors the protein to the outer membrane [32]. The passenger consists of three chains, which pass through the pore of the barrel and form an α-helical coiled-coil stalk followed by a sticky globular head at the N-terminus (Figure 1). YadA is a multifunctional protein that binds to host ECM components like fibrillar collagens such as types I, II, III, the network-forming collagen type IV, fibronectin and laminin [33-35]. The triple-helical conformation of collagen is required for YadA

132 binding, though a specific sequence is not necessary for its recognition [36]. Nonetheless, YadA binds
133 more tightly to regions of collagen rich in 4-hydroxyproline with a low net charge [37]. *Y.*
134 *enterocolitica* YadA shows higher affinity towards collagen and laminin compared to *Y.*
135 *pseudotuberculosis* YadA, which in turn binds very efficiently to fibronectin [38]. YadA of *Y.*
136 *pseudotuberculosis* mediates more efficient entry of bacteria into epithelial cells. This difference in
137 function has been attributed to the additional 31 residues present at the N-temini of the head
138 domain of *Y. pseudotuberculosis* YadA [38].
139 YadA mediates adherence to various cell types, including epithelial cells, neutrophils and
140 macrophages [39]. *Yersinia* infection involves tight contact of the bacteria with the host cells, which
141 is mediated by InvA (see section 2.3.1) and YadA by binding to β_1 integrins. In the case of YadA, this is
142 assumed to occur through a bridging ECM molecule [40]. Type III effector proteins (*Yersinia* outer
143 proteins or Yops) are then injected into the host cells to disrupt the cytoskeleton and prevent
144 phagocytosis [41,42]. YadA has co-evolved to match the length of the injectisome needle of the type
145 III secretion system, and altering the length of either without simultaneously changing the other
146 prevents Yop injection into host cells [43].
147 Further activities of YadA include autoaggregation of bacterial cells [44]. Electron micrographs show
148 the formation of a zipper-like structure between YadA-expressing cells [31]. YadA promotes serum
149 resistance by eluding the complement system of the host, which is the first line of defense against
150 micro-organisms. The complement system is activated by three different pathways: the classical,
151 lectin and alternative pathways [45]. All the three pathways lead to formation of opsonin C3b which
152 deposits on the bacterial surface and is recognized by phagocytes. YadA plays a major role in
153 promoting serum resistance [46]. YadA binds to Factor H (FH), a negative regulator of the alternative
154 complement pathway [47]. YadA also plays a role in the interaction of *Yersinia* with complement
155 component 4-binding protein (C4bp), which is a negative regulator of both the classical and lectin
156 pathways [48]. A recent study showed that YadA recruits C3b and iC3b (the cleavage product of C3b)
157 to the bacterial surface, which causes further recruitment of FH. FH acts as a cofactor in mediating

158 the cleavage of C3b to iC3b, which prevents the formation of the membrane attack complex that
159 leads to bacterial lysis [49]. Conversely, YadA makes *Yersinia* more susceptible to killing by neutrophil
160 extracellular traps (NETs). NETs are extracellular fibres formed by protein (including collagen)
161 granules and chromatin released from neutrophils. YadA mediates binding of *Yersinia* to NETs and
162 thereby exposes the bacteria to antimicrobial peptides present in the traps [50].

163 2.2.2 YabB and YadC

164 YadB and YadC are TAAs present in *Y. pestis* and *Y. pseudotuberculosis* [51].These proteins have an
165 architecture similar to that of YadA. YadB (35 kDa per monomer) has a small head region (only 62
166 residues long), whereas YadC is larger (61.6 KDa) and its head region does not show any sequence
167 similarity to YadA [51].

168 Neither protein is very strongly expressed in *Y. pestis* [51]. Unlike YadA, they do not seem to play a
169 role in adherence to epithelial cells. Deletion of *yadBC* led to a slight reduction (60% compared to the
170 wild-type) in invasion of epithelial cells [51]. Additionally, YadBC increase the uptake of bacteria by
171 phagocytes by 60%, confirming their role in invasion [52].

172 YadBC appear not to be involved in eliciting pneumonic plague, and their role in bubonic plague is
173 very subtle [51]. However, *yadBC* are highly expressed in fleas [53] but do not seem to play a role in
174 flea colonization [52]. Nonetheless, absence of these genes leads to two- to four-fold less recovery of
175 *Y. pestis* from infected skin, indicating a role in promoting bacterial survival during the initial stages
176 of infection [52]. Furthermore, these proteins reduce the levels of the chemoattractant CXCL-1,
177 which is produced by macrophages, neutrophils and epithelial cells and attracts polymorphonuclear
178 cells [52]. Thus, YadBC might help the bacteria survive during the transition from a flea to a human
179 host.

180

2.3 Type Ve adhesins in Yersiniae

2.3.1 Invasin

InvA, in addition to YadA, is the major adhesin required for establishing the initial bacterial infection. InvA is important in the first phase of infection, allowing bacterial cells to adhere and invade microfold (M) cells. The *invA* gene encoding the surface-exposed outer membrane protein, homologous to intimin found in enterohemorrhagic *Escherichia coli*, is located on the chromosome [54,54].

Adhesion to and internalization of enteropathogenic *Yersiniae* into Peyer's patches is mediated by InvA, which binds to β_1 integrins, specifically $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_V\beta_1$ integrins, found on the apical surface of M cells [55]. This process leads to cytoskeletal rearrangements, where focal adhesion complexes are formed. This is followed by internalization of the bacterium by a zipper mechanism, which triggers the production various pro-inflammatory cytokines such as interleukin-8, monocyte chemotactic protein-1, tumor necrosis factor-α, granulocyte-macrophage colony stimulating factor, and others [56]. Though InvA plays a major role in binding and invasion of M cells [57], YadA can substitute for these functions, though the process is slow [58]. A recent study showed that InvA, in addition to YadA, induces production of NETs in a β_1 integrin-dependent manner [59].

invA encodes a 92-kDa (835-residue) and 103-kDa (986-residue) protein in *Y. enterocolitica* and *Y. pseudotuberculosis*, respectively. InvA is anchored in the outer membrane with its transmembrane β-barrel domain [60]. The extracellular C-terminal region consists of up to five domains (Figure 1). Domains D1-D4 resemble immunoglobulin superfamily domains, whereas the C-terminal D5 domain has a C-type lectin-like fold [61]. InvA from *Y. pseudotuberculosis* is composed of five extracellular domains, while *Y. enterocolitica* InvA lacks the D2 domain [62]. This domain promotes self-association, resulting in InvA multimerization and a higher avidity for host cells. Lack of the D2 domain decreases the efficiency of bacterial uptake [63]. The D4-D5 domains play a critical role in integrin binding. Interestingly, InvA binds to integrins with an affinity 100-fold times higher than the

natural ligand, fibronectin [64]. Surprisingly, the production of InvA by *Y. pestis* is abrogated due to the insertion of an IS*200* element in the *invA* gene [65].

Regulation of InvA expression depends on various factors, among which temperature and the transcriptional regulator, RovA, play a major role [66]. *invA* is maximally expressed at environmental temperature (25 °C), whereas only low amounts of InvA are detectable at 37 °C [4]. Recently, *invA* expression was shown to be up-regulated during persistent infection [67]. However, *invA* expression also depends on the strain in question. In particular, InvA production is inhibited at 37 ˚C in *Y. enterocolitica* serotype O:8 due to rapid degradation of the temperature-sensitive RovA and silencing of *invA* transcription by H-NS (the histone-like nucleoid structuring protein) [68,69]. H-NS binds to regions within the *rovA* promoter and forms a regulatory complex with YmoA, which prevents RNA polymerase from binding to the *invA* promoter [70]. Likewise, the amount of InvA synthesis is reduced at 37 ˚C in *Y. enterocolitica* serotype O:9 [71]. In contrast, InvA is efficiently produced by *Y. enterocolitica* O:3 even at 37 °C. In this serotype, RovA is only weakly temperature-dependent due to a single proline to serine (P98S) substitution [72]. In addition, insertion of an IS*1667* element at the *invA* promoter in *Y. enterocolitica* O:3 leads to constitutive production of InvA [72].

2.3.2 Other inverse autotransporter adhesins in *Yersiniae*

Recent genome analyses show that there are several others invasin-like autotransporters among the *Yersiniae* that mediate adhesion to host cells and promote colonization of different host tissues. *Y. pseudotuberculosis* encodes three additional inverse ATs: Ifp (InvB), InvC and InvD [73]. The *Y. pestis* orthologue of InvC is referred to as Ilp (intimin/invasin-like protein) [74]. These proteins have a similar structural organization to InvA. The protein called Ifp (intimin family protein) is present in all *Y. pseudotuberculosis* strains [75]. Interestingly, in *Y. pestis*, the predicted Ifp sequence is disrupted by an IS*285* insertion element, with the exception of strain 91001, where it is altered by a point mutation. *ifp* is maximally expressed at 37 °C in the late exponential phase or early stationary phase [75]. Invasion and adhesion assays confirmed that Ifp and InvC are able to bind and mediate invasion of human, murine and porcine epithelial cells. In addition, the loss of Ifp and InvC leads to the

recruitment of a higher number of immune cells to Peyer's patches [73,75]. In *Y. pestis*, Ilp-deficient mutants showed reduced adhesion to and internalization by HEp-2 cells. Furthermore, mice challenged with *ilp* mutants demonstrated a significant delay in time to death and reduced bacterial dissemination to the liver, kidney and lungs [74].

Environmental representatives of the *Yersiniae*, such as *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii*, and *Y. ruckeri* also possess one or more inverse AT genes in their genome [76,77]. However, these proteins and their roles in infection processes (e.g. of fish in the case of *Y. ruckeri*) have not been investigated.

3. Small β-barrel proteins

3.1 Ail

The Ail (Attachment and Invasion locus) adhesin belongs to a family of outer membrane proteins distributed in organisms such as the pathogenic *Yersiniae*, *Salmonella enterica* (PagC and Rck) or *Escherichia coli* (OmpX) [78]. This small, chromosomally encoded protein is an important *Yersinia* virulence factor. The crystal structure of *Y. pestis* Ail revealed an eight-stranded transmembrane β-barrel with four extracellular loops [79] (Figure 1). Many of its functions, including serum resistance, cell adhesion, cell invasion, and promotion of Yop delivery into host cells have been well characterized [80-84,84].

Ail plays a role in serum resistance in all three human pathogens, especially in *Y. pestis*, where deletion of *ail* leads to almost complete serum sensitivity [85,86]. Ail can recruit the complement-regulatory proteins FH and C4bp, which confers significant protection against killing by complement [46,48]. However, the activity of Ail, due to its small size, is usually masked by the lipopolysaccharide (LPS) outer core oligosaccharide and O-antigen in *Y. enterocolitica* O:3 [47] or O-antigen in *Y. pseudotuberculosis* YPIII [87]. Thus, Ail only displays full biological activity in strains with rough LPS, such as *Y. pestis*; however, as the expression of O-antigen and outer core in *Yersinia* is temperature-

regulated, it is plausible that in vivo the O-antigen and/or outer core expression is repressed, thus unmasking Ail.

In *Y. enterocolitica* and *Y. pestis*, Ail mediates binding to various epithelial cell lines and ECM proteins, including laminin, fibronectin, vitronectin and heparan sulfate proteoglycans [4,79,81,86,88,89]. Binding to laminin and fibronectin facilitates close contact with host cells and thus promotes injection of Yops [79]. The binding site for Ail in fibronectin has been mapped to the ninth FNIII repeat [90]. In contrast to *Y. pestis* and *Y. enterocolitica*, Ail from *Y. pseudotuberculosis* has been reported to lack adhesion and invasion capacity [87,91]. Interestingly, the sequence of Ail from *Y. pestis* is almost identical to that of *Y. pseudotuberculosis*, differing at only two positions located in extracellular loops, suggesting these residues might play a significant role in binding to cell components such as fibronectin [87]. Furthermore, Ail mediates autoaggregation of *Y. pestis* [86].

ail is highly expressed at 37°C under reduced oxygen levels in *Y. enterocolitica*, but not at lower temperatures [84,92]. In contrast, *ail* is also expressed at 26 °C in *Y. pestis*, albeit at lower levels than at 37 °C, probably as an adaptation to the different infection route of this organism [85,86]. In addition, the expression levels of *ail* are much higher in *Y. pestis* than in *Y. pseudotuberculosis*; in the former, 20-30 % of the outer membrane proteome consists of Ail at 37 °C [85,93]. *Y. pestis* and *Y. pseudotuberculosis* contain three additional *ail* paralogues, *y1682* (OmpX), *y2304* and *y2446*, but these do not contribute to serum resistance [85].

3.2 Plasminogen activator

Pla has proteolytic and adhesive activity critical for the progression of bubonic and pneumonic plague [94]. It is a member of the omptin family of β-barrel proteins [95]. Pla is encoded by the *pla* gene located on the small plasmid pPCP1 (also called pPla or pPst) exclusive to *Y. pestis* [96]. *pla* was detected in ancient DNA samples from the Bronze Age, showing that pPCP1 was an early acquisition in *Y. pestis* [3]. Pla consists of 10 antiparallel transmembrane β-strands with five extracellular loops; the catalytic residues are located at the top of the β- barrel [97,98] (Figure 1).

The main pathogenic function of Pla is cleavage of plasminogen into its active form, plasmin [99,100]. Plasmin is a serine protease that degrades fibrin clots. The degradation of these clots enhances the dissemination of *Y. pestis* into host tissue as well as an inhibition of immune cell recruitment [100]. In addition, plasmin cleaves ECM components such as laminin and fibronectin and activates pro-matrix metalloproteinase, which also enhances faster bacterial dissemination [100]. Pla was shown to facilitate bacterial dissemination from the primary site of infection to the lymph nodes in bubonic plague; during pneumonic plague, it is required for bacterial outgrowth in airways [100,101]. Pla mediates adhesion to and invasion of macrophages via the DEC-205 receptor, which leads to dissemination of *Y. pestis* in a murine infection model [102]. However, in contrast to these reports, a recent study showed that Pla neither promotes dissemination to the lymph nodes nor causes organ destruction, but it does promote bacterial multiplication and helps to protect *Y. pestis* cells against host defence [103].

Recent studies have shown the protective role of Fas ligand (FasL), degraded by Pla, in the induction of host immunity during *Y. pestis* lung infections [104]. FasL is a membrane protein required for host cell death and it acts as a protective molecule during bacterial pneumonia. Mice challenged with wild-type *Y. pestis* showed a decreased level of FasL, in contrast to *pla* mutants, demonstrating that the degradation of FasL changes host inflammatory responses and facilitates *Y. pestis* outgrowth in the lungs [104]. The activity of Pla may also play a role in complement evasion by inactivating the complement factor C3, which results in inhibition of opsonophagocytosis [100].

Pla is also an adhesin that contributes to Yop delivery and cell invasion, with the strongest effect demonstrated at 28 ˚C and 37 ˚C at neutral pH [105,106]. Pla is present at both temperatures, but is twice as abundant at 37 °C, and Pla is also more active at this temperature [93,107,108]. Pla mediates attachment to (and even lead to invasion of) eukaryotic cells and binds ECM components such as collagen type IV, laminin and heparan sulfate proteoglycan [109-111]. Moreover, the presence of rough LPS is critical for the proteolytic and adhesive activity of plasminogen [107,112].

4. Fimbrial adhesins

4.1 Chaperone-usher fimbriae

Fimbriae and pili are long, linear appendages protruding from the cell surface formed of multiple subunits. These structures may be involved in several cellular processes, including adhesion and biofilm formation, DNA uptake by naturally competent bacteria, some forms of motility, and conjugation. Many fimbrial structures, particularly those involved in adhesion, are assembled by the chaperone-usher (C-U) pathway [113]. *Y. pestis* produces two well-characterised C-U -assembled adhesin structures, the pH 6 antigen (Psa) and the cluster fraction 1 antigen (F1 antigen or Caf) [114,115]. In contrast to type I and P pili, Psa and Caf do not form distinct fimbriae but rather thin filaments or a capsule-like mesh on the cell surface, respectively. Furthermore, Psa does not have a single adhesive subunit at its tip, but rather all pilin subunits have adhesive activity, thus making the Psa filaments polyvalent adhesins [116].

Caf is encoded by a plasmid specific to *Y. pestis*, pFra. Though not an adhesin as such, Caf is an important virulence factor that aids in resisting phagocytosis and evading the innate immune system by binding to the proinflammatory cytokine interleukin-1β during early stages of infection [117,118]. Caf is expressed at mammalian body temperature; however, Caf may also play a role in transmission through flea bites to the mammalian host [119].

In contrast to Caf, Psa is chromosomally encoded, and orthologous loci are found in both *Y. pseudotuberculosis* and *Y. enterocolitica* [91,120]. In the latter, Psa is referred to as mucoid factor (Myf). In *Y. pestis*, Psa is an important adhesin mediating attachment to host cells via β1-linked galactosyl residues in glycosphingolipids [121] and can promote Yop delivery [105,122]. Phosphatidyl choline was identified as another receptor for Psa on alveolar epithelial cells [123] , and Psa binds to low-density lipoprotein by interacting with the lipid component [124]. The *Y. pestis* PsaA pilin contains distinct but adjacent binding sites for both galactose and choline [125] (Figure 1). The choline-binding motif in Myf is disrupted, which could explain why it does not agglutinate erythrocytes; Psa-mediated hemagglutination is dependent on phosphocholine binding in *Y. pestis*

[125]. Psa also aids in immune evasion by binding to the Fc portion of IgG, possibly through interactions with the carbohydrate moiety of Fc [126,127]. Furthermore, Psa promotes biofilm formation [128]. As its common name suggests, *psa* is expressed at low pH (<6) and high temperature (37 °C) [129], though more recent data point to *psa* also being expressed at 28°C in minimal medium [128]. Interestingly, *psa* is expressed and Psa is present at higher levels in *Y. pestis* than in *Y. pseudotuberculosis* [130]. *Y. pestis* coexpresses *psa* and *caf*, with the adhesive properties of the former dominating the phenotype [131]. Interestingly, both Psa and Caf appear to inhibit invasion of epithelial cells by *Y. pestis* [131].

Genome sequencing has uncovered eight additional chromosomal loci encoding putative C-U fimbrial systems. However, two of these have disrupted usher genes, and so are unlikely to be functional [132]. The six intact loci (Table 1) all produced pilus-like structures when heterologously expressed in *E. coli*, though only one, encoded by the *y0561-0563* locus, promoted adhesion to epithelial cells and significantly promoted biofilm formation at 28 °C [128]. However, deletion of this locus had no appreciable effect on the adhesion of *Y. pestis*. Deletion of another fimbrial locus, *y1858-1862*, displayed a modest reduction in *Y. pestis* virulence in mice when introduced intravenously and resulted in somewhat reduced adhesion to a macrophage cell line, suggesting this fimbria might have a role in immune evasion [128]. A later study found that also *y0348-0352* and *y1869-1873* had similar effects in an intranasal infection model [132].

A C-U fimbria widespread among *Y. enterocolitica* strains is the mannose-resistant haemagglutinin (MRHA). MRHA fimbriae are channelled structures approximately 8 nm in diameter that mediate agglutination of erythrocytes from several animal species at environmental temperatures [133,134]. The major pilin subunit, MrpA, is homologous to the pilin of the mannose-resistant fimbriae of *Proteus mirabilis* [135]. Recently, a MRHA orthologue in *Y. intermedia* was found to be downregulated under anaerobiosis [136].

4.2. Type IV pili

Another class of fimbrial adhesins are type IV pili, which are retractable surface appendages that confer twitching motility on a number of bacterial species [137]. In contrast to C-U systems, type IV pili are assembled by a protein complex spanning both the inner and the outer membrane, related to type II secretion systems [138]. Many strains of *Y. pseudotuberculosis* harbour a genetic locus (*pil*) encoding a type IV pilus system that forms polar bundles when heterologously expressed in *E. coli* [139]. This is located on a pathogenicity island, YAPI, present in *Y. enterocolitica* and *Y. pseudotuberculosis*, but missing in *Y. pestis* [140]. *pil* expression is upregulated under high temperature and osmolarity conditions, and deletion of the *pil* locus results in reduced virulence in a mouse model [139].

A second type IV pilus locus is *tad* (for Tight Adhesion), encoding the fimbiral low-molecular-weight protein (Flp) pilus [141]. The *tad* locus is widespread in Gram-negative bacteria, and the locus is present in all pathogenic *Yersiniae* [142]. However, in *Y. pestis*, it is most likely inactive due to a deletion of the major pilin gene *flp* and a frameshift mutation in another gene encoding a putative secretin [143]. In *Y. enterocolitica*, Flp pili are detectable only in a subset of the population, but they appear to be involved in microcolony formation at 26 °C [142]. The *tadD* gene of the fish pathogen *Y. ruckeri* is expressed in the host during infection; Flp may thus play a role in the virulence of this organism [144].

5. Other adhesins

A constitutively expressed outer membrane protein of *Vibrio parahaemolyticus*, multivalent adhesion molecule 7 (MAM7), was identified as mediating initial attachment to host cells [145]. This protein consists of seven repeated mammalian cell entry domains, and is widespread in Gram-negative bacteria; an orthologous gene is present in all three pathogenic *Yersinia* species (Table 1). *V. parahaemolyticus* MAM7 binds to fibronectin and phosphatidic acid, with significantly higher affinity for the latter [145,146]. MAM7-negative *Y. pseudotuberculosis* adhered significantly less to

fibroblasts and was less cytotoxic than the wild-type and complemented mutant strain [146] Furthermore, *E. coli* expressing MAM7 from *Y. pseudotuberculosis* was able to adhere to HeLa cells and could compete with *Y. pseudotuberculosis* for binding. These results suggest MAM7 plays a role in *Y. pseudotuberculosis* virulence.

An important stage in the life cycle of *Y. pestis* is infection of the flea proventriculus and formation of an occluding biofilm [147]. This is dependent on the hemin storage locus, the operon *hmsHFRS*, which is active at 26 °C but not at 37 °C [148]. This operon produces and exports an extracellular polysaccharide, poly-*β*-1,6-*N*-acetyl-D-glucosamine (PGA), which forms the matrix of the biofilm [149]. Biofilm production is enhanced in *Y. pestis* due to a frameshift arising from an internal duplication in the *rscA* gene, a negative regulator of biofilm production in *Y. pseudotuberculosis* [150]. In addition, LPS itself can act as an adhesin. The core oligosaccharide of *Y. pestis* LPS can interact with a lectin expressed by antigen-presenting cells called DC-SIGN (dendritic cell-specific intercellular adhesion molecule-grabbing non-integrin) [151]. This interaction may allow *Y. pestis* to invade antigen-presenting cells such as dendritic cells and macrophages, which *Y. pestis* could use as a pathway to disseminate to lymph nodes from the primary site of infection.

6. Conclusions and future perspectives

The *Yersiniae* comprise a medically important, environmentally ubiquitous and biologically fascinating genus of bacteria. They have been used extensively as model organisms for extracellular infection, type III secretion system effector delivery, immune evasion, and adhesion. For a long time, the major adhesion phenotype of the enteropathogenic *Yersiniae* was believed to be solely due to YadA, InvA and, to a lesser extent, Ail. Though these are still unquestionably the major adhesins in these organisms, recent studies have highlighted the role played by other autotransporters, fimbriae and other types of adhesins in the virulence of these organisms. *Y. pestis*, which produces neither YadA nor InvA, has been long known to contain alternative adhesins such as Psa, but even in this

412 bacterium, numerous adhesins and potential adhesins have recently been uncovered by genome
413 sequencing.
414 A remarkable feature of the virulence phenotype in *Y. enterocolitica* is the dominance of YadA. In
415 most other bacterial pathogens, including *Y. pseudotuberculosis* and *Y. pestis*, no single adhesin has
416 such a profound effect on not only the adhesive properties of the bacteria, but also on serum and
417 phagocytosis resistance. In many cases, the effects of a single adhesin are difficult to establish due to
418 functional redundancy among adhesion molecules, as exemplified by *Salmonella,* where a multitude
419 of adhesins have been described, but none of such central importance for virulence have been
420 identified [152]. YadA in *Y. enterocolitica* is thus quite exceptional.
421 A major regulator of *Yersinia* virulence traits is temperature. It is now clear that, at different
422 temperatures, *Yersiniae* elaborate very different surfaces (Figure 2). This applies not only to the
423 assortment of adhesins expressed, but also to other surface molecules such as the Ysa and Ysc type
424 III secretion systems, flagella and LPS [27,153-156]. Though some proteins appear to dominate the
425 adhesive phenotype at certain temperatures (specifically InvA at environmental temperatures and
426 YadA at mammalian body temperature in the enteropathogenic *Yersiniae*), several adhesins are
427 expressed concomitantly at any given temperature, and there even seems to be some overlap among
428 differentially expressed adhesins. However, only a few studies have addressed the interplay of
429 adhesins in adherence functions or immune evasion [e.g.47,131,157-161]. Though more challenging,
430 these kinds of studies are needed to fully delineate the in vivo roles of the adhesins, which – despite
431 a great deal of experimental data on individual adhesins – remain elusive.
432 Additionally, different adhesins may have differing roles in different host organisms, as exemplified
433 by the importance of InvA in swine, a notable reservoir for *Y. enterocolitica* O:3 [162]. Thus, to gain a
434 full understanding of the functions of individual adhesins or adhesins acting in concert, it is not
435 sufficient to study just one host organism. *Y. enterocolitica* and *Y. pseudotuberculosis* are both
436 capable of infecting not only various mammals, but also insects and nematodes, and can additionally
437 be found free-living in the environment [153,163-166]. For *Y. pestis*, colonising the flea is a major

stage in the infectious cycle, and studying this interaction has provided much data on the factors required for survival in the flea, biofilm formation and transmission to mammalian hosts [167].

To further complicate matters, it has become clear that even closely related adhesins from different strains can have significantly different functions [38,62,87]. Thus, not all results from a single adhesin orthologue may be applicable to the same adhesin from other strains, not to mention other species. Therefore, we urge future studies to include a comparative element to assess the generality of novel findings. It might be particularly fruitful to compare species that are not pathogenic to humans or mammals, such as *Y. ruckeri* (a fish pathogen) and *Y. entomophaga* (an insect pathogen), with the classical human pathogenic *Yersiniae*. This could potentially shed light on the pathogenesis of both groups of organisms, and provide insight into the mechanisms of virulence in different hosts.

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867 **Table 1.** Adhesins of human pathogenic *Yersiniae*

Adhesin Class	Adhesin	Function(s)	Presence in species[a]			Ref.
			Y. enterocolitica	*Y. pseudo-tuberculosis*	*Y. pestis*	
Autotransporter adhesins						
Type Va	YapA	Not known	-	x (Q667Z2)	+ (Q9F292)	[12]
	YapB1	Not known	-	x (Q667Z0)	o	[12]
	YabB2	Not known	-	x (Q667Z1)	-	[12]
	YapC	Autoagglutination, binding to epithelial cells and macrophages, biofilm formation.	-	x (Q66DI5)	+ (Q9F290)	[15]
	YapE	Binding to eukaryotic cells, autoaggregation	x (A1JSQ7)	x (Q664E)	+ (Q9F288)	[18]
	YapF	Not known	-	x (Q665R2)	+ (Q9F287)	[12]
	YapG	Not known	-	x (Q665P5)	+ (Q9F286)	[16]
	YapH	Not known	-	x (Q666F5)	+ (Q9F285)	[12]
	YapJ	Not known	-	-	+ (Q0WGA9)	[12]
	YapK	Not known	-	x (Q66FH2)	+ (Q0WJZ8)	[12]
	YapL	Not known	-	x (Q668J2)	+ (Q7CJH7)	[12]
	YapM	Not known	-	x (Q667C1)	+ (Q0WIL1)	[12]
	YapN	Not known	-	x	+	[12]

				(A0A0U1QUE7)	(Q0WID7)	
	YapV	Interacts with actin-polymerizing factor N-WASP	-	x (Q666H3)	+ (Q8CZT5)	[10]
	YapX	Not known	-	x (Q666H2)	o	[11]
Type Vc	YadA	Binding to ECM components, epithelial cells, macrophages and neutrophils, mediates serum resistance and autoagglutination	+ (P31489)	+ (K7ZVF1)	o	[168]
	YadB	Promotes survival in skin after flea bite	-	+ (Q66CJ1)	+ (Q7CHJ4)	[52]
	YadC	Promotes survival in skin after flea bite	-	+ (Q7CHJ5)	+ (Q7CHJ5)	[52]
Type Ve	InvA	Adhesion to and invasion of epithelial cells via β_1 integrins	+ (A1JT35)	+ (P11922)	o	[56]
	Ifp/InvB	Adhesion to and invasion of epithelial cells	x (A0A0H3NUI2)	+ (Q66C38)	o	[75]
	InvC/Ilp	Adhesion to and invasion of host cells	-	+ (A0A0H3AYF9)	+ (Q7CFY4)	[73]
	InvD	Not known	-	x (A0A0H3B1G5)	-	[73]
Fimbrial adhesins						
C-U fimbriae	Psa/Myf	Binding to galactose and phosphocholine, biofilm formation	+ (P33408)	+ (Q56983)	+ (P31527)	[115]
	Caf	Protection from phagocytosis, binding to interleukin-1β	-	-	+ (P26949)	[114]
	y0348-0352	Ahdesion to macrophages	-	x (Q66G26)	+ (Q7CKZ7)	[132]
	y0561-0563	Biofilm formation (?)	-	x (Q66FH7)	+ (Q7CKQ0)	[128]

	y1858-1862	Adhesion to macrophages	x (A1JM00)	x (Q669U8)	+ (Q7CIW9)	[128]
	y1869-1873	Adhesion to macrophages	-	x (Q669W0)	+ (Q7CIW3)	[132]
	y2388-2392	Not known	x (A1JTK2)	x (Q66B61)	+ (Q9ZC30)	[128]
	y3478-3480	Not known	-	x (Q665Z6)	+ (Q7CGJ4)	[128]
	MRHA	Mannose-resistant hemagglutination	+ (A1JJU6)	-	-	[134]
Type IV pili	Pil	Not known	+	+	-	[139]
	Flp	Microcolony formation	+ (A1JQP1)	x (Q665Z1)	o	[142]
Small β-barrels						
OmpX family	Ail	Adhesion to and invasion of epithelial cells, promotes serum resistance	+ (P16454)	+ (Q56957)	+ (Q0WCZ9)	[78]
	OmpX	Not known	x (A1JU26)	x (Q669E5)	+ (Q8D0S1)	[85]
	y2304	Not known	-	x (Q66AY4)	x (Q7CI97)	[85]
	y2446	Not known	-	x (Q66BP0)	x (Q7CI12)	[85]
Omptin family	Pla	Plasminogen activation, complement inactivation, adhesion to and invasion of epithelial cells	-	-	+ (E5GAD2)	[169]
Other adhesins						
	MAM7	Binding to fibronectin and phosphatidic acid	x (A1JM37)	+ (A0A0H3B2K4)	x (Q8D0N9)	[146]
	PGA	Biofilm formation; produced by the *hmsHRSF* locus	x (A1JSA3)	+ (Q66B31)	+ (Q56939)	[149]

	LPS core oligosaccharide	Binding to DC-SIGN on antigen-presenting cells	+	+	+	[151]

[a] + = gene present and expressed; x = gene present (expression status unknown); o = pseudogene; - = not present. Where information on the presence of a particular adhesin gene is not available in the literature, we used bioinformatics tools (e.g. BLAST [170] and GCview [171]) to determine whether a gene is present in one or more genomes from the species in question. For intact genes, we have included a UniProt accession code for a representative sequence (in parentheses). In the case of fimbrial adhesins, the accession code is for the usher protein. For type IV pili, the accession code is for the major pilin subunit. For PGA, the accession code is for the HmsH protein, and for LPS we have not included an accession code.

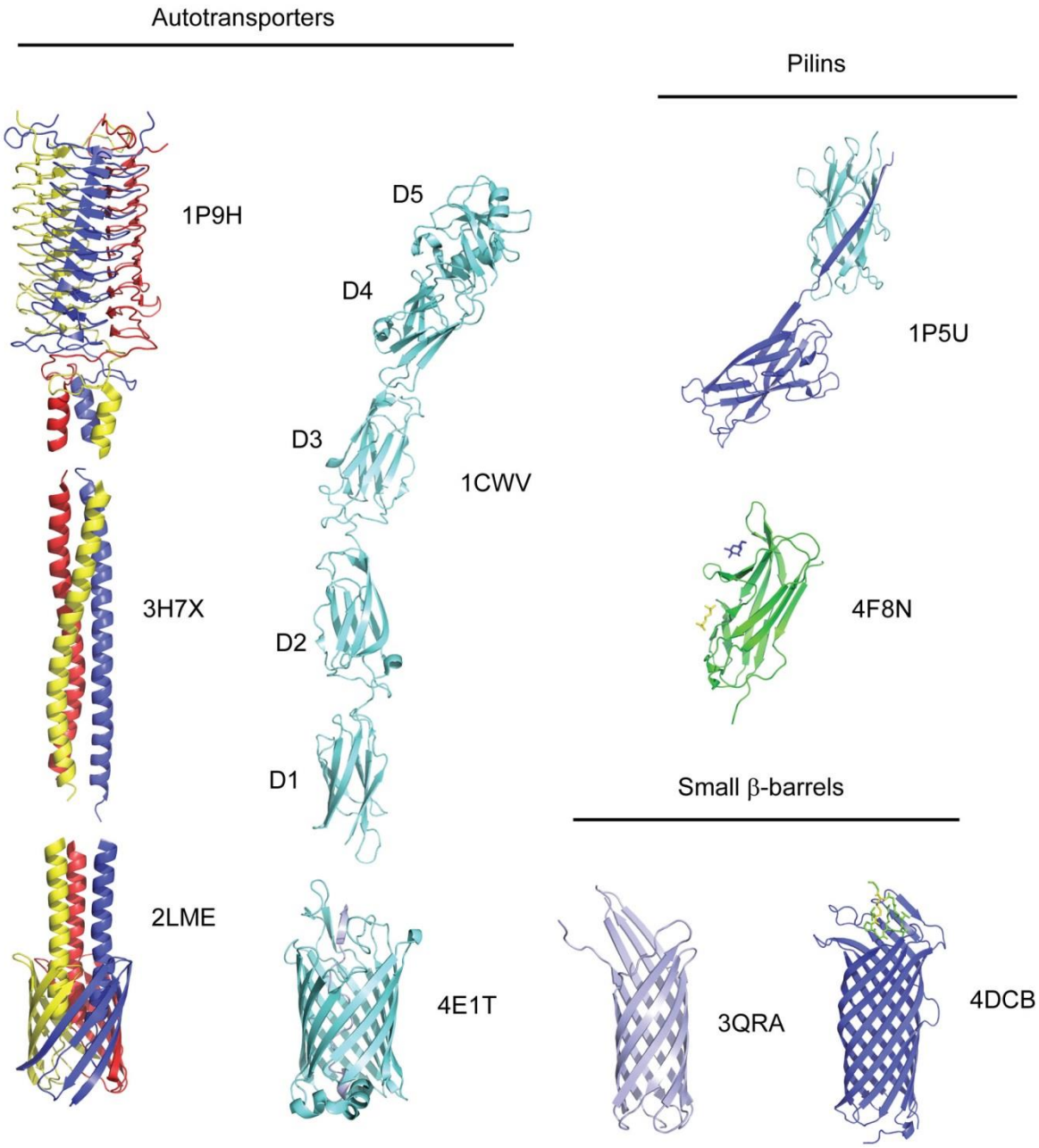
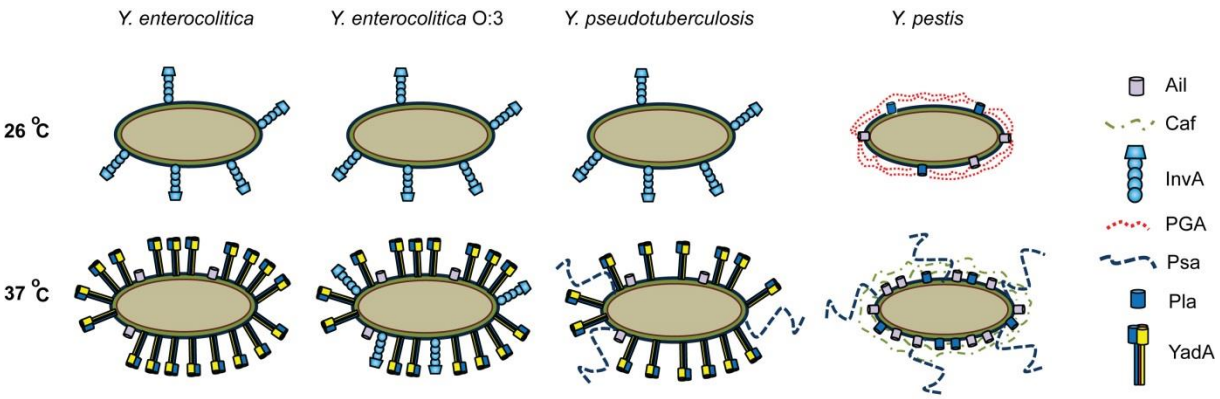


Figure 1. Experimental structures of *Yersinia* adhesins. The structures depicted for YadA (from *Y. enterocolitica*) are the collagen-binding head domain (PDB ID 1P9H), a segment of the stalk (3H7X), and the C-terminal membrane anchor (2LME). In these structures, the three chains are coloured differently. For InvA from *Y. pseudotuberculosis*, the structures of the N-terminal membrane anchor domain (4E1T) and the passenger (1CWV) are shown; the domains D1-D5 of the passenger are indicated. The structures of the small β-barrel proteins Ail (3QRA) and Pla (4DCB) are both from *Y. pestis*. Pla (in blue) is shown in complex with the activation loop peptide of human plasminogen (in yellow). The pilin subunit PsaA (4F8N, in green) of pH 6 antigen from *Y. pestis* is shown in complex

884 with galactose (blue) and phosphocholine (yellow). A minifibre of two Caf1 subunits (1P5U) from *Y.*
885 *pestis* is shown with one subunit in dark blue and one in light blue, with a the light blue subunit
886 complemented with the donor strand from the dark blue subunit. The structures are shown to
887 approximate scale.
888



889

890 **Figure 2.** Effect of temperature shifts between 26 °C and 37 °C on the adhesins displayed on the
891 surface of *Yersiniae*. The major adhesins present at these temperatures are displayed. In *Y.*
892 *pseudotuberculosis* and most *Y. enterocolitica* strains, the major adhesin at 26 °C is InvA, but this is
893 repressed at 37 °C. In contrast, in *Y. enterocolitica* serotype O:3, InvA is also expressed efficiently at
894 37 °C [72]. YadA is expressed by both species at 37 °C. *Y. pestis* lacks both InvA and YadA, but
895 expresses several other adhesins in a temperature-dependent manner, including Ail, Caf and Psa at
896 37 °C. The biofilm-promoting exopolysaccharide PGA is expressed at 26 °C. Pla is present at both
897 temperatures, but more abundant at 37 °C.

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