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Expression, refolding, and initial structural characterization of the *Y. pestis* Ail outer membrane protein in lipids

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ABSTRACT

Ail is an outer membrane protein and virulence factor of *Yersinia pestis*, an extremely pathogenic, category A biothreat agent, responsible for precipitating massive human plague pandemics throughout history. Due to its key role in bacterial adhesion to host cells and bacterial resistance to host defense, Ail is a key target for anti-plague therapy. However, little information is available about the molecular aspects of its function and interactions with the human host, and the structure of Ail is not known. Here we describe the recombinant expression, purification, refolding, and sample preparation of Ail for solution and solid-state NMR structural studies in lipid micelles and lipid bilayers. The initial NMR and CD spectra show that Ail adopts a well-defined transmembrane β-sheet conformation in lipids.

1. Introduction

The *Yersinia* species of Gram-negative bacteria include three strains that are pathogenic for humans, each causing diseases ranging from gastroenteritis (*Yersinia pseudotuberculosis*, *Yersinia enterocolitica*) to plague (*Yersinia pestis*), one of the most deadly human infectious diseases [1–4]. Of these, *Y. pestis* is extremely pathogenic, causing disease with only a few cells, and is easily transmittable from rats to humans through the bite of an infected flea or from human to human through the air during widespread infection. *Y. pestis* has a long history of precipitating devastating human pandemics on a scale unmatched by any other infectious agent, including through its use in bioterrorism (e.g., during Black Death), and plague is still responsible for human outbreaks in endemic areas throughout the world, including the US, where more than 90% of human plague occurs in the Southwest, especially New Mexico, Arizona, California, and Colorado. Because *Y. pestis* spreads very easily and kills very quickly, it is classified as a "category A biothreat agent", and plague is recognized as a re-emerging disease by the World Health Organization. The potential of bio-engineered antibiotic resistance and the lack of a vaccine providing protection from aerosolized *Y. pestis* further underscore the need to identify new drugs [5].

Based on epidemiological observations and historical records, three *Y. pestis* biotypes have been associated with the three major human pandemics: biotype Antiqua with the Justinian Plague during the first millennium, biotype Medievalis with the Black Death from the 12th up to the 19th century, and biotype Orientalis with the third pandemic, which started during the 19th century and is still widespread and associated with modern plague [6]. The recently sequenced genomes of three *Y. pestis* strains, one from Orientalis (CO92) obtained from a clinical isolate in the United States, one from Medievalis (KIM), and one from Microtus (9100), which constitutes a fourth biotype, have provided a wealth of information about mechanisms of infection and pathogenesis [7–9].

The pathogenicity of *Y. pestis* stems from its striking ability to overcome the defenses of the mammalian host and overwhelm it with its massive growth, as well as its ability to survive in a variety of diverse environments (flea, mammalian host tissues, macrophages, and blood), which is critical for transmission from host to host. All three pathogenic *Yersinia* establish infection by adhering to the host and injecting cytotoxic *Yersinia* outer proteins (Yops) into host cells via a type III secretion system. Once in the host cell, Yops interfere with the host signaling pathways required for bacterial phagocytosis, actively blocking host-defense-mediated bacterial phagocytosis and lysis (reviewed in Refs. [2,10,11]). Establishing contact with the host cell is essential for engagement of secretion apparatus.

The outer membrane protein Ail (Attachment Invasion Locus; gene *y1324*) is a key virulence factor of *Y. pestis*, important for colonization,

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and pathogenesis [12–16]. Ail mediates bacterial adhesion to host epithelial cells through its association with the extracellular matrix component fibronectin [12–14,16], facilitates Yop delivery to host cells [14,15], and confers resistance to host serum [12,13]. Deletion of the *ail* gene significantly attenuates the lethality of *Y. pestis*, increasing the pathogen's median lethal dose by >3000-fold. Furthermore, the *ail* deletion mutant is rapidly killed by exposure to animal sera, exhibits reduced binding to human-derived cell lines, and colonizes host tissue at much lower levels, compared to the wild-type strain [13,14]. The expression and function of Ail are sensitive to temperature, reflecting sensitivity to the bacterial host environment [13,17]. At 26 and 37 °C, the ambient temperatures of the flea vector and its mammalian hosts, Ail is expressed at high levels and is required for resistance to complement-mediated killing. However, expression is minimal at 6 °C, i.e., outside the host. Furthermore, the serum-sensitive *Escherichia coli* strain DH5α becomes serum-resistant when it is transformed with an Ail expression vector, confirming that Ail confers serum resistance independent of other *Y. pestis* virulence factors [13].

Y. pestis, diverged from its closest ancestor *Y. pseudotuberculosis* only 1500 to 20,000 years ago [18], and the Ail proteins from *Y. pseudotuberculosis* (gene *YPTB2867*) and *Y. enterocolitica* (gene *YE1820*) also mediate cell adhesion and invasion [16,19] and confer serum resistance by binding to host complement regulatory proteins [20–23]. However, the single amino acid difference between *Y. pestis* Ail (Phe101) and *Y. pseudotuberculosis* Ail (Val101) is reported to reduce the adhesion and invasion activities in the latter [16].

Ail belongs to a family of outer membrane proteins (Ail/Lom; pfam: PF06316) that function, at least in part, to protect bacteria from complement-mediated host defense [24–29]. The family members share amino acid sequence homology and are predicted to have similar membrane topologies, exemplified by the structure of *E. coli* OmpX [30], also a family member, which consists of eight transmembrane amphipathic β-strands and four extracellular loops (Fig. 1). The regions of greatest sequence homology are concentrated in the predicted membrane-spanning segments, while the extracellular loops of Ail are significantly longer than those of OmpX. The second and third extracellular loops (EL2, EL3) of Ail are reported to play a critical role in *Y. enterocolitica* cell adhesion [31] and are also predicted to be important for the functions of Ail from *Y. pestis* and *Y. pseudotuberculosis*. Interestingly, these loops exhibit significant amino acid sequence similarity to those in the unstructured/β-strand extracellular regions of bacterial fibronectin-binding proteins (Fig. 1D), which mediate bacterial adhesion to host by binding to the Fn type 1 (F1) domains in the N-terminus of mammalian fibronectin [32]. The sequence similarity is somewhat stronger for Ail from *Y. pestis* and *Y. pseudotuberculosis* than for *Y. enterocolitica*, suggesting a potential explanation for the somewhat weaker adhesion activity of the latter. Given the predicted integral membrane β-barrel structure of Ail, we anticipate that its mode of binding fibronectin will be different from that observed for other bacterial fibronectin-binding proteins [32].

Outer membrane proteins are often the first line of communication with the extracellular environment, with prominent roles in molecular transport, cellular homeostasis, and bacterial pathogenesis, and therefore, represent important targets for structural and functional characterization [33]. The structures of about twenty β-barrel membrane proteins have been determined in crystals by X-ray diffraction (reviewed in Refs. [33–36]), the structures of five proteins (OmpX, OmpA, PagP, OmpG, VDAC) have been determined in micelles by solution NMR [37–41] and three proteins (OmpG, OmpA, OmpX) have been structurally characterized by solid-state NMR in lipid bilayers [42–45]. Lipid bilayers have the important advantage of providing an environment that closely resembles the cellular membrane, and structures determined in this environment are highly representative of the native *in vivo* conformations. Although samples of membrane proteins in lipid bilayers are too large for solution NMR structural studies, they are suitable for solid-state NMR studies where macroscopic alignment of the samples in the magnetic field provide very high resolution, orientation-dependent restraints for the determination of both protein structure and protein orientation within the membrane [46–48].

The structure of Ail is not known but is essential for understanding the molecular mechanism underlying its interaction with fibronectin and cell adhesion. Here we describe the expression, purification, refolding, and sample preparation of Ail for solution and solid-state NMR structural studies of the protein in micelles and lipid bilayers.

2. Materials and methods

2.1. Protein expression and purification

The gene encoding mature Ail from *Y. pestis* KIM 10 (gene *y1324*, without the signal sequence) was cloned in the *Nde*I and *Bam*HI restriction sites of the *E. coli* plasmid vector pET-3b (Novagen). Deletion of the signal sequence directs the expression of Ail into inclusion bodies. The Ail-bearing plasmid was transformed in *E. coli* C41(DE3) cells [49] (Lucigen), and positive clones were grown in M9 minimal medium, at 37 °C, to a cell density of $OD_{600} = 0.6$, before induction with 1 mM IPTG (isopropyl 1-thio-β-D-galactopyranoside) for 6 h. Cells were harvested by centrifugation (10,000×*g*, 4 °C, 30 min) and stored at −80 °C overnight. For ^{15}N and ^{13}C isotopic labeling, the growth medium was prepared with $(^{15}NH_4)_2SO_4$ and

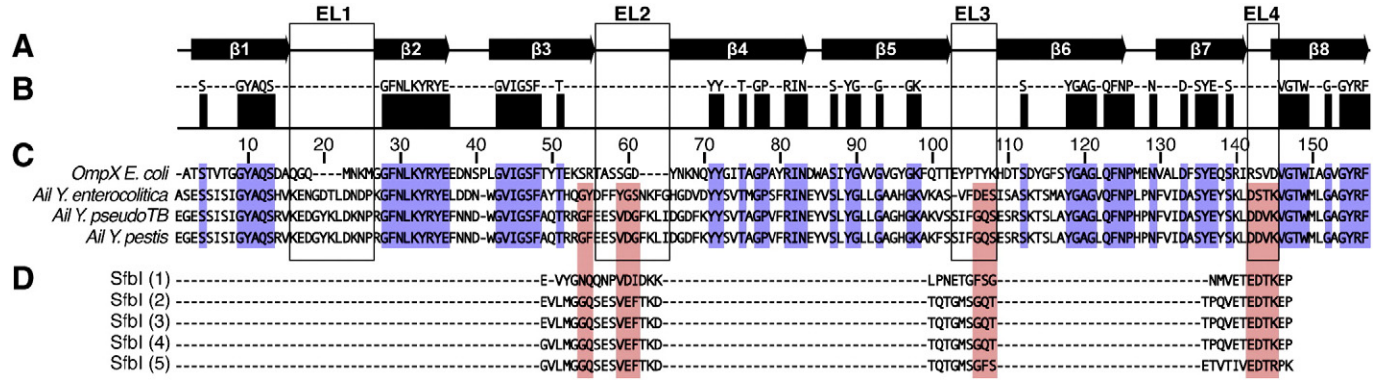

Fig. 1. Multiple sequence alignment of Ail, OmpX, and fibronectin-binding proteins. (A) Secondary structure of OmpX [30]. (B) Consensus sequence for Ail and OmpX. (C) Alignment of mature Ail from *Y. pestis* (gene *y1324*), *Y. pseudotuberculosis* (gene *YPTB2867*), and *Y. enterocolitica* (gene *YE1820*), and OmpX from *E. coli* (gene *c0900*). The Ail sequences from *Y. pestis* and *Y. pseudotuberculosis* are identical except for the F101V mutation before the predicted extracellular loop EL3. Amino acids identical in all four proteins are in blue. (D) Sequences of fibronectin F1 binding domains in the C-terminal region of the fibronectin-binding protein SfbI from *Streptococcus pyogenes*. Amino acids important for fibronectin binding, sharing similarity with the extracellular loops of Ail, are in red. Alignments were produced with ClustalW and rendered with Jalview.