

1 **Correlating the structure and activity of *Y. pestis* Ail in a bacterial cell** 2 **envelope**

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8 9 10 **ABSTRACT**

11 Understanding microbe-host interactions at the molecular level is a major goal of fundamental biology and
12 therapeutic drug development. Structural biology strives to capture biomolecular structures in action, but the
13 samples are often highly simplified versions of the complex native environment. Here we present an *E. coli*
14 model system that allows us to probe the structure and function of Ail, the major surface protein of the deadly
15 pathogen *Yersinia pestis*. We show that cell surface expression of Ail produces *Y. pestis* virulence phenotypes
16 in *E. coli*, including resistance to human serum, co-sedimentation of human vitronectin and pellicle formation.
17 Moreover, isolated bacterial cell envelopes, encompassing inner and outer membranes, yield high-resolution
18 solid-state nuclear magnetic resonance (NMR) spectra that reflect the structure of Ail and reveal Ail sites that
19 are sensitive to the bacterial membrane environment and involved in the interactions with human serum
20 components. The data capture the structure and function of Ail in a bacterial outer membrane and set the stage
21 for probing its interactions with the complex milieu of immune response proteins present in human serum.

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23

24 SIGNIFICANCE

25 Ail is a critical virulence factor of *Y. pestis*, and its interactions with human serum are central for promoting
26 the immune resistance of bacteria to the human host defenses. Here we capture the action of Ail in a functional
27 bacterial environment and set the stage for probing its interactions with the complex milieu of immune response
28 proteins present in human serum. The development of an *E. coli* model system of *Y. pestis* for biophysical
29 studies is new and biologically important. Finally, the work extends the range *in-situ* NMR spectroscopy to
30 include models of microbial infection.

31

32 INTRODUCTION

33 The protein and lipid components of bacterial outer membranes work together to support microbial cell survival
34 in a wide range of host environments and represent key virulence factors. This phenomenon is especially
35 striking in the case of *Yersinia pestis* – the agent responsible for multiple devastating human plague pandemics
36 throughout history. The *Y. pestis* outer membrane protein Adhesion invasion locus (Ail) and lipopolysaccharide
37 (LPS) have co-evolved to enhance microbial resistance to human innate immunity, enabling the bacterium to
38 produce high-level septicemia in its mammalian hosts (1-11). Ail and LPS jointly promote evasion of the host
39 immune defenses and adhesion/invasion of host cells (9-11). Ail and LPS mutants exhibit altered sensitivity to
40 human serum, antibiotics and cell wall stress (12, 13) and we have identified LPS-recognition motifs on the
41 surface of Ail important for establishing mutually reinforcing Ail-LPS interactions that promote microbial
42 survival in human serum, antibiotic resistance and cell envelope integrity (14). These features highlight the role
43 of the outer membrane environment as a critical regulator of Ail activity and underscore the importance of
44 characterizing the native Ail-membrane assembly as a whole, rather than its individual components.

45 Previous structural studies have relied on purified Ail refolded in detergent micelles, detergent-free lipid
46 nanodiscs or liposomes for X-ray diffraction and NMR (15-19). The structure of Ail – an eight-stranded β -
47 barrel with four extracellular loops and three intracellular turns – is conserved in all cases, but these chemically
48 defined samples preclude NMR studies aimed at probing the molecular interactions of Ail with either bacterial
49 membrane components like LPS, or human ligands, in the context of the native environment. Importantly, this
50 situation negates a major advantage of NMR compared to other structure determination technologies: the high
51 sensitivity of NMR signals to their local environment, which makes them extremely useful for characterizing
52 even weak inter-molecular interactions by direct spectroscopic detection.

53 We have shown that nanodiscs and liposomes can incorporate Ail with various types of LPS, including native
54 *Y. pestis* LPS (14, 19), but even though these detergent-free lipid platforms represent important advances in
55 membrane complexity they remain distant substitutes for the native outer membrane, which is highly
56 anisotropic, heterogeneous, and connected to the cytoskeletal peptidoglycan layer. The emerging field of *in-*
57 *situ* NMR (20-26) presents new opportunities for examining the structural properties and interactions of Ail in
58 a native bacterial outer membrane.

59 Here we describe an *E. coli* model system where Ail is natively folded in the bacterial outer membrane for
60 parallel solid-state NMR structural studies and microbial functional assays, *in-situ*. Using this model, we
61 demonstrate that Ail expression produces *Y. pestis* virulence phenotypes in *E. coli*, and that isolated cell
62 envelopes expressing ¹⁵N and ¹³C labeled Ail yield atomic-resolution NMR spectra that allow us to probe the
63 structure of Ail in a native bacterial membrane and its interactions with human serum components. This model
64 sheds light on the interactions of Ail with components of human serum and provides a platform for advancing
65 structure-activity NMR studies of Ail in the native environment, including its various physiological
66 modulations that can be elicited by factors such as temperature, antibiotics, or exposure to serum.

67

68 MATERIALS AND METHODS

69 **Expression of Ail in the *E. coli* outer membrane.** The gene encoding mature *Y. pestis* Ail was cloned in the
70 *NcoI* and *XhoI* restriction sites of the *E. coli* plasmid pET-22b(+), downstream of the signal sequence of pectate
71 lyase B (27). This plasmid was transformed into *E. coli* cells by heat shock and positive clones were selected
72 by plating on LB-agar with ampicillin (100 µg/mL) and chloramphenicol (35 µg/mL), in the case of
73 Lemo21(DE3) cells. Three *E. coli* cell strains derived from BL21(DE3) (28) were tested: C41(DE3) (29),
74 BL21(DE3)ΔACF (30) and Lemo21(DE3) (31). Transformed cells were cultured overnight, at 30°C, with
75 vigorous shaking, in 50 mL of M9 minimal media supplemented with Basal Medium Eagle vitamin solution
76 (1% by vol.), ampicillin (100 µg/ml), and chloramphenicol (50 µg/mL). This overnight culture was used to
77 inoculate 500 mL of fresh M9 media (supplemented as above), and grown from OD₆₀₀ ~0.05 to OD₆₀₀ ~0.4, at
78 30°C, at which point the cells were harvested by low-speed centrifugation (5,000 g, 4°C, 20 min) and then
79 resuspended in 500 mL of fresh supplemented M9 media. Protein expression was induced by adding 0.4 mM
80 isopropyl β-D-1-thiogalactopyranoside (IPTG), after which the shaking speed was reduced, and the temperature
81 lowered to 25°C. The cells were cultured for 20 min before adding rifampicin (100 µg/mL), and then for an
82 additional 20 hours, in the dark. The cells were harvested by low-speed centrifugation, and resuspended in
83 HEPES buffer (10 mM, pH 7.4).

84 To obtain uniformly ¹⁵N,¹³C labeled Ail, the cells were grown in unlabeled M9 media and only transferred to
85 ¹⁵N,¹³C labeled M9, prepared with (¹⁵NH₄)₂SO₄ (1 g/L) and ¹³C₆-glucose (5 g/L), before induction with IPTG.
86 To obtain ²H labeling of endogenous *E. coli* biomolecules, cells were grown in 100 mL of ²H₂O and then
87 switched to H₂O before induction with IPTG.

88 **Isolation of bacterial membrane fractions.** Ail-expressing cells were lysed by three passes through a French
89 Press. After removing cellular debris by centrifugation (20,000 g, 4°C, 1 h), the total cell envelope fraction,
90 including inner and outer membranes, was harvested by ultracentrifugation (100,000 g, 4°C, 1 h), washed three
91 times with sodium phosphate buffer (20 mM, pH 6.5), and then collected by ultracentrifugation. To further
92 isolate the outer membrane fraction, the total cell envelope was suspended in 7 mL of buffer I (10 mM HEPES,
93 pH 7.4, 3.4 mM N-lauroylsarcosine) and then separated by ultracentrifugation. The supernatant containing
94 detergent-solubilized inner membrane was removed, while the pellet was washed three times with sodium
95 phosphate buffer (20 mM, pH 6.5) and then collected by ultracentrifugation. All three fractions – total cell
96 envelope, inner membrane and outer membrane – were analyzed by polyacrylamide gel electrophoresis (PAGE)
97 in sodium dodecyl sulfate (SDS), and immune-blotting with the α-Ail-EL2 antibody (17).

98 **NMR sample preparation.** Samples for solid-state NMR studies were packed into 4 mm, 3.2 mm or 1.3 mm
99 magic angle spinning (MAS) rotors. For samples incubated with human serum (NHS or HIS), isolated cell
100 envelopes expressing ¹³C,¹⁵N Ail were suspended in 7 mL of serum and gently mixed for 4 h, at room
101 temperature, before harvesting and washing with sodium phosphate buffer by ultracentrifugation and packing
102 into the MAS rotor. The preparation of Ail liposome samples has been described (18).

103 **Solid-state NMR spectroscopy.** Solid-state NMR experiments with ¹³C detection were performed on a
104 750 MHz Bruker Avance III HD spectrometer with a Bruker 3.2 mm ¹H,¹³C,¹⁵N E-free MAS probe, or a
105 500 MHz Bruker Avance spectrometer with a Bruker 4 mm ¹H,¹³C,¹⁵N E-free MAS probe, at an effective
106 sample temperature of 7±5°C, with a spin rate of 11,111 Hz. Typical π/2 pulse lengths for ¹H, ¹³C and ¹⁵N were
107 2.5 µs, 2.5 µs, and 5 µs, respectively. ¹H decoupling was implemented with the SPINAL64 sequence, with a
108 radio frequency (RF) field strength of 90 kHz during acquisition. Two-dimensional ¹⁵N/¹³C NCA spectra were
109 acquired using the SPECIFIC-CP pulse program, with contact times of 2 ms and a 70-100% ramp, for ¹H/¹⁵N
110 cross polarization (CP), and 4.9 ms for ¹⁵N/¹³C CP. Two-dimensional ¹³C-¹³C PDS (proton driven spin
111 diffusion) spectra were acquired with a ¹H-¹³C contact time of 1 ms, a ¹³C-¹³C mixing time of 50 ms, and 20 ms
112 of SWf-TPPM ¹H decoupling.

113 Experiments with ¹H detection were performed on a 900 MHz Bruker Avance III HD spectrometer equipped
114 with a Bruker 1.3 mm MAS probe, operating at an effective sample temperature of 30 ± 5°C, with a spin rate

115 of 57,000±15 Hz. Two-dimensional $^1\text{H}/^{15}\text{N}$ CP-HSQC spectra were acquired as described previously (18),
116 using the MISSISSIPPI sequence (32) for water suppression.

117 **Bacterial cell assays.** *E. coli* Lemo21(DE3) cells transformed with Ail-expressing plasmid pET-22b(+) or with
118 empty plasmid were cultured as described above, then harvested by low-speed centrifugation, washed twice in
119 ice-cold phosphate buffer saline (PBS), and resuspended in ice-cold PBS to $\text{OD}_{600}=1.0$.

120 To assay binding to human Vn, washed bacteria (250 μl) were added to an equal volume of either NHS (Sigma-
121 Aldrich H4522), HIS (Sigma-Aldrich H3667), purified full-length Vn (1 μM in PBS; Sigma-Aldrich
122 SRP3186), or purified Vn-HX (2 μM in PBS) prepared as described (33). The binding reactions were incubated
123 for 30 min at 37°C, then placed on ice. Bacterial cells and bound proteins were then collected by centrifugation
124 (6,000 g, 5 min, 4°C), washed three times with 1 mL of ice-cold PBS containing 0.1% Tween-20, and lysed by
125 boiling in 100 μL of SDS-PAGE sample buffer (50 mM Tris-HCl, 2% SDS, 5% glycerol, 1% β -
126 mercaptoethanol, pH 6.8). Bacterial cell lysates and co-sedimented proteins were subjected to SDS-PAGE and
127 immunoblot analysis with rabbit polyclonal anti-Vn (R12-2413, Assay Biotech) and anti-Ail-EL2 (34)
128 antibodies.

129 To assay serum resistance, *E. coli* cells collected after incubation with NHS or HIS were diluted 100-fold with
130 PBS, and 25 μL of this dilution mixture were plated on LB-agar supplemented with ampicillin (100 $\mu\text{g}/\text{mL}$)
131 and chloramphenicol (35 $\mu\text{g}/\text{mL}$). After incubating overnight at 37°C overnight, the percentage of cell survival
132 was estimated by counting the number of bacterial colonies present on each plate, using ImageJ (35). The
133 percent survival represents the number of colonies that survive in NHS divided by the number that survive in
134 HIS.

135 To assay pellicle formation, *E. coli* cells were harvested, then resuspended in 2 mL of M9 minimal media to
136 $\text{OD}_{600} = 0.5$ in 15 x 100 mm glass tubes, and incubated for 16 h at 37°C. After removing the cells by
137 centrifugation, the interior glass walls were treated with methanol and air-dried overnight, then washed three
138 times with PBS and again air-dried overnight. The tubes were treated with 2.5 mL of crystal violet solution
139 (0.1 %) for 10 min, then washed with PBS and air-dried. Pellicle formation was detected qualitatively as a
140 violet-stained rim at the air-liquid interface.

141

142 RESULTS AND DISCUSSION

143 **Production of folded Ail in the outer membrane of *E. coli*.** High-resolution, multi-dimensional NMR studies
144 require isotopically ^{15}N and ^{13}C labeled biomolecules. For studies of proteins in native cell membranes, targeted
145 isotopic labeling is critical for suppressing background NMR signals from other cellular components, and solid-
146 state NMR methods are needed to overcome the correlation time limitations posed by samples that are
147 effectively immobilized on a time scale $\geq \mu\text{sec}$. Moreover, the inherently low sensitivity of NMR necessitates
148 samples that are enriched in target protein. This requirement is compatible with the properties of Ail, which
149 naturally comprises more than 30% of the *Y. pestis* outer membrane proteome at the mammalian infection
150 temperature of 37°C (6, 9, 36-38).

151 *E. coli* is widely used as a model for assaying the functions of *Y. pestis* proteins, including Ail. *E. coli* strains
152 derived from BL21(DE3) have a rough-type LPS that lacks an extended O-antigen, similar to that of *Y. pestis*,
153 and we have shown that *E. coli* rough-type LPS and *Y. pestis* LPS both perturb the NMR spectra of Ail in a
154 similar manner (14). The use of *E. coli* also facilitates isotope labeling for NMR.

155 To drive the production of folded, ^{15}N , ^{13}C -Ail in the *E. coli* outer membrane, we cloned the sequence of the
156 pectate lyase B (PelB) leader peptide (27) before the N-terminus of mature Ail, and relied on the *E. coli* native
157 β -barrel assembly machinery (BAM) to insert Ail across the bacterial outer membrane. The Ail-enriched outer
158 membrane or the entire cell envelope – including inner and outer membranes – were isolated and taken directly
159 for NMR (Fig. 1A). To reduce isotopic labeling of endogenous *E. coli* components, the cells were grown in
160 unlabeled minimal media and transferred to isotopically labeled media only before induction with IPTG. We

161 tested Ail expression in three derivative strains of *E. coli* BL21(DE3) (28), where recombinant protein synthesis
 162 is controlled by a chromosomally-encoded, IPTG-inducible polymerase from bacteriophage T7 and its plasmid-
 163 encoded T7 promoter.

164 Our initial attempts utilized C41(DE3) cells (29), which promote folded protein insertion in the *E. coli*
 165 membrane due to a mutation that weakens the production of T7 polymerase (31). These cells yield high
 166 expression of properly folded Ail (Fig. 1B), but also produce appreciable amounts of endogenous *E. coli*
 167 proteins that are isotopically labeled. Background NMR signal can be reduced by expressing Ail in
 168 BL21(DE3) Δ ACF, a mutant cell strain that lacks the major *E. coli* outer membrane proteins OmpA, OmpC and
 169 OmpF (30). Compared to C41 strains, this mutant is compatible with the use of rifampicin, a potent inhibitor
 170 of *E. coli* RNA polymerase (39) that suppresses endogenous protein production was first used to enable targeted
 171 isotopic labeling of heterologous proteins for solution (40) NMR studies *in situ*. These cells produce very high
 172 levels of Ail (Fig. 1C), but their usefulness is limited by the production of large amounts of misfolded protein,
 173 detectable as a band migrating just above 17 kDa in sodium dodecyl sulfate polyacrylamide gel electrophoresis
 174 (SDS-PAGE) that is distinguishable from folded Ail at 14 kDa (17). The addition of rifampicin to
 175 BL21(DE3) Δ ACF cells reduces protein expression levels overall, but does not enhance the proportion of folded
 176 to unfolded Ail. Although endogenous background signal can be reduced by isolating the outer membrane from
 177 the cell envelope, this process exposes the sample to the compromising effects of detergent on biomolecular
 178 structure and activity (41, 42). The two-dimensional (2D) $^{15}\text{N}/^{13}\text{C}$ NCA solid-state NMR spectra from outer
 179 membrane preparations of either C41(DE3) or BL21(DE3) Δ ACF cells reflect the overall signature of Ail (17-
 180 19) but suffer from sub-optimal resolution and sensitivity (Fig. S1).

181 To avoid these complications we tested Lemo21(DE3) cells (31), where T7 RNA polymerase can be controlled
 182 by its natural inhibitor T7 lysozyme, and rifampicin can be used to effectively halt transcription of the *E. coli*
 183 genome. By initially growing the cells in unlabeled minimal media, then transferring them to isotopically
 184 labeled media supplemented with both rifampicin and IPTG, expression of $^{15}\text{N}/^{13}\text{C}$ -Ail was induced and
 185 allowed to continue under control of the bacteriophage T7 promoter, while endogenous protein production was

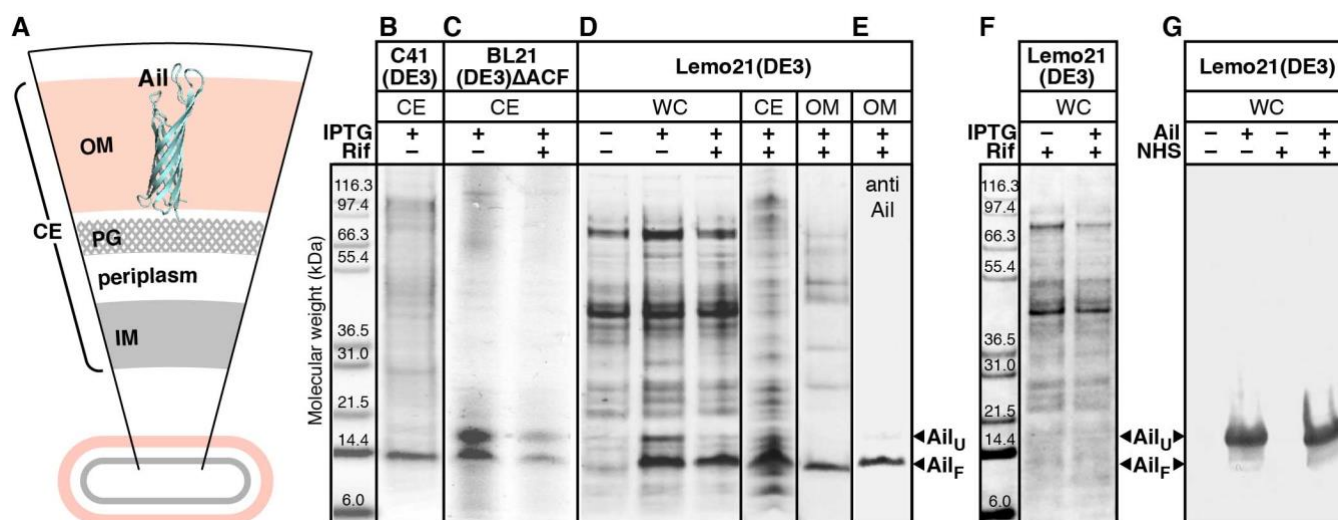


Figure 1. Production of folded Ail in *E. coli* and in situ NMR of Ail in *E. coli* cell envelopes. (A) Depiction of the bacterial cell envelope (CE) fraction isolated for NMR studies, including the outer membrane (OM) with embedded Ail (cyan), inner membrane (IM), peptidoglycan layer (PG), and periplasm. **(B-F)** SDS-PAGE analysis of cell envelope (CE), whole cell (WC), or outer membrane (OM) fractions isolated from *E. coli* C41(DE3), BL21(DE3) Δ ACF, or Lemo21(DE3) cells transformed with Ail plasmid (B-E) or empty plasmid (F). Cells expressing Ail (+) or empty plasmid (-) were incubated with NHS for 30 min and then analysed by SDS-PAGE and immunoblotting after heat denaturation (G). Cells were grown with or without rifampicin and induced with IPTG. Ail was visualized with Coomassie stain (B-D, F) or immunoblotting with Ail-specific antibody (E, G).

186 blocked. As an additional important advantage, we found that rifampicin moderates the level of Ail
187 overexpression and reduces protein misfolding to negligible levels, resulting in cell envelope and outer
188 membrane fractions that are highly enriched in folded Ail (Fig. 1D, E).

189 **Solid-state NMR of Ail in bacterial cell envelopes.** Cell envelope preparations from Lemo21(DE3) cells yield
190 high quality 2D $^{15}\text{N}/^{13}\text{C}$ solid-state NMR spectra, with respect to both signal intensity and resolution (Fig.
191 2A, black). The line widths of well-resolved signals, such as those from Ala and Gly (Fig. S2), are in the range
192 of 0.6–0.8 ppm for ^{13}C and 1.3–1.8 ppm for ^{15}N . They compare favorably with those measured from purified
193 Ail reconstituted in liposomes (18), demonstrating that the combined use of this cell strain with rifampicin is
194 highly beneficial for producing folded, isotopically labeled Ail. Based on comparison of the 1D cross-sections
195 of the $^{15}\text{N}/^{13}\text{C}$ peaks (Fig. S2, S6) with those from Ail-liposomes (18), we estimate that the cell envelope sample
196 in the 3.2 mm MAS rotor contains approximately 0.7 mg of Ail or 25% of the Ail-liposomes.

197 Notably, the 1D ^{13}C spectra (Fig. S3) acquired for *E. coli* cell envelopes prepared from Ail(–) bacteria,
198 transformed with empty plasmid but otherwise grown in an identical manner as Ail(+) bacteria, show no
199 evidence of protein signals when compared to the spectra of Ail(+) preparations. These cells do not express Ail
200 (Fig. 1F) and, together with the NMR data, they demonstrate that the expression system is tightly regulated and
201 leads to highly selective isotope labeling of the target Ail, with minimal background.

202 Many resonances in the 2D $^{15}\text{N}/^{13}\text{C}$ spectrum can be assigned by direct comparison with the solid-state and
203 solution NMR spectra of purified Ail reconstituted in liposomes (Fig. 2A, red) or nanodiscs (18, 19), indicating

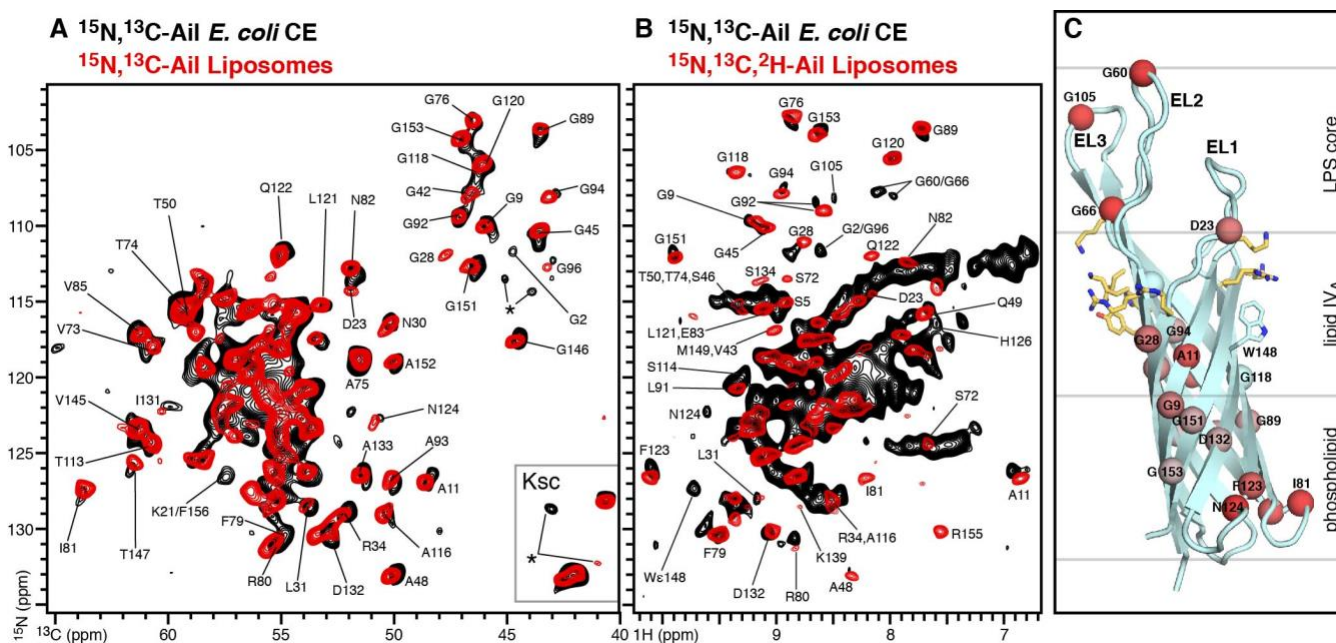


Figure 2. Solid-state $^{15}\text{N}/^{13}\text{C}/^1\text{H}$ NMR spectra of Ail in *E. coli* cell envelopes. (A) 2D $^{15}\text{N}/^{13}\text{C}$ NCA spectra of $^{15}\text{N}/^{13}\text{C}$ -Ail in *E. coli* cell envelopes (black) or reconstituted liposomes (red). Spectra were recorded at 750 MHz, 7°C, with a MAS rate of 11 kHz and 640 transients for the cell envelope samples or 128 transients for the liposome samples. Resolvable assigned peaks are marked. Asterisks denote new unassigned peaks. (B) ^1H -detected $^1\text{H}/^{15}\text{N}$ CP-HSQC spectra of ^{15}N -Ail in *E. coli* cell envelopes (black) or in reconstituted liposomes (red). Spectra were recorded at 900 MHz, 30°C, with a MAS rate of 57 kHz and 1,600 transients for the cell envelope sample or 160 transients for the liposome samples. (C) Structural model of Ail embedded in the *Y. pestis* outer membrane taken from previous MD simulations (14). Spheres denote resolved and assigned amide N atoms that undergo $^1\text{H}/^{15}\text{N}$ chemical shift perturbations from 0 ppm (cyan) to 0.15 ppm (red), between the cell envelope and liposome environments. Sidechains forming two clusters of LPS-recognition motifs are shown as yellow sticks. The boundaries of the outer membrane phospholipid and LPS layers are marked (gray lines). Residue numbers, from E1 to F156, corresponds to the mature sequence of Ail.

204 that the same overall structure – an eight-stranded β -barrel with four extracellular loops (EL1-EL4) and three
205 intracellular turns (T1-T3) (15, 16, 19) – is preserved in the bacterial outer membrane. Within the 2D $^{15}\text{N}/^{13}\text{C}$
206 spectrum, there are some new signals and 47 cross-peaks are sufficiently resolved to facilitate the detection of
207 site-specific chemical shift perturbations (relative to liposomes) for residues dispersed across the Ail sequence
208 and its β -barrel topology (Fig. S4A).

209 The ^1H , ^{15}N and ^{13}C chemical shifts from backbone sites are sensitive to chemical environment, ligand binding
210 and protein conformational changes (43). To examine the effects of the native membrane environment on Ail,
211 we acquired a ^1H -detected $^1\text{H}/^{15}\text{N}$ cross polarization (CP) solid-state NMR spectrum of Ail in bacterial cell
212 envelopes (Fig. 2B, black). Typically, ^2H labeling of side chain protons is needed to achieve line narrowing and
213 high sensitivity in ^1H -detected solid-state NMR experiments with MAS rates ~ 60 kHz (44, 45). For purified
214 and reconstituted membrane proteins, this can be achieved by labeling with ^2H and back-exchanging the amide
215 hydrogens to ^1H during reconstitution with lipids. This approach, however, is not readily applicable to cellular
216 membrane proteins where membrane-embedded segments remain water-inaccessible throughout the sample
217 preparation protocol. In this study, we prepared the sample by initially growing cells in $^2\text{H}_2\text{O}$, and then
218 transferring the culture to $^1\text{H}_2\text{O}$, with ^{15}N salts and rifampicin, for Ail induction. The goal was to silence the
219 background by ^2H labeling ($\sim 70\%$) of endogenous *E. coli* components, whilst achieving targeted ^{15}N labeling
220 of Ail. The resulting 2D CP-HSQC spectrum is strikingly good, despite the lack of Ail deuteration.

221 The cell envelope spectrum, obtained at 900 MHz ^1H frequency and 30°C , compares favorably with the CP-
222 HSQC solid-state NMR spectrum of fractionally deuterated ($\sim 70\%$) Ail in liposomes (Fig. 2B, red), which was
223 obtained at the same field and temperature (18), and also with the TROSY-HSQC solution NMR spectrum of
224 uniformly deuterated ($\sim 99\%$) Ail in nanodiscs, obtained at 800 MHz and 45°C (Fig. S5) (18, 19). Individual
225 resonances have line widths in the range of 0.15 ppm for ^1H and 1.5 ppm for ^{15}N , in line with recent reports of
226 ^1H -detected spectra of membrane proteins in native cell membranes (46). A total of 22 $^1\text{H}/^{15}\text{N}$ peaks can be
227 resolved and assigned by direct comparison with the previously assigned solution NMR spectra of nanodiscs
228 and solid-state NMR spectra of liposomes (18, 19). Improvements in $^1\text{H}/^{15}\text{N}$ resolution should be available
229 through the implementation of solid-state NMR experiments with MAS rates greater than 60 kHz as described
230 (44, 45), and ^2H labeling schemes such as those that enable selective visualization of the water-accessible loops,
231 obtained by growing cells in ^2H -labeled media followed by incubation in H_2O to back-exchange water-
232 accessible sites.

233 Previously, we showed that Ail acquires conformational order in the presence of LPS, leading to substantial
234 enhancement of solid-state NMR CP signal intensity (19). Moreover, all-atom MD simulations (14) indicate
235 that the extracellular loops of Ail exhibit dramatically reduced root mean square fluctuations in a native
236 bacterial membrane. In line with these earlier observations, the NCA and CP-HSQC spectra of Ail in cell
237 envelopes contain some new signals, which were not previously observed in the spectra from reconstituted
238 liposomes. These include HN peaks assigned in the solution NMR spectra of nanodiscs (G2, G60, G66, G105,
239 S114, and the side chain of W148), as well as new unassigned peaks. These results suggest that Ail has more
240 restricted conformational dynamics in *E. coli* outer membranes leading to enhanced CP transfer efficiency and
241 signal intensity.

242 Spectral comparisons reveal chemical shift differences between the chemically defined liposome membrane
243 environment and the bacterial cell envelope (Fig. 2C; Table S1). Independent spectroscopic assignments will
244 be needed to fully and precisely map the effects of the bacterial membrane environment on the protein, but
245 some prominent differences are detectable at this stage. These map to sites in the extracellular loops (D23, G60,
246 G66, G105) and near the extracellular membrane-water interface (A11, G28, A48, G92, G94), just below the
247 two clusters of positively charged residues that we have identified as LPS-recognition motifs on opposite poles
248 of the Ail β -barrel (14): cluster I (R14, K16, K144 and K139), which is tightly localized to the base of the two
249 short extracellular loops EL1 and EL4, and cluster II, which occupies a broader region on the barrel surface
250 extending from the base (R27, R51, H95) to the outer extremities (K69, K97, K99) of the two long loops EL2
251 and EL3.

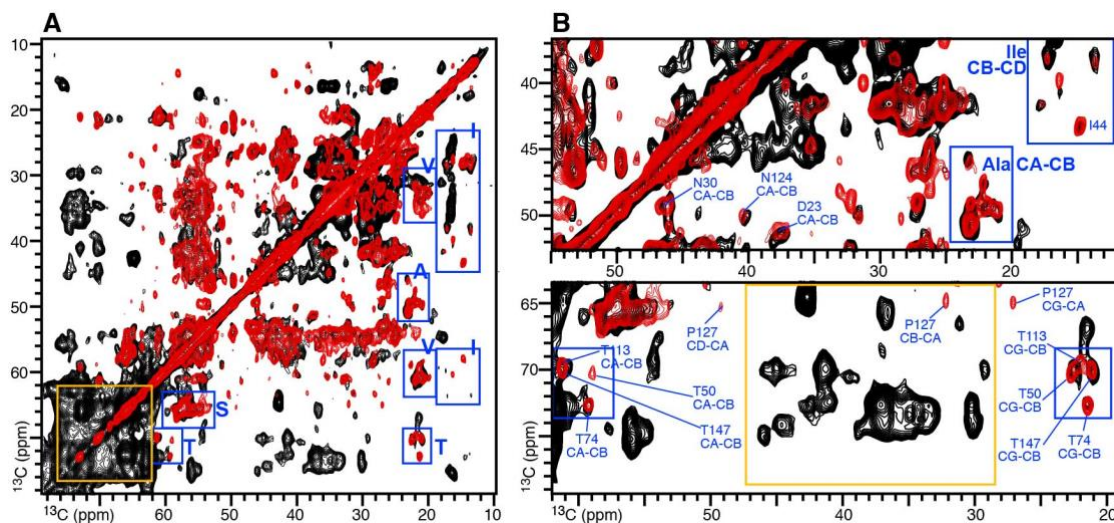


Figure 3. Solid-state $^{13}\text{C}/^{13}\text{C}$ PDSD NMR spectra of $^{15}\text{N}, ^{13}\text{C}$ -Ail in *E. coli* cell envelopes. Resolvable signals from Ala Ile, Ser, Thr, and Val residues of Ail (blue) and non-Ail bacterial cell envelope components (gold) are marked. **(A)** Aliphatic region of the 2D spectra from *E. coli* cell envelopes (black) or reconstituted liposomes (red). Spectra were recorded at 750 MHz, 7°C, with a MAS rate of 11 kHz, 204 t1 increments and 304 transients for the cell envelope sample, or 512 t1 increments and 64 transients for the liposome sample. **(B)** Expanded spectral regions.

252 Notable spectral differences are also observed for membrane-embedded sites of the Ail β -barrel (G9, G89,
253 D132, G151, G153) and sites in the intracellular turns (R80, I81, F123, N124), suggesting that the entire protein
254 senses the physical-chemical properties of its membrane environment. The results are in line with our recent
255 report (14) that specific interactions of outer membrane LPS with cluster I and cluster II Ail sites enhance the
256 conformational order of Ail, dampen LPS dynamics and cause an overall thickening of the outer membrane
257 around its β -barrel, while mutations in the Ail binding sites for LPS compromise cell envelope integrity, reduce
258 *Y. pestis* survival in serum, and enhance antibiotic susceptibility.

259 To further examine the side chain conformations of Ail in the bacterial cell envelope, we acquired a 2D $^{13}\text{C}/^{13}\text{C}$
260 correlation PDSD spectrum (Fig. 3 black). The spectrum, obtained in 25 hours, has excellent resolution. The
261 overall pattern of cross peaks is conserved between bacterial cell envelope and liposome preparations (Fig. 3,
262 black), indicating that the side chain conformations are broadly similar. Resolved signals from Ala, Ile, Pro,
263 Ser, Thr and Val spin systems can be easily identified based on their chemical shifts, which reflect the β -barrel
264 structure of Ail, and 54 intra-residue cross-peaks can be assigned (Fig. S6) by direct transfer from the liposome
265 NMR data which was assigned previously (18). By contrast, the spectrum of Ail(-) cell envelopes contains no
266 protein signals (Fig. S6), confirming the isotopic labeling selectivity of the expression system.

267 The $^{13}\text{C}/^{13}\text{C}$ correlation PDSD spectrum also contains signals from cell envelope components other than Ail,
268 including lipids, LPS and peptidoglycan, which make up a large proportion of the bacterial cell mass and
269 incorporate ^{13}C even with the use of rifampicin, due to relatively fast doubling time of *E. coli* cells and the lag
270 time between IPTG induction and rifampicin addition. While the flexible regions of these molecules are
271 detectable only by through-bond polarization transfer, their more rigid groups are visible in the NMR spectra
272 obtained with CP (23, 47) and are present in the PDSD spectra of both Ail(+) and Ail(-) cell envelopes (Fig.
273 S6).

274 Treatment with cerulenin, an inhibitor of fatty acid biosynthesis has been used to inhibit ^{13}C labeling of *E. coli*
275 lipids and simplify $^{13}\text{C}/^{13}\text{C}$ correlation spectra of proteins (22). Moreover, spectroscopic approaches have been
276 developed to silence lipid signals based on ^{15}N -filtering (48), and NMR data acquisition above the gel-to-liquid

277 phase transition of the lipids has also been shown to suppress lipid signal intensity (49). These cell envelope
278 signals, however, also present an opportunity to probe specific interactions of Ail with the cell envelope.

279 Notably, a set of $^{13}\text{C}/^{13}\text{C}$ cross peaks observed in the region between 30-45 ppm and 60-75 ppm of the Ail(+)
280 cell envelope spectrum are absent from the Ail(-) spectrum. Resonance assignments will be needed to
281 determine the network of inter-atomic interactions in the Ail outer membrane, but there are two plausible
282 explanations for this apparently Ail-dependent observation. Firstly, these resonances could reflect contacts
283 between LPS sugar moieties (60-75 ppm) and His, Lys, Arg and Tyr sidechains of Ail (30-45 ppm) in line with
284 previous observations based on chemical shift perturbation analysis (14). Secondly, they could reflect inter- or
285 intra-molecular contacts of LPS and lipids, resulting from the general ordering effect of Ail on the outer
286 membrane of *E. coli*. These effects need not be exclusive but could synergize such that Ail-LPS interactions
287 rigidify bound LPS molecules thereby enhancing LPS intra-molecular CP transfer.

288 **Emergence of *Y. pestis* phenotypes in *E. coli* through Ail expression.** One important advantage of *in-situ*
289 NMR spectroscopy is the ability to probe the structural underpinnings of biology by assaying protein activity

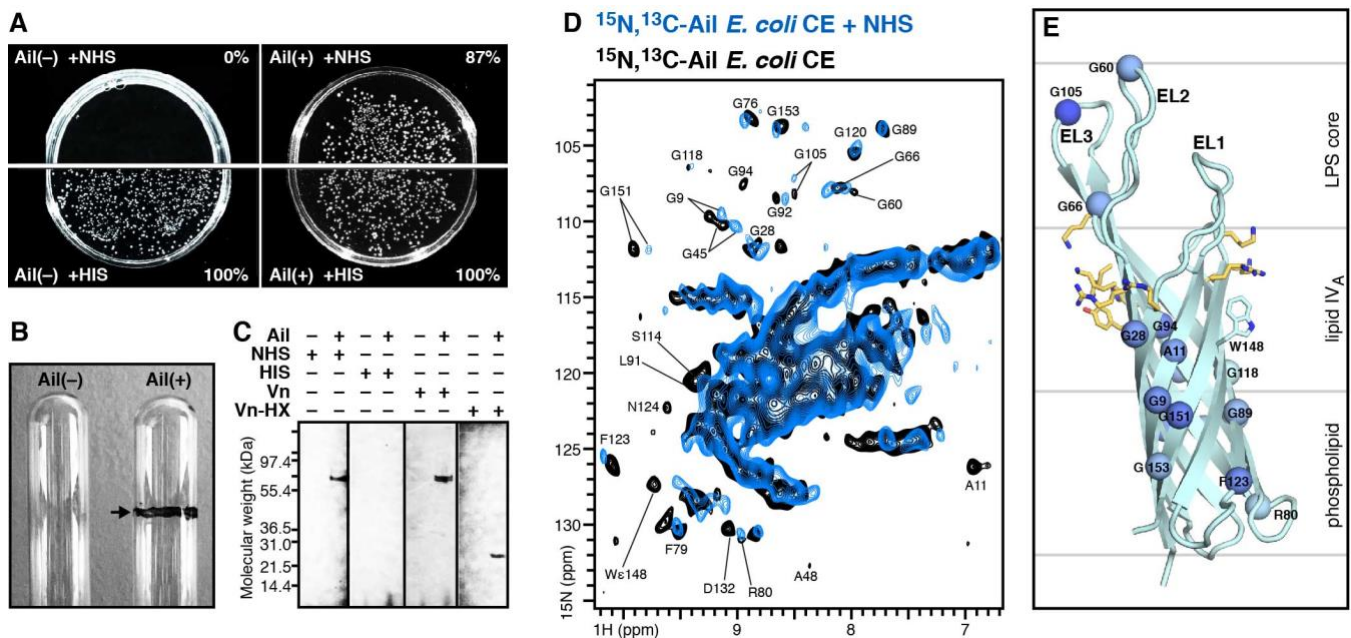


Figure 4. *Y. pestis* phenotypes induced in *E. coli* by Ail expression and interaction of Ail-expressing *E. coli* cells with human serum. Assays were performed with *E. coli* Lemo21(DE3) cells transformed with Ail-bearing plasmid, Ail(+), or with empty plasmid, Ail(-). Each data set is representative of at least triplicate experiments. **(A)** Survival of *E. coli* cells in normal human serum (NHS) relative to heat-inactivated serum (HIS) was assayed by incubating cells with either serum, then plating on agar and counting the surviving bacterial colonies. Survival is reported as percent serum resistance relative to the number of surviving colonies in HIS. **(B)** Pellicle formation observed in Ail(+) but not Ail(-) cells. Cells were suspended in 2 mL of M9 minimal media ($\text{OD}_{600} = 0.5$, in 15 x 100 mm glass tubes) and incubated for 16 h at 37°C. After removing the cells by centrifugation, the interior glass walls were treated with methanol and air-dried overnight, then washed three times with buffer and air-dried overnight. Finally, the tubes were treated with 2.5 mL of crystal violet solution (0.1% for 10 min) then washed with buffer and air-dried. Pellicle formation is detected as a violet-stained rim at the air-liquid interface (arrow). **(C)** Vn binding activity assayed by co-sedimentation of Ail(+) or Ail(-) bacterial cells, with NHS, HIS, purified full-length Vn, or purified Vn-HX. Immunoblots were probed with anti-Vn or anti-Ail antibodies. **(D)** ^1H -detected $^1\text{H}/^{15}\text{N}$ CP-HSQC solid-state NMR spectra of Ail in *E. coli* cell envelopes acquired before (black) or after (blue) incubation with NHS. Spectra were recorded at 900 MHz, 30°C, with a MAS rate of 57 kHz, and 1,600 (black) or 2048 (blue) transients. **(E)** Structural model of Ail embedded in the *Y. pestis* outer membrane taken from a recent MD simulation (14). Spheres denote resolved and assigned amide N atoms that undergo $^1\text{H}/^{15}\text{N}$ chemical shift perturbations from 0 ppm (cyan) to 0.20 ppm (blue), in the presence of NHS. The boundaries of the outer membrane phospholipid and LPS layers are marked.

290 in the same samples that are analyzed by high resolution spectroscopy. Having confirmed the structural integrity
291 of Ail expressed in *E. coli* cells, we asked whether Ail also presents its characteristic virulence phenotypes
292 observed in *Y. pestis*.

293 The role of Ail in providing serum resistance to *Y. pestis* is well known, and expression of Ail in *E. coli* DH5 α
294 cells is sufficient to render them immune to serum mediated killing (34). Similarly, we found that Ail(+) Lemo-
295 21(DE3) cells, expressing plasmid-encoded Ail, exhibit $87 \pm 7\%$ survival after incubation with normal human
296 serum (NHS) relative to heat-inactivated serum (HIS). By contrast, Ail(-) cells, transformed with empty
297 plasmid, formed no colonies after incubation with NHS (Fig. 4A).

298 Ail is also known to promote bacterial cell auto-aggregation, pellicle formation and flocculent bacterial growth
299 (50), three phenotypes that are associated with virulence in *Y. pestis* strains and many other microbial
300 pathogens. In the case of Ail, this property is thought to be related to the distribution of charged amino acids in
301 its extracellular loops. Notably, Ail(+) Lemo21(DE3) cells displayed marked auto-aggregation, and formed a
302 pellicle at the air-liquid interface that can be readily visualized by staining with crystal violet (Fig. 4B). By
303 contrast, neither Ail(-) or native non-transformed cells formed pellicles.

304 Finally we tested the ability of Ail(+) Lemo-21(DE3) cells to bind known ligands of Ail. Previous studies have
305 shown that *Y. pestis* relies on Ail to associate with many human host proteins, including fibronectin (51), C4b-
306 binding protein (52), and vitronectin (Vn) (33, 34). Incubation of Ail(+) cells with either NHS, purified full-
307 length Vn, or purified Vn-HX – corresponding to the hemopexin-like (HX) domain of Vn (33) – resulted in Vn
308 co-sedimentation in all three cases (Fig. 4C). By contrast, no co-sedimentation was observed for either Ail(-)
309 cells or Ail(+) cells incubated with HIS where Vn is presumably denatured by heat treatment (34). We conclude
310 that Ail confers specific virulence-related *Y. pestis* phenotypes to Lemo21(DE3) cells. Notably, this implies
311 that the samples for structural analysis are taken from a biologically active cellular system.

312 **Interaction of Ail with human serum components.** Identifying specific Ail sites involved in serum resistance
313 is of high biomedical importance. To reconstitute the host-pathogen interface that exists when *Y. pestis* enters
314 the blood stream and study this microenvironment at atomic resolution, we incubated Ail(+) cell envelopes
315 with NHS, washed extensively with buffer to eliminate non-specific binding, and then transferred the sample
316 to the NMR rotor for spectroscopic analysis. Previous studies (34) have shown no evidence of Ail proteolysis
317 in the presence of serum and we confirmed that this was indeed the case by immunoblot analysis of Ail after
318 incubation with NHS (Fig. 1G; note that the sample was heat-denatured prior to SDS-PAGE and
319 immunoblotting, thus Ail migrates at the apparent molecular weight of unfolded protein). Incubation with NHS
320 produced several chemical shift perturbations in the $^1\text{H}/^{15}\text{N}$ spectrum of Ail (Fig. 4D, E; Fig. S4B).

321 Ail is known to bind at least two serum components C4BP (34, 52) and Vn (33, 34). While additional studies
322 with purified Ail ligands will be needed to assign the observed perturbations to specific serum molecules, it is
323 notable that a few $^1\text{H}/^{15}\text{N}$ signals are appreciably perturbed. These include signals from G66, G92, G94, residues
324 that localize near F54, F68, S102, and F104 which have been shown to be critical for binding serum Vn and for
325 the serum resistance phenotype (53). Interestingly, intra-membrane perturbations, near the center of the Ail
326 transmembrane β -barrel, are also observed, indicating that the interactions of serum molecules with the
327 extracellular loops of Ail may relay allosterically to membrane-embedded sites. Moreover, it is possible that
328 the Ail β -barrel senses outer membrane perturbations caused by the association of serum components with the
329 bacterial cell surface.

330 **Conclusions.** The ability to probe the molecular structure and interactions of Ail in its native cellular
331 environment is critical for gaining mechanistic insights into Ail-mediated pathogenesis and for developing
332 medical countermeasures. Previously, using NMR and MD simulations with artificial membranes of defined
333 molecular composition, we identified specific molecular interactions between Ail and LPS that are important
334 for *Y. pestis* resistance to serum and antibiotics (14). Here, we have shown that Ail and its virulence phenotypes
335 can be expressed in a bacterial outer membrane for parallel, *in situ* microbiology assays and solid-state NMR
336 structural studies with atomic-resolution. These tools allow us to expand the size and complexity of the Ail

337 microenvironment further than previously possible, and identify key residues that are sensitive to both the
338 bacterial membrane environment and the interactions of human serum.

339 In NMR studies with bacterial cell envelopes, we detect specific Ail signals that firmly establish the sensitivity
340 of the protein's conformation to its microenvironment. Perturbations across distinct topological regions of Ail
341 can be detected as a response to its expression in the asymmetric bacterial outer membrane, and while the
342 overall structure of Ail is unchanged, the native membrane appears to have subtle ordering effects on the
343 extracellular loops. The NMR data further reveal sensitivity of specific Ail sites to serum exposure, and while
344 the identity of the serum components responsible for these effects remains to be determined, human Vn is a
345 good candidate as it is avidly recruited by Ail to the bacterial cell surface. Finally, the NMR data indicate that
346 Ail may sense serum-induced perturbations of the surrounding membrane environment. While the mechanism
347 underlying these observations is unknown, the recruitment of complement components at the cell surface could
348 be responsible for such an effect.

349 The present results provide a molecular window to the Ail-membrane assembly. They reaffirm the importance
350 of the membrane environment as a key regulator of biological function and underscore the importance of
351 characterizing the native assembly as a whole, rather than its individual components.

352

353

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359

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361 JEK, SJO, GVP, FMM: research design; data analysis; writing and editing

362

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364

365 **Data Availability:** All data needed are present in the paper and/or the Supplementary Materials. Additional
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369 REFERENCES

370 1. Perry, R. D., and J. D. Fetherston. 1997. *Yersinia pestis*--etiologic agent of plague. *Clin. Microbiol. Rev.* 10(1):35-66.

371 2. Parkhill, J., B. W. Wren, N. R. Thomson, R. W. Titball, M. T. Holden, M. B. Prentice, M. Sebaihia, K. D. James, C. Churcher, K.
372 L. Mungall, S. Baker, D. Basham, S. D. Bentley, K. Brooks, A. M. Cerdeno-Tarraga, T. Chillingworth, A. Cronin, R. M. Davies, P.
373 Davis, G. Dougan, T. Feltwell, N. Hamlin, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Leather, S. Moule, P. C. Oyston, M. Quail,
374 K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S. Whitehead, and B. G. Barrell. 2001. Genome sequence of *Yersinia pestis*,
375 the causative agent of plague. *Nature* 413(6855):523-527.

376 3. Deng, W., V. Burland, G. Plunkett, 3rd, A. Boutin, G. F. Mayhew, P. Liss, N. T. Perna, D. J. Rose, B. Mau, S. Zhou, D. C. Schwartz,
377 J. D. Fetherston, L. E. Lindler, R. R. Brubaker, G. V. Plano, S. C. Straley, K. A. McDonough, M. L. Nilles, J. S. Matson, F. R.
378 Blattner, and R. D. Perry. 2002. Genome sequence of *Yersinia pestis* KIM. *J. Bacteriol.* 184(16):4601-4611.

- 379 4. Knirel, Y. A., and A. P. Anisimov. 2012. Lipopolysaccharide of *Yersinia pestis*, the Cause of Plague: Structure, Genetics, Biological
380 Properties. *Acta Naturae* 4(3):46-58.
- 381 5. Miller, V. L., J. B. Bliska, and S. Falkow. 1990. Nucleotide sequence of the *Yersinia enterocolitica* ail gene and characterization
382 of the Ail protein product. *J. Bacteriol.* 172(2):1062-1069.
- 383 6. Kolodziejek, A. M., D. R. Schnider, H. N. Rohde, A. J. Wojtowicz, G. A. Bohach, S. A. Minnich, and C. J. Hovde. 2010. Outer
384 membrane protein X (Ail) contributes to *Yersinia pestis* virulence in pneumonic plague and its activity is dependent on the
385 lipopolysaccharide core length. *Infect Immun* 78(12):5233-5243.
- 386 7. Hinnebusch, B. J., C. O. Jarrett, J. A. Callison, D. Gardner, S. K. Buchanan, and G. V. Plano. 2011. Role of the *Yersinia pestis*
387 Ail protein in preventing a protective polymorphonuclear leukocyte response during bubonic plague. *Infect Immun* 79(12):4984-
388 4989.
- 389 8. Hinnebusch, B. J., C. O. Jarrett, and D. M. Bland. 2017. "Fleaing" the Plague: Adaptations of *Yersinia pestis* to Its Insect Vector
390 That Lead to Transmission. *Annu. Rev. Microbiol.* 71:215-232.
- 391 9. Bartra, S. S., K. L. Styer, D. M. O'Bryant, M. L. Nilles, B. J. Hinnebusch, A. Aballay, and G. V. Plano. 2008. Resistance of *Yersinia*
392 *pestis* to complement-dependent killing is mediated by the Ail outer membrane protein. *Infection and Immunity* 76(2):612-622.
- 393 10. Felek, S., and E. S. Krukonis. 2009. The *Yersinia pestis* Ail protein mediates binding and Yop delivery to host cells required for
394 plague virulence. *Infection and Immunity* 77(2):825-836.
- 395 11. Kolodziejek, A. M., C. J. Hovde, and S. A. Minnich. 2012. *Yersinia pestis* Ail: multiple roles of a single protein. *Front Cell Infect*
396 *Microbiol* 2(103):103.
- 397 12. Knirel, Y. A., S. V. Dentovskaya, O. V. Bystrova, N. A. Kocharova, S. N. Senchenkova, R. Z. Shaikhutdinova, G. M. Titareva, I.
398 V. Bakhteeva, B. Lindner, G. B. Pier, and A. P. Anisimov. 2007. Relationship of the lipopolysaccharide structure of *Yersinia pestis*
399 to resistance to antimicrobial factors. *Advances in Experimental Medicine and Biology* 603:88-96.
- 400 13. Felek, S., A. Muszynski, R. W. Carlson, T. M. Tsang, B. J. Hinnebusch, and E. S. Krukonis. 2010. Phosphoglucosyltransferase of
401 *Yersinia pestis* is required for autoaggregation and polymyxin B resistance. *Infection and Immunity* 78(3):1163-1175.
- 402 14. Singh, C., H. Lee, Y. Tian, S. Schesser Bartra, S. Hower, L. M. Fujimoto, Y. Yao, S. A. Ivanov, R. Z. Shaikhutdinova, A. P.
403 Anisimov, G. V. Plano, W. Im, and F. M. Marassi. 2020. Mutually constructive roles of Ail and LPS in *Yersinia pestis* serum
404 survival. *Mol. Microbiol.* 114(3):510-520.
- 405 15. Yamashita, S., P. Lukacik, T. J. Barnard, N. Noinaj, S. Felek, T. M. Tsang, E. S. Krukonis, B. J. Hinnebusch, and S. K. Buchanan.
406 2011. Structural insights into Ail-mediated adhesion in *Yersinia pestis*. *Structure* 19(11):1672-1682.
- 407 16. Marassi, F. M., Y. Ding, C. D. Schwieters, Y. Tian, and Y. Yao. 2015. Backbone structure of *Yersinia pestis* Ail determined in
408 micelles by NMR-restrained simulated annealing with implicit membrane solvation. *J. Biomol. NMR* 63(1):59-65.
- 409 17. Ding, Y., L. M. Fujimoto, Y. Yao, G. V. Plano, and F. M. Marassi. 2015. Influence of the lipid membrane environment on structure
410 and activity of the outer membrane protein Ail from *Yersinia pestis*. *Biochim Biophys Acta* 1848(2):712-720.
- 411 18. Yao, Y., S. K. Dutta, S. H. Park, R. Rai, L. M. Fujimoto, A. A. Bobkov, S. J. Opella, and F. M. Marassi. 2017. High resolution solid-
412 state NMR spectroscopy of the *Yersinia pestis* outer membrane protein Ail in lipid membranes. *J. Biomol. NMR* 67(3):179-190.
- 413 19. Dutta, S. K., Y. Yao, and F. M. Marassi. 2017. Structural Insights into the *Yersinia pestis* Outer Membrane Protein Ail in Lipid
414 Bilayers. *J Phys Chem B* 121(32):7561-7570.
- 415 20. Serber, Z., L. Corsini, F. Durst, and V. Dotsch. 2005. In-cell NMR spectroscopy. *Methods Enzymol.* 394:17-41.
- 416 21. Fu, R., X. Wang, C. Li, A. N. Santiago-Miranda, G. J. Pielak, and F. Tian. 2011. In situ structural characterization of a recombinant
417 protein in native *Escherichia coli* membranes with solid-state magic-angle-spinning NMR. *J. Am. Chem. Soc.* 133(32):12370-
418 12373.
- 419 22. Mao, L., K. Inoue, Y. Tao, G. T. Montelione, A. E. McDermott, and M. Inouye. 2011. Suppression of phospholipid biosynthesis by
420 cerulenin in the condensed Single-Protein-Production (cSPP) system. *J. Biomol. NMR* 49(2):131-137.
- 421 23. Renault, M., R. Tommassen-van Boxtel, M. P. Bos, J. A. Post, J. Tommassen, and M. Baldus. 2012. Cellular solid-state nuclear
422 magnetic resonance spectroscopy. *Proc. Natl. Acad. Sci. U. S. A.* 109(13):4863-4868.

- 423 24. Baker, L. A., G. E. Folkers, T. Sinnige, K. Houben, M. Kaplan, E. A. van der Crujisen, and M. Baldus. 2015. Magic-angle-spinning
424 solid-state NMR of membrane proteins. *Methods Enzymol.* 557:307-328.
- 425 25. Warnet, X. L., A. A. Arnold, I. Marcotte, and D. E. Warschawski. 2015. In-Cell Solid-State NMR: An Emerging Technique for the
426 Study of Biological Membranes. *Biophys. J.* 109(12):2461-2466.
- 427 26. Luchinat, E., and L. Banci. 2018. In-Cell NMR in Human Cells: Direct Protein Expression Allows Structural Studies of Protein
428 Folding and Maturation. *Acc. Chem. Res.* 51(6):1550-1557.
- 429 27. Sockolosky, J. T., and F. C. Szoka. 2013. Periplasmic production via the pET expression system of soluble, bioactive human
430 growth hormone. *Protein Expr. Purif.* 87(2):129-135.
- 431 28. Studier, F. W., and B. A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of
432 cloned genes. *J. Mol. Biol.* 189(1):113-30.
- 433 29. Miroux, B., and J. E. Walker. 1996. Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some
434 membrane proteins and globular proteins at high levels. *J. Mol. Biol.* 260(3):289-298.
- 435 30. Meuskens, I., M. Michalik, N. Chauhan, D. Linke, and J. C. Leo. 2017. A New Strain Collection for Improved Expression of Outer
436 Membrane Proteins. *Front Cell Infect Microbiol* 7:464.
- 437 31. Wagner, S., M. M. Klepsch, S. Schlegel, A. Appel, R. Draheim, M. Tarry, M. Hogbom, K. J. van Wijk, D. J. Slotboom, J. O.
438 Persson, and J. W. de Gier. 2008. Tuning *Escherichia coli* for membrane protein overexpression. *Proc. Natl. Acad. Sci. U. S. A.*
439 105(38):14371-14376.
- 440 32. Zhou, D. H., and C. M. Rienstra. 2008. High-performance solvent suppression for proton detected solid-state NMR. *J Magn Reson*
441 192(1):167-172.
- 442 33. Shin, K., B. C. Lechtenberg, L. M. Fujimoto, Y. Yao, S. S. Bartra, G. V. Plano, and F. M. Marassi. 2019. Structure of human
443 Vitronectin C-terminal domain and interaction with *Yersinia pestis* outer membrane protein Ail. *Sci Adv* 5(9):eaax5068.
- 444 34. Bartra, S. S., Y. Ding, L. Miya Fujimoto, J. G. Ring, V. Jain, S. Ram, F. M. Marassi, and G. V. Plano. 2015. *Yersinia pestis* uses
445 the Ail outer membrane protein to recruit vitronectin. *Microbiology* 161(11):2174-2183.
- 446 35. Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*
447 9(7):671-675.
- 448 36. Anisimov, A. P., R. Z. Shaikhutdinova, L. N. Pan'kina, V. A. Feodorova, E. P. Savostina, O. V. Bystrova, B. Lindner, A. N.
449 Mokrievich, I. V. Bakhteeva, G. M. Titareva, S. V. Dentovskaya, N. A. Kocharova, S. N. Senchenkova, O. Holst, Z. L. Devdariani,
450 Y. A. Popov, G. B. Pier, and Y. A. Knirel. 2007. Effect of deletion of the *lpxM* gene on virulence and vaccine potential of *Yersinia*
451 *pestis* in mice. *J. Med. Microbiol.* 56(Pt 4):443-453.
- 452 37. Sebbane, F., N. Lemaitre, D. E. Sturdevant, R. Rebeil, K. Virtaneva, S. F. Porcella, and B. J. Hinnebusch. 2006. Adaptive
453 response of *Yersinia pestis* to extracellular effectors of innate immunity during bubonic plague. *Proc. Natl. Acad. Sci. U. S. A.*
454 103(31):11766-11771.
- 455 38. Chauvaux, S., M. A. Dillies, M. Marceau, M. L. Rosso, S. Rousseau, I. Moszer, M. Simonet, and E. Carniel. 2011. In silico
456 comparison of *Yersinia pestis* and *Yersinia pseudotuberculosis* transcriptomes reveals a higher expression level of crucial
457 virulence determinants in the plague bacillus. *Int. J. Med. Microbiol.* 301(2):105-116.
- 458 39. Hartmann, G., K. O. Honikel, F. Knusel, and J. Nuesch. 1967. The specific inhibition of the DNA-directed RNA synthesis by
459 rifamycin. *Biochim Biophys Acta* 145(3):843-844.
- 460 40. Almeida, F. C., G. C. Amorim, V. H. Moreau, V. O. Sousa, A. T. Creazola, T. A. Americo, A. P. Pais, A. Leite, L. E. Netto, R. J.
461 Giordano, and A. P. Valente. 2001. Selectively labeling the heterologous protein in *Escherichia coli* for NMR studies: a strategy
462 to speed up NMR spectroscopy. *J Magn Reson* 148(1):142-146.
- 463 41. Zhou, H. X., and T. A. Cross. 2013. Influences of membrane mimetic environments on membrane protein structures. *Annu Rev*
464 *Biophys* 42:361-392.
- 465 42. Chipot, C., F. Dehez, J. R. Schnell, N. Zitzmann, E. Pebay-Peyroula, L. J. Catoire, B. Miroux, E. R. S. Kunji, G. Veglia, T. A.
466 Cross, and P. Schanda. 2018. Perturbations of Native Membrane Protein Structure in Alkyl Phosphocholine Detergents: A Critical
467 Assessment of NMR and Biophysical Studies. *Chem. Rev.* 118(7):3559-3607.

- 468 43. Williamson, M. P. 2013. Using chemical shift perturbation to characterise ligand binding. *Prog. Nucl. Magn. Reson. Spectrosc.*
469 73:1-16.
- 470 44. Schubeis, T., T. Le Marchand, L. B. Andreas, and G. Pintacuda. 2018. ¹H magic-angle spinning NMR evolves as a powerful
471 new tool for membrane proteins. *J Magn Reson* 287:140-152.
- 472 45. Cala-De Paepe, D., J. Stanek, K. Jaudzems, K. Tars, L. B. Andreas, and G. Pintacuda. 2017. Is protein deuteration beneficial for
473 proton detected solid-state NMR at and above 100 kHz magic-angle spinning? *Solid State Nucl. Magn. Reson.* 87:126-136.
- 474 46. Baker, L. A., T. Sinnige, P. Schellenberger, J. de Keyzer, C. A. Siebert, A. J. M. Driessen, M. Baldus, and K. Grunewald. 2018.
475 Combined ¹H-Detected Solid-State NMR Spectroscopy and Electron Cryotomography to Study Membrane Proteins across
476 Resolutions in Native Environments. *Structure* 26(1):161-170 e163.
- 477 47. Laguri, C., A. Silipo, A. M. Martorana, P. Schanda, R. Marchetti, A. Polissi, A. Molinaro, and J.-P. Simorre. 2018. Solid State NMR
478 Studies of Intact Lipopolysaccharide Endotoxin. *ACS chemical biology* 13(8):2106-2113.
- 479 48. Baker, L. A., M. Daniels, E. A. van der Cruijssen, G. E. Folkers, and M. Baldus. 2015. Efficient cellular solid-state NMR of
480 membrane proteins by targeted protein labeling. *J. Biomol. NMR* 62(2):199-208.
- 481 49. Shahid, S. A., M. Nagaraj, N. Chauhan, T. W. Franks, B. Bardiaux, M. Habeck, M. Orwick-Rydmark, D. Linke, and B. J. van
482 Rossum. 2015. Solid-state NMR Study of the YadA Membrane-Anchored Domain in the Bacterial Outer Membrane. *Angew. Chem.*
483 *Int. Ed. Engl.* 54(43):12602-12606.
- 484 50. Kolodziejek, A. M., D. J. Sinclair, K. S. Seo, D. R. Schnider, C. F. Deobald, H. N. Rohde, A. K. Viall, S. S. Minnich, C. J. Hovde,
485 S. A. Minnich, and G. A. Bohach. 2007. Phenotypic characterization of OmpX, an Ail homologue of *Yersinia pestis* KIM.
486 *Microbiology* 153(Pt 9):2941-2951.
- 487 51. Tsang, T. M., S. Felek, and E. S. Krukoni. 2010. Ail binding to fibronectin facilitates *Yersinia pestis* binding to host cells and Yop
488 delivery. *Infection and Immunity* 78(8):3358-3368.
- 489 52. Ho, D. K., M. Skurnik, A. M. Blom, and S. Meri. 2014. *Yersinia pestis* Ail recruitment of C4b-binding protein leads to factor I-
490 mediated inactivation of covalently and noncovalently bound C4b. *Eur. J. Immunol.* 44(3):742-751.
- 491 53. Thomson, J. J., S. C. Plecha, and E. S. Krukoni. 2019. Ail provides multiple mechanisms of serum resistance to *Yersinia pestis*.
492 *Mol. Microbiol.* 111(1):82-95.
493