



Rationally designed FimH variants with enhanced functional immunogenicity as vaccine candidates for ExPEC urinary tract infections



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Extraintestinal pathogenic *Escherichia coli* (ExPEC) is a major cause of urinary tract infections (UTIs) and invasive disease. Rising antimicrobial resistance highlights the need for preventive strategies such as vaccination. The conserved D-mannose-specific adhesin FimH is essential during early UTI pathogenesis, making it an attractive vaccine target. We rationally designed a FimH lectin domain antigen stabilized in a low-affinity, tense conformation by two amino-acid substitutions (^{LA}FimH_{LD}), thereby exposing epitopes within the mannose-binding pocket relevant for functional immunity. Multiple vaccine formats incorporating ^{LA}FimH_{LD} were generated, including fimbrial tip constructs, carrier protein fusions or conjugates and a nanoparticle. In rats, monomeric ^{LA}FimH_{LD} induced functional antibody responses comparable to a clinically tested FimCH reference, while four ^{LA}FimH_{LD} variants elicited superior inhibition of ExPEC binding to human bladder cells. A multimeric ^{LA}FimH_{LD}–CRM₁₉₇ conjugate further demonstrated robust functional immunogenicity in minipigs. These data show enhanced FimH vaccine potency and identify candidates for UTI prevention.

Extraintestinal pathogenic *Escherichia coli* (ExPEC) is a leading cause of bacteremia and sepsis worldwide^{1–3}. Often, these severe and potentially life-threatening conditions result from an initial urinary tract infection (UTI)³. Uropathogenic ExPEC strains (also known as UPEC) are responsible for up to 80% of urinary tract infections⁴. UTIs are among the most common bacterial infections that predominantly affect women and frequently recur. At least 30% of women who experience an initial UTI will experience a second infection within 12 months^{4–6}. The global burden of UTIs continues to grow, with an estimated 400 million cases occurring annually^{7–9}, and the increasing prevalence of antimicrobial-resistant uropathogens further adds to the clinical and economic impact¹⁰. The frequent treatment of UTIs with antibiotics contributes to the increase in antimicrobial resistance (AMR), which complicates treatment and increases the risk of developing invasive disease. Preventing infections through vaccination is therefore crucial to

reduce antibiotic use and limit AMR. However, an effective vaccine to prevent systemic or mucosal ExPEC infections has not yet been developed. This may in part reflect the versatility and redundancy of virulence factors and immune resistance mechanisms that characterize ExPEC^{11,12}. Additionally, development of an effective UTI vaccine is particularly challenging due to the difficulties to overcome mucosal barriers. The urothelium forms a highly impermeable layer, and the continuous flow of urine and shedding of urothelial cells further limit antigen persistence and immune activation at the mucosal surface. The local immune environment of the urinary tract is hyporesponsive, with limited resident immune cell populations and active suppression of pro-inflammatory signaling. Importantly, the immune correlates of protection for UTI remain poorly defined, including the relative contribution of locally produced mucosal antibodies versus systemic IgG reaching the mucosa through transudation.

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Adherence of bacteria to urothelial cells in the bladder is an important primary step in UTI pathogenesis. Among the multitude of ExPEC virulence factors, type 1 fimbriae with the highly conserved mannose-specific tip adhesin FimH play a crucial role in this process^{13,14}. FimH facilitates adherence mainly via the mannosylated uroplakin-1a receptor that is abundantly present on the apical surface of urothelial cells¹⁵. FimH has also been implicated in biofilm formation^{16,17}, intestinal colonization¹⁸, and invasion of bladder epithelial cells leading to the establishment of intracellular communities that serve as protected reservoirs contributing to infection recurrence^{19–21}.

The FimH protein consists of two structural domains; the N-terminal mannose-binding lectin domain (FimH_{LD}) and the C-terminal pilus-anchoring pilin domain (FimH_{PD}), connected by a short linker. Inter-domain interactions enable the protein to adopt two distinct structural conformations: tense and relaxed, characterized by a wide or shallow mannose binding pocket and by low or high affinity to mannose, respectively²². This conformational switch mechanism promotes tight bacterial adhesion under the mechanical forces of urine excretion while allowing bacterial motility and colonization of new tissues under static conditions. This process is also referred to as catch-bond formation^{22,23}. Because the interdomain interactions and binding pocket specificity of FimH are crucial for protein function and thus for bacterial colonization and pathogenicity potential, sequence variation within the protein is constrained and primarily reflects pathoadaptive changes^{24,25}. Among UTI and bacteremia ExPEC isolates, several prevalent FimH subtypes such as FimH30, FimH41, and FimH22, exemplify distinct sequence signatures that appear optimized for adhesion and persistence^{25,26}.

The conformation of FimH is not only crucial for its biological function, but also a major consideration for vaccine antigen design. Antibodies elicited by a FimH_{LD} subunit variant stabilized in a tense, low-affinity conformation were shown to block and even reverse bacterial binding to urothelial cells, inhibit biofilm formation, and reduce bladder CFU in a mouse *E. coli* UTI infection model²⁷. In the same study, immunization with wild-type FimH_{LD}, which naturally adopts the relaxed, high-affinity conformation in absence of the pilin domain, did not trigger these effects²⁷. The epitopes of inhibitory antibodies were found to be predominantly localized within the mannose binding pocket²⁸. Elicitation of such antibodies requires display of a wide binding pocket, which in lectin domain-only constructs can be achieved by applying specific mutations to stabilize the tense, low-affinity state. Several mutations have been reported to bias FimH_{LD} towards this conformation, including Arg60Pro, Val27Cys/Leu34Cys²⁹, and Gly15Ala/Gly16Ala/Val27Ala³⁰. However, these variants can still shift to a relaxed, higher-affinity state upon mannoside binding. Notably, recent evidence from monoclonal antibody studies suggests that antibodies targeting epitopes outside the mannose-binding pocket on relaxed-state FimH_{LD} can also confer some degree of protection, likely through steric interference³¹. These results indicate that multiple inhibitory mechanisms may exist. For a vaccine approach aimed at eliciting a polyclonal response enriched for functionally inhibitory antibodies, deliberate exposure of the binding pocket through tense-state stabilization represents a rational antigen design strategy.

Due to its role in infection, FimH has historically been identified as a key therapeutic target to prevent (recurrent) ExPEC infections. Several pre-clinical animal studies have reported functional immunogenicity and reduced bladder colonization or bacteriuria following immunization with FimH-based vaccine candidates^{32–36}. A vaccine phase 1 clinical trial using wild-type FimH complexed with its natural chaperone FimC (FimCH), in which FimH is structurally forced in a relaxed, high-affinity conformation, also showed promising immune responses³⁷. However, development of a similar FimCH vaccine by MedImmune in the early 2000s was discontinued after failure to demonstrate clinical efficacy in a phase 2 UTI trial³⁸.

The research on FimH performed over the past decade has generated in-depth knowledge on its conformational state, stabilization options, and potential to elicit inhibitory antibodies. These findings open opportunities for further optimization of FimH-based vaccine antigens to enhance

protective immune responses targeting the mannose-binding pocket. In particular, stabilization of FimH_{LD} in a tense, low-affinity conformation enhances exposure of functionally relevant epitopes within the mannose-binding pocket and can thereby improve induction of inhibitory antibody responses. In this study, we applied a structure-guided approach to design a conformationally stabilized FimH_{LD} antigen and evaluated its immunogenic potential across multiple vaccine formats, including native fimbrial tip assemblies, carrier protein-based constructs, and multivalent nanoparticle presentations. By assessing functional antibody responses in relevant animal models, we aimed to identify optimized FimH-based vaccine candidates for the prevention of UTIs caused by ExPEC.

Results

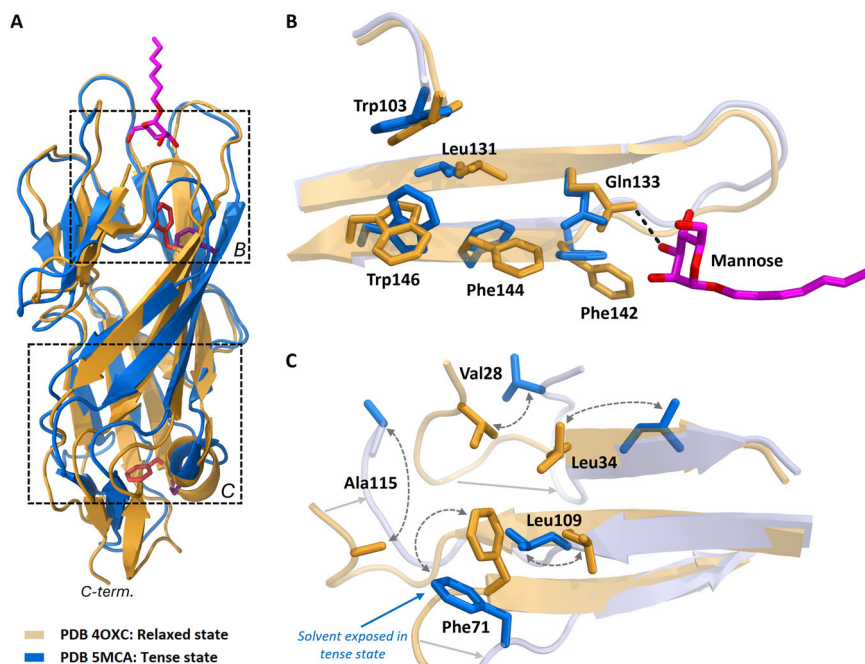
Structure-guided identification of allosteric mutations stabilizing FimH_{LD} in a low-affinity state

We used an *in silico* 3D structure-based approach to select amino acid substitutions in FimH_{LD} expected to stabilize the tense, low-affinity conformation and thereby enhance induction of mannose-binding pocket-specific inhibitory antibodies upon immunization. Previous X-ray and NMR studies have elucidated key features of the FimH_{LD} conformational switch, providing a structural framework for antigen design²⁹. Building on this knowledge, we further interrogated the structural boundaries of the switch to identify residues that could be leveraged to bias conformational selection toward the low-affinity state. To this end, we superimposed two FimH_{LD} structures representing its conformational boundaries: the mannoside-bound wild-type relaxed (high-affinity) form (PDB: 4XOC) and the mannoside-free Arg60Pro tense (low-affinity) form (PDB: 5MCA) (Fig. 1A). Despite a pronounced twist of the central β -sheet that complicates overlay, this comparison revealed two prominent differences that could be exploited for allosteric stabilization of the tense, low-affinity state. First, we identified a 90° flip of the side chain of the centrally positioned, buried Trp146 near the mannose-binding pocket, which is associated with a concerted motion of conformational changes involving Leu131, Phe144, Phe142 and Gln133 that positions residues more favorably for ligand binding in the relaxed state (Fig. 1B). Because Trp146 (and nearby Trp103) is predicted to be important for structural integrity, we prioritized disruption of this Trp-flip mechanism by introducing a Phe144Val substitution, reasoning that the smaller valine side chain cannot support the flip-driven rearrangement while largely preserving hydrophobic core interactions. Second, we identified a hydrophobic pocket formed by hydrophobic residues across four loop regions (residues 22–34, 64–71, 111–120, and 150–158). This pocket is located at the opposite side of FimH_{LD}, distant from the ligand-binding site, and undergoes concerted rearrangement between conformations (Fig. 1C). In the tense state, several of these residues are solvent-exposed, whereas in the relaxed state they become buried within the pocket. To bias conformational selection toward the tense, low-affinity state via an allosteric effect outside the mannose-binding site, we introduced a minimal Phe71Tyr substitution, reasoning that addition of an OH group would sterically hinder formation of the pocket architecture observed in the relaxed state. Together, these considerations led us to prioritize Phe144Val and Phe71Tyr, individually and in combination, as candidate mutations to lock FimH_{LD} in the tense, low-affinity conformation by disruption of two largely independent, allosteric switch mechanisms.

Biophysical characterization of mutated FimH_{LD} variants

To assess the effect of the two amino acid substitutions on the mannoside binding affinity and conformational state of FimH_{LD}, we produced FimH_{LD} antigens in *E. coli* with single or combined Phe144Val and Phe71Tyr mutations. We applied these mutations to the amino acid sequence of FimH30, a subtype which is common among clinically relevant ExPEC lineages including O25-ST131³⁹. Using Surface Plasmon Resonance (SPR) we observed a 150-fold reduced binding affinity to immobilized mannoside for the Phe71Tyr mutated FimH_{LD} variant as compared to the wild-type (Fig. 2A, Supplementary Fig. S1). For the Phe144Val single and Phe71Tyr/

Fig. 1 | Structure-guided identification of allosteric mutations stabilizing FimH_{LD} in the tense, low-affinity state. **A** Superimposition of the crystal structures of liganded FimH_{LD} WT (yellow, PDB-ID 4XOC) and the ligand-free R60P mutant (blue, PDB-ID 5MCA), representing the high-affinity relaxed state and low-affinity tense state, respectively. The inset-panels indicate the regions near the mannose binding site (magenta) and the C-terminal domain end showing substantial structural deviation between both states, employed for vaccine antigen design through mutation of two phenylalanines (red and purple sticks). **B** Detailed view of hydrophobic core near the ligand binding site, showing the rotamer flip (~90°) of Trp146, and adjacent Leu131, Phe144, Phe142, Gln133; the latter two thereby altering their side chain position towards the mannose ligand. Mutation of Phe144 to a smaller valine without capacity to support the full flipping mechanism constraints formation of strong interactions with the ligand. **C** Detailed view of the remote hydrophobic pocket near the C-terminus, showing drastic conformational changes of several loops (28–34, 71–75, 109–115) between the relaxed and tense state, alongside with a hydrophobic pocket reorganization as indicated by (among others) residues Val28, Leu34, Phe71, Leu109, and Ala115. The Phe71 side chain is surface accessible in the tense state, efficiently allowing incorporation of an extra OH-group through mutation to a tyrosine, whereas being pocket buried in the relaxed state, it possesses insufficient space to accommodate the OH-group and thus favoring the tense conformation.



Phe144Val double mutated FimH_{LD} variants, no mannoside binding was detected up to 10 μ M protein. Binding pocket integrity was confirmed by binding of mAb926 and mAb475, monoclonal antibodies specific to epitopes located within or close to the mannose binding pocket²⁸, in a bio-layer interferometry assay using FimH_{LD} variant-coated biosensors (Fig. 2A).

We performed conformational analysis of the FimH_{LD} variants by two-dimensional Nuclear Magnetic Resonance (2D-NMR) using reporter signals for Ala2, Lys4, Asp54, and Gln133, residues previously identified to be indicative for the conformational switch between the tense and relaxed state of the FimH binding pocket²⁹ (Supplementary Fig. S2). We found that in the apo state, both Phe144Val and Phe71Tyr single variants and the Phe71Tyr/Phe144Val double mutated FimH_{LD} variant adopt a tense conformation, whereas wild-type FimH_{LD} adopts the relaxed state (Fig. 2B, C, Supplementary Fig. S3). In the presence of tenfold molar excess mannoside ligand, Phe144Val and Phe71Tyr single mutants and wild-type FimH_{LD} switch to the relaxed state, whereas the Phe71Tyr/Phe144Val double mutant remains in a tense conformation (Fig. 2C). Together, these results indicate that Phe144Val and Phe71Tyr amino acid substitutions in FimH_{LD} favor a tense, low-affinity state in ligand-free conditions, but a conformational switch is still possible in the presence of excess mannoside. The combination of both mutations, however, stabilizes the tense, low-affinity state and locks FimH_{LD} permanently in this conformation. From here onwards, FimH_{LD} variant with the combination of Phe71Tyr and Phe144Val mutations, stabilized in a tense, low-affinity state, is referred to as ^{LA}FimH_{LD}.

Immunogenicity of low-affinity stabilized FimH_{LD}

Immunogenicity of ^{LA}FimH_{LD} was tested in rats using different dose levels (3, 10, and 30 μ g) of ^{LA}FimH_{LD} in combination with AS01_B, a liposome-based adjuvant known to promote Th1-biased immune responses that are considered important for mucosal immunity⁴⁰. ^{LA}FimH_{LD}-binding IgG

antibodies were detected after two immunizations at all dose levels and were highest after three immunizations (Fig. 3A). A modest dose effect was observed. The lowest dose level already elicited robust ^{LA}FimH_{LD}-binding IgG antibodies and was therefore selected for further analysis. Serum antibodies from rats immunized with 3 μ g ^{LA}FimH_{LD} also recognized ^{WT}FimH_{LD} and full-length FimH, both without (^{WT}FimH) and with low-affinity mutations (^{LA}FimH) (Fig. 3B). To test whether the antibodies induced upon three immunizations were functional, FimH-mediated bacterial binding of a fluorescently labeled *E. coli* UTI89 strain to human bladder epithelial cells was measured in the absence or presence of serum (in a so-called adhesion inhibition assay (AIA)). Bacterial adhesion in this assay was confirmed to be predominantly FimH-dependent, as a UTI89 Δ FimH strain showed substantially reduced adhesion to bladder cells across all bacterial densities tested (Supplementary Fig. S4). Inhibition of adhesion was expressed as a potency value relative to mAb475, a reference monoclonal antibody known to inhibit the interaction of FimH with mannosylated substrates²⁷. mAb475 was included as an internal reference on each plate to correct for plate-to-plate and run-to-run variation, enabling reliable comparison of relative potency titers across different assay runs. A significant increase in relative potency titers was observed when animals were immunized with 3 μ g ^{LA}FimH_{LD} compared to saline (Fig. 3C). These results show that ^{LA}FimH_{LD} is immunogenic and induces functional antibodies capable of inhibiting binding of *E. coli* to human bladder cells.

Design of vaccine constructs incorporating low-affinity stabilized FimH_{LD}

The FimH_{LD} subunit is a relatively small (18 kDa) protein, which could benefit from optimization by design to increase its immunogenicity for use as a vaccine antigen. Increasing antigen size, addition of T-cell epitopes and multimerization are common strategies to enhance the potency of vaccine

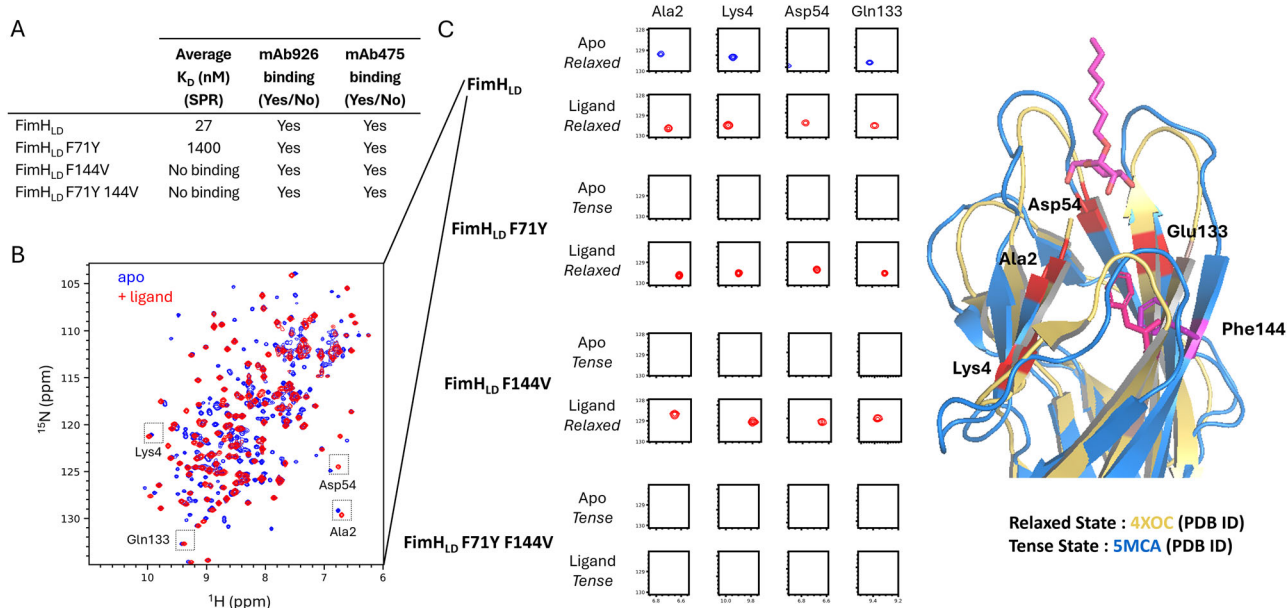


Fig. 2 | Mannose binding pocket characterization of structurally stabilized FimH_{LD} variants. A Surface plasmon resonance (SPR) analysis showed significant decreased mannose binding capabilities of the mutated FimH_{LD} variants compared to the wild-type, while integrity of the binding pocket was retained as indicated by binding of mAb475 and mAb926 using biolayer interferometry (BLI). B 2D-NMR analysis of the FimH_{LD} wild-type variant in the presence (red) or absence (blue) of mannose with four residues previously identified as reporter signals for the conformational state of the lectin domain shown in a square box²⁹ (Supplementary

Fig. S2). C Overview of the four reporter signals for all FimH_{LD} variants analyzed in the presence of ligand or in the absence of (apo) mannose. Absence of the signal means the structure is in the tense state whereas presence of the signal means the structure is in the relaxed state²⁹. The location of each reporter residue is highlighted in red in the FimH_{LD} structure in tense (blue color) and relaxed (yellow color) state. Full spectra are available in Supplementary Fig. S3.

antigens and induce long lasting functional immune responses. We applied these strategies to the ^{LA}FimH_{LD} construct and designed five additional candidate vaccine antigens (Fig. 4). These constructs included two type 1 fimbriae tip variants containing full-length FimH, either alone (^{LA}FimH) or in combination with FimG (^{LA}FimGH), a fusion to genetically detoxified *P. aeruginosa* exoprotein A (^{LA}FimH_{LD}-EPA), a chemical conjugate using CRM₁₉₇ as a carrier protein (^{LA}FimH_{LD}-CRM₁₉₇) and a ferritin-based nanoparticle (^{LA}FimH_{LD}-NP) (Fig. 4A). Designs that included full-length FimH or FimG were stabilized using a previously described donor strand complementation strategy⁴¹. A mimic of a FimCH protein-chaperone complex that has previously been proven immunogenic in a Phase I trial³⁷, was included as a control antigen (Fig. 4B).

Manufacturability and quality of ^{LA}FimH_{LD}-based vaccine constructs

The ^{LA}FimH_{LD}-based vaccine constructs were produced using expression platforms selected based on manufacturability, and assessed for quality in terms of concentration, purity, and host cell impurities as summarized in Supplementary Table S1. The purity of the constructs varied from 78 to 100% according to CE-SDS and SEC-MALS with acceptable levels of high Mw species ($\leq 11\%$), endotoxin (≤ 63 EU/mg), and DNA ($\leq 0.04\%$ w/w) (Supplementary Table S1). The measured molecular masses (Fig. 5A) and radii (Fig. 5B) of all antigen variants corresponded well with the theoretical values based on amino acid sequence for all variants except for the ^{LA}FimH_{LD}-NP construct. For monomeric ^{LA}FimH_{LD}-NP, MALDI-TOF-MS analysis showed a mass of 41.4 kDa (theoretical protein mass: 37.3 kDa) and for multimeric ^{LA}FimH_{LD}-NP, a mass of 1199.7 kDa was measured by SEC-MALS (theoretical mass for 24-mer: 895.2 kDa). The larger size and mass measured for this construct can likely be explained by the presence of high-mannose N-glycans on three potential N-glycosylation sites (Asn7, Asn78, Asn190), resulting from the mammalian expression system. For the ^{LA}FimH_{LD}-CRM₁₉₇ construct, we used the mass measured with SEC-MALS (i.e. 134.3 kDa) to calculate an average of 3.4 ^{LA}FimH_{LD} subunits per

CRM₁₉₇ carrier protein, which is in line with MALDI-TOF MS results showing a range of 1–5 ^{LA}FimH_{LD} subunits conjugated to CRM₁₉₇ (Fig. 5C). All constructs, except FimCH, were stabilized in the low-affinity conformation based on their inability to bind a mannose ligand (Fig. 5D). Binding of mannose binding pocket-specific antibodies mAb926 and mAb475 confirmed epitope accessibility for all constructs except for ^{LA}FimH_{LD}-EPA and ^{LA}FimH_{LD}-NP, that did not show binding to mAb475 (Fig. 5D). mAb926 recognizes an epitope that is on top of the lectin domain and close to the mannose binding pocket of FimH, while mAb475 recognizes an epitope that overlaps with the mannose binding pocket and directly competes with the mannose ligand²⁸. The lack of binding of mAb475 to ^{LA}FimH_{LD}-EPA may be due to partial reshuffling of the intramolecular disulfide bridge in the binding pocket of ^{LA}FimH_{LD} and formation of disulfide bridges with cysteines of EPA that was observed by LC-MS (Supplementary Table S3). For ^{LA}FimH_{LD}-NP, potential N-glycosylation in the binding pocket (on Asn7) may play a role, although we have not investigated this in detail.

Functional immune responses elicited by ^{LA}FimH_{LD}-based vaccine constructs

Immunogenicity of the generated ^{LA}FimH_{LD}-based vaccine constructs was tested in two separate rat studies, both including a group that received ^{LA}FimH_{LD} as a benchmark. Rats were immunized with 3 doses of AS01B-adjuvanted protein, using an equal 3 μ g dose of FimH_{LD} for each construct based on calculated molar mass. ^{WT}FimH-specific IgG binding antibodies showed a sharp rise already after one immunization for all constructs except for ^{LA}FimH_{LD}, for which antibodies became detectable only after a second dose (Fig. 6A). The highest ^{WT}FimH binding antibody titers were observed after three immunizations (day 42) for all constructs. After each immunization, ^{WT}FimH binding IgG titers were significantly higher for all constructs tested compared to the group immunized with ^{LA}FimH_{LD}, except for ^{WT}FimCH, which showed comparable binding antibody titers after three immunizations (Fig. 6A, B).

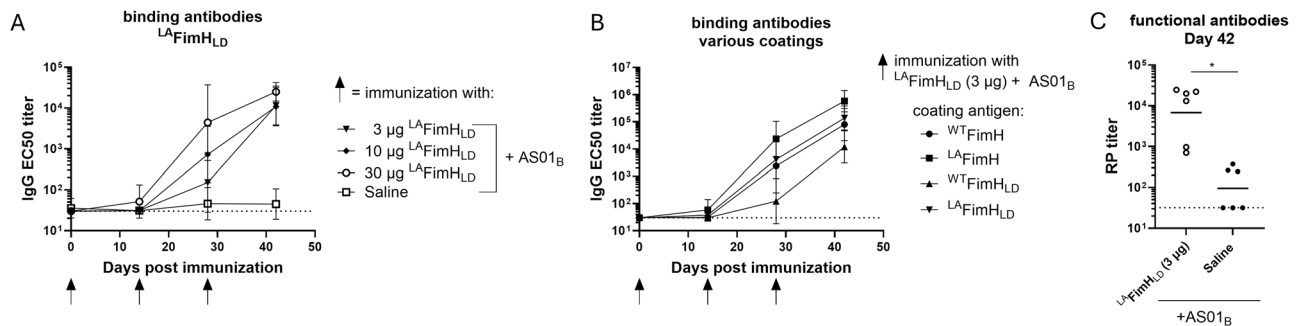


Fig. 3 | Immunogenicity of ^{LA} FimH_{LD} in rats. A Rats were immunized IM with different dose levels (3, 10, or 30 µg) of ^{LA} FimH_{LD} ($N = 6$ per group), or received saline ($N = 10$), all in combination with AS01_B, on days 0, 14, and 28 (indicated with an arrow). Day 0, 14, 28, and 42 serum samples were measured for ^{LA} FimH_{LD}-binding IgG antibodies by ELISA using a ^{LA} FimH_{LD} coating concentration of 1 µg/mL. The geometric mean with the geometric standard deviation of the EC50 titers per group is shown. The lower limit of detection (LLOD) was 30 EC50 and is indicated with a dotted line. B IgG binding antibodies to ^{WT} FimH, ^{WT} FimH_{LD}, ^{LA} FimH and ^{LA} FimH_{LD} were determined by ELISA using serum samples of rats immunized with 3 µg ^{LA} FimH_{LD} + AS01_B ($N = 18$) and a 2 µg/mL coating

concentration of FimH variants. The geometric mean with the geometric standard deviation of the EC50 titers for each coating antigen is shown with a line. The LLOD was 30 EC50 and is indicated with a dotted line. C Day 42 samples from rats immunized with 3 µg ^{LA} FimH_{LD} or saline ($N = 6$), both in combination with AS01_B were tested in an adhesion inhibition assay, using human bladder epithelial 5637 cells and mCherry expressing UTI89 *E. coli*. Relative Potency (RP) titers were calculated and are shown per animal. Line indicates group geometric mean. The lower limit of detection was 32 RP titer and is indicated with a dotted line. The Tobit model was used to determine statistically significant differences. P values < 0.05 were considered significant.

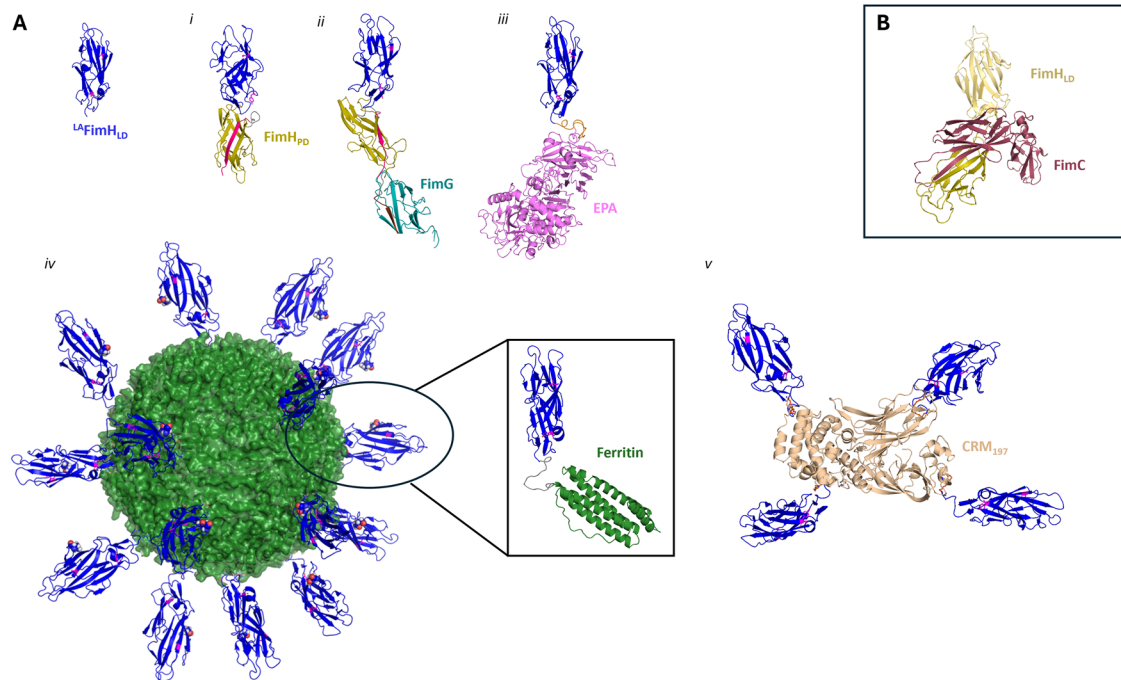


Fig. 4 | Structural overview of ^{LA} FimH_{LD}-based vaccine constructs and FimCH control. Shown are (A) visualizations of the FimH lectin domain with the two low-affinity, tense state locking mutations (^{LA} FimH_{LD}), integrated into natural type 1 pili tip variants containing the FimH pilin domain (^{LA} FimH (i)) and additionally FimG (^{LA} FimGH (ii)), a fusion to genetically detoxified *P. aeruginosa* exoprotein A (^{LA} FimH_{LD}-EPA (iii)), a ferritin-based nanoparticle with the exposed asparagine, which is a potential target for host cell N-glycosylation, highlighted (^{LA} FimH_{LD}-NP

(iv)) and a chemical conjugate using CRM197 as a carrier protein (^{LA} FimH_{LD}-CRM197 (v)). Additionally, B the periplasmic chaperone complex FimCH control antigen is shown. FimCH does not contain low-affinity stabilizing mutations in the FimH lectin domain (^{WT} FimH)). All structures were created with Bioluminate and PyMOL (Schrodinger) using PDB entries 1KLF (FimCH), 4XOD (^{LA} FimH), 4XOC (^{LA} FimH_{LD}), 3JWN and 3BFQ (^{LA} FimGH), 1IKQ (EPA), 7RRW (CRM197), 3BVE (Ferritin).

E. coli adhesion inhibition assays were performed to assess antibody functionality. Interestingly, while the potency values of serum from animals immunized 3 times with ^{LA} FimH_{LD}, ^{LA} FimH, and ^{WT} FimCH were similar, ^{LA} FimGH, ^{LA} FimH_{LD}-EPA, ^{LA} FimH_{LD}-NP, and ^{LA} FimH_{LD}-CRM197 showed a superior ability to induce antibodies that could prevent adhesion of *E. coli* to human bladder cells (Fig. 6C). Serum antibodies were also able to inhibit adhesion of *E. coli* to yeast mannan in an in vitro binding assay, indicating

that the inhibitory effect is specific for the interaction of FimH and its mannose substrate (Supplementary Fig. S5).

While ^{LA} FimH_{LD}, ^{LA} FimH_{LD}-EPA, and ^{LA} FimH_{LD}-CRM197 could be produced using bacterial expression platforms, ^{LA} FimGH, ^{LA} FimH and ^{LA} FimH_{LD}-NP required mammalian expression due to insufficient scalable production in *E. coli*, consistent with previous findings for FimH antigens³⁰. To assess potential platform effects, we compared immunogenicity of

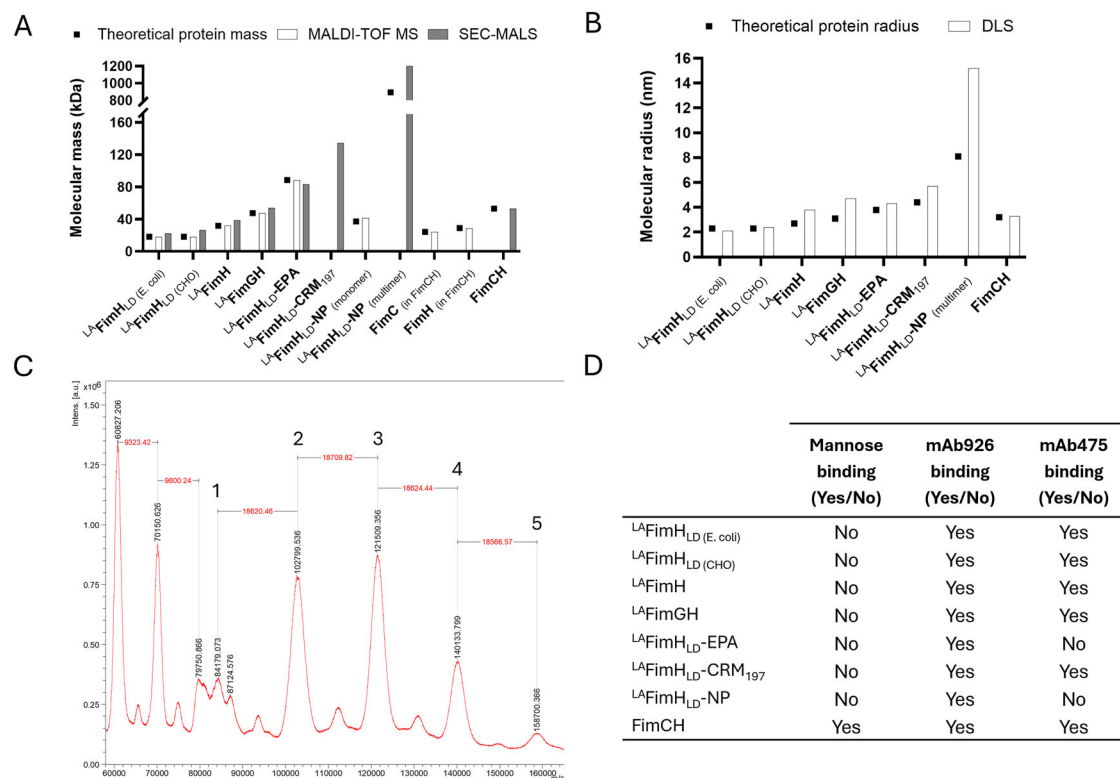


Fig. 5 | Biophysical characterization of ^{LA} FimH_{LD}-based vaccine constructs and FimCH control. Analytical evaluation shows that the molecular structure of each construct is intact and in line with its molecular design (mass, size and multimerization). **A** Theoretical protein masses compared with actual measured masses with MALDI-TOF MS and SEC-MALS. Note that the MALDI-TOF MS spectrum of the ^{LA} FimH_{LD}-CRM₁₉₇ construct did not show one molecular mass but a size distribution presented separately in Fig. 5C. Also, MALDI-TOF MS analysis dissociates ^{LA} FimH_{LD}-NP into monomers and FimCH into FimC and FimH. SEC-MALS analyzes the ^{LA} FimH_{LD}-NP and FimCH constructs in native (multimeric) state. **B** Theoretical protein radii compared with actual radii measured with DLS. ^{LA} FimH_{LD}-NP is likely glycosylated (as seen from its measured mass and radius) and

displays on average 29 ^{LA} FimH_{LD} molecules per nanoparticle according to the multimer mass from SEC-MALS divided by the monomer mass from MALDI-TOF MS. See Supplementary Table S2 for an overview of the dataset that was used for the graphs in Fig. 5A and Fig. 5B. **C** MALDI-TOF intact mass spectrum of ^{LA} FimH_{LD}-CRM₁₉₇. The conjugate displays a range of 1–5 ^{LA} FimH_{LD} molecules coupled to the carrier protein CRM₁₉₇ according to MALDI-TOF MS, with an average of 3.4 ^{LA} FimH_{LD} molecules per CRM₁₉₇ according to SEC-MALS. **D** The low-affinity conformation (i.e., lack of mannose binding) is confirmed with BLI for all constructs containing ^{LA} FimH_{LD} or ^{LA} FimH. All constructs showed binding to mAb926 and mAb475, except for ^{LA} FimH_{LD}-EPA and ^{LA} FimH_{LD}-NP, which did not show binding to mAb475.

^{LA} FimH_{LD} produced in bacterial or mammalian cells. Our results showed that ^{WT} FimH binding antibody titers were comparable between the two different platforms (Supplementary Fig. S6A and S6B). However, functional antibody responses were significantly lower in the group that received mammalian-produced ^{LA} FimH_{LD} in comparison to the group that received bacterial-produced ^{LA} FimH_{LD} (Supplementary Fig. S6C).

To allow for comparison across FimH constructs tested in separate rat immunization experiments, ^{WT} FimH-binding and functional antibody response were normalized to the corresponding benchmark ^{LA} FimH_{LD} construct within each study. We found that ^{LA} FimH_{LD}-CRM₁₉₇ induced the largest relative increase in FimH binding antibodies, whereas ^{LA} FimH_{LD}-NP induced the largest increase in the functional antibody response (Fig. 6D). Functional antibody responses increased disproportionately relative to binding titers, suggesting improved antibody quality, particularly for ^{LA} FimH_{LD}-CRM₁₉₇ and ^{LA} FimH_{LD}-NP.

Collectively, these results demonstrate that optimization of vaccine potency of FimH was successfully achieved through rational design strategies combining conformational stabilization with increasing antigen size, addition of T-cell epitopes, or multimeric display, leading to the identification of four promising ^{LA} FimH_{LD}-based vaccine candidates.

^{LA} FimH_{LD}-CRM₁₉₇ induces a functional immune response in minipigs

As humans share more similarities with pigs than traditional rodent models in terms of urogenital anatomy, renal physiology, and immunology, pigs

and minipigs have shown promise as animal models to study UTIs^{42–46}. Therefore, we evaluated ^{LA} FimH_{LD}-CRM₁₉₇, our novel vaccine antigen construct, which showed promising results in the rat immunization studies, for its potential to elicit a functional immune response in minipigs. ^{LA} FimH_{LD}-CRM₁₉₇ was selected for this purpose based on material availability and the promising rat immunization data available at the time of study initiation. Although ^{LA} FimH_{LD}-NP demonstrated superior functional antibody titers in rats, we reasoned that immunogenicity findings with ^{LA} FimH_{LD}-CRM₁₉₇ would be informative for the construct class as a whole. Immunization with AS01B-adjuvanted ^{LA} FimH_{LD}-CRM₁₉₇ dosed at 100 μ g FimH_{LD} showed a robust increase in ^{WT} FimH-binding IgG antibody titers after two doses, with a booster effect after the third dose (Fig. 7A). These serum antibodies were able to inhibit adhesion of *E. coli* to human bladder epithelial cells (Fig. 7B and Supplementary Fig. S7A) and yeast mannann (Supplementary Fig. S7B). Our results confirm functional immunogenicity for the optimized FimH vaccine antigen construct ^{LA} FimH_{LD}-CRM₁₉₇ in a second animal model.

Discussion

Over the past two decades, the fimbrial tip adhesin FimH has been a central focus for the development of preventive strategies against *E. coli* UTIs due to its critical role in the establishment of infection, biofilm formation, and recurrence⁹. FimH-containing vaccines have been shown to elicit inhibitory antibodies and reduce bladder colonization or bacteriuria in preclinical models^{32–37,47}. The mannose binding pocket present in the lectin domain of

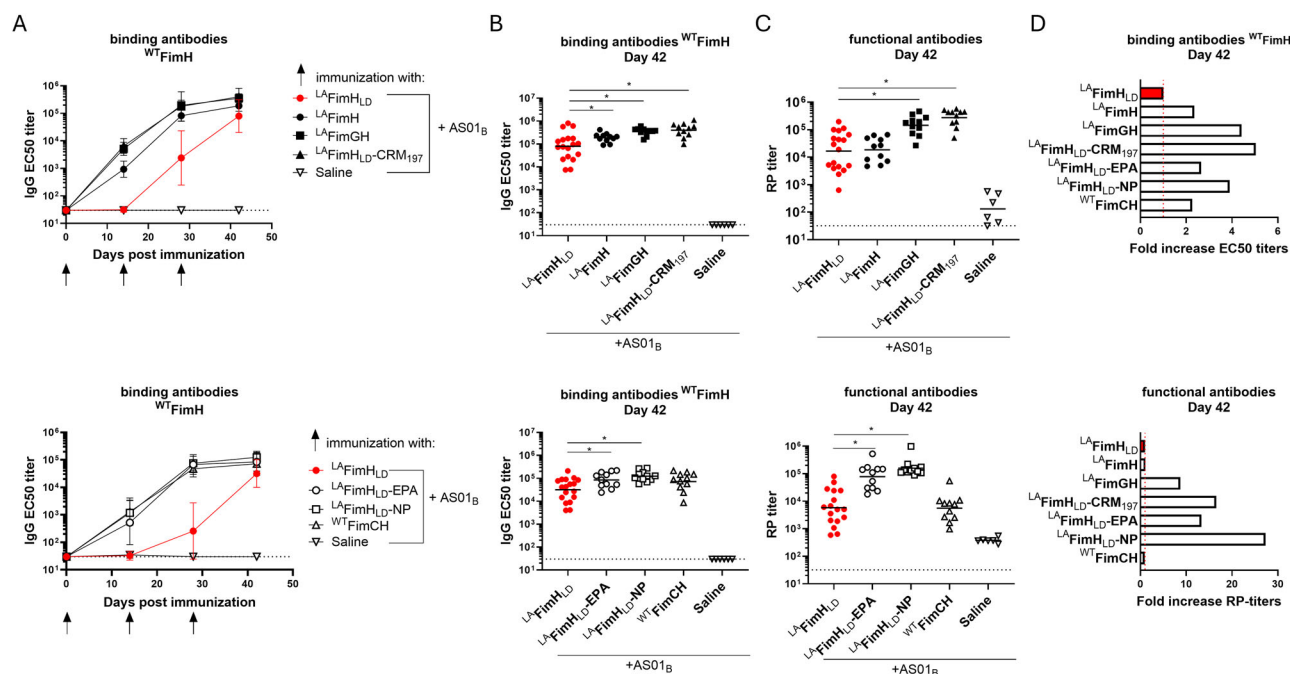


Fig. 6 | Immunogenicity of ^{LA}FimH_{LD}-based vaccine constructs and FimCH control in rats. Rats were immunized IM with ^{LA}FimH_{LD} ($N = 18$) or different FimH variants ($N = 11$ per group), or received saline ($N = 6$), all in combination with AS01_B, on days 0, 14, and 28 (indicated with arrows). The upper and lower panel in Figs. 6A, 6B and 6C display results from two independent experiments, each including a separate ^{LA}FimH_{LD} control (A) Day 0, 14, 28, and 42 serum samples were measured for ^{WT}FimH-binding IgG antibodies by ELISA. The geometric mean with the geometric standard deviation of the EC50 titers of each group is shown. The lower limit of detection (LLOD) was 30 EC50 and is indicated with a dotted line. B ^{WT}FimH binding IgG EC50 titers at day 42 are shown. Each dot represents an individual animal. Line indicates group geometric mean. C Day 42 samples were

tested in an adhesion inhibition assay, using human bladder epithelial 5637 cells and mCherry expressing UTI89 *E. coli*. Relative Potency (RP) titers were calculated and are shown per animal. Line indicates group geometric mean. The lower limit of detection was 32 RP titer and is indicated with a dotted line. The Tobit model, including a Bonferroni correction, was used to compare the groups that received an optimized FimH construct with the group immunized with ^{LA}FimH_{LD} (indicated in red). Adjusted P values < 0.05 were considered significant. D Fold increase in the geometric mean of the ^{WT}FimH binding Ab EC50 titers and the functional Ab RP-titers of each group compared to the response of the ^{LA}FimH_{LD} group within the same experiment is shown.

the FimH protein contains crucial epitopes for inhibitory antibodies²⁷, making the FimH_{LD} subunit of particular interest for vaccine development. Here, we have shown that the FimH_{LD} subunit can be permanently stabilized in the low-affinity conformation, with optimal display of mannose binding pocket epitopes associated with inhibitory antibodies, by applying a combination of Phe71Tyr and Phe144Val mutations. These amino acid substitutions stabilize the low-affinity conformation by combining two distinct structural mechanisms, leading to a permanent inability of the FimH_{LD} to bind mannose while retaining its structural integrity. This feature may be advantageous for vaccination by limiting potential masking of binding pocket epitopes by mannoside ligands present in the host and preserving accessibility for B-cell receptors.

Immunogenicity of vaccine antigens is crucial for efficacy. The experimental vaccine antigen constructs we created based on the low-affinity stabilized FimH_{LD} subunit optimize antigen size, display and/or T-cell help. Four out of five optimized constructs demonstrated a superior ability to induce adhesion blocking antibodies compared to monomeric ^{LA}FimH_{LD}, with the exception of full-length ^{LA}FimH. Interestingly, the clinical reference antigen ^{WT}FimCH, which forces FimH into a relaxed, high-affinity conformation in which the mannose-binding pocket is structurally occluded, did not elicit superior functional antibody responses despite its larger size and provision of T-cell help, although some inhibitory activity was observed. These findings suggest that antigen conformation and specifically display of an open binding pocket, is an important determinant of inhibitory antibody induction. Beyond conformation, the choice of antigen format and the breadth of epitopes presented may further influence the quality and magnitude of the immune response. Native type I pili constructs such as full-length FimH and the FimGH fusion have the advantage of

providing additional relevant *E. coli* epitopes that can be recognized by the immune system upon infection. Wild-type full-length FimH, which is naturally in a low-affinity conformation in absence of substrate, has been evaluated as a vaccine antigen by others and induced functional antibodies and protective efficacy in preclinical animal models^{30,35}. In our preclinical rat model, we did not find an additional benefit of permanently locked low-affinity full-length FimH over the lectin domain subunit in its potential to elicit inhibitory antibodies. The FimGH fusion construct however, did elicit increased functional antibody titers and might be an interesting construct for further investigation. Both constructs could not be expressed in *E. coli* and required a mammalian expression system for production, which could negatively impact economic viability of a potential vaccine. Additionally, specialized production hosts or specific mutations in the antigen construct are required to limit or avoid glycosylation associated with mammalian production platforms. Although we did not observe significant differences in binding antibody levels in response to ^{LA}FimH_{LD} produced in the bacterial and mammalian platform, antibodies elicited with mammalian produced ^{LA}FimH_{LD} showed a significantly lower inhibitory potential. Possibly, the two Asn-to-Ser substitutions introduced to prevent mammalian production host-related N-glycosylation may have locally altered the surface properties of the antigen, potentially reducing accessibility of epitopes associated with adhesion-inhibitory antibodies or shifting immunodominance toward non-inhibitory epitopes. This is an important consideration for future construct optimization and should be addressed in follow-up studies.

The potential limitations associated with production of bacterial antigens in the mammalian platform are not applicable to the ^{LA}FimH_{LD}-EPA construct, which could be produced at high titers in the periplasm of *E.*

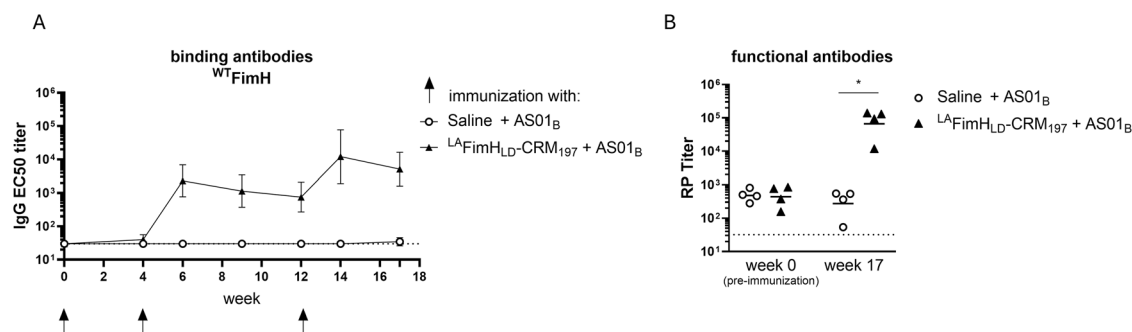


Fig. 7 | Immunogenicity of ^{LA}FimH_{LD}-CRM₁₉₇ in minipigs. Minipigs ($N = 4$ per group) were immunized IM with ^{LA}FimH_{LD}-CRM₁₉₇ (black triangles) or saline (open circles), both adjuvanted with AS01_B, on week 0, 4, and 12 (indicated with arrows). **A** Serum samples were measured by ELISA for total IgG antibodies binding to ^{WT}FimH at indicated timepoints. Geometric mean with geometric SD per group is indicated. The lower limit of detection (LOD) was 30 EC50 and is indicated with a dotted line. **B** Serum samples obtained pre-immunization (week 0) and 5 weeks post

the third immunization (week 17) were tested in an adhesion inhibition assay, using human bladder epithelial 5637 cells and mCherry expressing UTI89 *E. coli*. Relative Potency (RP) titers were calculated and are shown per animal. Line indicates group geometric mean. The lower limit of detection was 32 RP titer and is indicated with a dotted line. The Tobit model was used to determine statistically significant differences between the groups. P values < 0.05 were considered significant.

coli host cells (Supplementary Table S1), allowing low-cost manufacturing. Fusion to the EPA carrier protein significantly increased functional immunogenicity of ^{LA}FimH_{LD}. Clinical trials have shown safety and immunogenicity of the EPA protein^{48–50}, that can induce a strong T-helper cell response when used as a carrier protein. EPA can additionally facilitate incorporation of bacterial polysaccharides via N-linked glycosylation⁵¹, supporting broader vaccine antigen designs. One limitation of the current ^{LA}FimH_{LD}-EPA design is the partial reshuffling of the intramolecular disulfide bridge in the FimH mannose binding pocket and formation of unintended disulfide bonds with cysteine residues in EPA, as observed by LC-MS. This may explain the absence of mAb475 binding to this construct and may limit accessibility of binding pocket epitopes. Unwanted reduction or disulfide scrambling of one or more of the four cysteine pairs of EPA is a known hurdle when using it as a carrier protein⁵². Targeted mutagenesis of the relevant cysteine residues in EPA represents a straightforward optimization strategy to prevent unwanted crosslinking and potentially further enhance the functional immunogenicity of this construct. Despite this limitation, ^{LA}FimH_{LD}-EPA still elicited functional antibodies capable of inhibiting bacterial adhesion, demonstrating that inhibitory immunity can be achieved through epitopes beyond the mAb475 binding site.

Whereas ^{LA}FimH_{LD}-EPA is a 1:1 fusion construct, the ^{LA}FimH_{LD}-CRM₁₉₇ antigen we generated has an average of 3.4 FimH_{LD} particles conjugated to the CRM₁₉₇ carrier protein allowing for multimeric display. Of all antigens we tested, ^{LA}FimH_{LD}-CRM₁₉₇ showed the greatest fold increase in binding antibody titers relative to ^{LA}FimH_{LD} and a significant inhibitory potential. CRM₁₉₇, a detoxified form of diphtheria toxin, is an established carrier protein that contains potent T-cell epitopes and is used in multiple licensed conjugate vaccines^{53,54}. We found that the small ^{LA}FimH_{LD} subunit can be expressed and secreted at high titers in a bacterial expression system (Supplementary Table S1), making it an economically attractive manufacturing platform.

Multivalent presentation of FimH_{LD} was also achieved using a ferritin nanoparticle format (^{LA}FimH_{LD}-NP). Ferritin self-assembles into a 24-mer, resulting in a ~1200 kDa particle when ^{LA}FimH_{LD} is displayed on the surface, compared with 18–130 kDa for the other antigens we tested (Figs. 4A and 5A). Such differences in size, display, and structure can affect receptor-mediated uptake and trafficking and may contribute to improved immunogenicity. An mRNA construct encoding a ferritin-based nanoparticle containing full-length FimH has recently been found to induce robust levels of functional antibodies in rats³⁶. Here, we show that a purified nanoparticle containing ^{LA}FimH_{LD} presented on ferritin also induces functional antibodies and of all constructs tested in rodents elicited the greatest relative increase in functional antibody titers relative to binding titers, suggesting it holds particular promise for further evaluation. Unlike other mammalian-

expressed constructs, Asn-to-Ser substitutions were not introduced in ^{LA}FimH_{LD}-NP. Expi293F GnTI cells were selected as expression host based on optimal yield and nanoparticle assembly, and as these cells produce simpler high-mannose N-glycans, the native FimH_{LD} sequence was retained. However, the observed lack of binding of binding-pocket specific mAb475 to ^{LA}FimH_{LD}-NP suggests potential masking of the epitope by N-glycosylation on Asn7 located in the binding pocket. Together with our observations on mammalian-produced ^{LA}FimH_{LD}, our data suggest that both the presence of N-glycans and the sequence changes introduced to avoid them can independently affect epitope accessibility in the binding pocket, underscoring N-glycosylation as an important consideration for mammalian production of FimH-based vaccine antigens.

Interestingly, while ^{LA}FimH_{LD}-CRM₁₉₇ induced the greatest relative increase in binding antibody titers, ^{LA}FimH_{LD}-NP excelled in functional antibody induction, suggesting that differences in valency and particle size may drive qualitatively distinct immune responses. The substantially higher valency and larger size of ^{LA}FimH_{LD}-NP could promote stronger selective pressure during antibody maturation, enriching for clones targeting functionally relevant epitopes, whereas ^{LA}FimH_{LD}-CRM₁₉₇ may recruit a broader response accumulating higher binding titers.

The molecular and immunological mechanisms underlying the differences in functional immunogenicity between constructs remain to be fully elucidated and represent an important area for follow-up research. Epitope mapping of polyclonal antibodies induced by the most promising ^{LA}FimH_{LD}-based constructs would provide valuable insight into the repertoire of inhibitory antibodies and the binding pocket residues they target, particularly for ^{LA}FimH_{LD}-EPA and ^{LA}FimH_{LD}-NP for which mAb475 accessibility was absent. The mechanism by which the antibodies elicited by these constructs inhibit FimH-mediated adhesion is likely multifaceted and warrants further investigation. In addition to direct competition for the mannose-binding site, inhibition may involve parasteric mechanisms as well as nonspecific steric interference with fimbrial engagement of urothelial receptors. This is consistent with recent monoclonal antibody studies demonstrating that protective anti-FimH antibodies can act through steric interference outside the mannose binding pocket³¹, and is further supported by our observation that ^{WT}FimCH, which presents FimH in the relaxed conformation, also elicited some functional antibodies in our rat model. Taken together, these findings suggest that while mannose binding pocket-specific antibodies are likely the primary mediators of inhibition in our vaccine antigen-induced polyclonal responses, additional inhibitory mechanisms may contribute to overall protection.

While optimized FimH vaccine constructs can elicit antibodies that block *E. coli* adhesion to bladder epithelial cells, effective inhibition may require near-complete saturation of FimH on multiple type 1 fimbriae

across infection stages, a challenge compounded by generally low antibody levels in the bladder. It remains unclear whether potential FimH vaccine-based protection would rely solely on adhesion blockade or whether additional mechanisms such as opsonophagocytosis, which may operate at lower antibody densities, could also contribute. CD4 + T-cell driven immunity may also play a role, by production of cytokines such as interferon- γ and interleukin-17 that mediate the recruitment and activation of phagocytic cells. While the current study was not designed to quantify these cellular mechanisms, their potential contribution to protection represents an important avenue for future investigation. Proper routing of the immune response to the mucosal surfaces of the urinary tract is an additional consideration for vaccine-mediated protection. Intramuscular immunization with AS01B predominantly drives systemic IgG responses, and whether sufficient antibody concentrations reach the urothelial surface through transudation to confer protection remains to be established. Locally produced secretory IgA may also contribute to mucosal protection, though its relative importance remains unclear given the poorly defined immune correlates of protection for UTI. Exploration of alternative administration routes or adjuvant formulations that optimally support protective immunity at the urinary mucosa therefore represent an important area for future research. Protective immunity against UTI may require multiple complementary modes of action and targeting of several bacterial factors, particularly when antigen expression is phase dependent. In this context, combining FimH with a mucosal adjuvant and additional antigens with limited phase-variation such as iron acquisition factors and polysaccharides, which are critical for bacterial fitness during infection, may represent an effective multicomponent strategy. Preclinical studies have demonstrated the immunogenicity and protective potential of iron acquisition factors and polysaccharide-based antigens against ExPEC infections, supporting their inclusion in a multicomponent vaccine strategy^{48,50,55}.

Our immunization experiments in rats showed the successful optimization of FimH_{LD} vaccine potency by low-affinity conformation stabilization combined with an increase of particle size, antigen display, and immunogenic epitopes. We confirmed functional immunogenicity of the ^{LA}FimH_{LD}-CRM₁₉₇ conjugate in minipigs. The results obtained in this larger animal model support further evaluation of ^{LA}FimH_{LD}-CRM₁₉₇, as well as the additional ^{LA}FimH_{LD}-based antigens identified in this study, in a translational porcine UTI challenge model that closely resembles human urinary tract anatomy, physiology and immune responses^{45,56}. Assessment of these candidates, alone or in combination with complementary non-FimH UTI antigens, will be important to inform the development of multicomponent vaccine strategies likely required for effective protection against *E. coli* UTI. In this context, complementary preclinical studies should include mechanistic analyses of adhesion inhibition, opsonophagocytosis, and T-cell responses. Validation of these findings in relevant animal challenge models such as (mini)pigs will be essential to support translation towards clinical evaluation and the design of human studies assessing protection against UTIs.

Methods

Ethics statement

All animal studies were reviewed and approved by the Johnson & Johnson Spring House Institutional Animal Care and Use Committee under AUP IDV510S (rats) and AUP IDV560S (minipigs), and housed in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International)-accredited facility. Studies are reported in accordance with ARRIVE guidelines. Randomization and blinding were not performed.

Antigen design

The antigen design strategy to identify amino acid substitutions that bias the FimH_{LD} conformation towards the low-affinity state was based on structural insights from Rabbani et al.²⁹, who used X-ray crystallography and NMR to elucidate key features of the FimH_{LD} conformational switch. The published structures of the mannoside-bound wild-type relaxed (high-affinity) form (PDB: 4XOC) and the mannoside-free Arg60Pro tense (low-

affinity) form (PDB: 5MCA) were superimposed in PyMOL, to identify residues within the FimH lectin domain that, when substituted with specific amino acids, would be expected to stabilize the low-affinity conformation.

Antigen production

The selection of expression system for each construct was primarily driven by manufacturability. Constructs that could be produced at sufficient yield and quality in bacteria were expressed in *E. coli*. For native type 1 pili variants ^{LA}FimH and ^{LA}FimGH, which could not be produced adequately in bacteria, mammalian expression in CHO cells was employed, with potential N-glycosylation sites removed by Asn-to-Ser substitutions to prevent host-related glycosylation that could interfere with epitope accessibility. For ^{LA}FimH_{LD}-NP, Expi293F GnTI cells were identified as the optimal expression host. As these cells produce simpler high-mannose N-glycans compared to standard mammalian hosts, the native FimH_{LD} sequence was retained for this construct.

FimH_{LD} variants for lead selection. FimH_{LD} variants designed for lead selection were gene-synthesized (GenScript) on a pET22b vector with a periplasmic PelB signal peptide and C-terminal His₆-tag (see Supplementary Data S1 (Sequence list) for the amino acid sequence). Antigens were expressed in *E. coli* C43 (DE3) shaker flask cultures at 30°C in M9 medium supplemented with 2 mM MgSO₄, 0.2 mM CaCl₂, trace elements, BME vitamin mix (Sigma), 1 g/L NH₄Cl, 4 g/L D-glucose and 100 mg/L ampicillin⁵⁷. For uniformly labeled [¹⁵N] proteins, 1 g/L ¹⁵NH₄Cl was used as the sole nitrogen source. Antigen was extracted from the periplasm by incubating the pelleted cells with lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mg/mL polymyxin sulfate) for 2 h at 4 °C followed by centrifugation and buffer exchange of the supernatant containing the periplasmic extract into 50 mM NaH₂PO₄, 300 mM NaCl, pH 8. Purification was performed from the periplasmic extract by Immobilized metal affinity chromatography (IMAC) using a Talon crude resin (GE Healthcare). The elution fractions containing FimH_{LD} were pooled and buffer exchanged into 20 mM NaH₂PO₄, 50 mM NaCl, pH 7.4.

Mammalian expressed constructs. ^{LA}FimH_{LD}, ^{LA}FimH, and ^{LA}FimGH variants designed for expression in mammalian cells were codon-optimized and gene-synthesized (GenScript) on a pcDNA3.4 vector with a secretory murine IgK signal peptide (METDTLLLVWVLLWVPGSTG), FimH mutations Asn7Ser, Asn70Ser, Asn228Ser, and Asn235Ser, and FimG mutation Asn22Ser to avoid N-glycosylation by the eukaryotic production host, which could sterically interfere with epitope accessibility and potentially alter the immunogenicity of the construct, and a C-terminal His₆-tag or CG-His₈-tag with thrombin cleavage site. For protein stabilization of FimH, a donor peptide from FimG (ADVTTITVNGKVVAK) preceded by a 7GS linker was added at the C-terminus of FimH⁴¹. The FimGH fusion protein design included N-terminal FimH followed by a 7GS linker and C-terminal FimG sequence. For protein stabilization, a donor peptide from FimF (ADSTITIRGYVRDNR) preceded by a DNQK linker was added at the C-terminus of FimG. Antigens were expressed at 200 mL scale in transfected CHO cells, purified by one-step Ni-NTA affinity chromatography and buffer exchanged in 25 mM Tris-HCl, 150 mM NaCl, pH 7.4. ^{LA}FimH_{LD}-NP designed for nanoparticle expression in mammalian cells was codon-optimized and gene-synthesized (GenScript) on a pcDNA3.4 vector with a secretory murine IgK signal peptide (METDTLLLVWVLLWVPGSTG), an N-terminal ^{LA}FimH_{LD} subunit, an SGS-His₈-GGG linker followed by a 10 AA short leader sequence and C-terminal *H. pylori* ferritin protein sequence. Nanoparticles were expressed at 500 mL scale from transfected Expi293F GnTI cells, purified by one-step Ni-NTA affinity chromatography and buffer exchanged in PBS pH 7.2.

^{LA}FimH_{LD}-EPA. ^{LA}FimH_{LD}-EPA fusion protein was gene-synthesized (GenScript) on an arabinose-inducible pEC415 vector with a periplasmic PelB signal peptide, an N-terminal ^{LA}FimH_{LD} subunit, a 14 AA linker

followed by genetically detoxified *Pseudomonas aeruginosa* exotoxin A (EPA) protein including motifs for N-linked glycosylation, and a C-terminal His₆-tag. Antigens were expressed in *E. coli* K-12 W3110 host cells in Soy-LB fed-batch bioreactor cultures. Antigens were released from the bacterial periplasm by osmotic shock and purified by multistep chromatographic purification as described⁴⁸, and subsequently buffer exchanged in 20 mM NaH₂PO₄, 50 mM NaCl, pH 7.4.

^{WT}FimCH. The ^{WT}FimCH dimer was gene-synthesized (GenScript) on a pET-DUET vector with an *E. coli* PelB signal peptide for both FimC and FimH, and a C-terminal His₆ tag separated by a Thrombin cleavage site for FimC. Antigens were expressed in *E. coli* C43 (DE3) shaker flask cultures at 30°C in M9 medium supplemented with 2 mM MgSO₄, 0.2 mM CaCl₂, trace elements, BME vitamin mix (Sigma), 1 g/L NH₄Cl, 4 g/L D-glucose, and 100 mg/L ampicillin. Antigen purification was performed from the periplasmic extract by Immobilized metal affinity chromatography (IMAC) using a Talon crude resin (GE) as described for the FimH_{LD} variant. The purified dimer was buffer exchanged in PBS pH 7.4.

^{LA}FimH_{LD}-CRM₁₉₇. ^{LA}FimH_{LD}-CRM₁₉₇ was produced by chemical conjugation of ^{LA}FimH_{LD} produced by an *E. coli* secretory bioreactor process (Premas Biotech, India) to the commercially available carrier protein CRM₁₉₇ (Pfenex, Primrose Bio, Inc.) using HiPEG™ conjugation technology (Abzena, Cambridge, UK). In short, *E. coli* BL21 (DE3) and C43 (DE3) strains for expression of ^{LA}FimH_{LD}-His₆ with a PelB secretion signal were grown at 30 °C in a bioreactor fed-batch fermentation process with chemically defined medium (33 g/L glucose, 13.3 g/L K₂HPO₄, 6.67 g/L (NH₄)₂SO₄, 2.83 g/L citric acid monohydrate, 0.67 g/L MgSO₄, 0.17 g/L FeCl₃·6H₂O, 23 mg/mL MnSO₄·H₂O, 20 mg/L ZnSO₄·7H₂O, 15 mg/L EDTA free acid, 5 mg/mL H₃BO₃, 4 mg/L CoCl₂·6H₂O, 4 mg/L Na₂MoO₄·2H₂O, 2.5 mg/L CuCl₂·2H₂O, pH 7.0 with H₃PO₄ and NH₄OH) containing 100 µg/mL carbenicillin. In these conditions, the ^{LA}FimH_{LD} protein was spontaneously released from the cell to allow antigen capture from clarified and filtered culture supernatant using IMAC. After polishing by anion exchange chromatography in flow-through mode, the antigen was buffer exchanged in a formulation buffer (20 mM Na₂HPO₄, 50 mM NaCl, pH 7.4) compatible with the conjugation chemistry. The free amine groups of surface exposed lysine residues from a 1 to 1.2 mg/mL (16.7–20 µM) solution of CRM₁₉₇ protein were functionalized with a 15–25 molar equivalent of HiPEG™-PEG6-NHS ester linker (933.05 Dalton, synthesized by Abzena, Cambridge, UK) in PBS pH 7.4, 10% propylene glycol, 10% DMSO, under constant agitation for 3 h at 22 °C. After removal of unreacted linker and CRM₁₉₇ protein by preparative SEC (Superdex 200 10/300 GL with phosphate buffer pH 7.4 as the isocratic elution buffer), functionalized CRM₁₉₇ with a degree of labeling (DoL) of ~7 was used for the conjugation reaction with purified ^{LA}FimH_{LD}. To that end, the HiPEG™ functionalized CRM₁₉₇ at a concentration of 11 µM was mixed with 12–18 times the molar equivalent of ^{LA}FimH_{LD} in PBS, pH 7.4, under constant agitation for 18 h at 22 °C. The His₆ tag reacted with the HiPEG™-PEG6 linker as described previously^{58,59}. After removal of unreacted ^{LA}FimH_{LD} and functionalized CRM₁₉₇ protein by preparative SEC (Superdex 200 10/300 GL with phosphate buffer pH 7.4 as the isocratic elution buffer) the resulting ^{LA}FimH_{LD}-CRM₁₉₇ showed an average DoL of 3.4 ^{LA}FimH_{LD} molecules per CRM₁₉₇ molecule determined by SDS-PAGE and SEC-MALS.

Construct protein sequences are available in Supplementary Data S1.

Characterization of vaccine antigens

Antigen integrity, oligomeric state, conformational state, and mannose-binding pocket accessibility were characterized using SPR, NMR, SEC-MALS, MALDI-TOF MS, DLS, and BLI.

SPR. Binding affinity of FimH_{LD} variants to a mannoside ligand was determined by surface plasmon resonance at 25 °C using HBS-EP (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20, GE Healthcare) as running buffer. A carboxyl-functionalized mannoside 5 (8-(α-D-mannopyranosyloxy)-octanoic acid, synthesized at Mercachem) was immobilized on a CM4 sensor chip via an ethylene-diamine linker as described previously²⁹. FimH_{LD} variants were injected as analytes and association/dissociation responses were recorded.

NMR. ¹H, ¹⁵N HSQC and TROSY experiments were recorded on Bruker 600 MHz and 850 MHz spectrometer equipped with a 5 mm TXI 13 C Z-grad and a 5 mm CPTCI 1H-13C/15 N/D Z-grad cryoprobe, respectively. For HSQC experiments a standard Bruker pulse sequence (fhsqc3gp) was used and for TROSY experiments a pulse sequence with band-selective inversion pulse (developed by Tammo Diercks) was used. Uniformly ¹⁵N-labeled FimH_{LD} variants were measured in the absence and presence of mannoside 1 (n-heptyl-α-D-mannopyranoside, synthesized at Mercachem) to assess structural differences on a residue-based level. Protein was concentrated and measured at 176–584 µM in 20 mM phosphate buffer, pH 7.4, 50 mM NaCl, 0.1% H₂O₂, with 5% D₂O. Mannoside 1 was dissolved in H₂O and added stepwise to the protein up to a tenfold molar excess. The spectra of each sample were compared to reference spectra²⁹, including previously assigned residues, to assess whether a conformational change had occurred upon interaction with mannoside 1.

SEC-MALS. Molecular mass and oligomeric state of vaccine antigens were determined by SEC-MALS using an Ultimate 3000 HPLC system with DAD detector (Thermo Scientific) coupled to a DAWN NEON MALS detector and Optilab NEON RI detector (Wyatt Technology). Samples (40 µg protein) were separated on an XBridge Premier Protein SEC (7.8 × 300 mm) column and a MaxPeak Premier Protein SEC Guard using 2x PBS (pH 7.4) as a running buffer with a flow rate of 0.5 mL/min and UV detection at 280 nm. The molecular weight of the main peak was calculated using Wyatt ASTRA 8 software with antigen-specific extinction coefficients and dn/dc = 0.185 mL/g.

MALDI-TOF MS. Intact protein mass was determined by MALDI-TOF MS analysis after buffer exchange of the antigens into Milli-Q water and direct spotting with a 2,5-dihydroxyacetophenone (DHAP) / diammonium hydrogen citrate (DAC) mixture as matrix. Solutions of 20 mg/mL DHAP in ethanol and 18 mg/mL DAC in water were prepared and mixed in a 3:1 volume ratio to obtain the matrix solution. A sample aliquot of 2 µL was mixed with 2 µL of 2% trifluoroacetic acid and 2 µL matrix solution. Spotted samples (0.75 µL) were analyzed on a Rapiflex MALDI-TOF MS instrument (Bruker) using the linear positive detection mode at a power of 70–90% and overlaying between 10,000 and 20,000 profiles (1000–2000 profiles per shot). The instrument was calibrated with carbonic anhydrase II on the calibration spot closest to the sample prior to the measurements.

DLS. The hydrodynamic radius of the antigens was measured in their respective formulation buffer (20 mM sodium phosphate with 50 mM NaCl at pH 7.4 (^{LA}FimH_{LD} (*E. coli*), ^{LA}FimH_{LD}-EPA), 25 mM Tris-HCl with 150 mM NaCl at pH 7.4 (^{LA}FimH_{LD} (CHO), ^{LA}FimH, ^{LA}FimGH), DPBS at pH 7.4 (^{LA}FimH_{LD}-CRM₁₉₇), PBS at pH 7.2 (^{LA}FimH_{LD}-NP), or PBS at pH 7.4 (FimCH)) at 25 °C with a DynaPro Plate Reader III (Wyatt Technology), auto-attenuation enabled and 20 acquisitions (of 3 s each) per DLS measurement. A sample volume of 25 µL was loaded in triplicate in a 384-well DLS plate, which was measured three times. The theoretical protein radius was calculated based on the theoretical protein mass assuming a globular protein as described⁶⁰. BLI. Mannose binding was

assessed by biolayer interferometry on Octet RED384 (Sartorius) in kinetics mode, using biotinylated mannose (0.4 µg/mL) captured on streptavidin biosensors. A blocking step with biocytin (25 µg/mL) was included, followed by association to the FimH samples (392 nM). 1x Kinetics buffer (Sartorius) was used as sample diluent and assay buffer, and ¹²⁵I-FimH_{LD} as positive control (i.e., binding to mannose). Reactivity of mAb926 and mAb475 towards the vaccine antigens was evaluated by BLI in kinetics mode using Ni-NTA biosensors and 1x kinetics buffer (Sartorius). The HIS-tagged FimH variants were loaded at a concentration of 100 nM and a blocking step with a hexa-his protein was included, followed by association to mAb926 (25 µg/mL) or mAb475 (25 µg/mL). Octet BLI analysis software (version 12.2.2.4) was used for kinetic analysis of the association step, to obtain response values in nm and report the presence or absence of binding.

Protein concentration, purity and impurities. Protein concentration was measured with a NanoDrop Eight spectrophotometer (Thermo Scientific) and antigen buffer as blank. The UV signal at 340 nm was subtracted from the UV signal at 280 nm and for each antigen the extinction coefficient and theoretical protein mass were calculated based on its amino acid sequence (Supplementary Data S1)⁶¹. Purity (250 µg/mL) was evaluated with CE-SDS under denaturing and reducing conditions using a Maurice OBM Protein Analyzer (ProteinSimple) and CE-SDS PLUS Size Application Kit (ProteinSimple). Molecular weight species were separated using a load time of 20 to 40 s, load voltage of 4600 V, separation time of 35 min and separation voltage of 5750 V and detected with UV absorbance at 220 nm. Purity was defined as the main peak area percentage. Endotoxin levels were measured with an Endosafe nexgen-MCS (Charles River) and disposable LAL test cartridges of 5 to 0.05 EU/mL or 10 to 0.1 EU/mL (Charles River). Host cell DNA levels were measured with a Quant-iT PicoGreen dsDNA kit and reagent (Thermo Scientific) and a BioTek Synergy H1 microplate reader (Agilent) with fluorescence excitation at 480 nm and emission at 520 nm.

Cysteine bridging analysis by LC-MS. Sample preparation, LC-MS analysis, and data processing were performed by ProtaGene GmbH. Non-reduced and dithiothreitol reduced samples were analyzed by HPLC-ESI-MS and MS/MS. From collected MS data of non-reduced samples the bridged cysteine residues were determined by detection of linked (Cys-Cys) di-peptides using MS data evaluation software. The identification was based on MS masses of di-peptides and sequencing of the amino acid residues by MS/MS spectra annotation. The protein samples were proteolytically cleaved with chymotrypsin and AspN. One-half of each sample was reduced with dithiothreitol and carbamidomethylated using iodoacetamide. The other half was kept untreated. HPLC-ESI-MS and -MS/MS mass spectra were obtained using an Ulti-Mate® 3000 system (Thermo Fisher Scientific) coupled to a Q Exactive Orbitrap Plus mass spectrometer (Thermo Fisher Scientific). The separation of the peptides was performed with reversed phase (RP) chromatography on an Accucore RP-MS LC column (2.1 × 100 mm, 2.6 µm particle size, Thermo Fisher Scientific). Water with 0.1% formic acid and acetonitrile with 0.1% formic acid were used as eluents and a gradient was applied with a flow rate of 0.4 mL/min. MS and MS/MS spectra were recorded in positive ion mode with internal mass calibration. The datasets of the non-reduced samples were searched with ProteinMetrics Byonic™ against the amino acid sequences. The annotated MS spectra were evaluated for fragment ions of crosslinked peptides. Only linkages including cysteines present in the FimH binding pocket (Cys3 and Cys44) were considered. The levels of expected and shuffled disulfides were assigned by comparing the peak area of these dipeptides. The sum of the peak areas for the linkages of each cysteine was set to 100%. No correction for different ionization efficacy of the peptides was done; the level of a cysteine linkage in percent does not exactly reflect the amount of the peptide.

Immunization

Female Sprague Dawley rats, around 7 weeks of age, received 3 intramuscular immunizations in the hindleg with a FimH vaccine antigen combined with AS01_B. Formulations were administered in a 100 µL volume, which consisted of 50 µL of a 3.373 µM FimH variant mixed with 50 µL of AS01_B (unless otherwise stated), corresponding to 5 µg MPL and 5 µg QS-21 as active ingredients. The negative control group received saline (PBS, pH 7.4) in combination with 50 µL AS01_B. The immunizations were administered 2 weeks apart (day 0, 14, and 28) and two weeks post the third immunization, animals were euthanized (day 42). Blood was collected before each immunization to obtain serum. Rats were not sedated for immunizations or blood collection. Rats were anesthetized with CO₂ and euthanized by cutting the diaphragm.

Female Göttingen minipigs 5–6 months of age were intramuscularly immunized with 100 µg ¹²⁵I-FimH_{LD}-CRM₁₉₇ (based on FimH) or saline, both in combination with 250 µL AS01_B, in a total volume of 500 µL. Minipigs received a second and third dose 4- and 12-weeks post the first immunization. Blood was collected pre-immunization, at weeks 4 (before the second immunization), 6, 9, 12 (before the third immunization), 14, and 17 for serum analysis. Minipigs were sedated with Telazol (3.5 mg/kg, intramuscular) for immunizations and blood collection. Prior to terminal sample collection, animals were sedated by intramuscular administration of 0.044 mL/kg of a mixture containing tiletamine, zolazepam, ketamine, and xylazine (50 mg/mL each) and maintained under isoflurane anesthesia. After sample collection, animals were euthanized while still sedated via intravenous administration of 125 mg/kg euthanasia solution (390 mg/mL pentobarbital sodium, 50 mg/mL phenytoin sodium) through the right cranial vena cava. Death was confirmed by cardiac auscultation.

ELISA

Total IgG responses were quantified by ELISA using rat or minipig sera collected at the indicated time points. Nunc Maxisorp plates were coated overnight at 4 °C with 1–2 µg/mL FimH antigen (FimH30 wild-type or Phe71Tyr/Phe144Val; full-length or lectin domain), blocked with 2.5% skimmed milk in PBS, and incubated with serially diluted serum samples (duplicate wells). Bound IgG was detected using HRP-conjugated goat anti-rat IgG (1:2000; BioLegend) or rabbit anti-pig IgG (1:10000; Sigma Aldrich), developed with TMB, and read at 450 nm. EC50 titers were calculated using a 4-parameter logistic regression model.

Cellular adhesion inhibition assay

Antibody functionality was evaluated in a cellular adhesion inhibition assay (AIA) using human bladder epithelial 5637 cells (ATCC) and an mCherry-expressing *E. coli* UTI89 strain. For assay development, FITC-labeled *E. coli* UTI89 and a UTI89ΔFimH strain, generated by lambda red recombination-based gene disruption⁶², were used to confirm predominantly FimH-dependent bacterial adhesion to bladder cells and to select an optimal bacterial density for the assay (Supplementary Fig. S4). Overnight bacterial cultures were diluted to OD₆₀₀ 0.25 in PBS and pre-incubated with serially diluted serum samples (duplicate wells) prior to addition of 5637 cells (50,000 cells/well) and incubation at 37 °C. After washing, the percentage of mCherry-positive cells was quantified by flow cytometry (LSR Fortessa, BD). mAb475 was included as an internal reference on each plate. Relative potency titers were derived as the difference in log₁₀ IC50 between the reference mAb and test samples.

Mannose adhesion inhibition assay

A complementary mannan-based adhesion inhibition assay was performed using *S. cerevisiae* mannan-coated white Maxisorp plates (20 µg/mL, overnight at 4 °C). *E. coli* UTI89-mCherry was diluted to OD₆₀₀ 2 in PBS containing 0.1% BSA and incubated with serum dilutions, mAb926 (20 µg/mL), or methyl α-D-mannopyranoside (100 mM, Sigma) prior to addition to mannan-coated wells. After static incubation at 37 °C followed by washing, bound bacteria were quantified by fluorescence (Ex 590 nm/Em 620 nm; Tecan).

Statistical analysis

The Tobit model was used to assess statistically significant differences in FimH binding and functional antibody titers between the group immunized with ^{LA}FimH_{LD} and the groups immunized with the different FimH constructs. Bonferroni correction was applied to correct for multiple comparisons where applicable. Adjusted *P* values < 0.05 were considered significant. Sample sizes for the rat studies were determined by power calculation (80% power, $\alpha = 0.05$, with Bonferroni correction for multiple comparisons); minipig group sizes were determined based on feasibility and resource availability. No animals were excluded from the analysis.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files. The DNA sequences encoding the FimH vaccine constructs described in this study have been deposited in GenBank under accession numbers PZ710045 – PZ710052.

Received: 10 April 2026; Accepted: 23 August 2026;

Published online: 02 September 2026

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Acknowledgements

We thank Joan van Kregten, Jeroen Zeijpveld, Martijn Kremer, Aditya Perkasa, Timothy van Eijl, Paolo D'arrigo, Rosanne Loos, Theo Schouten, and Ashmita Arjoon for performing in vitro experiments. We thank Kaila Orlandini, Isabella Goebel, Jessica Henn, Matthew Wilms, Emily Podob, Kirsten Bair, and Jeffrey Fernandez for performing and supervising in vivo experiments. We thank Meissa Rocha Essensfelder Abrahao, Gerrard Perdok, Sjoerd Renger, Roxanne Douwes, Nienke Prins, and Hein Trip for producing the FimH variants in the *E. coli* expression platform, and Marco Glandrup, Nikkie Nassette, Hanna Hanzha, Lydia van de Beek, and Nidhi Saikhedkar for analytical measurements and preparation of samples for preclinical assessment. We thank Aldert Zomer for bioinformatics support. We thank Jan Serroyen and Leacky Muchene for statistical support. We thank Joyce Lübbers, Evelyn van der Aa, and Germie van den Dobbelaars for scientific discussions. We thank Dan Chan and Masa Kobayashi from

ZoBio and Richard Spears from Abzena for their support in production and analysis of vaccine antigen constructs.

Author contributions

E.W. and J.G. designed the study. J.G. and P.B. produced the antigens. M.v.B. led the antigen characterization experiments. S.W. and L.v.d.F. led the immunogenicity studies. P.W. performed the cellular AIA assay. L.F. and G.T. performed structure-based analysis. ST led the characterization experiments supporting initial antigen design. E.H., C.A., J.G.e., K.F. and J.P. provided advisory support. E.W. and J.G. drafted the manuscript. All authors contributed to data interpretation, manuscript revision, and approved the final version.

Competing interests

E.W., J.G., S.W., M.v.B., P.B., E.H., P.W., L.v.d.F., G.T., C.A., K.F., J.G.e., and J.P. are former employees of Janssen Vaccines & Prevention B.V. (Johnson & Johnson) and may own stock or stock options. JGe is currently an employee of Sanofi B.V. and may own company stock (options). J.G., E.W., J.G.e, K.F., and L.F. are named inventors on two pending patent applications filed by Janssen Pharmaceuticals, Inc. (WO 2021/144369 A1 and WO 2022/153166 A1), both relating to FimH-containing polypeptides carrying mutations that lock the binding pocket in a favorable conformation, as described in this manuscript. The applications are at different stages of prosecution internationally, with some claims granted and others still under examination. The other authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41541-026-01571-0>.

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