

Correction

MICROBIOLOGY

Correction for “Early evolutionary loss of the lipid A modifying enzyme PagP resulting in innate immune evasion in *Yersinia pestis*,” by Courtney E. Chandler, Erin M. Harberts, Mark R. Pelletier, Iyarit Thaipisuttikul, Jace W. Jones, Adeline M. Hajjar, Jason W. Sahl, David R. Goodlett, Aaron C. Pride, David A. Rasko, M. Stephen Trent, Russell E. Bishop, and Robert K. Ernst, which was first published August 31, 2020; 10.1073/pnas.1917504117 (*Proc. Natl. Acad. Sci. U.S.A.* **117**, 22984–22991).

The authors note that, due to a printer’s error, ‘2’ and ‘3’ should appear as ‘2 and 3’ in the following areas:

In the Abstract, page 22984, left column, sixth sentence. On page 22987, left column, second full paragraph, last sentence. And on page 22990, left column, fourth full sentence and in the right column, lines 1 and 7.

In addition, ‘1’ and ‘4’ should appear as ‘1 and 4’ in the second paragraph of the Introduction, page 22984, right column, third full sentence.

Finally, on page 22987, left column, second full sentence, the text that reads ‘non-Yp PagP model (“Restored” PagP or Pag+)’ should instead read ‘non-Yp PagP model (“Restored” PagP or PagP+)’.

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Early evolutionary loss of the lipid A modifying enzyme PagP resulting in innate immune evasion in *Yersinia pestis*

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Immune evasion through membrane remodeling is a hallmark of *Yersinia pestis* pathogenesis. *Yersinia* remodels its membrane during its life cycle as it alternates between mammalian hosts (37 °C) and ambient (21 °C to 26 °C) temperatures of the arthropod transmission vector or external environment. This shift in growth temperature induces changes in number and length of acyl groups on the lipid A portion of lipopolysaccharide (LPS) for the enteric pathogens *Yersinia pseudotuberculosis* (*Ypt*) and *Yersinia enterocolitica* (*Ye*), as well as the causative agent of plague, *Yersinia pestis* (*Yp*). Addition of a C16 fatty acid (palmitate) to lipid A by the outer membrane acyltransferase enzyme PagP occurs in immunostimulatory *Ypt* and *Ye* strains, but not in immune-evasive *Yp*. Analysis of *Yp pagP* gene sequences identified a single-nucleotide polymorphism that results in a premature stop in translation, yielding a truncated, nonfunctional enzyme. Upon repair of this polymorphism to the sequence present in *Ypt* and *Ye*, lipid A isolated from a *Yp pagP*+ strain synthesized two structures with the C16 fatty acids located in acyloxyacyl linkage at the 2' and 3' positions of the diglucosamine backbone. Structural modifications were confirmed by mass spectrometry and gas chromatography. With the genotypic restoration of PagP enzymatic activity in *Yp*, a significant increase in lipid A endotoxicity mediated through the MyD88 and TRIF/TRAM arms of the TLR4-signaling pathway was observed. Discovery and repair of an evolutionarily lost lipid A modifying enzyme provides evidence of lipid A as a crucial determinant in *Yp* infectivity, pathogenesis, and host innate immune evasion.

in the O-antigen gene pseudocluster, *Yp* isolates lack an O-antigen and produce lipid A capped by a core oligosaccharide (lipooligosaccharide [LOS]) (7). The immunostimulatory property of LPS lies in the lipid A component, which can be released into the environment after cell lysis for detection by the serum LPS-binding protein, thereby triggering an innate immune response via the TLR4/MD2/CD14 complex (8). The potent endotoxicity of *Escherichia coli* lipid A is a key etiologic step of sepsis and septic shock (9). The molecular structure of *E. coli* lipid A consists of a β-(1',6)-linked diglucosamine backbone, phosphate groups at the 1' and 4' positions, and various fatty acid chains connected to the diglucosamine backbone depending on the bacterial species and growth conditions (10–13). Immune stimulation depends on the lipid A structure, which can be modified in a number of ways including, but not limited to, the addition of carbohydrate moieties,

lipid A | evolution | pathogenesis | immune evasion | *Yersinia*

Yersinia pestis (*Yp*) is the causative agent of plague and responsible for the Black Death, one of the deadliest pandemics in human history (1). The *Yp* life cycle includes an arthropod vector, such as fleas, which feed on wild or domestic rodents that subsequently come into contact with humans (2, 3). In humans, *Yp* can cause three primary disease manifestations: bubonic, septicemic, and pneumonic plague. Bubonic plague is characterized by the appearance of bubos that result from inflammation of the lymph nodes draining the area associated with flea bites. These bubos form infection foci that allow the bacteria to spread out systemically, resulting in septicemic plague, which is 100% fatal if left untreated. Pneumonic plague occurs when the bacteria reach the lungs via the blood stream and can be spread person-to-person through aerosol droplets (4, 5).

Lipopolysaccharide (LPS) or endotoxin is the major component in the outer leaflet of the outer membrane of Gram-negative bacteria. LPS is composed of three structural regions: O-antigen, core polysaccharide, and lipid A (6). Due to mutations

Significance

Immune evasion is a hallmark of *Yersinia pestis* pathogenesis, including loss of pathogen-associated patterns recognized by Toll-like receptors. During its life cycle, *Y. pestis* alternates between mammalian hosts and arthropod transmission vectors and concurrently remodels its membrane, specifically modifying the structure of the lipid A portion of its lipopolysaccharide recognized by TLR4-MD2. Genomic analysis identified a single-nucleotide polymorphism that results in a premature stop in translation of the lipid A acyltransferase *pagP*, resulting in synthesis of a stealthy, hypoacylated lipid A structure absent in other *Yersiniaceae*. This provides evidence of lipid A as a crucial determinant in *Y. pestis* infectivity, pathogenesis, and host innate immune evasion and represents one of the earliest identified adaptations of *Y. pestis* from *Yersinia pseudotuberculosis*.

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The authors declare no competing interest.

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addition or removal of phosphate groups, as well as variation in length, number, and order of fatty acid chains (11–13). Each bacterial pathogen can modify their lipid A to exploit environmental niches to enhance the ability to colonize, spread to different tissue, and/or avoid the host's immune defense.

Yp is noted for its ability to evade the mammalian innate immune response, which allows it to colonize and infect a host before an effective immune response is mounted, leading to increased mortality (14, 15). A number of virulence factors have been shown to suppress the innate immune response and prevent activation of the adaptive immune response including the following: the capsular-like F1 antigen (16); LcrV, a protein involved in the formation of the type 3 secretion system (T3SS) needle tip (17, 18); the outer membrane protein Ail (19, 20); the T3SS effector *Yersinia* outer proteins (Yops) (21–24); and many others (14, 25, 26). Structural modifications to lipid A, the membrane anchor of LPS, also play a role in immune evasion (14, 27, 28).

When grown at 21 °C to 28 °C, which mimics the temperature in an arthropod vector, *Yp* predominantly produces a hexa-acylated lipid A molecule (Fig. 1); however, when the temperature shifts to 37 °C (the mammalian host temperature), lipid A production shifts to generate a hypo-acylated lipid A (Fig. 1, tetra-acylated) (28, 29). Like *E. coli* and other enterobacteria, hexa-acylated lipid A from *Yp* grown at a reduced temperature is a potent immune stimulator of the human TLR4-signaling pathway, whereas tetra-acylated lipid A is a weak agonist (6). This shift in lipid A composition results in decreased recognition mediated via TLR4, thereby dampening TLR4-driven innate immune responses and resulting in the ability of *Yp* to evade host defenses in the early stages of infection (28–32).

Lipid A extracted from *Yp* at mammalian temperature displays minimal endotoxic properties, providing a potential mechanism for the bacteria to avoid the host immune system (33). PagP is an

eight-stranded antiparallel β -barrel acyltransferase present in the outer membrane in a limited number of Gram-negative organisms (34). Enzymatically, PagP transfers palmitate (C16) from a phospholipid donor, delivered into the external leaflet of the outer membrane to the hydroxyl group of the *R*-3-hydroxymyristate (C14-OH) chain on lipid A. This reaction is unique because it occurs in the outer membrane without access to thiol-activated precursors located in the bacterial cytoplasm (34). PagP activity and palmitoylation of lipid A has been implicated in many processes including resistance to cationic antimicrobial peptides in *Salmonella*, increased biofilm formation in *Pseudomonas aeruginosa*, and infectivity in *Bordetella bronchiseptica* (35–38). Interestingly, lipid A isolated from *Y. pestis* is devoid of palmitoylated lipid A species, whereas it is observed in closely related *Yersinia pseudotuberculosis* (*Ypt*) or *Yersinia enterocolitica* (*Ye*) species, raising the possibility of potential functional differences in PagP between these species (32). Analysis of the *pagP* gene sequence from all sequenced *Yp* genomes identified a single-nucleotide polymorphism (SNP) that resulted in a premature stop in translation of the *Yp pagP* gene, yielding a nonfunctional enzyme.

In this study, we examine the impact of this SNP causing premature loss of translation by restoring it in the *Yp*-KIM6 *pagP* gene (a BSL-2 version of *Yp*) to the sequence present in all *Ypt* and *Ye* isolates. We demonstrate that *Yp* encoding a functional PagP results in a hexa-acylated lipid A structure with increased immunostimulatory properties, as compared to the wild type (WT) KIM6 tetra-acylated lipid A when grown at 37 °C. This increase in innate immune recognition via the TLR4-MD-2-signaling pathway could have implications in determining how downstream adaptive immune responses are altered and represents one of the earliest identified adaptations of *Yp* from *Ypt*.

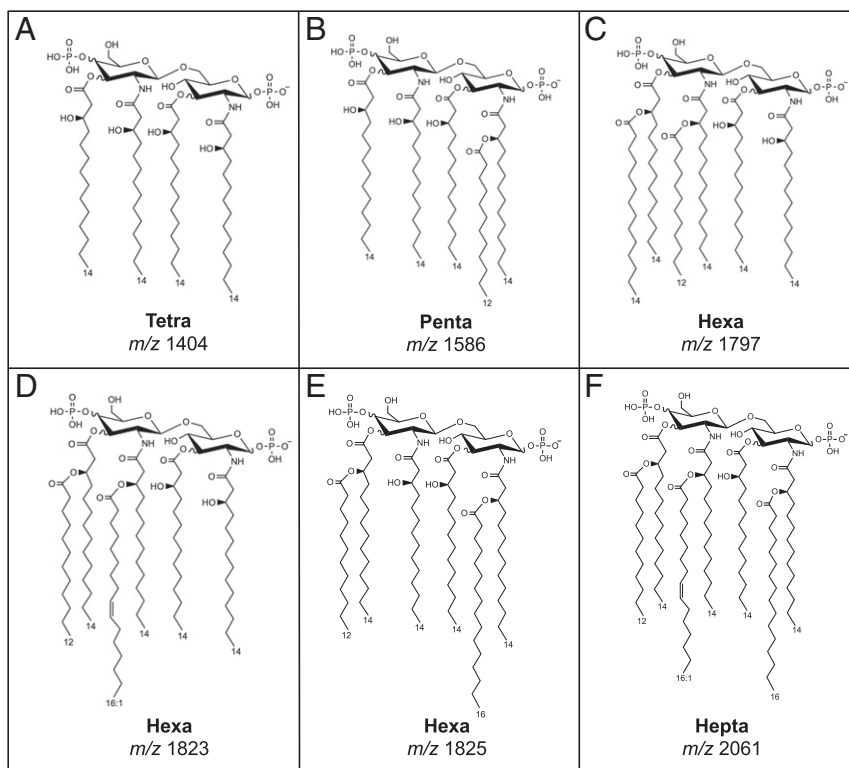


Fig. 1. Proposed structures of *Yersinia* lipid A molecules. *Yersinia* species are capable of producing a variety of lipid A structures that are influenced by their surrounding environments. (A) *Y. pestis* grown at 37 °C. (B) *Y. pseudotuberculosis* grown at 37 °C. (C) *Y. enterocolitica* grown at 37 °C. (D) *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* grown <26 °C, (E, F) PagP+ palmitoylated lipid A species.

Results

Bioinformatic Analyses of the *pagP* Gene. To identify differences in the *pagP* gene sequence among *Yersinia* species, a bioinformatics approach was utilized. Comparison of the *pagP* gene sequence from 19 individual *Yersinia* species identified two SNPs specific to the *Yp* isolates examined, when compared to the *pagP* of *Y. pseudotuberculosis* (SI Appendix, Fig. S1). The first *Yp*-specific SNP is synonymous and occurs at base 15, whereas the second (A→G) is located at base 599 and results in a premature stop codon (W→*) that truncates the PagP peptide by three amino acids at the C-terminal end of the protein in all *Yp* isolates. These amino acids are not part of any known functional motif; however, the truncated version of the polypeptide is likely to be structurally destabilized or not inserted in the outer membrane. This SNP was present in all *Yp* isolates examined, including those that are considered ancestral, suggesting that the truncating *pagP* SNP may be a very early evolutionary feature of *Yp* as a species. These mutations were examined in the genomic data available in

GenBank (39) as of March 2, 2020, and found to present with the same distribution along species lines. Finally, one additional SNP was specific to *Yp* Angola (base 424 [T→G]) that results in an apparently functionally neutral amino acid (aa) change (aa 142, W→G).

Modeling Suggests that *Yp* SNP Results in Structurally Altered PagP Protein. The amino acid sequence of the *Yp* PagP protein differs from other *Yersinia* species by only three C-terminal amino acid residues: tryptophan (W), glutamine (Q), and phenylalanine (F). The truncation is apparently sufficient to eliminate PagP function in *Yp* isolates yet lies in the terminal region of the protein, suggesting that the remainder of the protein could be synthesized but nonfunctional. To understand potential structural differences between *Yp* and other *Yersinia* species PagP proteins, the amino acid sequences were three-dimensionally modeled using the fold recognition bioinformatic software I-TASSER (15). The predicted secondary and tertiary structures (Fig. 2) reveal a β-barrel structure consistent with previous reports of PagP

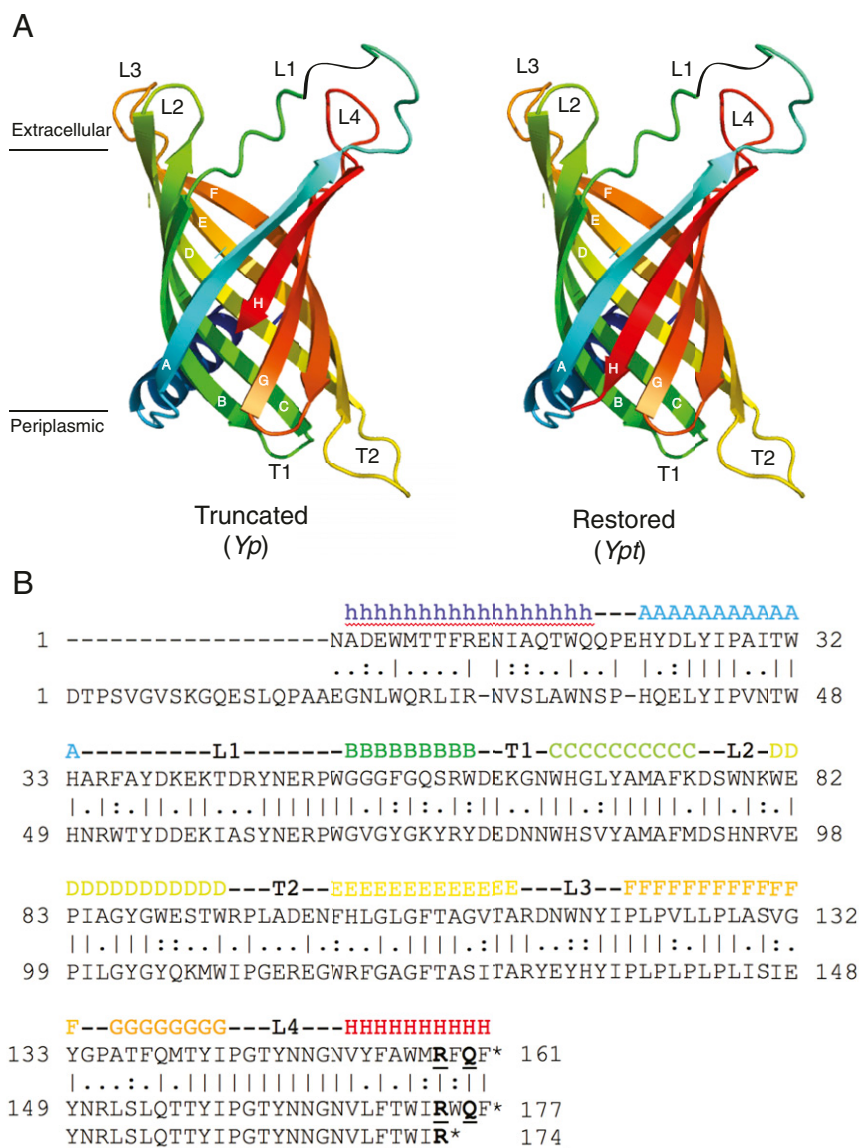


Fig. 2. Predicted tertiary and secondary structure of PagP homologs from *Y. pestis* and *Y. pseudotuberculosis*. (A) The molecular model is shown in A without the C-terminal three amino acid residues in β-strand H (Truncated-*Y. pestis*) and with these residues in place (Restored-*Y. pseudotuberculosis*). (B) The primary structure of *E. coli* PagP is presented with its known secondary structural elements indicated above and with the sequence of *Y. pseudotuberculosis* PagP aligned below.

structure (34). Importantly, the final strand of the β -barrel (strand H, red; Fig. 2A) is incomplete in the *Yp* model ("Truncated" PagP), yielding an incomplete and structurally altered protein. In the full-length, non-*Yp* PagP model ("Restored" PagP or Pag+), the final strand is complete and is able to form a complete β -barrel structure, allowing for folding of a functional enzyme. Additionally, the secondary structural elements of PagP were mapped onto the primary structure of *E. coli* PagP (Fig. 2B). The conserved penultimate glutamine residue (shown in boldface) is buried in the PagP interior while the flanking aromatic amino acid residues contribute to the continuous hydrophobic membrane surface potential. Without the three C-terminal residues, the new C terminus becomes the conserved interior arginine residue (shown in boldface). This arginine normally contacts several interior water molecules and the amide side chain of the penultimate glutamine residue. Therefore, the C-terminal three-amino-acid deletion exposes the arginine to the hydrophobic membrane surface, moves the α -carboxylate moiety from the membrane periphery to the membrane interior, and removes hydrogen-bonding interactions with three neighboring peptide bonds: two in β -strand A and one in β -strand G. These predictions suggest that a full-length protein is necessary for PagP function and that loss of function in *Yp* is due to structural alteration of the PagP protein that results from the SNP-induced early truncation.

Growth Temperature Impacts Lipid A Structures Isolated from WT *Yp*.

It has been previously demonstrated that growth temperature affects the overall structure of *Yp* lipid A (27, 29, 32). Lipid A isolated after growth at environmental temperatures results in the synthesis of a hexa-acylated structure consisting of a bisphosphorylated diglucosamine backbone with ester- and amide-linked 3-OH C14 fatty acids (29, 32). This base structure is further modified by the addition of acyloxyacyl-linked palmitoleic acid (C16:1) at the 2' position and lauric acid at the 3' position (Fig. 1). *Yp* lipid A isolated after growth at mammalian temperature is tetra-acylated, lacking these additional fatty acids (Fig. 1).

To determine the role of PagP in the biosynthesis of lipid A, we repaired the *pagP* SNP at base 599 to correct the *Yp* defect. The *Yp* BSL-2, nonselect agent KIM6 strain with a functional PagP enzyme was designated as *Yp*-KIM6-PagP+. To investigate temperature-specific lipid A remodeling in the presence and absence of PagP, WT *Yp*-KIM6 and *Yp*-KIM6-PagP+ were grown at mammalian (37 °C) and vector (26 °C) temperatures, and lipid A was isolated and analyzed by mass spectrometry (MS). After growth at 37 °C (Fig. 3C), we observed three major bisphosphoryl lipid A species in the *Yp*-KIM6-PagP+ strain: penta-acylated with one C16 fatty acid (*m/z* 1642) and hexa-acylated with two C16 fatty acids either without (*m/z* 1880) or with one aminoarabinose moiety (*m/z* 2011). Comparatively, WT *Yp*-KIM6 grown at 37 °C (Fig. 3A) contained lipid A species that lacked the C16 addition (higher *m/z* species *m/z* 1822 and *m/z* 1953 contain the C16:1 addition, which is mediated by LpxP, a lipid A palmitoleoyl transferase) (32). Unexpectedly, these data indicate that the functional *Yp pagP* gene encodes an enzyme capable of transferring two acyloxyacyl-linked palmitate chains at the 2' and 3' positions of lipid A.

PagP enzymatic activity was also observed in lipid A isolated from *Yp*-KIM6-PagP+ grown at 26 °C (Fig. 3D; *m/z* 1642, 1773, 1824, 1955, 2060, 2191, and 2322). The increased complexity in the MS spectra is consistent with the temperature-regulated activity of LpxL (HtrB) and LpxP that modifies the base tetra-acylated structure (*m/z* 1404, Fig. 3A) with a C12 or C16:1 fatty acid, respectively (40). Complete characterization of the 37 °C and 26 °C lipid A structures are presented as *SI Appendix, Methods, and Fig. S2*. Fig. 1 describes lipid A structures corresponding with the MS results in Fig. 3.

Gas Chromatography Demonstrates that *Yp*-KIM6-PagP+ Strain Lipid A Is Palmitoylated. To determine the effect of repairing the *pagP* gene in *Yp*-KIM6, lipid A from *Yp*-KIM6 and *Yp*-KIM6-PagP+

strains grown at 37 °C and 26 °C was extracted and subjected to gas chromatographic-flame ionizing detector (GC-FID) analysis. Individual fatty acids were assigned, and amounts of each fatty acid present on lipid A were calculated in reference to a pentadecanoic acid internal standard (Fig. 4). *Yp*-KIM6-PagP+ grown at 37 °C had a 5.7-fold increase in the percentage of lipid A C16 acylation, as compared to *Yp*-KIM6 grown at the same temperature (35.2% \pm 0.5 vs. 6.3% \pm 0.4 total fatty acids) (Fig. 4). *Y. pseudotuberculosis* and *Y. enterocolitica* displayed similar levels of palmitoylation as the *Yp*-KIM6-PagP+ (29). Basal levels of C16 in the 37 °C grown samples are possibly due to phospholipid contamination. A similar relative fold difference was observed when the bacteria were grown at 26 °C (4.1-fold increase in *Yp*-KIM6-PagP; 11.1% \pm 0.6 vs. 2.8% \pm 0.3 total fatty acids) (Fig. 4). These data show a significant ($P \leq 0.005$) increase in palmitoylation of lipid A in *Yp*-KIM6-PagP+ when grown at either temperature and provide evidence for a functional PagP enzyme in our *Yp* mutant strain.

***Yp* PagP Is a Palmitoyltransferase.** To confirm that PagP in the *Yp*-KIM6-PagP+ strain was the source of palmitoylation activity, membranes from *E. coli* expressing the *Yp* WT and PagP+ enzyme were isolated and assayed for palmitoyl transferase activity using the tetra-acylated lipid A precursor [4 - 32 P] lipid IV_A as an acceptor molecule. Briefly, *Yp* PagP proteins (WT and PagP+) and as a positive control, *E. coli* PagP, were expressed from the low copy vector pWSK29 in *E. coli* strain W3110 Δ *pagP*. Membranes isolated from *E. coli* Δ *pagP* expressing the WT *Yp pagP* gene did not result in any reaction products (Fig. 5, lane 4). As expected, complementation with *E. coli* PagP resulted in the production of a single, faster-migrating reaction product due to the addition of a single palmitate (C16:0) molecule (Fig. 5, lane 3). However, addition of membranes from *E. coli* expressing *Yp*-PagP+ resulted in two reaction products corresponding to the addition of two palmitate groups (Fig. 5, lane 5). The slower-migrating species is similar in size to the product from the *E. coli* PagP control and is interpreted as a single palmitoylation event. The second, faster-migrating reaction product is interpreted as a structure that has undergone dipalmitoylation, again confirming that the functional *pagP* gene encodes an enzyme capable of transferring two acyloxyacyl-linked palmitate chains.

Palmitoylated LPS Exhibits Greater Endotoxic Potential than Nonpalmitoylated

LPS. As the structure of the lipid A component of LPS is known to induce proinflammatory responses via the MD-2/TLR4 receptor complex, we hypothesized that the altered lipid A structure of the *Yp*-KIM6-PagP+ strain would display increased endotoxic potential. To demonstrate the endotoxic activity of the lipooligosaccharide (LOS) obtained from the *Yp*-KIM6 and *Yp*-KIM6-PagP+ strains, we measured cytokine production using promonocytic-vitamin D₃ differentiated human THP-1 and histiocytic-phorbol myristate-acetate differentiated U937 cells. The cytokines chosen were TNF- α and IL23p19, both driven through NF- κ B and RANTES, which are driven by a TRIF-TRAM response. As expected, LOS isolated from both *Yp*-KIM6 and *Yp*-KIM6-PagP+ cultures grown at 26 °C demonstrated high levels of endotoxic-like activity in THP-1 cells, which is attributed to the hexa-acylated lipid A structures present at this growth temperature. In contrast, LPS from *Yp*-KIM6-PagP+ grown at 37 °C was significantly more immunostimulatory than *Yp*-KIM6 grown at the same temperature for all cytokines measured (Fig. 6A–C). Similar results were shown using U937 cells (*SI Appendix, Fig. S3*).

To further demonstrate that human cells differentially respond to *Yp*-KIM6-PagP+ LOS, as compared to *Yp*-KIM6 LOS, we transiently transfected HEK-293 cells with human TLR4/MD2 followed by LPS stimulation. Results shown in Fig. 6D show a significant increase in NF- κ B gene-driven response to *Yp*-KIM6-PagP+ LPS grown at 37 °C when compared to *Yp*-KIM6

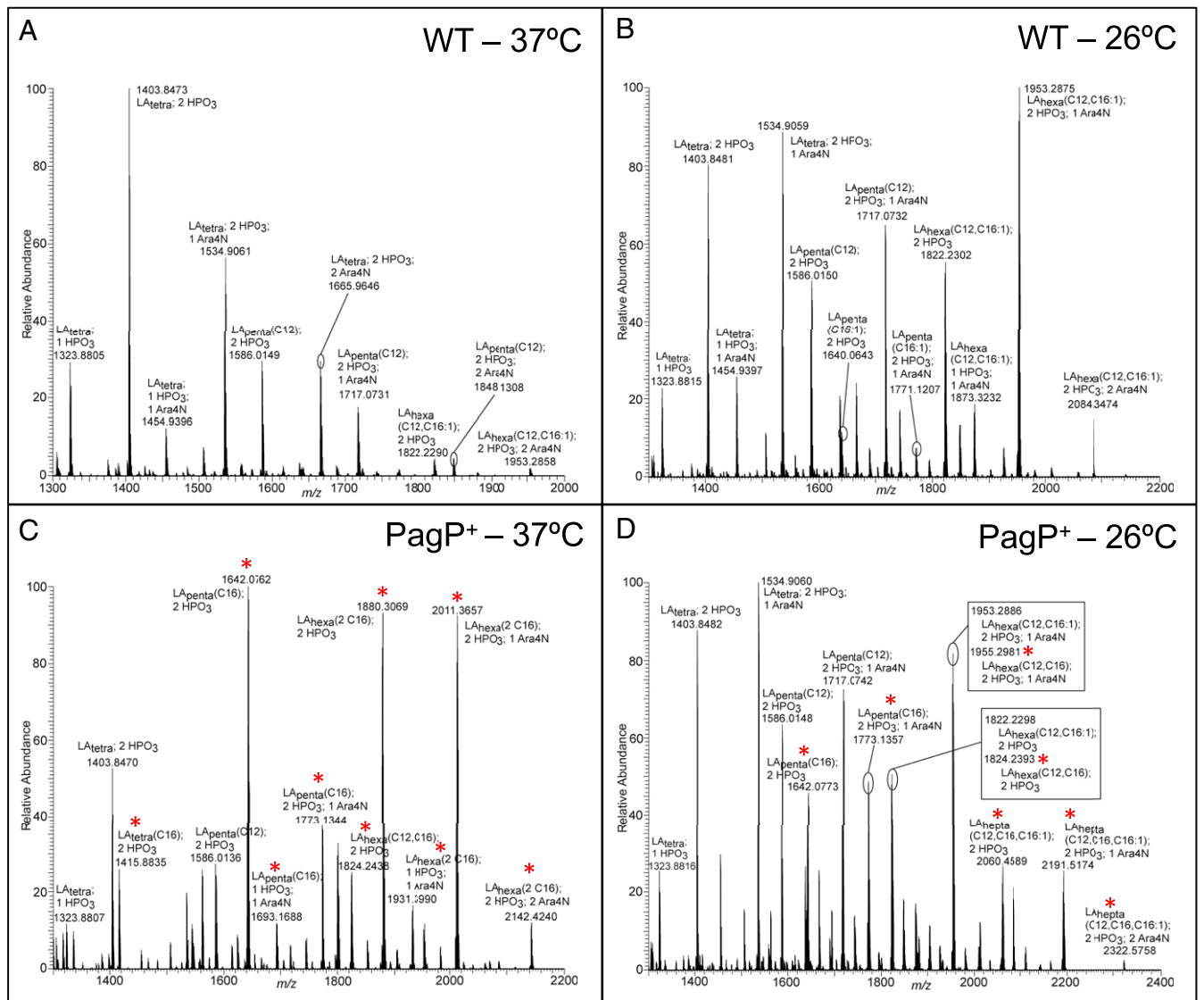


Fig. 3. MS analysis of PagP⁺ strains reveals lipid A palmitoylation. Negative ion mode ESI LQ-FT mass spectrum of lipid A from (A) *Yp*-WT at 37 °C, (B) *Yp*-WT-KIM6 at 26 °C, (C) *Yp*-KIM6-PagP⁺ at 37 °C, and (D) *Yp*-KIM6-PagP⁺ at 26 °C. All ions are singly deprotonated. Shorthand notation is as follows: Lipid A (LA); subscript indicates number of acyl chains; C12 indicates lauric acid; C16:1 indicates palmitoleic acid; HPO₃ indicates phosphate group; and Ara4N indicates aminoarabinose. Tetra-acylated *Yp* lipid A contains four primary 3-hydroxymyristic acid C14(3-OH) acyl chains and is identified as LA_{tetra}. *Yp* lipid A can also be penta-acylated ([LA_{penta}(C12)] or [LA_{penta}(C16:1)]) or hexa-acylated (LA_{hexa}[C12, C16:1]) with four primary C14(3-OH) acyl chains and secondary acyl chains of C12 and C16:1, respectively. *Yp* lipid A is predominantly diphosphorylated (2 HPO₃) and can be modified with the addition of one or two aminoarabinose (Ara4N) groups. In addition to the lipid A structures present in *Yp*-KIM6 at 37 °C, *Yp*-KIM6-PagP⁺ at 37 °C contain the addition of one or two palmitic acid (C16) acyl chains as secondary fatty acids, (LA_{penta}[C16], and LA_{hexa}[C12, C16], and LA_{hexa}[2 C16]), respectively, whereas *Yp*-KIM6-PagP⁺ at 26 °C contains the addition of a palmitic acid (C16) acyl chain as a secondary fatty acid, (LA_{penta}[C16], LA_{hexa}[C12, C16], LA_{hepta}[C12, C16, C16:1]), respectively. Red asterisks indicate palmitoylation. See *SI Appendix* for further details.

LPS grown at the same temperature. LPS grown at 26 °C showed similarly high levels of a NF-κB gene-driven response for both strains. The innate immune response of the LOS purified from *Y. pestis* wild-type (tetra-acylated) and PagP⁺ strains was also measured and compared to *E. coli* LPS in both mouse and human primary cells. Mouse ex vivo splenocytes from C57BL/6 and BALB/c backgrounds and human peripheral blood mononuclear cells from multiple healthy donors ($n = 3$) were evaluated for seven immune cytokine readouts (*SI Appendix*, Fig. S4). Results from these experiments support the conclusion that repairing the PagP enzyme in *Y. pestis* augments the immune response elicited by LOS. In summary, we demonstrate that mammalian temperature *Yp*-KIM6-PagP⁺ LPS can activate both the MyD88 and TRIF/TRAM arms of the TLR4-signaling pathway, which

supports the finding that the repaired strain results in production of LPS with higher immunostimulatory potential.

Discussion

Since its recent divergence from *Y. pseudotuberculosis* (*Ypt*) ~20,000 y ago, the *Y. pestis* (*Yp*) genome has undergone a significant number of gene inactivations, which likely played a prominent role in its pathogenic potential (27). In fact, as many as 13% of *Ypt* genes are no longer functional in *Yp*, whereas only 32 chromosomal genes and two virulence plasmids, important for pathogenesis, have been acquired since speciation (41, 42). Genes can become lost over time through genetic drift (43); however, genes may also remain inactivated if the loss allows for increased survival and propagation. We propose that *pagP* was under such selective pressures and that the lack of a

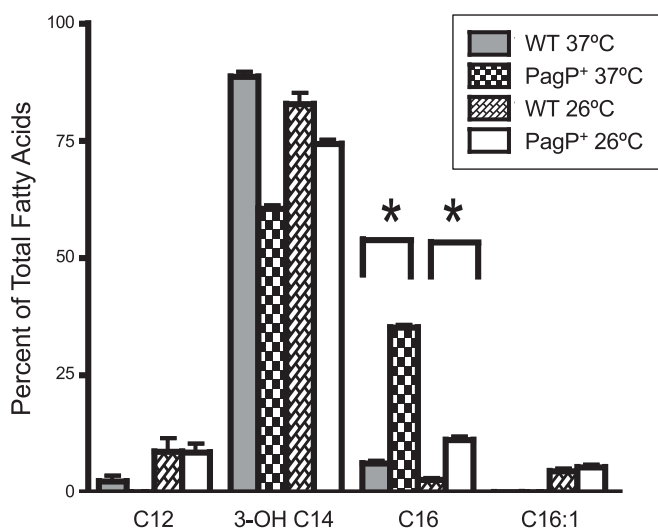


Fig. 4. Relative amounts of acyl groups (expressed as percentage of total fatty acid content) in lipid A from *Yersinia* strains grown at 37 °C or 26 °C. Data are from one representative experiment of three total experiments of $n = 3$. Error bars are SEM and represent the mean of triplicates; * $P \leq 0.005$.

functional PagP resulted in increased survival by evasion of the innate immune response, a critical step in pathogenesis.

PagP has been extensively studied because it is one of the few lipid A modification enzymes known to exist in the Gram-negative bacterial outer membrane (34, 44, 45). While the biological role of PagP has been studied in several bacterial species, the comparison of PagP homologs and the palmitoylation activities shown in this study provide interesting insights regarding the evolution in a particular organism. The *Yp pagP* gene sequence aligns closely with the *pagP* sequences from other Gram-negative bacteria, including the region coding the conserved active site His33 and Ser77 residues of *E. coli* PagP (46, 47). *Ypt* and *Yp pagP* homologs share 99% identity at the DNA level. They differ in the presence of a G-to-A transition mutation in *Yp* that converts the codon for Trp200 (TGG) into an amber stop codon (TAG), leading to a truncation of three carboxyl terminal amino acid residues. Interestingly, all *Yp pagP* sequences in the public domain contained the same SNP at position 599, suggesting that this mutation is advantageous and consistent across the *Yp* population from any of the past global pandemics.

Inspection of the *E. coli* PagP structure implicates the last three amino acids in the formation of hydrogen bonds that close the first and final β -strands into a β -barrel in the outer membrane (OM). These last three residues are expected to be particularly important in PagP because the β -bulge in the first β -strand changes the registration of hydrogen bonding with the final β -strand and causes sensitivity of the outer leaflet hydrogen bonds to the detergent environment (47). Consequently, the last three amino acids in the PagP sequence contribute two key hydrogen bonds in the inner-leaflet-exposed region that close the β -barrel structure. The absence of these three residues likely exposes the carboxyl-terminus and polar β -barrel interior region unfavorably to the phospholipid hydrocarbon chains in the OM inner leaflet. The thermodynamic cost of exposing charged amino acid residues to the membrane interior using the outer membrane β -barrel phospholipase OMP_{LA} as a model has been established (48). Although the penalty for exposing the α -carboxylate moiety to the membrane interior was not determined explicitly, it can be approximated from the penalty for exposing the side chain of aspartic acid to the membrane interior, which was measured to be 1.38 kcal/mol. The C-terminal arginine side chain was reported to

contribute 2.14 kcal/mol (48). It is well known that the per-residue free energy cost for disrupting peptide H-bonds in a membrane is about 4 kcal/mol (49). Without accounting for any stabilization contributed by the aromatic side chains of the penultimate tryptophan and C-terminal phenylalanine residues, the thermodynamic cost of deleting the C-terminal three amino acid residues from *Ypt* PagP (as is the case in *Yp* PagP), and of exposing the polar α -carboxylate and guanidinium moieties to the nonpolar membrane environment, is conservatively estimated to be about 16 kcal/mol. Additionally, PagP and many outer membrane proteins in general possess a carboxyl-terminal phenylalanine residue, which has been shown to be critical for correct OM assembly in studies of the PhoE porin (50, 51).

To demonstrate that the premature stop was responsible for gene inactivation, we repaired the SNP in the nonvirulent BSL-2 *Yp*-KIM6+ strain to that present in *Ypt* sequences. Lipid A isolated from the *Yp*-KIM6-PagP+ strain grown at 26 °C and 37 °C revealed the presence of palmitoylated lipid A at both temperatures (Fig. 2). Additionally, GC-FID results show a quantitative increase in palmitoylation of *Yp*-KIM6-PagP+ lipid A, as compared to *Yp*-KIM6, further demonstrating a functionally repaired PagP protein. Interestingly, the lipid A profiles at 37 °C contained spectra representing hexa-acylated lipid A with

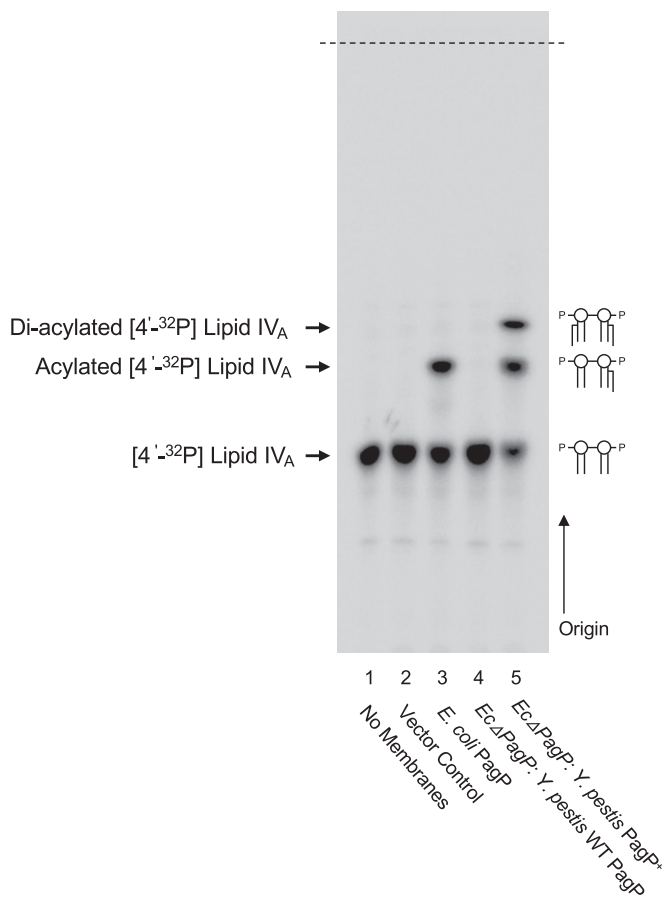


Fig. 5. In vitro assay of *Yp* PagP heterologous expression in *E. coli*. Both *Yp* WT (lane 4) and *Yp*-KIM6-PagP+ (lane 5) enzymes were expressed in *E. coli* strain W3110 Δ PagP, and *E. coli* membranes were isolated for the in vitro assay. The 32 P-labeled reaction products were separated by thin layer chromatography (TLC) and visualized by phosphorimaging analysis. Individual lipid A structures are shown next to their PagP enzymatic product with the "acylated" product having one and the "di-acylated" having two palmitates, respectively. WT *E. coli* W3110 with native *E. coli* PagP was also analyzed (lane 3). The solvent front is identified with the dashed black line.

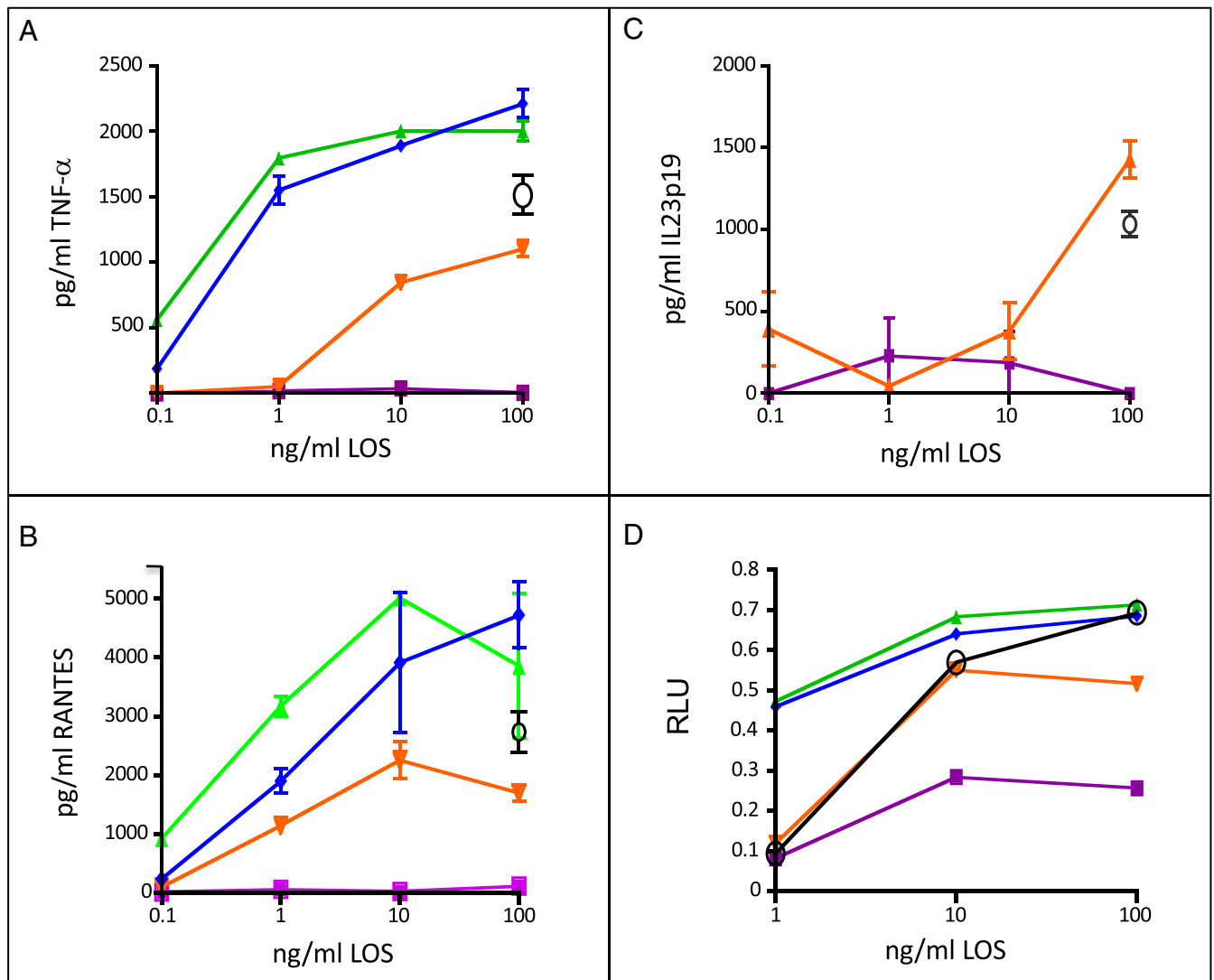


Fig. 6. Lipid A from *Yp*-KIM6-PagP+ shows significantly increased endotoxicity. Enzyme-linked immunosorbent assays were run for (A) TNF- α , (B) RANTES, and (C) IL-23p19 from supernatants of THP-1 cells stimulated with *Yp*-KIM6 and *Yp*-KIM6-PagP+ LPS both grown at 37 °C or 26 °C. *E. coli* O111:B4 LPS was used as a control. All stimulations were for 20 h. Data are from one representative experiment of three total experiments of $n = 3$. Error bars are SEM and represent the mean of triplicates. (D) Luminescence measurements read as relative light units (RLUs) from HEK293 cells transiently expressing human or murine TLR4/MD-2 stimulated with *Yp*-KIM6 and *Yp*-KIM6-PagP+ LPS grown at 37 °C or 26 °C. *E. coli* O111:B4 LPS was used as a control. All stimulations were for 4 h. Data are from one representative experiment of three total experiments of $n = 3$. Error bars are SEM and represent the mean of triplicates.

two palmitate chains. This indicates the addition of two palmitate moieties on the same lipid A molecule. Both *Ypt* and *Ye* have a single palmitate as their base structure is modified by the addition of acyloxyacyl-linked lauric acid (C12) at the 3' position. To confirm these results, we examined the detailed structure of doubly palmitoylated hexa-acylated lipid A using MS. The two palmitate residues were found attached to the hydroxyl group of *R*-3-hydroxymyristate chains at positions 2' and 3' (Fig. 1). The position of palmitoylation resembles the combination of each single palmitoylation in *E. coli*, *B. bronchiseptica*, and *P.*

aeruginosa, which occur at positions 2' and 3', respectively (29, 34). PagP enzymatic activity was confirmed by the membrane reconstitution experiment that demonstrated that two C16 fatty acids were added to tetra-acylated lipid A and suggests that the repaired enzyme is able to add two palmitate residues, as there are no other fatty acids to hinder their respective additions to the primary acyl chains at positions 2' and 3'.

Temperature-dependent lipid A modification has been proposed to be a key modulator in attenuation of *Yp* virulence and can alter its recognition by the host immune system (29). When the PagP

function is restored, the resulting lipid A becomes more proinflammatory due to enhanced TLR4-mediated signaling (Fig. 6). The LPS from *Yp-KIM6-PagP+* grown at 37 °C and utilized in a whole-cell stimulation assay shows a marked increase in both extracellular, MyD88-dependent, endosomal, and TRIF/TRAM-dependent signaling, providing evidence that hexa-acylated LPS produced at the mammalian temperature from *Yp-KIM6-PagP+* was more stimulatory than the predominantly tetra-acylated form from *Yp-KIM6*.

This study provides evidence that lipid A modifying enzymes can be used to engineer lipid A molecules that may have significant therapeutic use as potential vaccine adjuvants or antiseptics agonists. TLR4 ligands have now been described in the literature as having the ability to be used as adjuvants in multicomponent vaccines, suggesting decreased virulence via immune recognition (52–56). As LOS isolated from *Yp-KIM6-PagP+* can stimulate human cells through both the MyD88 and the TRIM/TRAF arms of the TLR4-signaling pathway, it may represent a candidate for vaccine adjuvant development.

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