**Bordetella parapertussis** PagP Mediates the Addition of Two Palmitates to the Lipopolysaccharide Lipid A

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**Bordetella bronchiseptica** PagP (PagP\(_{BB}\)) is a lipid A palmitoyl transferase that is required for resistance to antibody-dependent complement-mediated killing in a murine model of infection. *B. parapertussis* contains a putative pagP homolog (encoding *B. parapertussis* PagP [PagP\(_{BPa}\)]), but its role in the biosynthesis of lipid A, the membrane anchor of lipopolysaccharide (LPS), has not been investigated. Mass spectrometry analysis revealed that wild-type *B. parapertussis* lipid A consists of a heterogeneous mixture of lipid A structures, with penta- and hexa-acylated structures containing one and two palmitates, respectively. Through mutational analysis, we demonstrate that PagP\(_{BPa}\) is required for the modification of lipid A with palmitate. While PagP\(_{BB}\) transfers a single palmitate to the lipid A C-3' position, PagP\(_{BPa}\) transfers palmitates to the lipid A C-2 and C-3' positions. The addition of two palmitate acyl chains is unique to *B. parapertussis*. Mutation of PagP\(_{BPa}\) resulted in a mutant strain with increased sensitivity to antimicrobial peptide killing and decreased endotoxicity, as evidenced by reduced proinflammatory responses via Toll-like receptor 4 (TLR4) to the hypoacylated LPS. Therefore, PagP-mediated modification of lipid A regulates outer membrane function and may be a means to modify interactions between the bacterium and its human host during infection.

The genus *Bordetella* contains nine species, three of which have been studied in detail: *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*. *B. pertussis* and *B. parapertussis* are the causative agents of whooping cough. While long considered a disease of infants and children, it is now recognized that infections occur in adults, although they are milder than the classic whooping cough described in children (1–5). *B. pertussis* is considered the major pathogen of whooping cough; however, the prevalence of *B. parapertussis* in disease is not well understood but may be a significant contributor to the overall burden (6). Symptoms of *B. parapertussis* and *B. pertussis* disease are clinically indistinguishable and include a prolonged cough, whooping, paroxysms, vomiting, and cyanosis (7), leading to increased mortality in infected children; however, *B. parapertussis* is often considered to cause milder disease than *B. pertussis* (7). In many countries, whooping cough is controlled by vaccination against *B. pertussis*. However, many countries have recently suffered major outbreaks despite continuing high levels of vaccine coverage, initiating renewed interest in *B. parapertussis* vaccinology. The acellular pertussis vaccines (DTaP and Tdap) used in most developed countries do not result in protection against *B. parapertussis*, likely due to the *B. parapertussis* lipopolysaccharide (LPS) O antigen inhibiting the binding of antibodies to the bacterium (8).

LPS is the major component of the outer leaflet of the outer membrane of Gram-negative bacteria. The lipid A region imparts the endotoxin activity of LPS (9) that arises when lipid A binds to a host membrane complex that includes and activates signaling by Toll-like receptor 4 (TLR4) (10), resulting in the expression of many proinflammatory cytokines and chemokines in cells of the innate immune system. TLR4 signaling is also important for the activation of adaptive immunity, particularly through the activation of dendritic cells (DCs), which act as antigen-presenting cells (APCs) for T cells. Thus, lipid A-TLR4 interactions are central to the host immune response to Gram-negative bacteria, including *Bordetella* species.

**Bordetella** subspecies exhibit extensive heterogeneity in their lipid A structure, arising from both species-specific differences in and species-variable expression of specific lipid A-modifying enzymes (11, 12). Previously, we demonstrated that *B. bronchiseptica* pagP (pagP\(_{BB}\)) encodes a lipid A palmitoyl transferase that is responsible for the presence of palmitate in lipid A. The expression of pagP\(_{BB}\) is regulated by the Bvg two-component system (13) that regulates the expression of many *Bordetella* genes that are involved in pathogenesis (14). pagP\(_{BB}\) is maximally expressed in the Bvg+ (Bvg\(^{+}\)) phase that is adopted when the system is active and includes the expression of many of the toxins and adhesins considered important for infection. When the Bvg system is inactive, the bacteria adopt the Bvg- minus (Bvg\(^{-}\)) phase, and the expression of these virulence genes is downregulated. Thus, the Bvg+ and Bvg- phases are regarded as the states that the bacteria adopt upon entering the host respiratory tract and the environment, respectively (14). We also showed that pagP\(_{BB}\) is required for the persistence of *B. bronchiseptica* within the mouse respiratory tract through resistance to antibody-dependent complement-mediated killing (13, 15). This represented the first description of a...
direct role for the pagP gene in virulence for Bordetella. B. parapertussis also contains a pagP gene, but B. parapertussis lipid A contains two secondary palmitates, compared to a single palmitate residue in B. bronchiseptica (16). Here we present characterization of B. parapertussis pagP (pagP<sub>Bp</sub>), its role in lipid A biosynthesis, and the role of lipid A palmitoylation in B. parapertussis outer membrane function.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** *Escherichia coli* XL1-Blue (Agilent Technologies, Wokingham, United Kingdom) or Top10 (Invitrogen Life Technologies, Paisley, United Kingdom) cells were used for cloning and maintenance of plasmids. E. coli SM10<sub>apir</sub> (17) or CC118<sub>apir</sub> was used as the donor strain in conjugations. The pCR2.1 Topo or pCR8 Gateway entry vector (Invitrogen Life Technologies) was used to clone PCR products. pEX100T (18) was used as a suicide vector for allelic-exchange mutagenesis. The region encompassing pagP was cloned into the pCR8/GW/Topo cloning vector. The insert from either the putative tspc resistance cassette. pBBR1kan was used to introduce the pBBR1MCS is a broad-host-range vector that is capable of replication in *E. coli* (19). pBBR1kan was used to introduce a plasmid-borne pagP gene into the B. parapertussis wild type (WT) and the pagP mutant.

**Bacterial growth media and conditions.** B. parapertussis was grown on charcoal agar (BD, Oxford, United Kingdom) at 37°C. E. coli was grown on LB agar at 37°C. For liquid culture, B. parapertussis was grown in Stainer-Scholte broth (20) modified by the addition of Casamino Acids (50 g liter<sup>-1</sup>) at 37°C with shaking, and E. coli was grown in LB broth at 37°C with aeration. For growth of *B. parapertussis* in the Bvg<sup>+</sup> phase, MgSO<sub>4</sub> was added to growth medium to a concentration of 50 mM. Streptomycin (200 µg ml<sup>-1</sup>), ampicillin (100 µg ml<sup>-1</sup>), and kanamycin (50 µg ml<sup>-1</sup>) were used where appropriate.

**Chemicals and reagents.** Chemicals and reagents were obtained from BD, Fisher Scientific (Loughborough, United Kingdom), or Sigma-Aldrich (Gillingham, United Kingdom).

**DNA purification.** Plasmid DNA was purified by using an Invitrogen plasmid DNA purification kit according to the manufacturer’s instructions. Genomic DNA was purified by using the agarose plug method (21).

**DNA manipulation.** DNA manipulations were performed according to standard methods. DNA restriction and modifying enzymes were obtained from New England BioLabs (Hitchin, United Kingdom), Roche (Burgess Hill, United Kingdom), or Invitrogen Life Technologies.

**PCR and primers.** A genomic DNA template was made by resuspending plate-grown bacteria in 0.5 ml of water, boiling in a water bath for 5 min, spinning at top speed in a bench-top microcentrifuge for 2 min, and taking 0.2 ml of the supernatant. One microliter of supernatant was used per PCR. Each 50-µl PCR mixture was comprised of the genomic DNA template, buffer as directed by the manufacturer, deoxyribonucleoside triphosphates (dNTPs) (25 µM each), 20 ng of each primer, 5% (vol/vol) dimethyl sulfoxide (DMSO), 5 mM MgCl<sub>2</sub>, and 2.5 units of Taq polymerase (Promega, Southampton, United Kingdom). Primers used to amplify the *B. parapertussis* pagP locus were PagPF (5'-CTCCGGATCC AGGTAGGC-3') and PagPR (5’-TATGAACTCCAGCGGCGCAAC-3’). PCR mixtures were incubated at 94°C for 5 min followed by 30 cycles of 94°C for 75 s, 60°C for 75 s, and 72°C for 90 s, followed by a final step at 72°C for 7 min.

**Conjugations.** Conjugations were performed as described previously (22).

**Complementation of the pagP mutation.** The region encompassing the putative pagP promoter and the pagP coding sequence was amplified from either *B. parapertussis* or *B. bronchiseptica* by PCR, and the PCR product was cloned into the pCR8/GW/Topo cloning vector. The insert was subcloned into pBBR1kan that had been modified to a Gateway destination vector by using the Invitrogen Gateway cloning system. This construct was introduced into *B. parapertussis* by conjugation.

SDS-PAGE/silver stain analysis of LPS. Whole-cell-lysate LPS samples were prepared and analyzed by sodium dodecyl sulfate (SDS)-PAGE/silver staining, as previously described (23).

**Peptide killing assays.** Bacteria were grown on agar plates for 3 days and then resuspended in phosphate-buffered saline (PBS). The suspensions were diluted to 10<sup>6</sup> bacteria/ml. Ten microliters of PBS containing C18G at a final concentration of 0, 1, 2.5, 5, or 10 µg ml<sup>-1</sup> was added to each suspension, which was then incubated at 37°C for 30 min. Each peptide concentration was tested in triplicate. Suspensions were serially diluted in PBS, and 100 µl of dilutions was plated onto agar. Plates were incubated at 37°C for 3 days, and colonies were counted to enumerate the bacteria that had survived the assay. Differences in the numbers of surviving WT and mutant bacteria were compared by using a Student t test. The experiment was performed three times.

**TLR4 stimulation assays.** Reporter cell lines and stimulation assays were performed as described previously (20).

**Lipid A structural analyses.** (i) Matrix-assisted laser desorption ionization–time of flight/time of flight mass spectrometry. LPS was purified from 1 liter of cultures grown overnight, as described previously (24). Further treatment of LPS with RNase A, DNase I, and proteinase K ensured the removal of contaminating nucleic acids and proteins (25). Hydrolysis of LPS to isolate lipid A was accomplished with 1% SDS at pH 4.5, as described previously (26).

Lipid A was analyzed by matrix-assisted laser desorption ionization (MALDI) in the negative-ion mode on a 4700 proteomics analyzer (Applied Biosystems, Framingham, MA). Samples were dissolved in 10 µl of a mixture of 5-chloro-2-mercaptobenzothiazole (20 mg ml<sup>-1</sup>) in chloroform-methanol-water (4:4:1, vol/vol/vol), and 0.5 µl of the sample was analyzed by MALDI-time of flight/time of flight mass spectrometry (TOF/TOF MS). Both MS and tandem MS (MS/MS) data were acquired in the reflectron mode with a neodymium-doped yttrium aluminum garnet (Nd:YAG) laser with a 200-Hz repetition rate, and up to 4,000 shots were accumulated for each spectrum. The precursor isolation window was set to ±5 Da. Tandem MS spectra were acquired with collision energies of 1 keV, and air was used as the collision gas. Instrument calibration and all other tuning parameters were optimized by using HP Calmig (Sigma-Aldrich, St. Louis, MO). Data were acquired and processed by using Data Explorer (Applied Biosystems, Framingham, MA).

(ii) Electrospray ionization linear ion trap Fourier transform ion cyclotron resonance mass spectrometry. Lipid A was analyzed by electrospray ionization (ESI) in the negative-ion mode on a linear trap quadrupole Fourier transform (LTQ-FT) mass spectrometer (Thermo Fisher, San Jose, CA). Samples were diluted to ~0.5 to 1.0 mg/ml in chloroform-methanol (1:1) and infused at a rate of 1.0 µl/min via a fused silica capillary (75-µm internal diameter [i.d.]; 360-µm outside diameter [o.d.]) with an ~30-µm spray tip (New Objective, Woburn, MA). Instrument calibration and tuning parameters were optimized by using a solution of Ultramark 1621 (Lancaster Pharmaceuticals, PA). For experiments acquired in the ion cyclotron resonance (ICR) cell, resolution was set to 100K, and ion populations were held constant by automatic gain control at 1.0 × 10<sup>5</sup> and 5.0 × 10<sup>5</sup> for MS and MS/MS, respectively. For tandem mass spectra, the precursor ion selection window was set to 5 Da, and the collision energy was set to 30% on the instrument scale. Data from the collision-induced dissociation (CID) MS<sup>n</sup> analysis (where n represents the number of times an ion was subjected to MS) in the linear ion trap were acquired with an ion population of 1.0 × 10<sup>4</sup> and a maximum fill time of 200 ms. The subsequent MS<sup>2</sup> and MS<sup>3</sup> events had an isolation window of 4 Da with a collision energy of 25%. All spectra were acquired over a time period of 1 min and averaged. MS and MS<sup>2</sup> events were mass analyzed in the ICR cell, and MS<sup>3</sup> and MS<sup>4</sup> events were mass analyzed in the LTQ instrument. Data were acquired and processed by using Xcalibur, version 1.4 (Thermo Fisher), utilizing 7-point Gaussian smoothing.
RESULTS
Identification of pagP
pagP was identified by genome sequence analysis using the PagP
sequence (Swiss-Prot database accession number Q7WFT9.1). The B. parapertussis and B. pertussis genes were found to be nearly identical at the DNA level in both the promoter and coding sequence. A single G:A base change in the middle position of the 24th codon was observed, which resulted in an aspartate in PagPBPa, compared to a glycine in PagPBB. To investigate the role of pagP in overall LPS biosynthesis in B. parapertussis, a B. parapertussis pagP mutant was constructed and analyzed by using SDS-PAGE to determine the LPS profile compared to that of the WT (Fig. 1).

In these analyses, two main LPS types are visible: lipid A-core LPS and lipid A-core that is further substituted with O polysaccharide (O PS). The WT LPS profile differed between the Bvg+ and Bvg− phases. In the plus phase, core-lipid A LPS migrated as a diffuse smear, indicative of a heterogeneous nature of LPS structures being present in this region. The O PS-containing LPS separated as a series of bands, demonstrating that LPS molecules that differ in the number of O PS repeats were well separated (Fig. 1, lane 1). In the Bvg− phase, the O PS migrated as a compacted series of bands due to the action of the Bvg−-phase enzyme WbmE, which deaminates some of the O PS uronamide sugars (27). In addition, the core-lipid A LPS was present as a single intense band and just one or two other very faint bands, suggesting that a restricted number of core-lipid A structures were expressed in the minus phase compared to the plus phase (Fig. 1, lane 2).

The LPS profile of the pagP
mutant differed from that of the WT in the core-lipid A region. In the Bvg+ phase, the mutant profile comprised a doublet with near-equal-intensity bands, whereas in the minus phase, a single band was observed (Fig. 1, lanes 3 and 4). This suggests that PagP
expression is required for some of the core-lipid A structures observed in the WT and that Bvg activity affects the expression of PagP-dependent LPS structures. Importantly, these analyses demonstrate that mutant structures are incorporated into mature LPS and are likely incorporated into the outer membrane. This is important for interpreting the phenotype of the pagP
mutant (see below).

To confirm the role of pagP
in LPS biosynthesis, the pagP mutation was complemented in trans by expressing pagP from a plasmid using the native pagP promoter. The LPS profile of the complemented mutant in the Bvg+ phase comprised three well-resolved core-lipid A bands (Fig. 1, lane 6). This is different from the WT profile. We hypothesized that the multicopy nature of carrying the complementing gene on a plasmid was responsible for this difference. Thus, the complemented mutant profile was compared to that of the WT carrying the plasmid copy of pagP (Fig. 1, lane 5) and found to be the same. These data support the conclusion that the loss of core-lipid A bands from the profile of the pagP
mutant was due to a mutation of pagP (see also below).

Mass spectrometric analysis of lipid A isolated from WT B. parapertussis LPS. To define the specific structural differences between WT and mutant LPS structures, lipid A was purified from both strains and analyzed by mass spectrometry (MS). MALDI TOF/TOF and ESI LTQ-FT MS platforms were used to gain comprehensive structure determination. The negative-ion-mode MALDI-TOF mass spectrum of lipid A isolated from WT B. parapertussis LPS is shown in Fig. 2A. The most abundant ion, at m/z 1,332 (Fig. 2A), corresponded to a singly deprotonated lipid A structure that contained two phosphate groups and four acyl chains (i.e., diphasphoryl tetra-acylated lipid A). Two other prominent singly charged ions were recorded at m/z values of 1,570 (diphasphoryl penta-acylated lipid A) and 1,808 (diphasphoryl hexa-acylated lipid A). The ions at m/z 1,570 and 1,808 differed from the ion at m/z 1,332 by the addition of one and two secondary palmitate (C16) acyl chains, respectively. The proposed lipid A structures for the ions at m/z 1,332, 1,570, and 1,808 are displayed in Fig. 2. Structure determination was based on the tandem mass spectrometry experiments discussed in detail below. In addition to the m/z values mentioned above, a number of other m/z values were observed, which demonstrated the extensive heterogeneity of lipid A structures present in lipid A isolated from WT B. parapertussis LPS (Fig. 2A). These m/z values corresponded to differing phosphorylation and acylation patterns (see Table S1 in the supplemental material for proposed structures of these additional ions).

Mass spectrometric analysis of lipid A isolated from B. parapertussis pagP mutant LPS. The negative-ion-mode MALDI-TOF mass spectrum of lipid A isolated from B. parapertussis pagP mutant LPS is shown in Fig. 2B. This lipid A also displayed the most abundant ion, at m/z 1,332 (Fig. 2B), which corresponded to a singly deprotonated diphasphoryl tetra-acylated lipid A structure. Of particular note, ions at m/z 1,570 and 1,808, corresponding to the addition of one and two secondary C16 acyl chains, respectively, were not present in mutant lipid A. All m/z values for mutant lipid A were also observed for lipid A from WT LPS and corresponded to identical structures (see Table S1 in the supplemental material).

Determination of acyl chain positioning in lipid A isolated from WT and B. parapertussis pagP mutant LPS using tandem mass spectrometry. In order to confidently assign structures to specific m/z values, a series of tandem MS experiments using a MALDI-TOF/TOF mass spectrometer were performed. In addition, multistage tandem MS (MS”) experiments were performed on an ESI LTQ-FT MS platform to further validate structure assignment. The gas-phase dissociation of the precursor ion at m/z 1,332 determined via the MALDI-TOF/TOF platform revealed structure-specific, diagnostic product ions that allowed confident assignment of the four acyl chains along the lipid A glucosamine disaccharide (Fig. 3A). Gas-phase dissociation for structure determination of lipid A via tandem mass spectrometry, whereby structure-specific, diagnostic product ions are used for structure determination, was reported previously (12, 28). Of note, the collective
Analysis of several cross-ring, glycosidic, and competitive neutral losses of 1° acyl chains yielded a consistent pattern where the four acyl chains were confidently assigned to the following positions: amide-linked C14(3-OH) at the C-2 and C-2' positions, ester-linked C10(3-OH) at the C-3 position, and a secondary C14 ester linked via the 3-hydroxy position of the C-2 acyl chain (Fig. 2, structure at m/z 1,332). For example, the product ion at m/z 948.5 corresponded to a 0,2-2A2 cross-ring fragment that localized the 1° C10(3-OH) and C14(3-OH) acyl chains and the 2° C14 acyl chain to the distal (nonreducing) end of the disaccharide. Cross-ring and glycosidic product ions were named according to the nomenclature outlined by Domon and Costello (29), with the slight modification of designating the loss of neutral acyl chains if applicable. Next, the cross-ring product ion at m/z 796.4 [0,2A2-C10(3-OH)ket], presumably a consecutive dissociation from the ion at m/z 948.5, corresponded to the neutral loss of the 1° C10(3-OH) as a ketene from the 0,2A2 ion. These product ions determined that the C-3' position is occupied by the 1° C10(3-OH) acyl chain.
chain and that the C-2' position contained the 1° C14(3-OH) and 2° C14 acyl chains. In addition, the glycosidic product ion at m/z 484.2 (Y1) squarely indicated that the reducing end of the lipid A disaccharide contained a 1° C14 at the C-2 position. Further confirmation of the structure assignment for the ion at m/z 1,332 was demonstrated via the use of tandem mass spectrometry using an ESI LTQ-FT mass spectrometer, where MS^n experiments were performed (see Fig. S1 in the supplemental material). The precursor ions at m/z 1,332 from both the WT and the pagP mutant had identical structures.

Structure assignment for the precursor ion at m/z 1,570 corresponded to a diphosphoryl penta-acylated lipid A with acyl chain assignment as follows: amide-linked C14(3-OH) at the C-2 and C-2' positions, ester-linked C10(3-OH) at the C-3' position, secondary C14 ester linked via the 3-hydroxy position of the C-2 acyl chain, and one secondary C16 ester linked via the 3-hydroxy position of the C-2 or the C-3' acyl chain (Fig. 2, structure at m/z 1,570). As described above for the precursor ion at m/z 1,332, diagnostic product ions which included cross-ring, glycosidic, and competitive neutral losses of acyl chains were identified and used to

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**FIG 3** Negative-ion-mode MALDI-TOF/TOF mass spectra for precursor ions at m/z 1,332 (A), m/z 1,570 (B), and m/z 1,808 (C) isolated from WT B. parapertussis lipid A. Proposed lipid A structures for each precursor ion are shown in each spectrum, with dashed lines representing possible cleavage sites. Diagnostic cross-ring and glycosidic product ions provided unambiguous structure assignment and were named according to the nomenclature outlined by Domon and Costello (29), with the slight modification of designating the number of phosphate groups present and the loss of neutral acyl chains if applicable. All precursor ions were diphosphorylated with a heterogeneous mixture of bisphosphate and pyrophosphate. Only the bisphosphate configuration is displayed. C16, palmitic acid; C10(3-OH), 3-hydroxy capric acid; C10(3-OH)ket, neutral loss as a ketene; HPO_4, phosphate; H_3PO_4, phosphoric acid; [H_3P_2O_7]^- and [HP_2O_6]^-, pyrophosphate product ions; [H_2PO_4^-]^- and [PO_3]^-, phosphate product ions.
confidently assign the structure of the ion at m/z 1,570 (see Fig. 3B for identities and m/z values of several diagnostic product ions). Of particular note, the 2° C₁₆ acyl chain appeared to be localized to the reducing end (C-2 position), yet conclusive evidence for the exclusive positioning of the C₁₆ acyl chain was not obtained via MALDI-TOF/TOF experiments. Further experiments using an ESI LTQ-FT mass spectrometer capable of higher-order MSⁿ revealed that the 2° C₁₆ acyl chain was localized to either the C-2 or C-3’ position (see Fig. S2 in the supplemental material). Given the inherent difficulty of determining even relative amounts of isomeric structures during ESI mass spectrometry experiments, determination of the abundance of structures having a secondary C₁₆ at either the C-2 position or the C-3’ position was not attempted.

Structure assignment for the precursor ion at m/z 1,808 corresponded to a diphasphoryl hexa-acylated lipid A with acyl chain assignment as follows: amide-linked C₁₄(3-OH) at the C-2 and C-2’ positions, ester-linked C₁₀(3-OH) at the C-3’ position, secondary C₁₄ ester linked via the 3-hydroxy position of the C-2 acyl chain, and two secondary C₁₆ acyl chains ester linked via the 3-hydroxy position of the C-2 and C-3’ acyl chains (Fig. 2, structure at m/z 1,808). Evidence and reasoning analogous to those for the precursor ions at m/z 1,332 and 1,570 were used to determine the structure of this ion (Fig. 3C; see also Fig. S3 in the supplemental material). In effect, diagnostic product ions were identified to confidently assign the positions of the two 2° C₁₆ acyl chains.

These data demonstrate that *B. parapertussis* lipid A contains molecules with either one or two palmitates and that the presence of palmitate is dependent on pagP. In turn, this strongly suggests that *B. parapertussis* PagP transfers two palmitate moieties to lipid A. This is different from *B. bronchiseptica* PagP, which transfers only a single palmitate. It was possible that the two enzymes have different transferase abilities, presumably arising from the single-amino-acid difference between the two enzymes, or that the different lipid A substrates encountered by the enzymes in the two hosts direct different transferase activities. To investigate this, PagP_BB was expressed in the *B. parapertussis* pagP mutant. The *B. bronchiseptica* gene was carried on a plasmid and expressed from its natural promoter (the promoter regions of *B. bronchiseptica* and *B. parapertussis* are identical). This construct was previously used to complement a *B. bronchiseptica* pagP mutation (13). Complementation of the *B. parapertussis* pagP mutation by pagP_BB resulted in a lipid A-core profile identical to that produced by complementation with PagP_BB (Fig. 1, lanes 7 and 8), suggesting that both the *B. parapertussis* and *B. bronchiseptica* enzymes have the same activity in the *B. parapertussis* background. To confirm this, lipid A was purified from both complemented strains and analyzed by MS (Fig. 4). For both lipid A preparations, ions that corresponded to molecules containing either a single or two palmitate additions were detected, demonstrating that PagP_BB can add two palmitates to *B. parapertussis* lipid A. Thus, both PagP_BB and PagP_BB and have dual functionality, in that they can add palmitate to two different sites in lipid A. PagP_BB does this in *B. parapertussis* but not in *B. bronchiseptica*, and thus, we propose that the dual functionality is directed by the *B. parapertussis* lipid A acceptor substrate.

**pagP_BB** is required for WT levels of resistance to C₁₈G. The acylation pattern of lipid A is an important determinant of the integrity of the outer membrane of Gram-negative bacteria. In turn, this is important for a range of functions, including resisting antimicrobial molecules produced by hosts during infection. We hypothesized that PagP_BB contributes to the ability of the bacterium to resist host antimicrobial peptides, as observed for other bacterial species. To test this, we measured the level of killing of the WT and the *B. parapertussis* pagP mutant after exposure to C₁₈G, a synthetic α-helical antimicrobial peptide (30). Approximately 1 × 10⁴ CFU of the WT or mutant were incubated with the peptide (0 to 10 μg ml⁻¹) for 30 min at 37°C, and the number of surviving bacteria was enumerated. The results from a representative experiment are shown in Fig. 5. At each concentration of peptide, a significantly greater number of mutant bacteria than WT bacteria were killed. Overall, our data demonstrate that the WT level of resistance to C₁₈G-mediated killing is dependent on pagP_BB.

**TLR4 stimulation assays.** A key activity of lipid A during infection is stimulation of TLR4-mediated signaling. The acylation pattern of lipid A is a key determinant of this activity. The relative potencies for stimulation of TLR4-mediated signaling of lipid A isolated from the WT and pagP mutant strains were measured. A recombinant reporter system was used (20), expressing either human or murine TLR4 signaling complexes, as these two systems recognize and respond to different lipid A structures differently. Unstimulated cells acted as a negative control. Stimulation with a known agonist of NF-κB-mediated signaling, interleukin-1 (IL-1), resulted in relative light unit (RLU) values of 0.54 ± 0.03 and...
Stimulation with an *E. coli* lipid A (1,000 ng/ml) biosynthetic intermediate, lipid IVA, which is a strong agonist of murine but not of human TLR4 signaling, resulted in RLU values of 0.36 ± 0.03 and 0.05 ± 0.00 for murine and human TLR4-mediated signaling, respectively, and demonstrated that the experimental system could detect species-specific stimulation.

*B. parapertussis* WT lipid A was a more potent stimulant of murine than of human TLR4-mediated signaling (Fig. 6). *pagP* mutant lipid A was significantly less potent than the WT for stimulation of both murine (Fig. 6A) and human (Fig. 6B) TLR4-mediated signaling but was particularly attenuated for the human-derived system. These data demonstrate that PagPBPa-mediated modification of *B. parapertussis* lipid A significantly alters the endotoxin activity of lipid A toward the human TLR4 signaling complex.

**DISCUSSION**

Previously, we characterized *B. bronchiseptica* PagP as a Bvg-regulated lipid A palmitoyl transferase and determined that PagP-mediated palmitoylation was required for resistance to antibody-dependent complement-mediated killing (13,15). Here we show that *B. parapertussis* PagP is also a Bvg-regulated lipid A palmitoyl transferase but one that is able to transfer two palmitates to lipid A, a novel finding. When expressed in *B. parapertussis*, PagPBB also mediates the addition of two palmitates to lipid A. We propose that the lipid A acceptor substrate of *B. parapertussis* directs this dual functionality.

Interestingly, the MS analyses presented here demonstrated
considerable lipid A heterogeneity in addition to palmitoylation. Variability in the presence of C10(3-OH) was observed (Fig. 2). Ions at m/z 1,162 and 1,400 corresponded to derivatives of the major tetra- and penta-acylated molecules lacking C10(3-OH), whereas the ion at m/z 1,502 corresponded to a derivative of the tetra-acylated molecule that contained an additional C10(3-OH), presumably at the C-3 position as a result of incomplete deacylation by PagL (12). Furthermore, each of the peaks corresponding to the tetra-, penta-, and hexa-acylated species was surrounded by a series of peaks, each differing from the next by 14 mass units (Fig. 2), corresponding to a series of structures, each differing by one methylene (CH2) unit. This suggests that B. parapertussis incorporates a range of different acyl chains into its lipid A, including those containing an odd number of carbon atoms. Bordetella LpxA has relaxed acyl chain specificity that may contribute to this heterogeneity (31). In turn, this suggests that B. parapertussis lipid A contains not only three major molecular species but also numerous acylation variants of these major species. A full structural characterization of this heterogeneity is under way.

PagP-mediated palmitoylation results in an altered potency of B. parapertussis lipid A for stimulating signaling via TLR4. WT lipid A was more proinflammatory than pagP mutant lipid A, particularly via the human-derived signaling complex. When considering the basis for the effect of palmitoylation on lipid A potency, it is important to consider that the individual lipid A preparations are comprised of a mixture of lipid A molecules and that the overall potency is the sum of their individual potencies, which may include some antagonistic activities. Either way, the Bvg-regulated expression of pagP_bpa appears to confer on B. parapertussis a mechanism by which to regulate the potency of its lipid A for TLR4 stimulation. The Bvg+ phase, in which PagP_bpa appears to be maximally expressed, is thought to be that adopted by the bacteria during infection. In this case, our data suggest that both singly and doubly palmitoylated lipid A will be synthesized and that lipid A is at its most potent, compared to the Bvg-minus phase. However, B. parapertussis has low-potency lipid A compared to that of B. bronchiseptica, and it has been demonstrated in mice that B. parapertussis avoids invoking host immune responses through the lack of stimulation of TLR4-mediated signaling (33). This suggests that Bvg-mediated regulation of lipid A potency does not play a role during infection. Clearly, the in vitro system used here does not reveal the precise immune response stimulated by either palmitoylated or non-palmitoylated lipid A during infection, and thus, the consequences of regulating the level of lipid A palmitoylation are not clear. In addition, our data demonstrate that PagP activity regulates outer membrane function in other ways, such as resistance to host antimicrobial peptides, and that the bacterium may have to balance increased lipid A potency for TLR4 stimulation versus inherent resistance to immune effector molecules. In support of this, B. bronchiseptica pagP is required for persistent colonization of the mouse respiratory tract (13). However, a pagP_bsa mutant appears to colonize similarly to the WT during the early course of infection, with attenuation manifesting only between days 3 and 7 postinoculation, perhaps suggesting that there is little difference in early events when innate immunity resulting from TLR4 stimulation is operational. Furthermore, we demonstrated that pagP_bsa is required for resistance to antibody-dependent complement-mediated lysis (15), suggesting that the major role of pagP in B. bronchiseptica is to contribute to the integrity of the outer membrane.

It is also of interest to consider the difference in lipid A biosynthetic enzymes between Bordetella subspecies, B. pertussis, B. bronchiseptica, and B. parapertussis share a very similar set of lipid A biosynthesis and modification genes, but they each synthesize species-specific lipid A’s (34). Some genes are mutated in one species (e.g., pagP in B. pertussis (13)), while subtle allelic differences result in differences in activity among species, e.g., lpxA (31). Here our data suggest that slight alterations in the lipid A structure can direct different activities of enzymes common to more than one species. Presumably, the evolution of these three species in their different niches and different infection biology has selected for these subtly different combinations of lipid A biosynthesis activities. Thus, while the regulated expression of Bordetella pagP suggests that B. parapertussis may modulate its lipid A structure during infection, the extent to which this happens and the consequences of it require analyses in vivo, with consideration of the different potencies of stimulation of B. parapertussis lipid A toward TLR4 from different hosts.

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REFERENCES


