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Pasteurella multocida Expresses Two Lipopolysaccharide Glycoforms Simultaneously, but Only a Single Form Is Required for Virulence: Identification of Two Acceptor-Specific Heptosyl I Transferases▽

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Lipopolysaccharide (LPS) is a critical virulence determinant in *Pasteurella multocida* and a major antigen responsible for host protective immunity. In other mucosal pathogens, variation in LPS or lipooligosaccharide structure typically occurs in the outer core oligosaccharide regions due to phase variation. *P. multocida* elaborates a conserved oligosaccharide extension attached to two different, simultaneously expressed inner core structures, one containing a single phosphorylated 3-deoxy-D-manno-octulosonic acid (Kdo) residue and the other containing two Kdo residues. We demonstrate that two heptosyltransferases, HptA and HptB, add the first heptose molecule to the Kdo_1 residue and that each exclusively recognizes different acceptor molecules. HptA is specific for the glycoform containing a single, phosphorylated Kdo residue (glycoform A), while HptB is specific for the glycoform containing two Kdo residues (glycoform B). In addition, KdkA was identified as a Kdo kinase, required for phosphorylation of the first Kdo molecule. Importantly, virulence data obtained from infected chickens showed that while wild-type *P. multocida* expresses both LPS glycoforms in vivo, bacterial mutants that produced only glycoform B were fully virulent, demonstrating for the first time that expression of a single LPS form is sufficient for *P. multocida* survival in vivo. We conclude that the ability of *P. multocida* to elaborate alternative inner core LPS structures is due to the simultaneous expression of two different heptosyltransferases that add the first heptose residue to the nascent LPS molecule and to the expression of both a bifunctional Kdo transferase and a Kdo kinase, which results in the initial assembly of two inner core structures.

Pasteurella multocida is a gram-negative bacterial pathogen that causes disease in a wide range of mammals and birds. *P. multocida* is classified into serogroup A, B, D, E, or F based on capsular composition and is further classified into 16 Heddleston serotypes based on lipopolysaccharide (LPS) antigens. The type of disease that each strain can cause correlates to some degree with the type of capsule that is expressed, with serogroup A and occasionally serogroup F strains associated with fowl cholera outbreaks in birds, serogroup B and E strains with bovine hemorrhagic septicemia, and toxigenic serogroup D strains with atrophic rhinitis in pigs (5).

The expression of wild-type LPS is critical for the progression of fowl cholera (13). The structure of the LPS has been determined for two serotype A:1 fowl cholera strains, VP161 and X73, and the genome-sequenced strain Pm70 (28–30). The LPS expressed by *P. multocida* is similar to the LPS (often designated lipooligosaccharide) expressed by other mucosal pathogens, including species within the *Neisseria* and *Haemophilus* genera that have mono- and oligosaccharide extensions to the core structure but lack O-antigen polysaccharide repeating units (9, 10, 22, 26, 30). The LPS structures expressed by the three *P. multocida* strains VP161, X73, and Pm70 all share common regions but differ in their oligosaccharide extension and side branches (28–30). Unusually, two LPS glycoforms were expressed simultaneously by each strain, which differed in the inner core region but retained the same primary oligosaccharide extension (shown for VP161 in Fig. 1). Semiquantitative analysis of the LPS expressed by each strain showed that most glycoforms contained a single phosphorylated 3-deoxy-D-manno-octulosonic acid (Kdo) molecule that was frequently substituted with a phosphoethanolamine (PEtn) residue attached to the phosphate group (glycoform A). Furthermore, this single Kdo glycoform was shown to have a second glucose residue attached to the proximal heptose (Fig. 1). The alternative inner core LPS structure contained two unphosphorylated Kdo residues and lacked the second glucose residue on the proximal heptose (glycoform B).

To determine which genes were involved in the biosynthesis of each glycoform, we mutated two genes predicted to encode heptosyltransferases that attach the first heptose residues to Kdo and a gene predicted to encode a Kdo kinase required for phosphorylation of the lipid A-Kdo_1. The LPS structures expressed by these mutants were then elucidated, and the mu-

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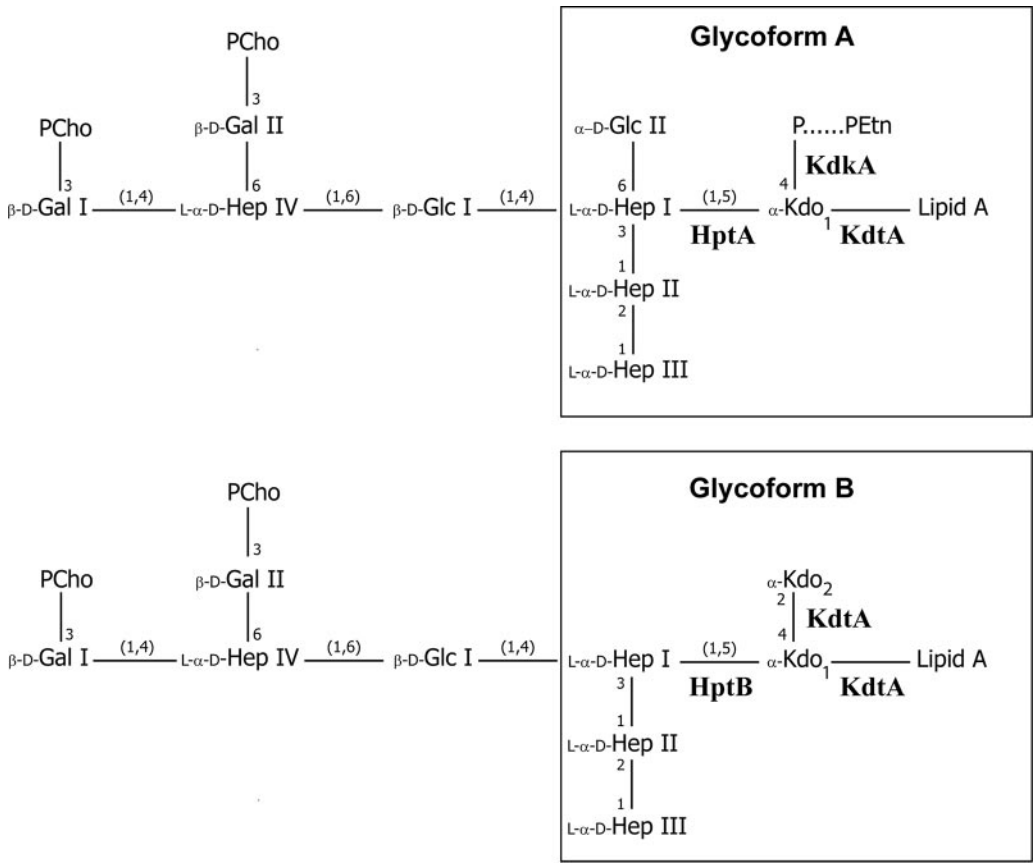

FIG. 1. LPS structure of *P. multocida* VP161 and predicted enzymes for selected biosynthetic steps. Two core types are observed, glycoforms A and B. *P. multocida* X73 has identical LPS glycoform structures, with the exception of an additional PEtn moiety attached to each terminal galactose residue. Glc, glucose; Hep, heptose; Gal, galactose; PCho, phosphocholine; Kdo, 3-deoxy-D-mannooctulosonate; P, phosphate.

tants were tested for their ability to cause disease in chickens, allowing us to determine for the first time whether expression of both glycoforms was required for virulence in the natural host. Finally, we assessed the role of LPS structure in conferring resistance to antimicrobial peptides which are predicted to be important components of the chicken innate immune system.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown routinely in Luria-Bertani broth. *P. multocida* strains were grown in either brain heart infusion (BHI) broth or nutrient broth (Oxoid, Basingstoke, U.K) supplemented with yeast extract (3% wt/vol). Solid media were obtained by the addition of 1.5% agar. When required, media were supplemented with spectinomycin (100 μg/ml), streptomycin (50 μg/ml), kanamycin (50 μg/ml), or tetracycline (2.5 μg/ml for routine culturing or 8 μg/ml for selection of *P. multocida* transconjugants). For structural studies on LPS isolated from in vitro-grown *P. multocida*, strains were grown on BHI plates with the appropriate antibiotics for 18 h at 37°C. For structural studies on LPS isolated from in vivo-grown *P. multocida*, 10^3 CFU of strain X73 were injected into the breast muscles of 14-week Hy-Line Brown chickens and disease allowed to progress for 14 to 18 h. When birds were showing clear clinical signs of fowl cholera, they were euthanatized by CO_2 asphyxiation and 10 ml of blood was recovered by cardiac puncture. Bacteria were purified from the blood as described previously (6). The cells were killed by addition of phenol to 2%, and at 3 h after phenol addition, 1 g of hyaluronidase (Roche Chemicals) was added and stirred for 1 h before cells were harvested by using a Sharples continuous-flow centrifuge.

DNA manipulations. Restriction digests and ligations were performed according to the manufacturers' instructions using enzymes obtained from New England Biolabs (Beverley, MA) or Roche Diagnostics GmbH (Mannheim, Germany). Plasmid DNA was prepared using alkaline lysis (4) and further purified using QIAGEN columns (QIAGEN GmbH, Germany), while genomic DNA was prepared using the cetyltrimethylammonium bromide method (2). PCR amplification of DNA was performed using *Taq* DNA polymerase or the Expand high-fidelity PCR system (Roche Diagnostics) and purified using the QIAGEN PCR purification kit. The oligonucleotides used in this study are listed in Table 2. DNA sequences were determined on an Applied Biosystems 3730S genetic analyzer and analyzed with Sequencher version 3.1.1 (Gene Codes, Ann Arbor, MI).

Construction of *P. multocida* mutants. The three *P. multocida* strains Pm70, X73, and VP161 have all been tested for amenability to mutant construction. To date, insertional inactivation of target genes has routinely been successful only for *P. multocida* strain VP161. For inactivation of each of the candidate glycosyltransferase genes in *P. multocida* strain VP161, we used a modification of the previously described single-crossover insertional mutagenesis method which utilizes the λ *pir*-dependent plasmid pUA826 (7). Internal DNA fragments of *kdkA* and *hptB* were amplified by PCR using the primers listed in Table 2, digested with SalI, and ligated into SalI-digested pUA826. As single-crossover insertion of pUA826 is likely to have polar effects on downstream genes, a different vector was constructed for inactivation of the *hptA* gene, which bioinformatic analysis suggested was cotranscribed with the gene PM1301. We modified pUA826 by insertion of the *P. multocida* constitutive *tpiA* promoter between the pUA826 PstI sites located at bp 4789 and 5472 (generating pUA826*tpi*). Thus, single-crossover insertion of derivatives of this plasmid would allow transcription of downstream genes through initiation of transcription from the *P. multocida tpiA* promoter located within the integrated plasmid. An internal fragment of *hptA* was amplified by PCR using the oligonucleotides listed in Table 2, digested with SalI, and ligated into SalI-digested pUA826*tpi*.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant description	Source or reference
Strains		
P. multocida		
X73	Serotype A:1	14
VP161	Serotype A:1, Vietnamese isolate from chickens	35
AL435	VP161 carrying a Tn*916* insertion in gene PM1417, fully virulent	This study
AL838	VP161 containing pAL99	This study
AL721	*kdkA* mutant of AL435	This study
AL839	AL721 containing pAL99	This study
AL840	AL721 containing pAL462	This study
AL690	*hptB* mutant of AL435	This study
AL844	AL690 containing pAL99	This study
AL845	AL690 containing pAL464	This study
AL836	Nonpolar *hptA* mutant of AL435	This study
AL846	AL836 containing pAL99	This study
AL847	AL836 containing pAL465	This study
E. coli		
DH5α	*deoR endA1 gyrA96 hsdR17* (r_K^- m_K^+) *recA1 relA1 supE44 thi-1* Δ(*lacZYA-argFV169*) ϕ80*lacZ*ΔM15 F^-	Bethesda Research Laboratories
Sm10 λ *pir*	Strain for propagation of pUA826 and its derivatives	23
Plasmids		
pUA826	Mob^+, R6K replicon, Ap^r Str^r Spc^r, single-crossover insertional mutagenesis vector	7
pUA826tpi	As for pUA826 but containing 240-bp PstI fragment containing *P. multocida tpiA* promoter region, nonpolar mutagenesis vector	This study
pAL99	*P. multocida* expression plasmid (Kan^r), constitutive *tpiA* promoter upstream of BamHI	13
pAL462	Complete *kdkA* gene cloned into pAL99	This study
pAL464	Complete *hptB* gene cloned into pAL99	This study
pAL465	Complete *hptA* gene cloned into pAL99	This study

To generate each donor strain, each ligation mix was transformed into *E. coli* SM10 λ *pir* cells and the transformants screened for recombinant pUA826 or pUA826*tpi*. The correct recombinant plasmids were then mobilized into the recipient *P. multocida* strain AL435 by conjugation. AL435 was used as the recipient strain, as it is resistant to tetracycline (due to integration of Tn*916* in gene PM1417), expresses wild-type LPS, and is fully virulent (see Table 4). For each mating, 1 ml of the recipient *P. multocida* strain AL435 and 1 ml of the donor SM10 λ *pir* strain, harboring the appropriate recombinant plasmid, were mixed and filtered through a 0.45-μm filter which was then placed on a blood agar plate and incubated at 30°C for 16 h. To recover the bacteria, filters were vortexed in 3 ml of BHI. *P. multocida* transconjugants were selected by plating the resuspended bacteria onto BHI containing tetracycline, streptomycin, and spectinomycin. Single-crossover insertion of the recombinant plasmid into each of the target genes was confirmed by PCR using flanking primers in combination with a primer located within the vector sequence (Table 2).

In *trans* complementation of mutants. The complete open reading frame for each gene to be complemented was amplified from *P. multocida* VP161 genomic DNA using flanking oligonucleotides (Table 2). The amplified DNA fragments were ligated into SalI- and BamHI-digested expression vector pAL99 (Table 1), such that transcription of the gene would be driven by the constitutive *P. multocida tpiA* promoter. The nucleotide sequence of each of the recombinant plasmids was determined to check fidelity of the cloned gene and then transformed into the corresponding *P. multocida* mutant, generating the complemented strains listed in Table 1. As a control, the vector pAL99 was transformed separately into each mutant (Table 1).

Assessment of virulence. All animal experiments were approved by the relevant Animal Ethics Committee. Groups of commercially obtained Hy-Line Brown chickens, aged 12 weeks, were infected with approximately 10^3 CFU (~100 times the 50% infective dose) of *P. multocida* by injection into the pectoral muscle, and the experiment was allowed to proceed for up to 39 h or until each

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence	Description
BAP3813	TTTGGAGTCGACAAGCGCGTATTCTTGGTT	*kdkA* internal forward primer for mutagenesis
BAP3814	CACTTTGTCGACGTTCACCAAGCTGATGTG	*kdkA* internal reverse primer for mutagenesis
BAP3914	AAAGGGATCCGTGGCCATTCGCTATCTCTG	*kdkA* flanking forward primer for expression
BAP3915	TATGTGTCGACCTTATGCTTGATATCCCGC	*kdkA* flanking reverse primer for expression
BAP3721	TATGTTGTCGACAAGTTGATTGGGTGGTCGAAG	*hptB* internal forward primer for mutagenesis
BAP3722	TAAATGGTCGACTCCGGTGTCAACAGAAACAA	*hptB* internal reverse primer for mutagenesis
BAP3745	TAATTGGGATCCAACCGGAATTGGTATTCG	*hptB* flanking forward primer for expression
BAP3746	TCAAGAGTCGACTGCCGTATAGGCAGTTTCAA	*hptB* flanking reverse primer for expression
BAP3719	CAAAACGTCGACGCAGTTACTGGCTGGTAT	*hptA* internal forward primer for mutagenesis
BAP3720	TCAGTAGTCGACAACTTACATTGGGCGAGGA	*hptA* internal reverse primer for mutagenesis
BAP3966	CACACAGGATCCATTGGACGAGATAATGATGCCG	*hptA* flanking forward primer for expression
BAP3967	ATAACAGTCGACGGTTCATCATTGAACGTC	*hptA* flanking reverse primer for expression
BAP2782	GCCCTACACAAATTGGGAGA	pUA826 primer near SalI site

TABLE 3. Negative-ion CE-ES-MS data and proposed compositions of LPS-OH from the *P. multocida* VP161 wild-type and mutant strains[a]

Strain (relevant description)	Observed ions (*m/z*)			Molecular ion (*m/z*)		Relative intensity	Proposed composition
	$(M - 4H)^{4-}$	$(M - 3H)^{3-}$	$(M - 2H)^{2-}$	Observed	Calculated		
VP161 (wild type)		992.4		2,980.2	2,977.6	0.1	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH
		999.6		3,001.8	2,999.5	0.3	2PCho, 4Hex, 4Hep, Kdo-P, lipid A-OH
		1,033.0		3,101.0	3,100.7	0.3	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn
		1,040.7		3,125.1	3,122.5	0.7	2PCho, 4Hex, 4Hep, Kdo-P, lipid A-OH-PEtn
		1,081.7		3,247.7	3,245.6	1.0	2PCho, 4Hex, 4Hep, Kdo-P-PEtn, lipid A-OH-PEtn
AL690 (*hptB*)		504.4	757.2	1,516.1	1,515.4	0.2	2Kdo, lipid A-OH-PEtn
	780.3	1,040.4		3,124.7	3,122.5	0.2	2PCho, 4Hex, 4Hep, Kdo-P, lipid A-OH-PEtn
	810.8	1,081.6		3,247.7	3,245.6	1.0	2PCho, 4Hex, 4Hep, Kdo-P-PEtn, lipid A-OH-PEtn
AL844 (*hptB* + vector)			757.5	1,516.1	1,515.4	0.2	2Kdo, lipid A-OH-PEtn
		1,040.8		3,124.7	3,122.5	0.3	2PCho, 4Hex, 4Hep, Kdo-P, lipid A-OH-PEtn
		1,081.7	1,622.7	3,247.7	3,245.6	1.0	2PCho, 4Hex, 4Hep, Kdo-P-PEtn, lipid A-OH-PEtn
AL845 (*hptB*, complemented)		1,033.0		3,101.0	3,100.7	0.2	2PCho, 3Hex, 4Hep, 2Kdo, Lipid A-OH-PEtn
		1,040.7	1,561.2	3,124.7	3,122.5	0.3	2PCho, 4Hex, 4Hep, Kdo-P, lipid A-OH-PEtn
		1,081.8	1,622.8	3,247.7	3,245.6	1.0	2PCho, 4Hex, 4Hep, Kdo-P-PEtn, lipid A-OH-PEtn
AL836 (*hptA*)			616.8	1,235.6	1,252.2	0.1	Kdo-P, lipid A-OH ($- H_2O$)
		451.6	678.4	1,358.8	1,375.2	0.5	Kdo-P-PEtn, lipid A-OH ($- H_2O$)
		1,033.4	1,549.9	3,102.6	3,100.6	1.0	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn
AL846 (*hptA* + vector)			616.8	1,235.6	1,252.2	0.1	Kdo-P, lipid A-OH ($- H_2O$)
		451.6	678.3	1,358.8	1,375.2	0.5	Kdo-P-PEtn, lipid A-OH ($- H_2O$)
	774.7	1,033.2	1,550.3	3,102.6	3,100.6	1.0	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn
AL847 (*hptA*, complemented)		1,033.3		3,101.0	3,100.7	0.5	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn
		1,040.7	1,561.2	3,124.7	3,122.5	1.0	2PCho, 4Hex, 4Hep, Kdo-P, lipid A-OH-PEtn
		1,081.5	1,622.4	3,247.7	3,245.6	0.9	2PCho, 4Hex, 4Hep, Kdo-P-PEtn, lipid A-OH-PEtn
AL721 (*kdkA*)	743.8	992.3		2,979.9	2,977.6	0.2	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH
	775.0	1,033.1		3,103.1	3,100.6	1.0	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn
	825.5	1,101.2		3,306.3	3,303.6	0.2	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn-P-PEtn
AL839 (*kdkA* + vector)		1,033.1	1,550.3	3,103.1	3,100.6	1.0	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn
AL840 (*kdkA*, complemented)		1,033.3		3,101.0	3,100.7	0.2	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn
		1,040.7	1,561.3	3,124.7	3,122.5	0.5	2PCho, 4Hex, 4Hep, Kdo-P, lipid A-OH-PEtn
		1,081.7	1,622.8	3,247.7	3,245.6	1.0	2PCho, 4Hex, 4Hep, Kdo-P-PEtn, lipid A-OH-PEtn

[a] Average mass units were used for calculation of molecular weights based on the following proposed composition: lipid A, 952.00; Hex, 162.15; Hep, 192.17; Kdo, 220.18; Kdo-P, 300.13; PEtn, 123.05; PCho, 165.05.

bird was deemed incapable of survival, when birds were euthanatized in accordance with animal ethics requirements. Blood samples for viable counts were obtained from all birds with late clinical signs of infection and from all healthy birds at 22 h postinfection. Postmortem samples of the site of injection were taken from a representative sample of birds within each group and plated onto BHI agar. Recovered bacteria were checked by PCR to confirm the identity of the *P. multocida* strains present.

Competitive growth assays. Competitive growth assays were performed as described previously (12) and used to quantify the relative growth rates of the *P. multocida* strains in vivo. Mutants were identified as attenuated if the relative competitive index (rCI) value was significantly less than 1.0 as determined by statistical analysis using the one sided *z* test ($P < 0.05$).

Antimicrobial peptide sensitivity assays. The chicken antimicrobial peptide fowlicidin-1 (RVKRVWPLVIRTVIAGYNLYAIKKK) (36) was synthesized at 96% purity by Auspep (Parkville, Australia). For peptide sensitivity assays, wild-type and mutant strains were grown to late exponential phase (optical density at 600 nm of 0.65), washed once and then diluted 1/10 in 10 mM phosphate (pH = 7.0), 10% BHI. Equal volumes of washed cells (100 μl) and diluted peptide (0 to 4 μg/ml in 10 mM phosphate [pH 7.0]) were mixed and incubated at 37°C for 1 h. Numbers of surviving bacteria were determined by viable counts on BHI agar.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining. Proteinase K-treated whole-cell lysates were analyzed on a Bio-Rad mini-protein gel apparatus, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (18). LPS was then visualized by carbohydrate silver staining (32).

Purification of LPS and LPS-OH. Cells were killed with 2% phenol, washed four times with water, lyophilized and resuspended in 200 μl of H_2O containing 5 μg of proteinase K, and incubated at 37°C for 5 h. The sample was heated to 70°C for 10 min, lyophilized, then dissolved in 200 μl of ammonium acetate buffer (20 mM, pH 7.4) containing 1 μg of RNase and 2 μg of DNase, incubated at 37°C for 5 h, and then lyophilized. The crude LPS-containing samples were O deacylated by dissolving in 200 μl of anhydrous hydrazine and incubating with stirring at 37°C for 1.5 h. Excess hydrazine was destroyed by addition of 5 volumes of ice-cold acetone to the chilled samples and repeated acetone

washes. The O-deacylated LPS (LPS-OH) pellet was redissolved in H_2O and lyophilized.

MS. Capillary electrophoresis-electrospray ionization-mass spectrometry (CE-ES-MS) was performed on a crystal model 310 CE instrument (AYI Unicam) coupled to an API 3000 MS (Perkin-Elmer/Sciex) via a microionspray interface (9). A sheath solution (isopropanol-methanol, 2:1) was delivered at a flow rate of 1 μl/min to a low-dead-volume tee (250-μm inner diameter; Chromatographic Specialties). All aqueous solutions were filtered through a 0.45-μm filter (Millipore) before use.

RESULTS

Bioinformatic prediction of inner core LPS biosynthesis genes. Putative LPS biosynthesis genes in *P. multocida* VP161 were identified based on their similarity to LPS transferases of known function in other bacteria using both BLAST (1) and the CAZY database of glycosyltransferases (8). Two candidate genes, *hptA* (annotated on the Pm70 genome as *opsX*) and *hptB* (PM1843), had been predicted to encode the 38.4- and 36.3-kDa heptosyltransferases required for the addition of the first heptose residue to either the lipid A-Kdo_1-Kdo_2 or the lipid A-Kdo_2-P core structure (Fig. 1) (30). A conserved domain search (21) showed that both had high levels of identity to heptosyltransferases belonging to pfam01075, the glycosyltransferase family 9, and COG0859, the RfaF group of transferases. An alignment of the two encoded proteins, HptA and HptB, showed only 21% identity. However, HptB has 53% identity with the heptosyl-I-transferase WaaC (RfaC), which adds heptose to the Kdo-Kdo LPS acceptor molecule in *Salmonella enterica* serovar Typhimurium (27), while HptA has 72% identity to OpsX, a heptosyl-I-transferase in *Haemophilus influenzae* which adds the first heptose to a singly phosphorylated Kdo acceptor molecule (11).

The protein encoded by the *P. multocida* VP161 gene *kdkA* (designated PM1303 on the Pm70 genome) shares 58% identity to the known Kdo kinase KdkA in *H. influenzae* (34) and belongs to the family of LPS kinases (pfam06293). It is therefore predicted to encode the kinase required for the addition of the phosphate group to the Kdo residue.

Sequencing of the VP161 genome within the regions that encoded HptA, HptB, and KdkA revealed that all genes were very highly conserved and that the genetic organization was identical to the corresponding regions in the annotated Pm70 genome. Each of the candidate genes identified in VP161 was mutated by insertional inactivation, and each mutant was complemented with the full-length gene cloned into the *P. multocida* expression vector pAL99 (Table 1).

Structural analysis of the mutants expressing modified LPS forms. CE-MS analyses of the LPS-OH isolated from the mutant strains are summarized in Table 3. The *hptA* mutant (AL836) produced a fully extended glycoform B (consisting of lipid A-Kdo_1-Kdo_2), but no fully extended glycoform A was expressed. Additionally, doubly and triply charged ions at *m/z* 678.4 and 451.6, respectively, indicated an accumulation of lipid A-Kdo_1-P truncated LPS (Fig. 2A). In contrast, analysis of the LPS from the *hptB* mutant (AL690) showed a fully extended glycoform A, and an accumulation of the lipid A-Kdo_1-Kdo_2 species was observed by virtue of the doubly and triply charged ions at *m/z* 757.2 and 504.4, respectively (Fig. 2B). The accumulation of the alternative truncated LPS in each of the mutants clearly suggests that HptA cannot utilize

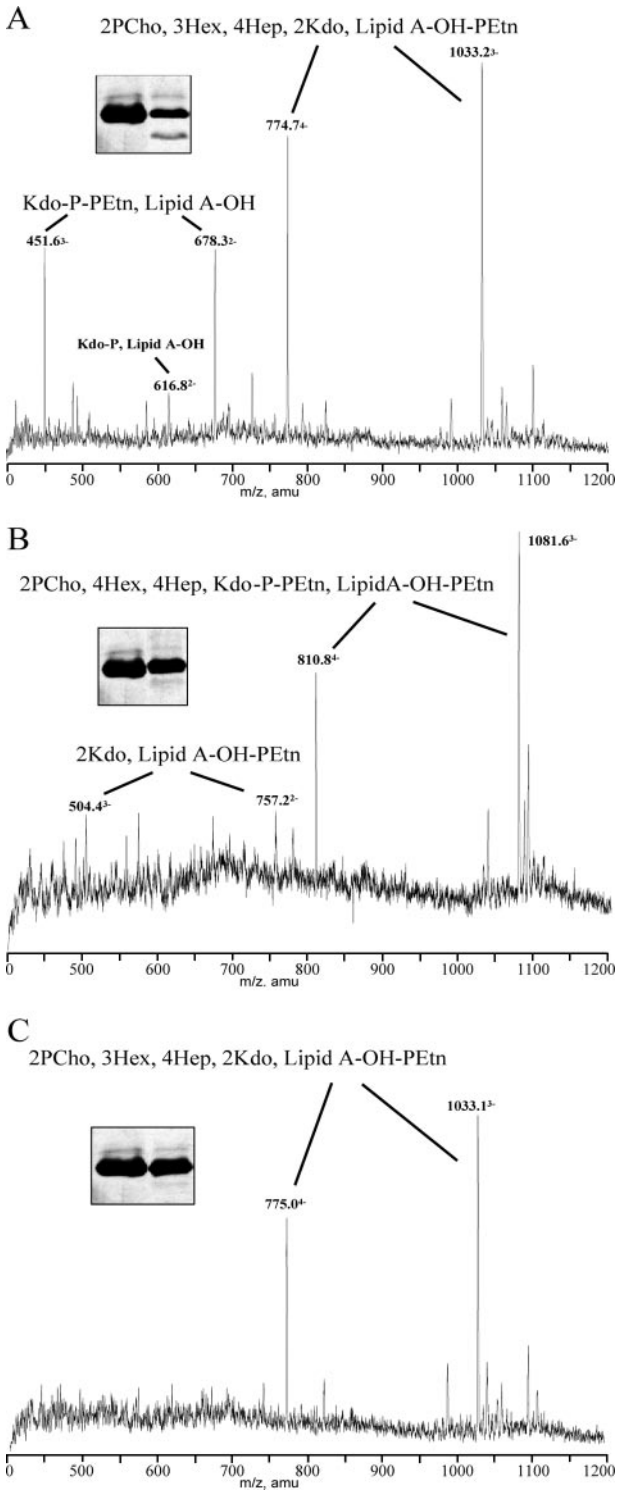

FIG. 2. Negative-ion CE-ES-MS of the LPS-OH from AL836 (*hptA*) (A), AL690 (*hptB*) (B), and AL721 (*kdkA*) (C), showing the relative abundances of truncated and full-length LPS. The inset within each panel shows a carbohydrate silver stain of proteinase K-treated whole-cell lysates with parent AL435 (left lane) and the relevant LPS mutant (right lane).

TABLE 4. Virulence of wild-type (VP161) and parent (AL435) strains and LPS mutants as determined by intramuscular challenge of 12-week-old Hy-Line Brown chickens

Strain (relevant genotype)	Dose (CFU)	Survival[a]	
		No. surviving/total	Time (h)
VP161	1.3×10^3	0/9	14–19
AL435	1.7×10^3	0/4	14–19
AL836 (*hptA*)	7×10^2	9/9	NA[b]
AL690 (*hptB*)	6×10^2	0/9	16–19
AL721 (*kdkA*)	1×10^3	0/4	14–20

[a] All birds that reached an agreed stage of the infection were euthanized in accordance with animal ethics requirements.
[b] NA, not applicable.

lipid A-Kdo_1-Kdo_2 and that HptB is unable to utilize lipid A-Kdo_1-P as an acceptor molecule. The Kdo kinase (KdkA) mutant (AL721) produced a fully extended glycoform B but no glycoform A and no accumulation of truncated LPS (Fig. 2C). These data indicate that an unphosphorylated Kdo on the acceptor molecule is freely available for the addition of a second Kdo residue allowing glycoform B to be produced. When complemented in *trans* with the appropriate genes, all mutant strains produced both wild-type glycoforms (Table 3).

Virulence of the mutants expressing modified LPS forms. To determine if any of the mutants were capable of causing fowl cholera, virulence assays were performed. All birds except those injected with the *hptA* mutant succumbed to fowl cholera infection and were euthanized (Table 4). Blood samples taken from all birds were plated onto BHI to determine levels of bacteremia. Blood samples taken from birds injected with VP161, AL435, AL690, and AL721 showed greater than 10^4 CFU/ml, but no bacteria were recovered from the blood of birds injected with the *hptA* mutant (AL836). Similarly, muscle was sampled at the site of injection from birds in all groups, and all birds except those from the AL836 group were positive for *P. multocida*.

The *hptA* gene is located upstream of PM1301, which encodes a putative ABC transporter. To rule out the involvement of PM1301 in the observed phenotype, the mutant was complemented in *trans* with an intact copy of *hptA* and tested by competition assay. The complemented mutant (AL847) was able to grow in vivo with an average rCI value of 0.73, whereas neither the mutant with vector (AL846) nor the *hptA* mutant (AL704) could grow (rCI = 0.0). Therefore, HptA activity is essential for *P. multocida* virulence.

Structural analysis of LPS expressed by *P. multocida* during in vivo growth. Based on negative-ion CE-ES-MS data obtained for the *P. multocida* strains VP161 (this study) and X73 (28), glycoform A is the dominant structure, comprising 70 to 80% of the total LPS produced by both strains. To determine if both glycoforms observed from in vitro-grown *P. multocida* were also expressed by the bacteria during growth in the host, chickens were infected with *P. multocida* strain X73. This serotype A:1 strain is closely related to VP161 and expresses very similar LPS structures (28), but it has the unique ability to grow to very high numbers (up to 5×10^{10} CFU/ml) in the blood of chickens, thus allowing analytical quantities of LPS to be isolated. Chickens were injected intramuscularly with *P. multocida* and the infection allowed to proceed until the birds showed late signs of fowl cholera infection. *P. multocida* was harvested from the blood and LPS isolated from the bacteria. Structural analysis of the LPS isolated from in vivo-grown bacteria showed that the type and amount of each LPS glycoform were the same as those observed from LPS isolated from in vitro-grown cells (data not shown) (28).

Sensitivity of LPS mutants to the antimicrobial peptide fowlicidin-1. To determine if *P. multocida* LPS mutants were more susceptible than the parent strain to chicken antimicrobial peptides, we performed bactericidal assays with the peptide fowlicidin-1 and compared the sensitivities of the parent strain AL435, the heptosyltransferase mutants (AL690 and AL836), and the Kdo kinase mutant (AL721) (Fig. 3). Both of the heptosyltransferase mutants were more sensitive to the effects of fowlicidin-1 than either parent strain or the Kdo kinase mutant. However,

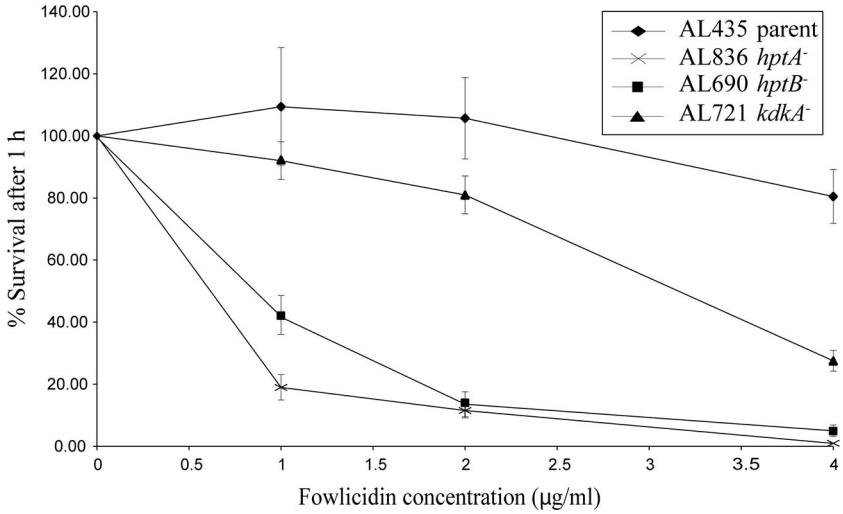

FIG. 3. Sensitivities of the *P. multocida* parent strain (AL435) and of LPS mutants AL836 (*hptA*), AL690 (*hptB*), and AL721 (*kdkA*) to the action of fowlicidin-1. Bacterial survival was determined by direct colony counts after incubation with various concentrations of synthetic fowlicidin-1 for 1 h at 37°C. Numbers are the mean percent survival for three replicates, and error bars are ±1 standard deviation.

there was no significant difference in sensitivity between the two heptosyltransferase mutants, AL690 and AL836.

DISCUSSION

The *P. multocida* fowl cholera-causing isolates VP161 and X73, when propagated in vitro in BHI, express two LPS glycoforms containing either a single Kdo residue, which we have designated glycoform A, or two Kdo residues, which we have designated glycoform B (28, 29). The Kdo_1 residue in glycoform A has a phosphate residue at the 4 position to which, in the majority of cases, a PEtn residue is attached. Glycoform A also has a glucose residue attached to the 6 position of the first heptose moiety (Fig. 1). In contrast, glycoform B contains two Kdo residues that lack the phosphate or PEtn residues and does not have the second glucose residue (Fig. 1). The expression of two very different Kdo region structures while the same oligosaccharide extension is retained is unusual. Other bacteria that express LPS lacking O polysaccharide include *Campylobacter jejuni*, *H. influenzae*, *Neisseria gonorrhoeae*, and *Neisseria meningitidis*. Typically, changes in structure occur in the outer core oligosaccharide extension and branch chains which lead to diversity in the LPS structure (reviewed in reference 24). Changes in LPS structure in these bacteria are often the result of phase variation caused by slipped-strand mispairing of homopolymeric tracts or multiple tandem repeats in DNA that results in the switching on or off of key LPS genes (16, 33). However, we have examined the annotated Pm70 genome and the sequenced LPS regions from a number of other *P. multocida* strains and have found no evidence of any genes that are subject to this type of phase variation.

Bioinformatic analysis of the genome of *P. multocida* strain Pm70 revealed two encoded proteins with similarity to known heptosyl-1-transferases. One of these, HptB (Pm1843 in Pm70), shares identity with WaaC, a transferase required for the addition of the first heptose residue to the lipid A-Kdo-Kdo acceptor molecule in bacteria belonging to the *Enterobacteriaceae* family (24, 27). The second transferase, HptA (annotated in Pm70 as OpsX), is similar to OpsX in *H. influenzae*, a novel type of heptosyltransferase that transfers the first heptose residue onto a Kdo-lipid A but only if the Kdo residue is phosphorylated (11). A search of all available bacterial genomes at NCBI revealed that only *Mannheimia haemolytica*, *Mannheimia succiniciproducens*, and *Actinobacillus pleuropneumoniae* harbor the genes for both types of heptosyltransferase I; these species are also members of the *Pasteurellaceae* family. Of these, only *M. haemolytica* has been shown to express more than one inner core LPS glycoform (19). The LPS structures of *A. pleuropneumoniae* strains examined so far produce only one inner core glycoform containing a single Kdo residue (31), while the composition of LPS expressed by *M. succiniciproducens* is unknown.

In addition to the identification of HptA and HptB as the predicted heptosyl-1-transferases, the gene PM1303 was also identified as encoding the putative Kdo kinase (KdkA) required for phosphorylation of the Kdo_1 residue in *P. multocida* VP161 (Fig. 1). To confirm the roles of HptA, HptB, and KdkA in LPS biosynthesis, we constructed mutants with mutations in each of the identified genes in strain VP161. Inactivation of *hptA* produced a mutant strain (AL836) that expressed glycoform B, but approximately 37% of the LPS consisted of the highly truncated species lipid A-Kdo_1-P (Table 3; Fig. 2A). This is consistent with *hptA* encoding the transferase required for the addition of the first heptose residue to the lipid A-Kdo_1-P core. The function of HptA was confirmed with structural data on the complemented mutant, AL847, which showed restoration of full-length LPS for both glycoforms.

Inactivation of *hptB* produced a mutant strain (AL690) that expressed both full-length glycoform A and a truncated LPS species, estimated at 15% of the total product, consisting of lipid A-Kdo_1-Kdo_2 (Table 3; Fig. 2B). Complementation of this mutant with a functional *hptB* gene restored expression of the entire glycoform B. These data support the hypothesis that *hptB* encodes the heptosyltransferase required for the addition of the first heptose to the lipid A-Kdo_1-Kdo_2 core.

Thus, HptB and HptA are both heptosyl-1-transferases, but each transferase exclusively recognizes different acceptor molecules, with HptA transferring heptose to lipid A-Kdo_1-P and HptB transferring heptose to lipid A-Kdo_1-Kdo_2. Virulence data showed that the *hptB* mutant, AL690, was still capable of causing lethal infection in chickens (Table 4). However, the *hptA* mutant, AL836, was fully attenuated. These data suggested that either glycoform A was critical for growth in vivo or AL836 was unable to survive in the host because of the amount of truncated LPS present on the surface; MS analysis indicated that the fully attenuated *hptA* mutant expressed more than twice the amount of truncated LPS as the virulent *hptB* mutant did (Fig. 2; Table 3).

To determine if expression of only glycoform B was sufficient for *P. multocida* growth in vivo, we constructed a Kdo kinase (*kdkA*) mutant. This kinase was predicted to add the phosphate residue to the first Kdo residue and thus should be essential for the synthesis of glycoform A. Structural analysis of the LPS expressed by the Kdo kinase mutant (AL721) indicated that it produced only glycoform B and not glycoform A, confirming the function of KdkA as the Kdo kinase (Table 3; Fig. 2C). Moreover, the Kdo kinase mutant expressed no truncated LPS, confirming that the lipid A-Kdo_1 structure acts as an acceptor molecule both for the addition of a second Kdo molecule and for phosphorylation by KdkA.

We predict that *P. multocida*, like *E. coli*, expresses a bifunctional Kdo transferase (KdtA) that can attach both the first and second Kdo residues to lipid A (3). Because of the predicted bifunctional nature of KdtA, we hypothesize that in wild-type *P. multocida* the Kdo kinase (KdkA) competes with the Kdo transferase (KdtA) for access to the lipid A-Kdo_1 acceptor molecule, and the predominance of glycoform A (70 to 80% of total LPS) suggests that phosphorylation of the first Kdo residue (by KdkA) occurs at a higher rate than the addition of the second Kdo residue by KdtA. Once phosphorylated, the lipid A-Kdo_1 is not recognized by the Kdo transferase as an acceptor molecule. However, in the *kdkA* mutant, which lacks a functional Kdo kinase, the Kdo transferase has unhindered access to the lipid A-Kdo_1, allowing all of the substrate to be converted to lipid A-Kdo_1-Kdo_2, the acceptor molecule recognized by the heptosyltransferase HptB. In order to confirm the function of KdtA in *P. multocida*, we attempted to construct a mutant with an insertion in the *kdtA* gene, but were unsuccessful. This is not surprising, as the lack of a functional Kdo

transferase would most likely result in a nonviable mutant that lacks any LPS on its surface (24).

Although both the *hptA* mutant (AL836) and the *kdkA* mutant (AL721) expressed full-length LPS glycoform B, the *kdkA* mutant was fully virulent while the *hptA* mutant was fully attenuated (Table 4). The virulence data obtained for the *kdkA* mutant indicate that *P. multocida* VP161 can grow in vivo while expressing only glycoform B LPS. Therefore, the attenuation observed in the *hptA* mutant is probably due to the expression of a large amount of truncated LPS that makes the bacteria more vulnerable to host defense mechanisms than strains expressing only full-length LPS.

Antimicrobial peptides are a critical component of the innate immune systems of higher organisms (17) and are known to interact with LPS. It has been demonstrated in other bacterial species that truncation or alteration of the LPS alters resistance to antimicrobial peptide activity (20, 25). A number of chicken antimicrobial peptides have been characterized, including members of the β-defensin (15) and cathelicidin (36) families. In this study, the resistance of each of the *P. multocida* LPS mutants to the cathelicidin fowlicidin-1 was determined. We found that although the *hptB* mutant could cause disease and the *hptA* mutant could not, bactericidal assays showed both the heptosyltransferase mutants were more sensitive to fowlicidin-1 than the parent strain AL435. Therefore, the profound difference in the abilities of these mutants to cause disease in the host does not correlate directly with sensitivity to the lytic peptide fowlicidin-1. Other antimicrobial peptides, in addition to the complement membrane attack complex and Toll-like receptors on macrophages and heterophils, are also likely to play a role in host defense. Moreover, the Kdo kinase mutant (AL721) was only slightly sensitive to fowlicidin compared to the parent strain (AL435) but was significantly more resistant than the *hptA* mutant. These two mutants both express full-length glycoform B, but AL836 also expresses a large amount of truncated LPS, indicating that susceptibility of the LPS mutant strains to fowlicidin-1 is directly related to the expression of truncated LPS.

It is interesting that *P. multocida* can cause disease in the host with only the expression of glycoform B, yet both glycoforms are expressed simultaneously in vitro. To determine if the expression of both glycoforms also occurred in vivo, we isolated bacteria from the blood of chickens infected with *P. multocida* strain X73 and analyzed the in vivo-expressed LPS by MS. The ratio and type of LPS expressed at a late stage of infection in chickens were very similar to those of LPS expressed in vitro. It is not clear whether *P. multocida* can cause disease with expression of only glycoform A, as we have been unable to construct a mutant that produced full-length glycoform A and no other LPS species. Such a mutant would require the addition of only the first Kdo residue, and we predict that the Kdo transferase, KdtA, is bifunctional, transferring both the Kdo_1 and Kdo_2 molecules. Therefore, we have no mechanism to prevent the addition of Kdo_2 while allowing the transfer of Kdo_1. It is probable that neither glycoform A nor B is deleterious for the bacteria growing systemically within the host, as both are clearly expressed in this niche in vivo, but perhaps the expression and regulation of two different glycoforms allow for *P. multocida* survival in different environments within the host. We are currently investigating this possibility.

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