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A Heptosyltransferase Mutant of *Pasteurella multocida* Produces a Truncated Lipopolysaccharide Structure and Is Attenuated in Virulence

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Pasteurella multocida **is the causative agent of fowl cholera in birds. In a previous study using signature-tagged mutagenesis, we identified a mutant, AL251, which was attenuated for virulence in mice and in the natural chicken host. Sequence analysis indicated that AL251 had an insertional inactivation of the gene *waaQ*$_{PM}$, encoding a putative heptosyl transferase, required for the addition of heptose to lipopolysaccharide (LPS) (M. Harper, J. D. Boyce, I. W. Wilkie, and B. Adler, Infect. Immun. 71:5440–5446, 2003). In the present study, using mass spectrometry and nuclear magnetic resonance, we have confirmed the identity of the enzyme encoded by *waaQ*$_{PM}$ as a heptosyl transferase III and demonstrated that the predominant LPS glycoforms isolated from this mutant are severely truncated. Complementation experiments demonstrated that providing a functional *waaQ*$_{PM}$ gene in *trans* can restore both the LPS to its full length and growth in mice to wild-type levels. Furthermore, we have shown that mutant AL251 is unable to cause fowl cholera in chickens and that the attenuation observed is not due to increased serum sensitivity.**

Pasteurella multocida is an encapsulated, gram-negative coccobacillus and is the causative agent of a wide range of animal diseases including avian fowl cholera. Several *P. multocida* virulence factors have previously been identified, including the capsule in serogroups A and B (4, 6), PMT toxin in strains causing atrophic rhinitis in pigs (18), putative filamentous hemagglutinins PfhB1 and PfhB2 (19), and several iron acquisition proteins such as TonB, ExbD and ExbB (3, 19, 27).

During infections by gram-negative bacteria, the presence of lipopolysaccharide (LPS) stimulates the innate immune system whereby the inflammatory response plays a critical role in helping to clear the bacteria and prevent infection. This initial response to gram-negative bacteria can be elicited by a number of bacterial components, the most potent being lipid A, a component of the core structure of LPS. If the inflammatory response in the host is unable to clear the bacteria and the infection is allowed to proceed, the presence of large amounts of systemic LPS can result in endotoxic shock, in which an overproduction of inflammatory mediators causes damage to tissues, septic shock, organ failure, and death (31).

LPS is considered to play an important role in the pathogenesis of disease due to *P. multocida.* Recently it has been shown that LPS from *P. multocida* assists in adhesion to neutrophils and transmigration through endothelial cells (20). However, there are conflicting reports about the endotoxic properties of LPS isolated from *P. multocida.* LPS isolated from a serotype B:2 strain was shown to be endotoxic, and intravenously administered LPS could reproduce clinical signs of hemorrhagic septicemia in buffalo (24). However, turkey poults were found to be relatively resistant to the lethal effects of LPS isolated from serogroup A strains of *P. multocida*, although the inflammatory response and microscopic hepatic lesions were similar to those observed in mammalian hosts (29, 34). In contrast, chicken embryos and mice were found to be highly susceptible to the toxic effects of *P. multocida* LPS (21).

It is clear that the LPS of *P. multocida* stimulates humoral immunity, and it is considered to be a protective antigen. *P. multocida* strains are classified into Heddleston serotypes based on the antibody responses to LPS, while antibodies raised against heat-killed *P. multocida* vaccines are directed primarily against LPS and protect the host against strains within the same serotype (5). Early studies demonstrated that LPS purified using the Westphal method and injected into mice and rabbits resulted in a poor antibody response and no protection against *P. multocida* infection. In contrast, LPS injected into chickens induced a good antibody response which passively protected recipients against disease (33). Monoclonal antibodies raised against the LPS from a serotype A strain were shown to be bactericidal and to completely protect mice against homologous challenge (43). In addition, an opsonic monoclonal antibody against LPS from a serotype B strain of *P. multocida* was shown to partially protect mice against *P. multocida* infection (32).

A modified LPS structure clearly affects the viability of *P. multocida* in vivo. Recently in our laboratory, we identified three strongly attenuated mutants that each had a single transposon insertion in the *pm1294* gene (designated *waaQ*$_{PM}$) (22).

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TABLE 1. Bacterial strains, plasmids and oligonucleotides used in this study

Strain, plasmid or oligonucleotide	Relevant description	Source or reference
Strains		
P. multocida		
VP161	Serotype A:1, Vietnamese isolate from chickens	44
AL251	VP161 Tn*916*EΔC $waaQ_{PM}$ mutant	22
AL298	AL251 with plasmid pAL170	This study
AL438	AL251 with plasmid pAL99	This study
E. coli		
DH5α	*deoR endA1 gyrA96 hsdR17*(r_k^- m_k^+) *recA1 relA1 supE44 thi-1* (*lacZYA-argFV169*) ϕ80*lacZ* ΔM15, F^-	Bethesda Research Laboratories
Plasmids		
pPBA1100	*P. multocida*/*E. coli* shuttle vector	23
pAL99	240-bp EcoRI fragment containing *P. multocida tpiA* promoter region cloned into pPBA1100 EcoRI site	This study
pAL170	pAL99 containing $waaQ_{PM}$ gene	This study
Oligonucleotides		
BAP2146	Forward primer for $waaQ_{PM}$ amplification; has BamHI site for cloning; GAGTAGGATCCTGAAACATGTTCCC	This study
BAP2147	Reverse primer for $waaQ_{PM}$ amplification; has SalI site for cloning; GGTTGGGTCGACCAAGCCACATTACTG	This study

This gene is predicted to encode a heptosyltransferase, based on 72% amino acid similarity to the heptosyltransferase III from *Haemophilus ducreyi*, a member of the *Pasteurellaceae* family (17). Furthermore, a *P. multocida galE* mutant has been constructed previously and was attenuated in mice (16). *galE* encodes an enzyme required for the epimerization of UDP-glucose to UDP-galactose prior to LPS assembly, and this mutant probably expresses an altered LPS, although no structural analysis of the LPS was reported (16).

The sugar composition of LPS isolated from two serotype A strains of *P. multocida* was analyzed previously, and a partial structure has been proposed that included the identification of a triheptose unit linked to a 2-keto-3-deoxyoctulosonic acid (KDO) residue (12–14). In this report we present the core structure of LPS from a highly virulent *P. multocida* serotype A:1 strain, VP161, that causes fowl cholera and compare it with the structure of LPS from a transposon mutant deficient in heptosyltransferase III. We also present virulence data that demonstrate a significant role for LPS in *P. multocida* disease progression.

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 (NB) supplemented with 3% yeast extract (Oxoid, Basingstoke, United Kingdom). Solid media were obtained by the addition of 1.5% agar. When required, the media were supplemented with tetracycline at 2.5 μg/ml. For structural studies, *P. multocida* strains VP161 and AL251 were grown in a 28-liter fermentor containing 24-liters of BHI broth for 18 h at 37°C with 20% dissolved-oxygen saturation. The cells were killed by addition of phenol to 2%, and 3 h after the phenol addition, 1 g of hyaluronidase (Roche Chemicals) was added; the mixture was stirred for 1 h before cells were harvested by using a Sharples continuous-flow centrifuge.

Transposon stability studies. *P. multocida* AL251 was grown in 10 ml of NB at 37°C with shaking. After approximately 10, 34, and 58 generations, samples of the culture were taken, diluted appropriately, and plated onto NB agar. After overnight incubation, 100 colonies were patched onto NB agar with tetracycline and incubated overnight at 37°C. Transposon loss was expressed as the percentage of tetracycline-sensitive colonies.

SDS-PAGE and silver staining. LPS was analyzed with a Bio-Rad mini protein gel apparatus, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (25). LPS was then visualized by silver staining (40).

DNA manipulations. Restriction digests, ligations, and PCR amplifications were performed as specified by the manufacturers, using enzymes obtained from NEB (Beverley, Mass.) or Roche Diagnostics GmbH (Mannheim, Germany). Plasmid DNA was prepared using alkaline lysis (2) and purified using Qiagen columns (Qiagen GmbH, Hilden, Germany) or by polyethylene glycol precipitation (1). Genomic DNA was prepared using the cetyltrimethylammonium bromide (CTAB) method (1). PCR amplification of DNA was performed using *Taq* DNA polymerase or the Expand High Fidelity PCR system (Roche Diagnostics), and the DNA was purified using the Qiagen PCR purification kit. The oligonucleotides used in this study are listed in Table 1. The DNA sequence was determined on a model 373A DNA sequencing system (Applied Biosystems) and analyzed with Sequencher version 3.1.1 (GeneCodes, Ann Arbor, Mich.).

In *trans* complementation of $waaQ_{PM}$. The complete $waaQ_{PM}$ gene was amplified from *P. multocida* VP161 genomic DNA using oligonucleotides BAP2146 and BAP2147 (Table 1). The amplified 1.1-kb DNA fragment was ligated to SalI- and BamHI-digested vector pAL99 (Table 1), such that transcription was driven by the *P. multocida tpiA* promoter. *E. coli* transformants were screened for the presence of the correct plasmid, and one, designated pAL170, was used to transform *P. multocida* AL251, generating strain AL298. As a control, the pAL99 vector was transformed separately into AL251, generating strain AL438 (Table 1).

Competitive growth assays. Competitive growth assays were performed as described previously (22) and were used to quantify the relative growth rates of the *P. multocida* LPS mutant AL251 and the complemented mutant AL298. The competitive index (CI) was determined by dividing the percentage of tetracycline-resistant colonies obtained from the output culture (in vitro or in vivo) by the percentage of tetracycline-resistant colonies obtained from the input culture. The relative competitive index (rCI), which measures the difference between growth in vivo and growth in vitro, was determined by dividing the in vivo CI by the in vitro CI. Mutants were identified as attenuated if the rCI value was significantly less than 1.0 as determined by statistical analysis using the one sided *z* test ($P < 0.05$).

Virulence trials. Groups of 10 commercially obtained Leghorn-cross chickens aged 12 weeks were infected with *P. multocida* VP161 or AL251 at two different doses by injection of 100 μl into the breast muscle. Blood samples were obtained at various time points after infection with AL251, and the birds deemed incapable of survival were euthanized in accordance with animal ethics requirements.

Blood samples were diluted twofold in BHI broth containing heparin and plated onto BHI plates. *P. multocida* colonies isolated from the blood were patched onto NB agar and NB agar with tetracycline.

Serum sensitivity assays. The sensitivity of *P. multocida* and *E. coli* to fresh chicken serum was determined as described previously (6).

Purification of LPS. *P. multocida* cells (210 g [wet weight] of VP161; 254 g [wet weight] of AL251) were freeze-dried, yielding 56 g of VP161 and 52 g of AL251. Freeze-dried cells were washed with organic solvents to remove lipids and other lipophilic components in order to enhance the efficiency of the LPS extraction (26). Washed cells (42 g of VP161; 50 g of AL251) were extracted by the hot-phenol–water method (42), and the aqueous phases were combined and dialyzed against running water for 48 h. The retentate was freeze-dried, made up to a 2% solution in water, and treated with DNase and RNase at 37°C for 4 h and then with proteinase K at 37°C for 4 h. Small peptides were removed by dialysis. After a freeze-drying step, the retentate was made up to a 2% solution in water and centrifuged at 8,000 × *g* for 15 min, and the supernatant was further centrifuged at 100,000 × *g* for 5 h. The pellet, containing purified LPS, was redissolved and freeze-dried. The core oligosaccharide (OS) was isolated by treating the purified LPS with 1% acetic acid (10 mg/ml) at 100°C for 1.5 h, with subsequent removal of the insoluble lipid A by centrifugation (5,000 × *g*).

Analytical methods. Sugars were identified on the basis of their alditol acetate derivatives (35) by gas-liquid chromatography and mass spectrometry (GLC-MS). LPS was hydrolyzed for 4 h using 4 M trifluoroacetic acid at 100°C, reduced overnight with $NaBD_4$ in H_2O, and then acetylated with acetic anhydride at 100°C for 2 h using residual sodium acetate as the catalyst. The GLC-MS apparatus was equipped with a 30-m DB-17 capillary column (180 to 260°C at 3.5°C/min), and MS was performed in the electron impact mode on a Varian Saturn II mass spectrometer. Methylation analysis was carried out by the NaOH-dimethyl sulfoxide-methyl iodide procedure (7) and analyzed by GLC-MS as above.

MS analysis. Capillary electrophoresis electrospray ionization MS (CE-ESI-MS) was performed on a crystal model 310 capillary electrophoresis (CE) instrument (AYI Unicam) coupled to an API 3000 mass spectrometer (Perkin-Elmer/Sciex) via a microIonspray interface (9). A sheath solution (isopropanol-methanol, 2:1 [vol/vol]) 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 pore-size filter (Millipore) before use.

Nuclear magnetic resonance. Nuclear magnetic resonance (NMR) spectra were acquired on a Varian Inova 500 MHz spectrometer using a 3-mm triple-resonance (^1H, ^{13}C, and ^{31}P) probe. The lyophilized sugar sample was dissolved in 140 μl of 99% D_2O. The experiments were performed at 25°C with suppression of the HOD (deuterated H_2O) signal at 4.78 ppm. The methyl resonance of acetone was used as an internal or external reference at 2.225 ppm for ^1H spectra and 31.07 ppm for ^{13}C spectra. Standard homo- and heteronuclear correlated two-dimensional pulse sequences from Varian, COSY, TOCSY, NOESY, ^{13}C-^1H HSQC, ^{13}C-^1H HSQC-TOCSY, and ^{13}C-^1H HMBC were used for general assignments.

RESULTS

An attenuated *P. multocida* mutant produces a truncated LPS that is restored to full-length LPS by complementation with a functional *waaQ*$_{PM}$ gene. We have previously used signature-tagged mutagenesis (STM) to identify mutants attenuated for growth in mice and chickens (22). During this previous analysis, a mutant was identified (designated AL251) that grew equally well as the wild type in vitro (mean CI = 0.96) but was attenuated in both chickens (mean CI = 0.01) and mice (mean CI = 0.59). Sequence analysis of the mutant revealed a single transposon insertion within the *waaQ*$_{PM}$ gene that is predicted to encode a heptosyltransferase, a glycosyltransferase responsible for the addition of heptose to LPS (17,22).

We compared the LPS profile of AL251 with that of its wild-type parent, VP161, and the complemented mutant, AL298, using SDS-PAGE followed by silver staining. The LPS from AL251 migrated further within the gel compared to wild-type LPS, indicating that the LPS produced by the mutant was significantly truncated (Fig. 1A). Furthermore, the LPS profile

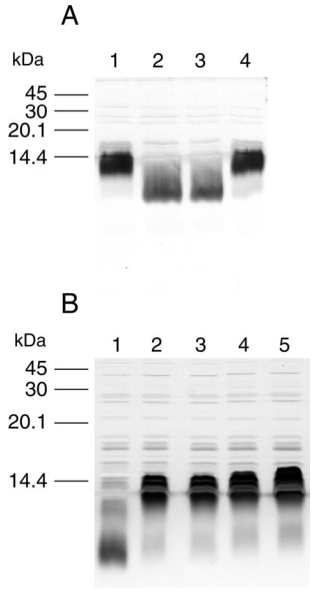

FIG. 1. Analysis of *P. multocida* LPS by SDS-PAGE and silver staining of whole-cell lysates. (A) Comparison of *P. multocida* LPS profiles from wild-type VP161 (lane 1), heptosyltransferase mutant AL251 (lane 2), control strain AL438 (AL251 containing vector plasmid pAL99) (lane 3), and the complemented mutant strain AL298 (lane 4). (B) Comparison of LPS profiles of *P. multocida* heptosyltransferase mutant AL251 (lane 1), wild-type VP161 (lane 2), and *P. multocida* wild-type revertants isolated from three different chickens inoculated with AL251 (lanes 3 to 5).

of the complemented mutant AL298 was identical to that observed for the wild type, indicating that complementation of the AL251 mutant with an intact *waaQ*$_{PM}$ gene was able to restore the synthesis of wild-type LPS (Fig. 1A).

Complementation of AL251 with *waaQ*$_{PM}$ also restores in vivo growth to wild-type levels. Since complementation of AL251 with *waaQ*$_{PM}$ restored production of wild-type LPS levels, we wanted to determine if complementing the inactivated *waaQ*$_{PM}$ also restored the mutant AL251 to wild-type levels of growth in vivo. Initial studies with the complemented mutant AL298 indicated that there was significant loss of the complementing plasmid pAL170 once antibiotic selection for the plasmid was removed (44% retention after 6 h). For this reason, mice were chosen instead of chickens for the competitive growth assay, since previous studies had demonstrated that the infection time required to harvest bacteria from mice was only 6 h compared with more than 12 h for infections in chickens (22). Three mice were injected with an equal mix of VP161 and the complemented strain AL298. As controls, two mice were injected with an equal mix of wild-type VP161 and the control strain AL438 (AL251 with the pAL99 vector). The complemented mutant AL298 was able to compete equally with wild-type VP161, with an average rCI value of 1.0, while the control strain AL438 had an average rCI value of 0.57 (P = 0.03), similar to the rCI values previously reported for AL251 in mice (22). These results demonstrate that *waaQ*$_{PM}$ is required both for production of full-length LPS and for normal growth during infection.

The *P. multocida waaQ*$_{PM}$ mutant is unable to cause disease in chickens. We had shown previously that strain AL251 dis-

TABLE 2. Virulence of VP161 and AL251 in groups of 10 chickens

Strain	Dose (CFU)	Mean time to death (range) (h)
VP161	1.5×10^2	<20 (ND[a])
	1.5×10^3	<20 (ND)
AL251	70	65 (33–120)
	7×10^2	30 (23–42)

[a] ND, not determined.

played a profoundly reduced growth rate in chickens (22); therefore, we wished to determine whether the mutant was still capable of causing disease in these hosts. Chickens were challenged with either VP161 or AL251 at two different doses (Table 2). All of the chickens challenged with wild-type VP161 died within 20 h. In contrast, most chickens challenged with AL251 remained well over the first 20 h but within 4 days all of the chickens inoculated with AL251, irrespective of dose, succumbed to fowl cholera infection. *P. multocida* was isolated from the blood of AL251-infected chickens in the late or terminal stages of the disease, and it was found that all of the isolated *P. multocida* colonies were tetracycline sensitive, indicating that the transposon was no longer present in the bacteria. Sequence analysis of $waaQ_{PM}$ from the recovered colonies indicated that in all cases the transposon had excised, thereby reconstituting a functional $waaQ_{PM}$ gene. Interestingly, for all but one isolate, the sequence analysis also revealed the presence of nucleotide substitutions at the point of transposon excision, resulting in two amino acid changes within $waaQ_{PM}$ (amino acids 88 and 89; Ser to Leu and Asp to Cys, respectively). These amino acid changes did not affect the function of $waaQ_{PM}$, since the LPS profiles of the *P. multocida* isolates recovered from the chickens challenged with AL251 were all identical to those for the wild type (Fig. 1B). Taken together, these results indicate that the later onset of fowl cholera observed in the chickens inoculated with AL251 was due entirely to wild-type revertants of AL251 and that strains with an inactivated $waaQ_{PM}$ gene are therefore incapable of causing disease.

Attenuation of the $waaQ_{PM}$ mutant in chickens is not due to increased sensitivity to chicken serum. To determine the relative sensitivity of the wild-type *P. multocida* strain VP161 and the LPS mutant AL251 to complement-mediated killing, mid-log-phase cells of each strain and a control strain, *E. coli* DH5α, were incubated in either normal or heat-treated chicken serum for 3 h. Wild-type *P. multocida* strain VP161 was able to grow at the same rate in both the heated and the unheated serum, indicating that it is fully serum resistant as previously reported for other *P. multocida* serotype A strains (11). The bactericidal activity of the chicken serum was confirmed using the *E. coli* control strain, which multiplied 19-fold in heat-treated serum whereas its viability was reduced approximately 9-fold in untreated serum. Interestingly, for the AL251 mutant, no difference in growth was observed between the heat-treated and normal serum, with approximately a 160-fold increase in growth in the heat-treated serum and an identical growth rate in the normal serum. These results indicate that the attenuation observed for the LPS mutant in chickens was not due to increased sensitivity to complement.

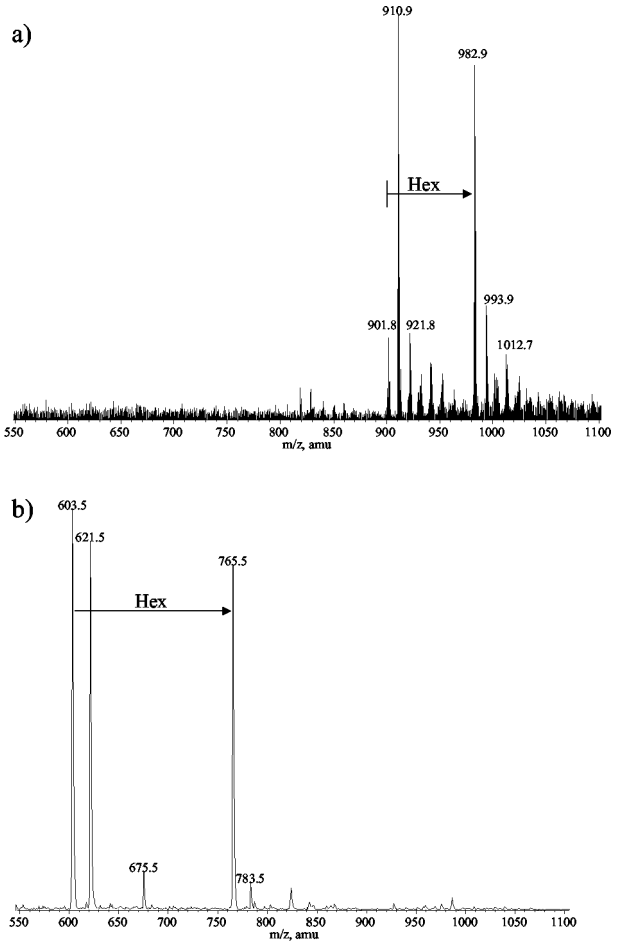

FIG. 2. Negative-ion CE-ESI-MS of *P. multocida* core OS. (a) Doubly charged region of core OS from parent strain VP161; (b) singly charged region of core OS from mutant strain AL251.

Structural analysis of the LPS from *P. multocida* VP161 and AL251. Sugar analysis of the LPS from the parent strain VP161 revealed glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), and L-*glycero*-D-*manno*-heptose (LD-Hep) in the ratio of 2:1:1:3. In contrast, the LPS from mutant strain AL251 revealed only Glc and LD-Hep in the ratio of 1:3 with traces of Gal and GlcNAc. It is possible that GlcNAc could be contributed by residual amounts of the hyaluronic acid capsule known for this serogroup in addition to the anticipated two residues in the lipid A region of each LPS molecule. CE-ESI-MS analyses of the core OS sample derived from the parent VP161 LPS revealed a simple mass spectrum (Fig. 2a) corresponding to two glycoforms with masses of 1,805 and 1,967 Da, the smaller glycoform being consistent with a composition of 2*P*Cho + 3Hex + 4Hep + KDO, where KDO is the unique LPS sugar 2-keto-3-deoxyoctulosonic acid and *P*Cho is the phosphate moiety phosphocholine. The larger glycoform contains an additional hexose species (Table 3; Fig. 2a). Consistent with SDS-PAGE analysis of the mutant AL251 LPS, CE-ESI-MS analyses of the core OS sample derived from the mutant AL251 LPS revealed a simple mass spectrum of a highly truncated molecule with a mass of 605 Da (Fig. 2b). This mass corresponds to a composition of 2Hep + KDO. A larger glycoform was ob-

TABLE 3. Negative-ion CE-MS data and proposed compositions for core OS from *P. multocida* strains VP161 (parent) and AL251 (mutant)[a]

Strain	$[M - H]^-$	$[M - H]^{2-}$	Observed mass of molecular ion	Calculated mass of molecular Ion	Relative intensity	Proposed composition
VP161	1,804.4	901.8	1,805.6	1,805.4	0.2	2*P*Cho + 4Hep + 3Hex + aKDO[b]
	1,822.4	910.9	1,823.1	1,823.4	1.0	2*P*Cho + 4Hep + 3Hex + KDO
		921.8	1,845.1	1,845.4	0.3	2*P*Cho + 4Hep + 3Hex + KDO + Na
	1,966.3	982.9	1,967.8	1,967.6	0.9	2*P*Cho + 4Hep + 4Hex + aKDO
	1,988.4	993.9	1,989.1	1,989.6	0.3	2*P*Cho + 4Hep + 4Hex + aKDO + Na
AL251	603.5		604.5	604.5	1.0	2Hep + aKDO
	621.5		622.5	622.5	0.9	2Hep + KDO
	765.5		766.5	766.7	0.8	Hex + 2Hep + aKDO
	1,447.5		1,448.5	1,448.2	0.1	*P*Cho + 3Hep + 3Hex + aKDO

[a] Average mass units were used for calculation of molecular weight based on proposed composition as follows: Hex, 162.15; Hep, 192.17; Kdo, 220.18; *P*Cho, 165.05. Relative intensity is expressed as relative height of either doubly or singly charged ions.
[b] aKDO, *anhydro*-KDO derivative.

served, consistent with the presence of an additional hexose residue (767 Da). Trace amounts of a more extended glycoform of 1,448 Da (*P*Cho + 3Hep + 3Hex + KDO) were observed, but no glycoforms were observed that contained the full parental complement of four Hep residues (Table 3). To characterize completely the nature of the truncation in the core OS of the mutant strain, ^{1}H-NMR experiments were performed on the parent- and mutant-derived core OS. Since MS data had suggested that a Hep residue was missing in the mutant OS and amino acid homology comparisons had suggested that $WaaQ_{PM}$ was a heptosyltransferase, close attention was paid to the resonances from the heptose residues of the molecule. Examination of the ^{1}H-NMR spectra revealed some heterogeneity for the heptose residues closest to KDO, as is often observed for core oligosaccharides due to rearrangement of the KDO residue under the acidic hydrolysis conditions used to obtain the core OS (Fig. 3). For the core OS derived from the parent strain, chemical shifts for the Hep II anomeric proton were identified at ~5.7 ppm, consistent with 2-substitution of this residue (Fig. 3a) (9). However, in the AL251 mutant OS ^{1}H-NMR spectrum (Fig. 3b), the anomeric resonance for the Hep II residue had shifted up-field about 0.5 ppm, consistent with this residue no longer being 2-substituted. The Hep II residue is now a terminal moiety, as we have observed previously for a *Haemophilus somnus* strain, and the chemical shifts to the H-4 resonance were consistent with this assignment (8). Definitive evidence for the structural nature of the mutation was obtained from two-dimensional NOESY experiments (Fig. 4). Characteristic nuclear Overhauser effects (NOEs) were observed for VP161 core OS between the Hep III and Hep II anomeric protons that are diagnostic for the α-1-2 linkage between the Hep III and Hep II residues (Fig. 4) (9). The NOESY spectrum of the AL251 mutant core OS confirmed the lack of 2-substitution of Hep II as the characteristic NOEs described above were absent, confirming that the

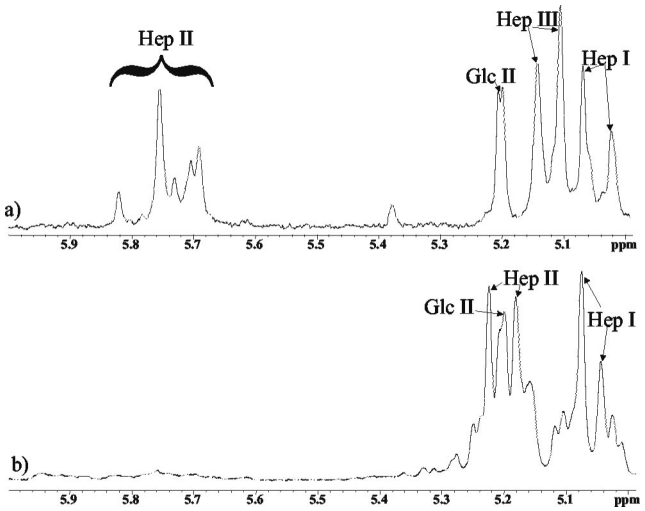

FIG. 3. Region of the ^{1}H-NMR spectrum of the core OS derived from the LPS of *P. multocida* parent strain VP161 (a) and *P. multocida* mutant strain AL251 (b). The spectra were recorded at 25°C and referenced against internal acetone at 2.225 ppm.

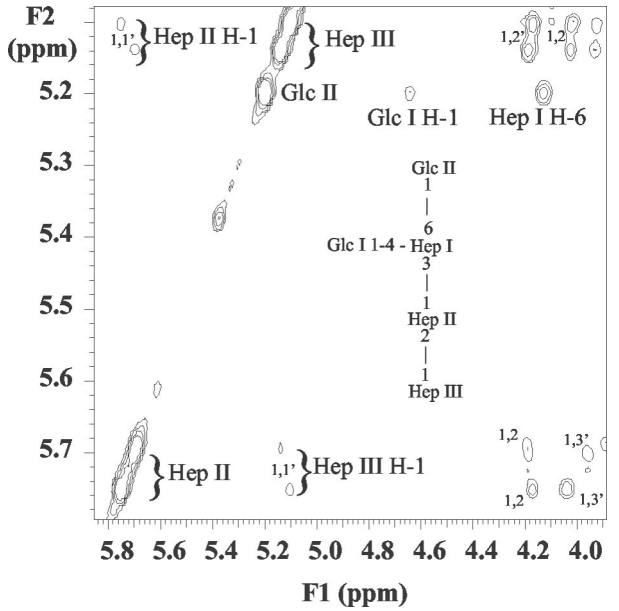

FIG. 4. Region of the NOESY spectrum of *P. multocida* VP161 core OS. NOE connectivities are as indicated. (Inset), Structure of the inner-core OS from VP161. The spectrum was recorded at 25°C and referenced against internal acetone at 2.225 ppm.

TABLE 4. ^{1}H-NMR chemical shifts for the Hep II and Hep III residues from the core OS derived from strains of *P. multocida* VP161 (parent) and AL251 (mutant)[a]

Strain and Hep	Chemical shift							NOEs[c]	
	H-1	H-2	H-3	H-4	H-5	H-6	H-7	Inter	Intra
VP161									
Hep II	5.76	4.17	3.85	3.83	3.60	4.05	3.76 3.65	5.11 Hep III H-1 4.04 Hep I H-3	4.17 H-2
Hep II	5.70	4.19	3.86	3.84	3.55	4.05	3.76 3.65	5.14 Hep III H-1 3.96 Hep I H-3	4.19 H-2
Hep III	5.14	4.02	3.87	3.83	3.78	4.05	3.77 3.65	5.70 Hep II H-1 4.19 Hep II H-2	4.02 H-2
Hep III	5.11	4.01	3.87	3.83	3.78	4.05	3.77 3.65	5.76 Hep II H-1 4.17 Hep II H-2	4.01 H-2
AL251									
Hep II	5.22	4.07	3.89	3.72	ND[b]	ND	ND	4.03 Hep I H-3	
Hep II	5.17	4.06	3.88	3.65	ND	ND	ND	ND	

[a] Recorded at 25°C in D_2O. Chemical shifts are referenced to internal acetone at 2.225 ppm. Two resonances were observed for each residue due to heterogeneity of the KDO molecule following core hydrolysis.
[b] ND, not determined.
[c] Inter, between residues; intra, with residues.

Hep III residue is no longer present in the mutant OS. Chemical shift and NOE data for the Hep II and Hep III residues for the parent OS and Hep II residue for the mutant OS are summarized in Table 4. Structural techniques have therefore demonstrated that the effect on the LPS structure of mutating gene *waaQ*$_{PM}$ is to preclude the addition of Hep III to Hep II (Fig. 5), and this function is consistent with strong similarity of the encoded protein to known heptosyltransferases.

DISCUSSION

The *P. multocida* LPS mutant AL251, first identified using STM in mice and chickens, was shown to have a transposon insertion in a predicted heptosyltransferase gene, *waaQ*$_{PM}$, and was significantly attenuated in chickens and mice (22). Silver-stained polyacrylamide gels of cell lysates from wild-type VP161 and AL251 showed that the LPS from the mutant was significantly truncated (Fig. 1A), consistent with *waaQ*$_{PM}$ encoding a heptosyltransferase responsible for the addition of a heptose molecule in the core region of the LPS structure.

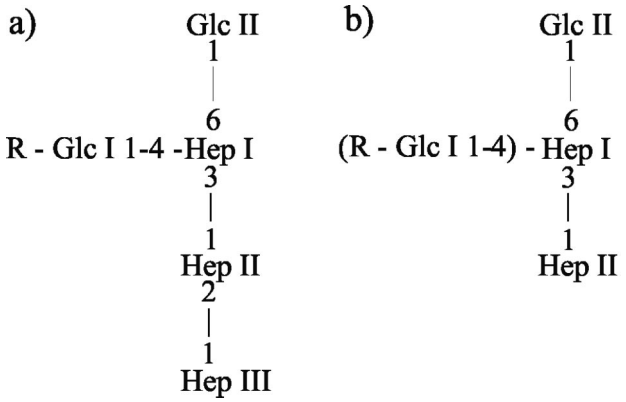

FIG. 5. Proposed structures of inner core LPS of *P. multocida* from parent strain VP161 (a) and mutant strain AL251 (b), where R is the OS chain extension beyond Glc. Based on negative-ion CE-MS data shown in Table 3, extension of the mutant LPS molecule to include the structures shown in parentheses occurs at only low frequency (less than 4%).

Analysis of the *P. multocida* Pm70 genome revealed that the *waaQ*$_{PM}$ gene was probably transcribed independently and therefore the truncated LPS structure and reduced growth in vivo in chickens and mice were due directly to the inactivation of *waaQ*$_{PM}$ and not to polar effects on downstream genes (27). This was confirmed by complementation, since the introduction of a wild-type *waaQ*$_{PM}$ gene in *trans* restored both the LPS structure (Fig. 1A) and wild-type levels of growth in mice.

Virulence trials using the LPS mutant AL251 in chickens resulted in a delayed onset of fowl cholera symptoms (Table 2), and *P. multocida* strains isolated from chickens with disease symptoms were tetracycline sensitive, indicating that they no longer carried the transposon. Nucleotide sequence data obtained from *P. multocida* DNA isolated from infected birds confirmed that the *waaQ*$_{PM}$ gene was intact and, in most cases, had nucleotide base changes at the point where the transposon had previously been inserted, resulting in two amino acid changes. The LPS from the *P. multocida* isolates recovered from the chickens challenged with AL251 showed that they produced wild-type LPS, confirming that the *waaQ*$_{PM}$ gene was functional despite the amino acid changes (Fig. 1B). Moreover, these results suggest that serine and aspartate, at amino acid positions 88 and 89, respectively, in WaaQ$_{PM}$, are not essential for full enzyme activity since nonconservative changes at these positions did not prevent the formation of full-length LPS. Taken together, these data indicate that the infection observed in chickens inoculated with AL251 was due only to revertant strains that had lost the transposon insertion. The wild-type *P. multocida* strain VP161 is a highly virulent organism, with fewer than 50 bacteria causing fowl cholera in chickens (44). Our observations measured the transposon excision rate from AL251 cells at 1% after the first 10 generations (overnight in vitro growth), rising to 4% after 58 generations (data not shown). This rate of excision would result in the generation and selection of wild-type revertants in chickens at a rate sufficient to cause lethal fowl cholera infection during the

course of the trial. We therefore conclude that a stable inactivation of $waaQ_{PM}$ would result in a *P. multocida* strain incapable of causing fowl cholera. However, a reliable method of constructing defined mutants in *P. multocida* has not yet been established.

Analysis of the *P. multocida* VP161 wild-type LPS indicated a "rough" LPS, similar to the LPS or lipooligosaccharide isolated from gram-negative mucosal pathogens such as *Haemophilus influenzae*, *H. ducreyi*, *Neisseria meningitidis*, and *N. gonorrhoeae*, with only a short nonrepeating polysaccharide unit attached to the lipid A (15). The inner-core structure of *P. multocida* LPS is similar to that described for *H. influenzae*, *Mannheimia haemolytica*, and *H. ducreyi*, with a triheptose unit linked via a KDO residue to lipid A (Fig. 4) (8, 28, 30). In the AL251 mutant, inactivation of $waaQ_{PM}$ resulted in the expression of a highly truncated LPS that lacked the third heptose molecule (Hep III) in the inner-core region (Fig. 2b). The most abundant glycoforms of LPS in the mutant also lacked all sugars distal to the first heptose, suggesting that the inactivation of $waaQ_{PM}$ prevented further sugar additions (Table 3). It is therefore probable that conformational changes in the LPS intermediates due to the lack of the third heptose largely prevented the action of subsequent transferases.

The loss of a full-length LPS molecule clearly affects the ability of the mutant AL251 to grow in mice and to cause disease in chickens. The specific reasons for this attenuation are not clear. However, in wild-type *P. multocida* VP161 LPS, two *P*Cho groups were identified, while the AL251 mutant contained only a single *P*Cho group in a very minor glycoform (Table 3). The presence of more than one *P*Cho residue in the VP161 LPS is unusual; bacteria with *P*Cho-decorated LPS usually have only a single residue attached, although the position of attachment onto the LPS structure varies. Only one other bacterium, a nontypeable *H. influenzae* strain, is known to have two *P*Cho residues attached to the LPS (M. K. Landerholm et al., unpublished observations). Interestingly, there are no *P*Cho groups on the LPS of the serotype A turkey isolate, PM70 (A. D. Cox, unpublished observations), and this strain is not virulent for chickens (I. W. Wilkie, unpublished observations).

*P*Cho groups are frequently attached to various bacterial structures on the surface of mucosal pathogens such as *H. influenzae*, *Actinobacillus actinomycetemcomitans*, *Streptococcus pneumoniae*, and *Neisseria* spp and play a key role in adhesion to and invasion of epithelial and endothelial host cells by binding to the platelet-activating receptor (10, 36–38). Nontypeable *H. influenzae* that has *P*Cho-positive glycoforms of LPS can attach to and invade human bronchial epithelial cells via a series of signaling events (38, 39). However, in both *H. influenzae* and *Neisseria* spp., although expression of *P*Cho on LPS was required for adhesion to and invasion of human epithelial cells, its presence reduced survival in some host niches, since strains expressing *P*Cho were more serum sensitive, mediated by binding of *P*Cho to C-reactive protein and subsequent activation of the complement system (37, 41). Interestingly, our studies have demonstrated that the LPS mutant AL251 is still highly resistant to the bactericidal action of complement in chicken serum, indicating that a completely wild-type LPS structure is not required for the bacteria to have full serum resistance. This is not surprising, since the $waaQ_{PM}$ mutant is still encapsulated and the hyaluronic acid capsule of *P. multocida* serotype A strains confers serum resistance (6).

Although it is likely that the observed attenuation of the $waaQ_{PM}$ mutant was due directly to the truncated LPS, we cannot completely exclude the possibility that pleiotropic effects, such as changes to the outer membrane structure, may play a role. However, a similar *E. coli* LPS mutant retained near-wild-type levels of outer membrane stability, as assessed by sensitivity to SDS and novobiocin (45).

In conclusion, we have characterized a *P. multocida* $waaQ_{PM}$ mutant that expresses a severely truncated LPS. We have determined the inner-core structure for both the wild-type *P. multocida* LPS molecule and the $waaQ_{PM}$ mutant molecule and demonstrated that a functional $waaQ_{PM}$ gene is required for the addition of the third heptose residue to the inner core of the LPS. Thus, the $waaQ_{PM}$ product has been identified as a heptosyltransferase III, and through virulence trials we have demonstrated that its activity is required for bacterial virulence in chickens, the natural host for this *P. multocida* strain. The inactivation of $waaQ_{PM}$ leads to the complete absence of the third heptose in the inner core and, as a result, to the fully wild-type glycoforms. The majority of glycoforms identified from the mutant LPS lack *P*Cho residues that in LPS of other gram-negative mucosal pathogens play a key role in bacterial virulence. Future work will focus on identifying the specific residues present on the LPS that are required for bacterial virulence.

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