

Pasteurella multocida Heddleston Serovar 3 and 4 Strains Share a Common Lipopolysaccharide Biosynthesis Locus but Display both Inter- and Intrastrain Lipopolysaccharide Heterogeneity

Marina Harper,^a Frank St. Michael,^b Marietta John,^a Evgeny Vinogradov,^b Jennifer A. Steen,^{a*} Lieke van Dorsten,^{b*} Jason A. Steen,^{a*} Conny Turni,^c Patrick J. Blackall,^c Ben Adler,^a Andrew D. Cox,^b John D. Boyce^{a,d}

Australian Research Council Centre of Excellence in Structural and Functional Microbial Genomics, Department of Microbiology, Monash University, Melbourne, Australia^a; Institute for Biological Sciences, National Research Council, Ottawa, Ontario, Canada^b; Queensland Alliance for Agriculture and Food Innovation, University of Queensland, Dutton Park, Brisbane, Australia^c; Department of Microbiology, Monash University, Melbourne, Australia^d

Pasteurella multocida is a Gram-negative multispecies pathogen and the causative agent of fowl cholera, a serious disease of poultry which can present in both acute and chronic forms. The major outer membrane component lipopolysaccharide (LPS) is both an important virulence factor and a major immunogen. Our previous studies determined the LPS structures expressed by different *P. multocida* strains and revealed that a number of strains belonging to different serovars contain the same LPS biosynthesis locus but express different LPS structures due to mutations within glycosyltransferase genes. In this study, we report the full LPS structure of the serovar 4 type strain, P1662, and reveal that it shares the same LPS outer core biosynthesis locus, L3, with the serovar 3 strains P1059 and Pm70. Using directed mutagenesis, the role of each glycosyltransferase gene in LPS outer core assembly was determined. LPS structural analysis of 23 Australian field isolates that contain the L3 locus revealed that at least six different LPS outer core structures can be produced as a result of mutations within the LPS glycosyltransferase genes. Moreover, some field isolates produce multiple but related LPS glycoforms simultaneously, and three LPS outer core structures are remarkably similar to the globo series of vertebrate glycosphingolipids. Our in-depth analysis showing the genetics and full range of *P. multocida* lipopolysaccharide structures will facilitate the improvement of typing systems and the prediction of the protective efficacy of vaccines.

Pasteurella multocida is a Gram-negative pathogen that causes a range of diseases in wild and domestic animals (1). These diseases include fowl cholera in poultry (2), hemorrhagic septicemia and shipping fever in ruminants (3), and atrophic rhinitis and respiratory disease in pigs (4), all of which cause significant economic losses to primary industries worldwide. *P. multocida* can also cause human infections following animal bites or close contact with domestic animals (5). Current classification of *P. multocida* strains combines Heddleston lipopolysaccharide (LPS) typing with Carter capsular typing, either by traditional serological methods or by multiplex PCR (6–8). *P. multocida* strains are currently classified into 5 capsular serogroups (A, B, D, E, and F) and 16 Heddleston LPS serovars (6, 8).

Protective immunity against *P. multocida* is generally considered to be mediated humorally, with protective antibodies directed primarily, but not exclusively, against the LPS antigen located on the surface of the *P. multocida* cell. Although both live and killed vaccines are available for protection against *P. multocida* infection, few afford good levels of protection against strains expressing different LPS (9, 10). Therefore, elucidating the full range of LPS structures expressed by *P. multocida* strains, including field isolates, and understanding the role of LPS in protective immunity are crucial for the formulation of effective, cross-protective vaccines.

The LPS structures produced by *P. multocida* type strains representing the Heddleston serovars 1, 2, 5, 8, 9, 13, and 14 have been determined, as well as the structure of the LPS expressed by the genome-sequenced serovar 3 strain Pm70 (11–17). Some *P. multocida* strains can produce two LPS inner core glycoforms simultaneously, termed glycoform A and glycoform B. The inner core

glycoform A is produced by all strains examined to date and contains a single phosphorylated 3-deoxy-D-manno-octulosonic acid (Kdo) residue that is substituted with a phosphoethanolamine (PEtn) residue (16). This glycoform also contains a second glucose residue (Glc II) attached to the 6 position of the first heptose (Hep I). The inner core glycoform B contains two Kdo residues and does not have the additional Glc on Hep I. LPS structural analysis of a range of *P. multocida* strains representing seven serovars has revealed that the most variable part of the molecule is the outer core (structure beyond Glc I). Analysis of the corresponding LPS outer core biosynthesis locus in each strain revealed that although some shared a nearly identical locus, they expressed different LPS molecules due to point mutations or deletions within LPS biosynthesis genes (11, 13). In this study, we report that the LPS outer core biosynthesis locus, first identified in Pm70 (17, 18) and named L3

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Address correspondence to John D. Boyce, john.boyce@monash.edu.

* Present address: Jennifer A. Steen, The University of Queensland, St. Lucia, Queensland, Australia; Jason A. Steen, The University of Queensland, St. Lucia, Queensland, Australia; Lieke van Dorsten, Eppendorf Nederland B.V., Nijmegen, Netherlands.

A.D.C. and J.D.B. are co-senior authors.

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in this study, is found in the serovar 3 and serovar 4 type strains (P1059 and P1662) and in 23 Australian field isolates. Each of these strains express the same conserved inner core structure, but the strains display significant variability in the length of the LPS outer core and in the number of LPS glycoforms produced simultaneously. We also determined the role of each of the L3 LPS outer core glycosyltransferase genes in the assembly of the LPS outer core.

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* was grown in nutrient broth, brain heart infusion (BHI), or heart infusion (HI) broth. Solid media were obtained by the addition of 1.5% (wt/vol) agar. When required, the media were supplemented with kanamycin (50 µg/ml), spectinomycin (50 µg/ml), or tetracycline (2.5 µg/ml). To isolate LPS from the *P. multocida* serovar 4 type strain (P1662), 2 liters of an early-log-phase culture of *P. multocida* (grown in BHI at 37°C with shaking at 200 rpm) was used to inoculate 24 liters of BHI in a 28-liter NBS fermenter. The culture was grown for 18 h at 37°C with shaking at 200 rpm, with aeration at 24 liters min⁻¹ and 20% O₂ saturation. Following treatment with hyaluronidase for 1 h (1 g; Sigma) to remove capsular material, phenol (2% final concentration) was added and the culture incubated at room temperature for 4 h to kill bacteria, which were then harvested with a Sharples continuous-flow centrifuge. For compositional analysis of LPS produced by the Heddleston serovar 3 type strain (P1059) and the Australian field isolates, small quantities of LPS were isolated from plate-grown cells as described previously (19).

DNA manipulations. Restriction digests and ligations were performed using enzymes and buffers obtained from NEB or Roche Diagnostics GmbH. Plasmid DNA and genomic DNA were prepared using a plasmid minikit from Qiagen and a genomic DNA extraction kit from RBC, respectively. PCR amplification of DNA was performed using *Taq* DNA polymerase or the Expand High Fidelity PCR system (Roche Diagnostics), and amplified fragments were purified using a Qiagen PCR purification kit. The oligonucleotides used in this study were synthesized by Sigma, Australia, and are listed in Table S1 in the supplemental material. Preparation of electrocompetent *P. multocida* and electroporation conditions were as described previously (20). DNA sequencing reactions and cycle conditions using either plasmid DNA or PCR products as templates were performed using BigDye Terminator, version 3.1 (Applied Biosystems), per the manufacturer's instructions, with the following modifications: 1.5 µl of 10× *Taq* PCR buffer (Roche) and 1 µl of BigDye Terminator, version 3.1, were used in a 15-µl (total volume) reaction mixture. For direct sequencing of *P. multocida* genomic DNA, reaction and cycle conditions were as described previously (21), with the following modifications: reactions were performed in a 40-µl volume with 3 µl 10× *Taq* PCR buffer (Roche), 13 pmol of oligonucleotide, and 4 µl of BigDye Terminator, version 3.1 (Applied Biosystems). All DNA sequences were determined on a capillary-platform genetic analyzer (model 3730; Applied Biosystems) and analyzed with Vector NTI Advance 11 (Invitrogen). Amino acid sequence alignments were conducted using BLAST and Clustal W2.

Construction of *P. multocida* TargeTron mutants. For inactivation of genes in *P. multocida* strain P1059, we used the TargeTron method (Sigma-Aldrich) of mutagenesis as described previously (20), but with the following modifications. To assist in the selection of *P. multocida* transformants containing an integrated intron, the plasmid pAL692 (20) was modified to introduce a kanamycin resistance gene, *aph3*, into the intron. Briefly, the *aph3* gene was amplified from pAL99 (Table 1) by using the primers BAP6796 and BAP6797 and then cloned into *Mlu*I-digested pAL692, generating the plasmid pAL953 (Table 1). Retargeting of the intron within pAL953 was performed per the instructions of the TargeTron user's manual, using oligonucleotides designed by the TargeTron design site (Sigma-Aldrich) (see Table S1 in the supplemental material). Each plasmid con-

taining an intron correctly targeted to the target gene (pAL1003, pAL1004, and pAL1006) (Table 1) was used to transform the *P. multocida* serovar 3 type strain (P1059) by electroporation; transformants containing the plasmid and/or integrated intron were selected on solid agar with kanamycin. Mutants were then cured of replicating plasmid by overnight growth in nutrient broth without antibiotic selection, and transformants containing the intron were identified by patching onto HI agar containing spectinomycin or kanamycin. Kanamycin-resistant, spectinomycin-sensitive transformants were then screened for the correct intron insertion by colony PCR using one oligonucleotide located within the target gene and one located within the intron (EBS universal). To confirm integration into the target gene and to determine if there were any additional intron insertions elsewhere in the genome, direct sequencing from genomic DNA was performed using the intron-specific EBS universal primer (Table 1) together with DNA isolated from each TargeTron mutant. Mutants that gave unambiguous sequencing data identical to the target gene sequence were selected for further study.

trans-Complementation of mutants. For complementation of the kanamycin-resistant TargeTron mutants, the *P. multocida* expression plasmid pAL99 was modified to remove the kanamycin resistance gene, *aph3*, and replace it with the *aadA* or *tetM* gene, conferring spectinomycin (pAL99S) or tetracycline (pAL99T) resistance, respectively (Table 1). The plasmid pAL99 was amplified using two outward-firing primers (BAP5358 and BAP5359 [see Table S1 in the supplemental material]) flanking *aph3*, digested with *EcoRV*, and then ligated to an *EcoRV*-digested PCR-amplified fragment containing either the *aadA* gene (amplified from pUA826 by using primers BAP5360 and BAP5361) or the *tetM* gene (amplified from Tn916 [in strain AL523] by using primers BAP7134 and BAP7135), generating pAL99S or pAL99T, respectively.

For complementation of each of the *P. multocida* mutants, PCR amplifications were performed using P1059 genomic DNA and the appropriate pair of oligonucleotides flanking the target gene (see Table S1 in the supplemental material). Each product was digested with the appropriate restriction enzyme and then ligated to appropriately digested pAL99, pAL99S, or pAL99T (Table 1), such that transcription of each gene would be driven by the constitutive *P. multocida* *tpiA* promoter. Each plasmid was then introduced into the corresponding *P. multocida* mutant, generating the complemented strain (Table 1). As a control, the appropriate empty vector was introduced separately into each mutant (Table 1).

Isolation and purification of lipopolysaccharide. LPS was isolated and purified as described previously (16). O-deacylated LPS (LPS-OH), core oligosaccharide (OS), and completely deacylated LPS were all isolated and purified from LPS as described previously (16).

Analytical methods, MS, and NMR spectroscopy. Sugars were determined as their alditol acetate derivatives, and linkage analysis was performed following methylation analysis by gas-liquid chromatography-mass spectrometry (GLC-MS) and electrospray mass spectrometry (ES-MS) as described previously. To determine exact locations and linkage patterns of residues, nuclear magnetic resonance (NMR) techniques were performed as described previously (16). The assignment of ¹H resonances was achieved using correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), and nuclear Overhauser effect spectroscopy (NOESY). The assignment of ¹³C resonances was achieved using ¹³C-¹H heteronuclear single-quantum coherence (HSQC) and ¹³C-¹H HSQC-TOCSY NMR. To identify the locations of PEtn substitutions, ³¹P-¹H heteronuclear multiple-quantum coherence (HMQC) experiments were performed (16).

Nucleotide sequence accession numbers. Sequence data for the P1059 and P1662 outer core biosynthesis loci have been deposited in GenBank under accession numbers KF314825 and KF314826, respectively.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant description	Source or reference ^a
Strains		
<i>E. coli</i> DH5 α	<i>deoR endA1 gyrA96 hsdR17</i> (r _K ⁻ m _K ⁺) <i>recA1 relA1 supE44 thi-1</i> Δ (<i>lacZYA-argF</i>)U169 ϕ 80d <i>lacZ</i> Δ M15 F ⁻	Bethesda Research Laboratories
<i>P. multocida</i> strains		
AL523	<i>P. multocida</i> VP161 strain containing Tn916 carrying <i>tetM</i>	This study
AL539	VP161 insertional <i>gatA</i> mutant generated using pAL290; no expression of <i>hptE</i> due to polar effects of the <i>gatA</i> insertion	This study
AL806	VP161 <i>gatA</i> mutant (AL539) containing pAL446	
AL2116	P1059 <i>gatF</i> (pm1141) TargeTron mutant of P1059	This study
AL2117	P1059 <i>natB</i> (pm1140) TargeTron mutant of P1059	This study
AL2155	P1059 <i>gatG</i> (pm1139) TargeTron mutant of P1059	This study
AL2192	P1059 <i>gatG</i> mutant containing pAL1022	This study
AL2297	P1059 <i>gatF</i> mutant containing pAL1046	This study
AL2299	P1059 <i>natB</i> mutant containing pAL1045	This study
AL2354	P1059 <i>natB</i> mutant containing vector pAL99T	This study
AL2355	P1059 <i>gatF</i> mutant containing vector pAL99T	This study
AL2395	P1059 <i>gatG</i> mutant containing vector pAL99S	This study
P1059	Heddlestone serovar 3 type strain	32
P1662	Heddlestone serovar 4 type strain	32
PM1	<i>P. multocida</i> turkey isolate	33
PM3	<i>P. multocida</i> turkey isolate	33
PM8	<i>P. multocida</i> turkey isolate	33
PM18	<i>P. multocida</i> chicken isolate	33
PM48	<i>P. multocida</i> chicken isolate	33
PM72	<i>P. multocida</i> chicken isolate	33
PM146	<i>P. multocida</i> chicken isolate	34
PM1075	<i>P. multocida</i> field isolate	MRG culture collection
PM1098	<i>P. multocida</i> field isolate	MRG culture collection
PM1099	<i>P. multocida</i> field isolate	MRG culture collection
PM1103	<i>P. multocida</i> field isolate	MRG culture collection
PM1120	<i>P. multocida</i> chicken isolate	MRG culture collection
PM1153	<i>P. multocida</i> chicken isolate	MRG culture collection
PM1205	<i>P. multocida</i> emu isolate	MRG culture collection
PM1258	<i>P. multocida</i> chicken isolate	MRG culture collection
PM1268	<i>P. multocida</i> chicken isolate	MRG culture collection
PM1320	<i>P. multocida</i> chicken isolate	MRG culture collection
PM1369	<i>P. multocida</i> chicken isolate	MRG culture collection
PM1434	<i>P. multocida</i> chicken isolate	MRG culture collection
PM1439	<i>P. multocida</i> chicken isolate	MRG culture collection
PM1441	<i>P. multocida</i> turkey isolate	MRG culture collection
PM1470	<i>P. multocida</i> turkey isolate	MRG culture collection
PM1474	<i>P. multocida</i> duck isolate	MRG culture collection
Plasmids		
pAL99	<i>P. multocida</i> expression plasmid (Kan ^r) which contains the <i>P. multocida</i> constitutive <i>Ptpi</i> promoter	35
pAL99S	<i>P. multocida</i> expression plasmid (Spec ^r); derivative of pAL99	This study
pAL99T	<i>P. multocida</i> expression plasmid (Tet ^r); derivative of pAL99	This study
pAL290	pUA826 derivative containing an internal fragment of <i>gatA</i>	This study
pAL446	Complete <i>gctC</i> and <i>hptE</i> genes from Pm70 cloned into pAL99	This study
pAL692	<i>P. multocida</i> plasmid (Spec ^r) containing TargeTron group II intron	20
pAL953	pAL692 with <i>aph3</i> (Kan ^r) inserted into TargeTron group II intron	This study
pAL1003	pAL953 retargeted to <i>natB</i> by using primers BAP6851/6852/6853	This study
pAL1004	pAL953 retargeted to <i>gatG</i> by using primers BAP6854/6855/6856	This study
pAL1006	pAL953 retargeted to <i>gatF</i> by using primers BAP6864/6865/6866	This study
pAL1022	Complete <i>gatG</i> gene cloned into pAL99S	This study
pAL1045	Complete <i>natB</i> gene cloned into pAL99T	This study
pAL1046	Complete <i>gatF</i> gene cloned into pAL99T	This study
pUA826	Mob ⁺ R6K replicon; single-crossover mutagenesis vector; Ap ^r Spc ^r	36

^a MRG, Microbiology Research Group, Queensland Alliance for Agriculture and Food Innovation, University of Queensland, Australia.

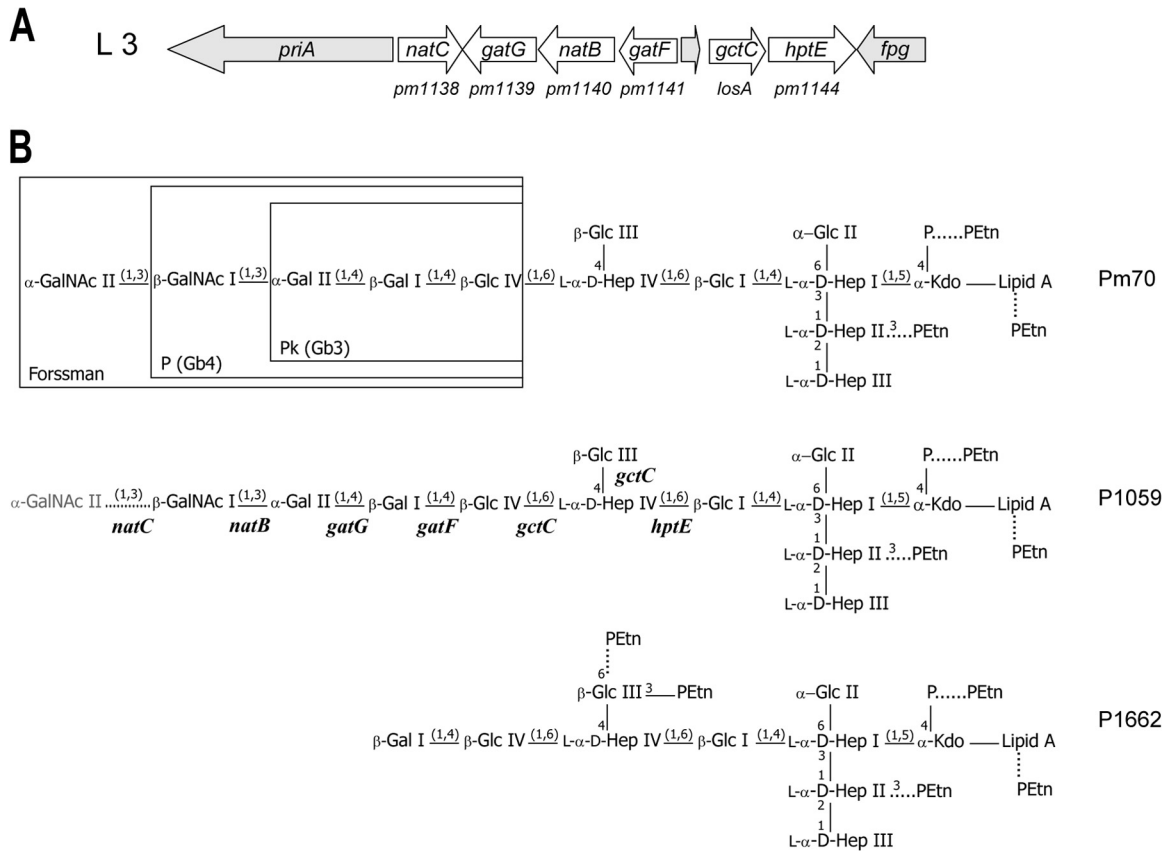


FIG 1 (A) Schematic representation of the genetic organization of the *P. multocida* L3 LPS outer core biosynthesis locus, which is common to serovars 3 and 4. Genes involved in LPS outer core assembly are shown in white. Conserved genes unrelated to LPS biosynthesis are shown in gray. Original gene annotation numbers for Pm70 are shown below the diagram. (B) Schematic representation of the LPS structure expressed by the *P. multocida* serovar 3 and 4 type strains (P1059 and P1662, respectively). Only inner core glycoform A is shown. The *P. multocida* glycosyltransferase genes predicted to be required for assembly of the outer core are shown below each linkage on the structure expressed by P1059. The rare addition of GalNAc II onto the P1059 LPS is shown with a dotted line and in gray. The specific number and position of phosphoethanolamine (PEtn) residues attached to each LPS structure are strain dependent; nonstoichiometric additions of PEtn are shown with dotted lines. Regions of the L3 LPS that are identical to the oligosaccharide components of the vertebrate glycosphingolipids, Forssman, P, and P^k, are shown boxed in the Pm70 structure. GalNAc, *N*-acetyl-galactosamine; Gal, galactose; Glc, glucose; Hep, heptose; PEtn, phosphoethanolamine; Kdo, 3-deoxy-*D*-manno-octulosonic acid; P, phosphate.

RESULTS

Analyses of LPS outer core biosynthesis loci. The region of the genome containing the LPS outer core biosynthesis locus in the serovar 3 and 4 type strains (P1059 and P1662, respectively) was sequenced, and the sequences were found to be 99% identical to each other and also to the equivalent locus in Pm70 (Fig. 1A) (18). However, the sequence of the locus in the serovar 4 type strain (P1662) contained a nonsense mutation within *natC* due to a C291A nucleotide substitution. In keeping with our function-based nomenclature of *P. multocida* LPS glycosyltransferases (19), we have named the orthologues of the Pm70 glycosyltransferase genes in the serovar 3 and 4 type strains *natC* (*pm1138*), *gatG* (*pm1139*), *natB* (*pm1140*), *gatF* (*pm1141*), *gctC* (*losA*), and *hptE* (*pm1144*) (Fig. 1A). The LPS outer core biosynthesis locus common to all three strains (Pm70, P1059, and P1662) was named L3 (Fig. 1A).

Structural analyses of *P. multocida* LPS derived from the serovar 3 and 4 type strains (P1059 and P1662). Sugar analysis of the purified LPS isolated from the serovar 4 type strain (P1662) revealed glucose (Glc), galactose (Gal), and *L*-glycero-*D*-mannoheptose (LD-Hep), in an approximate ratio of 2:1:3. A small

amount of *N*-acetylglucosamine (GlcNAc) was also identified, as is often observed for sugar analyses of LPS and presumably derives from lipid A. In order to separate and detect closely related glycoforms, capillary electrophoresis coupled with electrospray ionization mass spectrometry (CE-MS) was used to analyze the O-deacylated LPS (LPS-OH) (Table 2). This analysis revealed major triply charged ions at *m/z* 1,025.4 and 1,066.2, corresponding to a composition of 5Hex, 4Hep, Kdo-P, 2PEtn, and O-deacylated lipid A (lipid A-OH) for the smaller molecule and an additional PEtn moiety for the larger molecule. Glycoforms with one or no PEtn residues were also observed. Similarly, MS analysis of the nonfractionated core OS suggested a composition of 5Hex, 4Hep, 2PEtn, and Kdo as the major glycoform. Interestingly, there was no evidence of any glycoforms containing two Kdo residues (inner core glycoform B). Methylation analysis of the fractionated core OS (fraction 15) allowed determination of the linkage pattern of the molecule, which revealed the presence of terminal Glc, terminal Gal, 4-substituted Glc, and terminal LD-Hep in approximately equimolar amounts, with 6-substituted Glc, 2-substituted LD-Hep, 4,6-disubstituted LD-Hep, and 3,4,6-trisubstituted LD-Hep identified in smaller amounts.

TABLE 2 Negative-ion CE-ES-MS data and proposed compositions of O-deacylated LPS (LPS-OH) and core OS from the Heddleston serovar 3 and 4 type strains (P1059 and P1662), and proposed compositions of LPS-OH from the P1059 TargeTron mutants

Study and serovar (description)	Observed ions (<i>m/z</i>)				Molecular mass (Da) ^a		Relative intensity	Proposed composition
	(M - H) ⁻	(M - 4H) ⁴⁻	(M - 3H) ³⁻	(M - 2H) ²⁻	Observed	Calculated		
Serovar study								
Serovar 3 (P1059)			1,106.5		3,322	3,320.0	0.2	HexNAc, 6Hex, 4Hep, Kdo-P, PEtn, lipid A-OH
LPS-OH			1,147.3	1,721.2	3,444	3,443.0	1.0	HexNAc, 6Hex, 4Hep, Kdo-P, 2PEtn, lipid A-OH
		889.8	1,188.2		3,568	3,566.0	0.8	HexNAc, 6Hex, 4Hep, Kdo-P, 3PEtn, lipid A-OH
Serovar 4 (P1662)								
LPS-OH	707.4	943.5			2,833.6	2,831.6	0.2	5Hex, 4Hep, Kdo-P, lipid A-OH
	738.0	984.3		1,477.2	2,956.0	2,954.7	0.5	5Hex, 4Hep, Kdo-P, PEtn, lipid A-OH
	768.6	1,025.4		1,538.7	3,079.0	3,077.7	1.0	5Hex, 4Hep, Kdo-P, 2PEtn, lipid A-OH
	799.5	1,066.2			3,202.2	3,200.7	0.5	5Hex, 4Hep, Kdo-P, 3PEtn, lipid A-OH
Core OS				899.1	1,800.2	1,799.5	0.2	5Hex, 4Hep, aKdo ^b
				960.3	1,922.6	1,922.6	0.8	5Hex, 4Hep, PEtn, aKdo ^b
				1,022.1	2,046.2	2,045.7	1.0	5Hex, 4Hep, 2PEtn, aKdo ^b
GctC study								
AL539			709.7	1,064.9	2,131.9	2,130.9	0.2	Hex, 3Hep, 2Kdo, lipid A-OH
LPS-OH (<i>gatA</i> polar mutant)			717.0	1,075.9	2,153.9	2,152.9	1.0	2Hex, 3Hep, Kdo-P, lipid A-OH
LPS-OH			758.0	1,137.4	2,276.9	2,276.0	0.6	2Hex, 3Hep, Kdo-P, PEtn, lipid A-OH
			773.5	1,160.9	2,323.7	2,323.1	0.1	Hex, 4Hep, 2Kdo, lipid A-OH
			780.5	1,171.5	2,344.8	2,345.1	0.2	2Hex, 4Hep, Kdo-P, lipid A-OH
			822.1	1,233.2	2,469.2	2,468.2	0.1	2Hex, 4Hep, Kdo-P, PEtn, lipid A-OH
AL806 LPS-OH (AL539 + <i>gctC</i> , <i>hptE</i>)	691.8	923.2			2,772.1	2,770.5	0.5	3Hex, 4Hep, 2Kdo, PEtn, lipid A-OH
		930.4			2,794.2	2,792.4	0.3	4Hex, 4Hep, Kdo-P, PEtn, lipid A-OH
		971.4			2,917.6	2,915.5	1.0	4Hex, 4Hep, Kdo-P, 2PEtn, lipid A-OH
GatF study								
AL2116 LPS-OH (<i>gatF</i> mutant)	697.7	930.7	1,395.5		2,794.0	2,792.4	0.3	4Hex, 4Hep, Kdo-P, PEtn, lipid A-OH
	728.3	971.2	1,457.2		2,916.6	2,915.5	1.0	4Hex, 4Hep, Kdo-P, 2PEtn, lipid A-OH
AL2297 LPS-OH (<i>gatF</i> mutant + <i>gatF</i>)		1,174.0			3,525.0	3,523.2	0.4	2HexNAc, 6Hex, 4Hep, Kdo-P, PEtn, lipid A-OH
		1,215.0			3,648.0	3,646.3	1.0	2HexNAc, 6Hex, 4Hep, Kdo-P, 2PEtn, lipid A-OH
GatG study								
AL2155 LPS-OH (<i>gatG</i> mutant)	768.9	1,025.3			3,079.2	3,077.7	1.0	5Hex, 4Hep, Kdo-P, 2PEtn, lipid A-OH
	799.6	1,066.2			3,202.0	3,200.8	1.0	5Hex, 4Hep, Kdo-P, 3PEtn, lipid A-OH
AL2192 LPS-OH (<i>gatG</i> mutant + <i>gatG</i>)		1,106.5			3,322.5	3,320.0	1.0	HexNAc, 6Hex, 4Hep, Kdo-P, lipid A-OH
		1,147.5			3,445.5	3,443.1	0.9	HexNAc, 6Hex, 4Hep, Kdo-P, lipid A-OH
		1,174.0			3,525.0	3,523.2	0.3	2HexNAc, 6Hex, 4Hep, Kdo-P, PEtn, lipid A-OH
		1,215.0			3,648.0	3,646.3	0.4	2HexNAc, 6Hex, 4Hep, Kdo-P, 2PEtn, lipid A-OH
NatB study								
AL2117 LPS-OH (<i>natB</i> mutant)	768.7	1,025.4			3,079.0	3,077.7	0.8	5Hex, 4Hep, Kdo-P, 2PEtn, lipid A-OH
	809.4	1,079.5			3,241.5	3,239.9	1.0	6Hex, 4Hep, Kdo-P, 2PEtn, lipid A-OH
AL2299 LPS-OH (<i>natB</i> mutant + <i>natB</i>)		1,174.0			3,525.0	3,523.2	0.4	2HexNAc, 6Hex, 4Hep, Kdo-P, PEtn, lipid A-OH
		1,215.0			3,648.0	3,646.3	1.0	2HexNAc, 6Hex, 4Hep, Kdo-P, 2PEtn, lipid A-OH

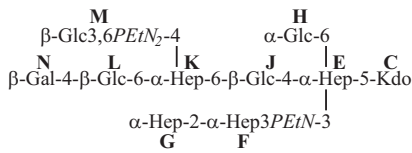
^a Average mass units were used for calculation of molecular mass based on the proposed composition, as follows: lipid A-OH, 952.00; Hex, 162.15; Hep, 192.17; Kdo, 220.18; anhydro Kdo (aKdo), 202.18; PEtn, 123.05; P, 79.95.

^b aKdo, hydrolysis products of Kdo formed during core hydrolysis.

In order to elucidate the exact locations and linkage patterns of the carbohydrate residues, NMR studies were performed on the core oligosaccharide fraction that gave the most resolved and homogeneous spectrum (fraction 14). The inner core oligosaccharide ¹H and ¹³C resonances were assigned with reference to the published data for the structurally related oligosaccharides from *P. multocida* strains belonging to serovars 1, 2, 3, and 5 and revealed that the serovar 4 strain elaborated the previously identified conserved inner core structure (Hep I to IV and Glc I and II) (Table 3) (12, 15–17).

The outer core residues of the oligosaccharide extension beyond Hep IV in strain P1662 were characterized by COSY, TOCSY, and NOESY experiments (Fig. 2). In addition to the residues of the conserved inner core structure, a glucose residue (Glc III) was identified, based on a characteristic spin system in TOCSY experiments. Two spin systems were actually identified for this residue, with proton resonances at H-1 (4.76 ppm), H-2 (3.53 ppm), H-3 (4.13 ppm), H-4 (3.67 ppm), H-5 (3.73 ppm) and H-6ab (4.23, 4.10 ppm) in one system and at H-1 (4.75 ppm), H-2 (3.51 ppm), H-3 (4.11 ppm), H-4 (3.60 ppm), H-5 (3.60 ppm),

and H-6ab (3.93, 3.75 ppm) in the other system. Both exhibited internuclear Overhauser effect (inter-NOE) connectivities from the anomeric protons to a resonance at 4.17 ppm (Fig. 2). This resonance was assigned as the proton at the 4-position of the Hep IV residue by virtue of ¹³C-¹H HSQC and ¹³C-¹H HSQC-TOCSY experiments (data not shown). The identification of two spin systems became apparent when ³¹P-¹H HMQC experiments were performed in conjunction with the homonuclear NMR data, which revealed the presence of two PEtn residues linked to both the H-3 (4.13 ppm) and H-6ab (4.23, 4.10 ppm) resonances in one spin system and to only the H-3 resonance (4.11 ppm) in the other system (Fig. 2). A further glucose residue (Glc IV) was identified, based on a characteristic spin system in TOCSY experiments, with H-1 (4.72 ppm), H-2 (3.44 ppm), H-3 (3.70 ppm), H-4 (3.68 ppm), H-5 (3.66 ppm), and H-6ab (3.98, 3.81 ppm) resonances being identified. An inter-NOE connectivity from the anomeric proton of Glc IV to a resonance at 4.33 ppm was identified (Fig. 2). This resonance was assigned as the proton at the 6-position of the Hep IV residue by virtue of ¹³C-¹H HSQC and ¹³C-¹H HSQC-TOCSY experiments (data not shown). This assignment, coupled

TABLE 3 ¹H- and ¹³C-NMR chemical shifts for the core OS from the *Pasteurella multocida* serovar 4 type strain, P1662^a

Residue	Chemical shift							Inter-NOE
	H-1 (C-1)	H-2 (C-2)	H-3 (C-3)	H-4 (C-4)	H-5 (C-5)	H-6 (C-6)	H-7 (C-7)	
Hep I (E)	5.03 (97.7)	3.97 (71.4)	3.89 (75.1)	4.22 (75.0)	3.65 (72.7)	4.16 (80.1)	3.88, 3.75 (63.3)	
Hep II ^b + 3-P (F)	5.82 (99.7)	4.28 (80.1)	4.42 (76.4)	4.08 (ND)	3.57 (ND)	ND	ND	3.89 Hep I H-3
Hep II, no 3-P (F')	5.65 (100.6)	4.18 (80.8)	3.89 (71.0)	3.89 (67.9)	3.57 (73.1)	4.04 (70.6)	3.75, 3.75 (64.6)	3.89 Hep I H-3
Hep III (G)	5.11 (102.8)	4.02 (71.7)	3.88 (71.8)	3.84 (67.3)	3.81 (72.8)	4.04 (70.6)	3.75, 3.75 (64.6)	4.18 Hep II H-2
Hep IV (K)	4.95 (100.3)	4.15 (70.6)	3.91 (70.7)	4.17 (78.1)	3.94 (71.3)	4.33 (80.2)	3.96, 3.78 (ND)	4.08 Glc I H-6a, 3.76 Glc I H-6b
β-Glc (Glc I) (J)	4.63 (104.4)	3.51 (74.4)	3.42 (78.0)	3.58 (70.8)	3.52 (74.9)	4.08, 3.76 (66.3)		4.22 Hep I H-4
α-Glc (Glc II) (H)	5.21 (102.5)	3.59 (73.1)	3.81 (74.2)	3.56 (69.8)	3.92 (72.8)	3.93, 3.76 (60.9)		4.16 Hep I H-6
β-Glc (Glc III) ^b + 3,6-P (M)	4.76 (103.4)	3.53 (74.0)	4.13 (82.0)	3.67 (69.7)	3.73 (75.4)	4.23, 4.1 (65.7)		4.17 Hep IV H-4
β-Glc (Glc III) ^b + 3-P (M')	4.75 (103.4)	3.51 (74.0)	4.11 (82.0)	3.60 (69.9)	3.60 (76.6)	3.93, 3.75 (61.9)		4.17 Hep I H-4
β-Glc (Glc IV) (L)	4.72 (104.5)	3.44 (74.2)	3.70 (75.4)	3.68 (79.7)	3.66 (75.8)	3.98, 3.81 (61.4)		4.33 Hep IV H-6
β-Gal (Gal I) (N)	4.76 (103.2)	3.56 (71.3)	3.67 (72.7)	3.94 (68.6)	3.73 (75.5)	3.80, 3.75 (62.0)		3.68 GlcIV H-4

^a Chemical shifts were recorded at 25°C and referenced to internal acetone at 2.225 ppm (¹H)/31.07 ppm (¹³C).

^b Signals for ethanolamine of phosphoethanolamine at F-3, M-3, and M-6 were observed at 4.19/63.1 and 3.31/41.3.

with the data for the Glc III residue, is consistent with the methylation analysis data which identified a 4,6-substituted LD-Hep residue. A terminal galactose residue (Gal I) was identified based on a characteristic spin system in TOCSY experiments, with H-1

(4.46 ppm), H-2 (3.56 ppm), H-3 (3.67 ppm), and H-4 (3.94 ppm) resonances being identified. An inter-NOE connectivity from the anomeric proton of Gal I to a resonance at 3.68 ppm was identified (Fig. 2). This resonance was assigned as the proton at the 4-position of the Glc IV residue by virtue of ¹³C-¹H HSQC and ¹³C-¹H HSQC-TOCSY experiments (data not shown). This assignment is also consistent with the methylation analysis data, which identified a 4-linked Glc residue and a terminal Gal residue. The identification of two PEtn residues substituting the 3- and 6-positions of Glc III, along with evidence for a PEtn residue at the 3-position of Hep II in some glycoforms, combined with the identification of only 2 PEtn residues in total in the core OS, led us to examine closely the ³¹P-¹H heteronuclear and homonuclear NMR data (Fig. 2). It became clear that the PEtn at the 6-position of the Glc III residue was present nonstoichiometrically; however, the PEtn residue at the 3-position was found to always be present. Furthermore, the PEtn residue was identified at the 3-position of the Hep II residue (in the inner core) approximately 20% of the time. However, the simultaneous expression of all three PEtn residues in the core OS was not identified, perhaps suggesting a mutual exclusivity. Taken together, these data show that the serovar 4 type strain (P1662) produces LPS that is a truncated derivative of the Pm70 LPS (17), with the exception of one or two additional PEtn residues on Glc III of the P1662 outer core (Fig. 1B).

As the full structure of the LPS core oligosaccharide from the serovar 3 strain Pm70 has been determined previously (17), only CE-MS analysis of LPS-OH was conducted on the serovar 3 type strain (P1059). Analysis of the P1059 LPS revealed that all LPS glycoforms lacked the terminal HexNAc; CE-MS analysis of LPS-OH revealed a major triply charged ion at *m/z* 1,147.3, corresponding to a composition of HexNAc, 6Hex, 4Hep, 2PEtn, Kdo, and lipid A-OH (Table 2). To confirm this finding, we undertook CE-MS analysis of LPS-OH isolated from two independent freeze-dried cultures of strain P1059 that had been sourced from different collections and found that the LPS composition consisted mainly of HexNAc, 6Hex, 4Hep, 2PEtn, Kdo, and lipid A-OH, but trace amounts of the 2HexNAc, 6Hex, 4Hep, 2PEtn,

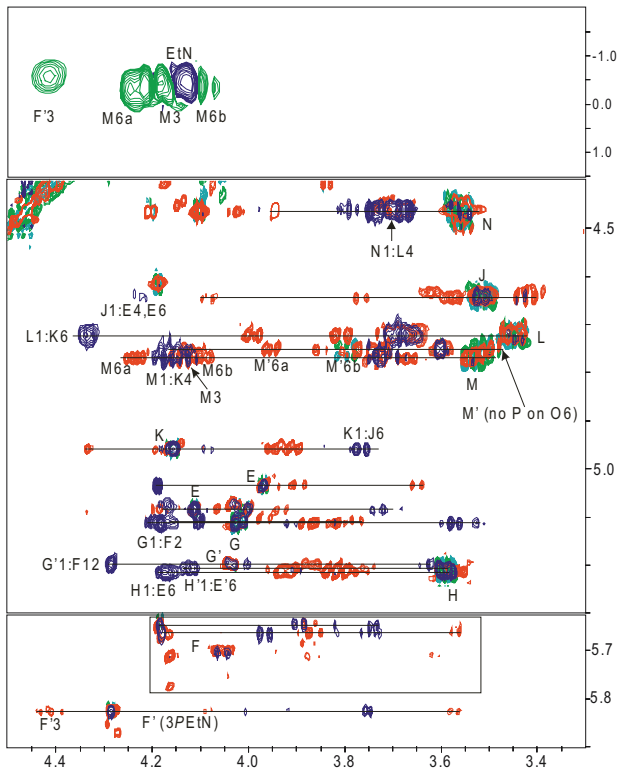


FIG 2 NMR analyses of core OS fraction 14 from *P. multocida* Heddleston serovar 4 strain P1662. Regions of overlapped COSY (green), TOCSY (red), and NOESY (purple) spectra (bottom two panels) and the ¹H-³¹P HMQC (green and purple) spectrum (top panel) of the core OS are shown. Residues are labeled as indicated in Table 3.

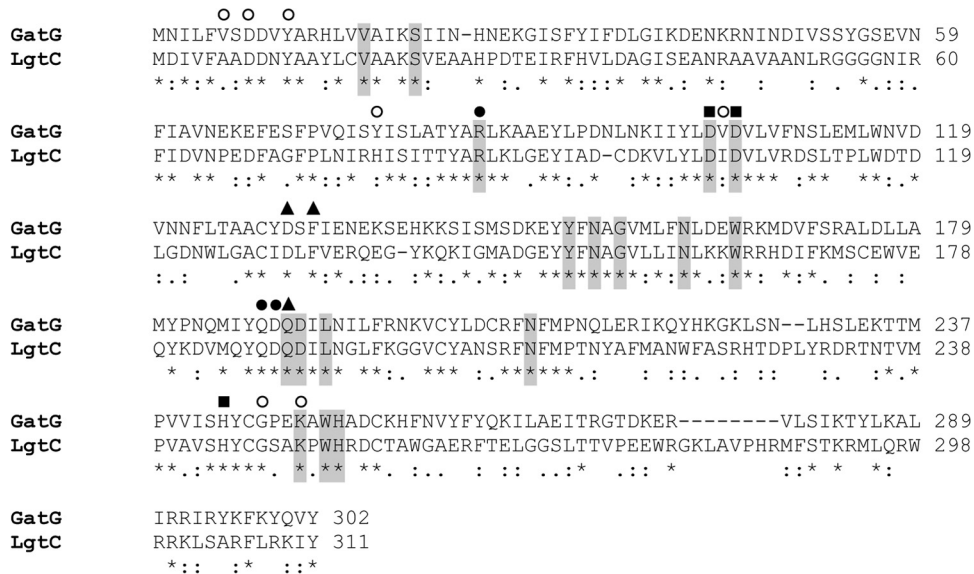


FIG 3 Amino acid sequence alignment of GatG (PM1139) from *Pasteurella multocida* strain Pm70 (Swiss-Prot accession no. Q9CLS0) aligned with the galactosyltransferase LgtC from *Neisseria meningitidis* (Swiss-Prot accession no. P96945). Amino acids that are highly conserved within LgtC-related glycosyltransferases are highlighted in gray (23). LgtC amino acids involved in binding to Mn^{2+} are marked with squares. Amino acids in LgtC known to interact with the UDP or galactose region of the nucleotide sugar UDP-galactose are marked with open or filled circles, respectively, while those involved in binding to the acceptor molecule are marked with triangles (23).

Kdo, and lipid A-OH glycoform were detected in both samples (data not shown). A trace amount of the inner core glycoform B (HexNAc, 5Hex, 4Hep, 2Kdo, PEtn, and lipid A-OH) was also detected in one of the samples (data not shown).

Characterization of the glycosyltransferases involved in biosynthesis of the L3 LPS outer core. The functions of two glycosyltransferases (HptE and NatC) encoded within the L3 LPS outer core biosynthesis locus were determined previously: HptE is required for the addition of the first outer core sugar, Hep IV (19), and NatC (annotated as PM1138 in strain Pm70) is an *N*-acetylgalactosyltransferase required for the addition of the last outer core sugar, GalNAc II (22) (Fig. 1). Previous bioinformatic analysis of the equivalent region in strain Pm70 indicated that *pm1141* (glycosyltransferase family 25) and *pm1139* (glycosyltransferase family 8) likely encode 1,4-galactosyltransferases that add Gal I and Gal II, respectively, and *pm1140* (glycosyltransferase family A) encodes an *N*-acetyl-galactosyltransferase that adds GalNAc I (17).

To confirm the predicted functions of these enzymes, the *pm1141*, *pm1139*, and *pm1140* orthologues (*gatF*, *gatG*, and *natB*, respectively) were separately inactivated in the serovar 3 type strain (P1059), and the effect of each mutation on LPS structure was determined (Table 2). To confirm that any LPS structural changes were due specifically to inactivation of the mutated glycosyltransferase, each mutant was complemented with a functional copy of the appropriate transferase provided in *trans*. Structural analysis of the mutants revealed that the *gatF* mutant (AL2116) was unable to assemble LPS beyond Glc IV and that the *gatG* mutant (AL2155) was unable to assemble LPS beyond Gal I (Fig. 1; Table 2), confirming bioinformatic predictions that *GatF* is the galactosyltransferase which adds Gal I to the 4th position of the Glc IV and *GatG* is a galactosyltransferase which adds Gal II to the 4th position of Gal I (Fig. 1). Similarly, analysis of the LPS produced by the P1059 *natB* mutant (AL2117) confirmed that *NatB* is the 1,3-*N*-acetyl-galactosyltransferase responsible for the

addition of GalNAc I to Gal II (Fig. 1; Table 2). Importantly, in each case, complementation experiments showed that mutants provided with a functional copy of the appropriate transferase in *trans* produced full-length LPS (Table 2), while mutants transformed with vector only did not (data not shown).

To determine the role of *gctC* in LPS outer core assembly, we used an LPS mutant, AL539, constructed in the serovar 1 strain VP161, which expresses only the highly conserved inner core LPS, including Glc I, which is the acceptor molecule for the HptE heptosyltransferase (19). This strain was transformed with the expression plasmid pAL446 containing a functional copy of the L3 glycosyltransferase genes *hptE* and *gctC* (PCR amplified from Pm70) (Table 1). MS (Table 2) and NMR structural analyses (data not shown) of the LPS produced by the complemented strain (AL806) revealed that it elaborated an LPS outer core of two terminal Glc residues linked to the 4- and 6-positions of Hep IV, confirming the role of the L3 HptE as the 1,6-heptosyltransferase and identifying that *GtcC* is a bifunctional glucosyltransferase that adds glucose to both the 4- and 6-positions of D-Hep IV.

Analysis of genetics underlying P1662 LPS truncation. The serovar 4 type strain, P1662, produced a truncated LPS structure terminating at Gal I, suggesting that the 1,4-galactosyltransferase, *GatG*, was nonfunctional in this strain. Analysis of the P1662 *gatG* sequence indicated that the translated product contained eight amino acid changes (R85K, V240A, A253D, N259K, R272K, E277K, V279M, and T284N) compared to the functional *GatG* glycosyltransferases expressed by Pm70 and P1059. The *Neisseria meningitidis* galactosyltransferase LgtC is 39% identical (57% conserved) to *GatG*, and the crystal structure of LgtC has been determined in complex with both its donor and acceptor molecules (23). Alignment of LgtC with other family 8 glycosyltransferases, including *GatG* (PM1139), identified a number of highly conserved amino acids critical for interaction with the donor molecule, the acceptor molecule, or the divalent cation Mn^{2+} (23)

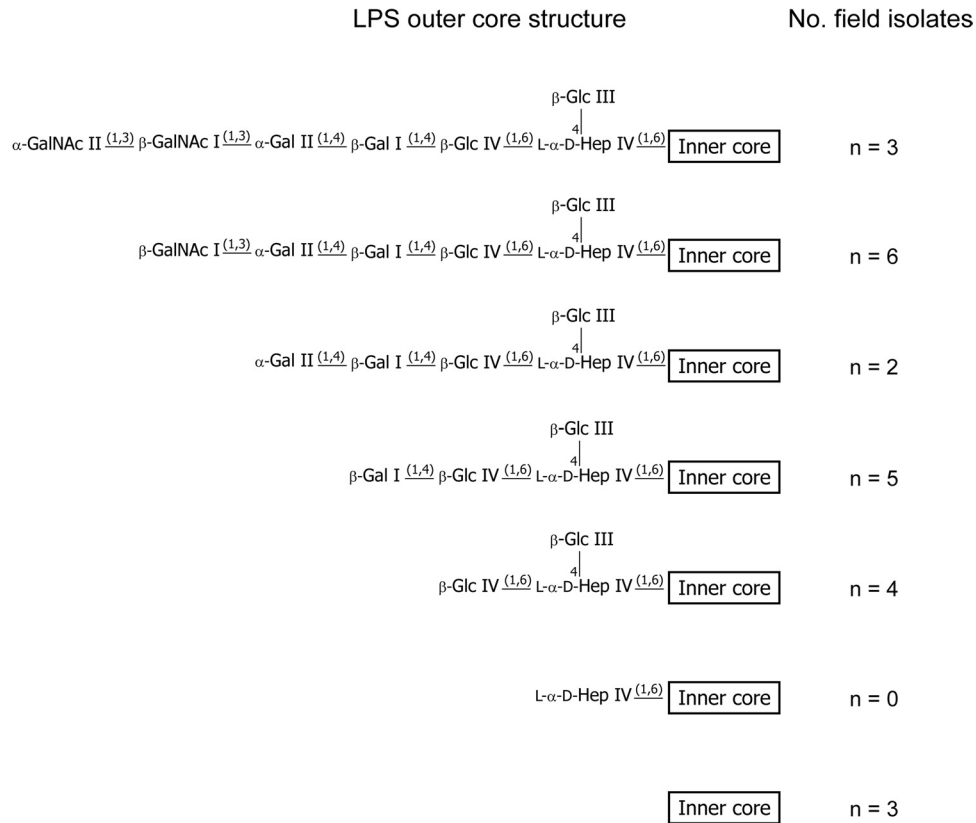


FIG 4 Schematic representation showing the range of LPS outer core structures expressed by the 23 Australian field isolates containing the L3 outer core biosynthesis locus. Only the longest LPS outer core structure is shown for strains expressing multiple LPS glycoforms. The specific number and position of phosphoethanolamine (PEtn) residues attached to each LPS structure are strain dependent and are not shown. GalNAc, *N*-acetyl-galactosamine; Gal, galactose; Glc, glucose; Hep, heptose.

(Fig. 3). Only one of the eight *GatG* substitutions identified in strain P1662 (R85K) was in a highly conserved position (R86 in *LgtC*), and this residue is critical for the interaction of *LgtC* with the galactose portion of the donor. Therefore, we suggest that the R85K mutation is responsible for the loss of *GatG* function in P1662.

Structural and genetic analyses of LPS biosynthesis in *P. multocida* field isolates belonging to genotype L3. To determine the diversity of LPS structures produced by *P. multocida* field isolates belonging to LPS genotype L3, we identified 23 *P. multocida* isolates from Australian poultry farms that contained L3 LPS outer core biosynthesis loci. Proposed compositions determined from CE-MS analysis of LPS-OH purified from each of the isolates (see Table S2 in the supplemental material) indicated that a number of different LPS structures were produced, ranging from inner core-only structures (terminating at Glc I) to full-length structures terminating at GalNAc II (Fig. 4; Table 4). Interestingly, 14 of the isolates simultaneously expressed multiple LPS glycoforms containing outer core sugar extensions ranging from 3 to 7 residues (see Table S2), and none of the field isolates analyzed elaborated an LPS structure terminating with Hep IV (Fig. 4; see Table S2).

To determine if the truncated LPS structures observed in the field isolates were the result of mutations within one of the glycosyltransferase genes in the L3 LPS outer core biosynthesis locus, the glycosyltransferase gene predicted to be responsible for the

arrested assembly in each case was amplified by PCR, and the nucleotide sequence was determined using the same oligonucleotides used for complementation experiments (Table 1). The translated amino acid sequences were then compared with those of the functional orthologs in P1059 and Pm70. In eight of the field isolates, there were clear mutations resulting in an immediate stop codon or a reading frameshift leading to a stop codon (PM48, PM1075, PM1120, PM1103, PM1205, PM1258, PM1434, and PM1439) (Table 4). In five field isolates (PM1, PM3, PM8, PM1369, and PM1474), there were no changes detected in the gene examined, although one isolate (PM1) had nucleotide changes and deletions in the predicted promoter region. However, seven of the field isolates contained one or more missense mutations in the genes encoding the predicted inactivated transferase; two strains (PM18 and PM146) contained mutations within *gatF*, two had changes in *natC* (PM1099 and PM1441), and three (PM1268, PM1320, and PM1098) contained mutations in *gatG* (Table 4).

As noted previously, the structure of *N. meningitidis* *LgtC* (a *GatG* ortholog) is known, and alignments of *GatG* from PM1098, PM1268, and PM1320 (each containing missense mutations) with *LgtC* revealed that there were amino acid changes located in close proximity to residues that are known to be critical in *LgtC*. These residues included those required for *LgtC* to interact with the UDP portion of the sugar donor (I104, G247, and K250), the divalent cation Mn^{2+} (D103, D105, and H244), or the acceptor

TABLE 4 Detected mutations in LPS outer core biosynthesis loci of *P. multocida* field isolates

Longest LPS outer core structure detected ^a	Predicted gene disrupted	Field isolate	Detected mutation(s) ^b
No detectable outer core	<i>hptE</i>	PM1075	Q229*
		PM1120	Q194*
		PM1258	Single nucleotide deletion at position 61
Hep·[Glc]·Glc	<i>gatF</i>	PM3	None detected
		PM8	None detected
		PM18	A205T
		PM146	S41P, V138M
Hep·[Glc]·Glc·Gal	<i>gatG</i>	PM1098	C128Y
		PM1205	Insertion of 42 nucleotides at position 353
		PM1268	I50V, K98R , A253D , D254N , N259K , R272K, E277K, V279M, T284N, A288D, F297L
		PM1320	I50V, K98R , A253D , D254N , N259K , R272K, E277K, V279M, T284N, A288D, F297L
PM1439	14 nucleotides deleted, positions 496–509		
Hep·[Glc]·Glc·Gal·Gal	<i>natB</i>	PM1369	None detected
		PM1474	None detected
Hep·[Glc]·Glc·Gal·Gal·GalNAc	<i>natC</i>	PM1	Nucleotide changes/deletions in promoter region
		PM48	S97*
		PM1099	I132N, I206V
		PM1103	Deletion/rearrangement between nucleotides 186 and 276
		PM1441	K3R, F4L, N7H, G33K, E49K, N62D, F78L, N80S, N105D, E115G, V120I, Y168D, K173N, Q176R, R193Q, I194V, Q207K, Q281K, I231V, E236K, E284D, K285Q, E271A, I292M, K296T, L312I, N396K, S397R
PM1434	S97*		
Hep·[Glc]·Glc·Gal·Gal·GalNAc·GalNAc	None	PM72	All genes functional
		PM1153	All genes functional
		PM1470	All genes functional

^a [Glc], residue is attached to the main chain residues via a 1,4 linkage to Hep.

^b Compared to nucleotide and amino acid sequences of the equivalent functional orthologues in Pm70 and/or P1059. *, stop codon. GatG residue changes shown in bold are in close proximity to amino acids predicted to interact with the donor or acceptor molecule or the divalent cation Mn²⁺.

molecule (D130 and F132) (23) (Fig. 3). Therefore, it is highly likely that the missense mutations in GatG abrogate transferase function in these field isolates. There are no data available on the structure of GatF and NatC homologs in other bacteria, so it is not possible to predict if the missense mutations identified in *gatF* and *natC* were responsible for the altered LPS production observed in those strains.

DISCUSSION

The strains belonging to the L3 LPS genotype include the first genome-sequenced *P. multocida* strain, Pm70, and the Hedleston serovar 3 and 4 type strains (P1059 and P1662, respectively). Using LPS structural analysis of directed mutants followed by *trans*-complementation, the role of each of the uncharacterized transferases in the L3 locus (NatB, GatG, GatF, and GctC) was defined, and previous functional predictions were confirmed (17). Interestingly, P1059 elaborates only trace quantities of LPS glycoforms containing GalNAc II. However, when the P1059 *gatF*, *gatG*, and *natB* mutants were complemented with the appropriate wild-type genes, all produced significant levels of the full-length glycoform containing the terminal GalNAc II (Table 2). This finding strongly suggests that the efficiency of addition of GalNAc II by NatC is dependent on the rate of production of the appropriate

LPS acceptor molecule (extended to GalNAc I), which in turn is dependent on the efficiency of the transferases involved in the earlier steps of LPS assembly.

LPS structural analysis of the strains belonging to LPS genotype L3, which includes the serovar 3 and 4 type strains, Pm70, and the 23 field isolates analyzed here, revealed that all produced LPS containing the inner core glycoform A (containing Kdo-P), which is the most common LPS inner core structure produced by *P. multocida*. However, the inner core glycoform B (containing Kdo-Kdo), previously reported by us to be expressed in significant quantities in the serovar 1 strains VP161 and X73 (15, 16), was not detected in strain P1662 or in any of the L3 Australian field isolates, although glycoform B was detected in trace amounts in strain P1059 and was previously found in LPS isolated from Pm70 (17). Although all strains share the same LPS outer core biosynthesis locus, L3, the length of the LPS outer core structure varied considerably; Pm70 produced significant quantities of the fully extended LPS structure, whereas nearly all LPS glycoforms in strain P1059 lacked the terminal GalNAc II, while the longest of the LPS glycoforms produced by P1662 terminated at Gal I. While some of this variation is due to clear mutations inactivating genes within the L3 locus, some field isolates produced a fully extended LPS glycoform as well as up to four shorter, but related, outer core

structures. This intrastrain variation in LPS structure as the result of truncations in the outer core region has previously been reported for *Haemophilus influenzae* (24) but has not been observed in strains belonging to any of the other *P. multocida* serovars examined to date. There is no genetic evidence of any phase variation in *P. multocida*, but it is possible that the expression of multiple glycoforms from the same locus could be controlled posttranscriptionally via an unknown mechanism. Alternatively, MsbA, the ABC transporter responsible for “flipping” LPS across the inner membrane (25), may transport an unusually large number of LPS glycoforms before the outer core region of the LPS has fully extended.

The LPS produced by strains belonging to the L3 LPS genotype contains outer core structures remarkably similar to the oligosaccharide component of P, P^k, and Forssman antigen, found on the surfaces of many cell types in vertebrates (26). In birds, Forssman antigen has been identified in the vascular endothelial tissue, perivascular connective tissue, and hematopoietic organs, including the spleen (27). Forssman antigen is also present on the surfaces of chicken erythrocytes (28). Notably, P and P^k antigen mimicry has been reported for other pathogens that express O-antigen-deficient LPS, including *Campylobacter jejuni* (29), *Haemophilus influenzae* (30), and *Neisseria* spp. (31).

In summary, we have shown that fowl cholera strains containing the LPS outer core biosynthesis locus L3 display a remarkable level of inter- and intrastrain LPS structural heterogeneity. This group includes the serovar 3 and 4 type strains (P1059 and P1662) as well as the first genome-sequenced strain, Pm70. This level of LPS diversity, particularly within a single strain and derived from a single LPS outer core biosynthesis locus, has not been observed in any other *P. multocida* strains examined to date (37). The role that these multiple LPS glycoforms play in disease progression and immunity is not known, but three of the outer core structures expressed are identical to the carbohydrate structure in the globo series of vertebrate glycosphingolipids, i.e., P, P^k, and Forssman antigen. LPS is considered a major immunogen of Gram-negative bacteria, but as the vertebrate immune system is tolerant of self-antigens, there may be a failure to respond appropriately to strains expressing these antigens. Thus, it is likely that the expression of L3 LPS molecules may aid the *in vivo* survival/persistence of bacteria through avoidance of the host immune system.

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