Structure and biosynthetic locus of the lipopolysaccharide outer core produced by *Pasteurella multocida* serovars 8 and 13 and the identification of a novel phospho-glycero moiety

Marina Harper1,2,3, Frank St Michael4, Evgeny Vinogradov4, Marietta John2,3, Jason A Steen2,5, Lieke van Dorsten4,6, John D Boyce2,3, Ben Adler2,3, and Andrew D Cox4

1Australian Research Centre for Excellence in Structural and Functional Microbial Genomics, Monash University, Melbourne 3800, Australia.
2Department of Microbiology, Monash University, Victoria, Australia; and 3Institute for Biological Sciences, National Research Council, Ottawa, ON, Canada K1A 0R6

Received on September 20, 2012; revised on October 24, 2012; accepted on October 24, 2012

*Pasteurella multocida* strains are classified into 16 Heddleston serovars on the basis of the lipopolysaccharide (LPS) antigens expressed on the surface of the bacteria. The LPS structure and the corresponding LPS outer core biosynthesis loci of strains belonging to serovars 1, 2, 3, 5, 9 and 14 have been characterized, revealing a clear structural basis for serovar classification. However, several of these serovars are genetically related, sharing the same LPS outer core biosynthesis locus, but producing different LPS molecules as a result of mutations within LPS assembly genes. In this article, we report that the *P. multocida* type strains belonging to serovars 8 and 13 share the same LPS outer core biosynthesis locus and produce structurally related LPS molecules. Structural analysis of the serovar 8 LPS revealed an inner core that is conserved among *P. multocida* strains and the following outer core structure:

\[
\alpha-(1\rightarrow6)-(15)\text{GalNAc}-(1\rightarrow4)\beta-(1\rightarrow3)\beta-(1\rightarrow6)\text{Gal}-(1\rightarrow4)\text{L}-(1\rightarrow6)\alpha-(1\rightarrow6)\text{Hep}-(1\rightarrow6)
\]

where X is a unique phospho-glycero moiety, 1-((4-aminobutyl)amino)-3-hydroxy-1-oxopropan-2-yl hydrogen phosphate, attached to the sixth position of (15)GalNAc. For serovar 13, the LPS structure is the same except for the absence of the terminal phospho-glycero moiety. Analysis of the common outer core biosynthesis locus from the serovar 8 and 13 type strains identified three genes that we predict are involved in the biosynthesis of this terminal moiety. Furthermore, bioinformatic comparisons with the characterized LPS outer core glycosyltransferases from *Actinobacillus pleuropneumoniae* serovar 1, strain 4074, allowed us to assign a function for each of the glycosyltransferases encoded within the serovar 8/13 LPS outer core biosynthesis locus.

Keywords: core oligosaccharide / genetics / LPS / *Pasteurella multocida* / structure

Introduction

*Pasteurella multocida* is a Gram-negative bacterium that is the causative agent of a number of economically important veterinary diseases, including fowl cholera (*Christensen and Bisgaard 2000*), hemorrhagic septicemia in cattle (*De Alwis 1992*) and atrophic rhinitis in pigs (*Chanter 1990*). *P. multocida* isolates are grouped into five Carter serogroups (A, B, D, E and F) on the basis of capsular antigens (*Carter 1952*) and into 16 Heddleston serovars on the basis of LPS antigens (*Heddleston et al. 1972*). Previously, we have determined the LPS structures and the genetic organization of the LPS outer core biosynthesis loci for strains representing serovars 1, 2, 3, 5, 9 and 14 (*St Michael, Li, Cox 2005; St Michael, Vinogradov et al. 2005; St Michael et al. 2009; Harper et al. 2011, 2012*). These data show that *P. multocida* LPS does not elaborate an O-antigen and that the outer core region is the most varied and distal part of the molecule. They also reveal that serovars 1 and 14 share the same outer core biosynthesis locus, but a mutation within the serovar 14 pcgA gene results in truncation of the LPS (Harper et al. 2011). Similarly, serovars 2 and 5 share the same LPS outer core biosynthesis locus and the LPS structures differ only in the presence or absence of phosphoethanolamine (PEtn) on the inner core heptose (Hep-II) (*St Michael et al. 2009*).

Previous compositional analysis of the LPS produced by the type strains belonging to serovars 8 and 13 identified glucose (Glc) and galactose (Gal) as common constituents but
t-glycero-d-manno-heptose (LD-Hep) was identified in the serovar 8 LPS but not in serovar 13 (Rimler et al. 1984). Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) separation of the LPS from the 16 different Heddleston serovars indicated that the serovar 8 and 13 type strains elaborate two of the largest LPS molecules (Rimler 1990). In this study, we report the full LPS structures of serovars 8 and 13 and show that LD-Hep is a component of the LPS elaborated by both strains. We also report the genetic organization of the LPS outer core biosynthesis locus, common to both serovars, that contains three genes predicted to be required for the biosynthesis and transfer of an unusual phospho-glycero moiety, 1-((4-aminobutyl)amino)-3-hydroxy-1-oxopropan-2-yl hydrogen phosphate. Furthermore, we identify a novel glycosyltransferase, NatA, predicted to be required for the addition of the open-chain galactosamine residue, (15)-2-acetamido-2-deoxy-d-galactose (15GalαNac).

Results
Structural analyses
Sugar composition analysis of the purified LPS from the type strains representing serovars 8 and 13 revealed the presence of Glc, Gal and LD-Hep in an approximate ratio of 2:1:3 in both strains.

Capillary electrophoresis-electrospray mass spectrometry (CE-MS) analyses of O-deacylated LPS (LPS-OH) from serovar 8 revealed a major triply charged ion at an m/z of 1131.23, which was assigned a composition of X, HexNAc, 4Hex, 4Hep, Kdo-P, 2PEtn, Lipid A-OH (Table I), where X was an unknown residue with a mass of ~275 atomic mass units. Larger and smaller glycoforms (m/z 1172.03 and 1090.23) consistent with the presence or absence of PEtn residues and glycoforms (m/z 1082.03 and 1123.53) consistent with 2Kdo, 3Hex glycoforms were also observed, but were in lower abundance. Similarly, MS analysis on the nonfractionated core OS suggested an OS composition of X, HexNAc, 4Hex, 4Hep, Kdo and X, HexNAc, 3Hex, 4Hep, Kdo with either one or two PEtn residues as the major glycoforms, consistent with the LPS-OH MS data (Table I).

CE-MS analyses of LPS-OH isolated from the serovar 13 type strain revealed a major triply charged ion at an m/z of 1039.23, which was assigned a composition of HexNAc, 4Hex, 4Hep, Kdo-P, 2PEtn, Lipid A-OH (Table I). Smaller amounts of a glycoform (m/z 1080.23) consistent with an additional PEtn residue and a glycoform (m/z 1032.03) consistent with a 2Kdo, 3Hex glycoform were also observed. Similarly, MS analysis on the nonfractionated core OS suggested a composition of HexNAc, 4Hep, 4Hex, Kdo and HexNAc, 3Hex, 4Hep, Kdo with either one or two PEtn residues, as the major glycoforms consistent with the LPS-OH MS data (Table I).

MS/MS studies were performed on the core OS from serovars 8 and 13 in positive ion mode. For serovar 8, selective ionization at an m/z of 1102.22, corresponding to the glycoform containing a single PEtn residue, resulted in a series of ions observed building up from the singly charged ion at an m/z of 257.04 that corresponds to the residue X (Figure 1A). For serovar 13, selective ionization at an m/z of 1044.22, corresponding to the glycoform containing two PEtn residues, resulted in a series of ions observed building up from the singly charged ion at an m/z of 204.04 that corresponds to a HexNAc residue (Figure 1B). An ion at an m/z of 286 was also identified and assigned as a Hex–PEtn moiety. The fragmentation pattern also indicated that the Hex–PEtn moiety was located between the Hep and Hex residues. Similar evidence for Hex–PEtn was observed for serovar 8 (data not shown).

The MS/MS data therefore gave a preliminary structure of HexNAc-Hex-(PEtn)-Hex-Hep-Hex extending from the first heptose residue in both serovars, with serovar 8 elaborating an additional residue X attached to the HexNAc residue.

Table I. Negative ion CE–ES–MS data and proposed compositions of O-deacylated LPS (LPS-OH) and core OS from the type strains representing P. multocida serovars 8 and 13

<table>
<thead>
<tr>
<th>Strain (serovar)</th>
<th>Observed Ions (m/z)</th>
<th>Molecular mass (Da)</th>
<th>Proposed composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(M-5H)-</td>
<td>(M-4H)-</td>
<td>(M-3H)-</td>
</tr>
<tr>
<td>P1581 (serovar 8)</td>
<td>–</td>
<td>811.0</td>
<td>1082.0</td>
</tr>
<tr>
<td>LPS-OH</td>
<td>–</td>
<td>817.4</td>
<td>1090.2</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>842.5</td>
<td>1123.5</td>
</tr>
<tr>
<td>678.4</td>
<td>–</td>
<td>848.2</td>
<td>1131.2</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>879.0</td>
<td>1172.0</td>
</tr>
<tr>
<td>Core OS</td>
<td>–</td>
<td>–</td>
<td>1027.9</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>–</td>
<td>1089.4</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>–</td>
<td>733.0</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>–</td>
<td>774.0</td>
</tr>
<tr>
<td>P1591 (serovar 13)</td>
<td>–</td>
<td>–</td>
<td>1032.0</td>
</tr>
<tr>
<td>LPS-OH</td>
<td>623.2</td>
<td>779.0</td>
<td>1039.2</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>809.8</td>
<td>1080.2</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>–</td>
<td>961.5</td>
</tr>
<tr>
<td>Core OS</td>
<td>–</td>
<td>–</td>
<td>970.5</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>–</td>
<td>980.8</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>–</td>
<td>1042.2</td>
</tr>
</tbody>
</table>

Average mass units were used for calculation of molecular weight based on proposed composition as follows: Lipid A-OH, 952.00; Hex, 162.15; Hep, 192.17; HexNAc, 203.19; Kdo, 220.18; PEtn, 123.05; X, 79.95. X = proposed phospho-glycero moiety (1-((4-aminobutyl)amino)-3-hydroxy-1-oxopropan-2-yl hydrogen phosphate).
Fig. 1. Positive-ion capillary electrophoresis-electrospray mass spectrum of *P. multocida* core OS: (A) MS/MS of an $m/z$ of 1102.2$^+$ from serovar 8; (B) MS/MS of an $m/z$ of 1044.0$^+$ from serovar 13. Inset are fragmentation patterns illustrating the fragment ions formed. For serovar 13, fragments from inner core fragmentation were also observed and are detailed.
Methylation analysis was performed on the fractionated core OS (Fr 16) from serovar 8 and Fr. 17 from serovar 13 in order to determine the linkage pattern of the molecule revealing similar profiles. The presence of terminal Glc, 6-substituted Glc, 3-substituted Gal, terminal LD-Hep, 4,6-di-substituted Gal and 4-substituted LD-Hep in approximately equimolar amounts was observed. Smaller amounts of 3,4-disubstituted LD-Hep and 3,4,6-trisubstituted LD-Hep were also observed.

In order to elucidate the exact locations and linkage patterns of the LPS from serovar 8, NMR studies were initially performed on a core OS fraction that gave the most resolved spectrum. The assignment of 1H resonances of the inner core residues for each serovar were achieved by COSY, TOCSY and NOESY experiments with reference to the published data for the structurally related oligosaccharides from P. multocida serovars 1, 3, 2 and 5 and revealed that the conserved inner core structure (Hep I-IV and Gic I-II) was present (Table II). The outer core residues of the oligosaccharide extension beyond the inner core in serovar 8 were characterized by COSY, TOCSY and NOESY experiments (data not shown) and the assignment of 13C resonances was achieved with 13C-1H HSQC (Figure 2) and 13C-1H HSQC-TOCSY experiments (Table II). This analysis revealed that the first three residues distal to Hep IV in the outer core of the serovar 8 LPS were identical to those found in the outer core of the LPS produced by the Actinobacillus pleuropneumoniae serovar 1 strain 4074 (St Michael et al. 2004). This trisaccharide of 1SGalaNAc-(1-4,6)-α-Gal II-(1-3)-β-Gal I linked to the 4-position of the Hep IV residue contains the rarely encountered open-chain galactosamine residue, 1SGalaNAc, that substitutes the adjacent Gal II residue at the 4- and the 6-positions. MS analyses had suggested that an unknown residue X was located distal to this trisaccharide, with an apparent molecular weight of 239/257/275. Chemical shifts not assigned to the conserved inner core and outer core trisaccharide were examined. This revealed signals consistent with the presence of dianobutane at 1.64 and 1.70 ppm correlating with carbon signals at 26.5 and 25.3 ppm, respectively, attributed to CH3 groups, along with signals at 3.02 and 3.33 ppm correlating with carbon signals at 40.4 and 39.6 ppm, respectively attributed to CH2NH2 groups. Furthermore, signals consistent with the presence of galactose were observed, namely a carbonyl moiety at 173.3 ppm, a substituted CHOH group with a proton resonance at 4.59 ppm and a carbon signal at 77.8 ppm and a CH2OH group with proton resonances at 3.92 ppm and a carbon signal at 63.8 ppm. Taken together, these data suggest that the 6-position of the N-acetylgalactosamine is further substituted to produce residue X (1-((4-aminobutyl) amino)-3-hydroxy-1-oxopropan-2-yl hydrogen phosphate). It was apparent from comparison of the H-6/C-6 resonances of the serovar 8 1SGalaNAc (3.96, 3.96/68.4) with the H-6/C-6 resonances of the terminal 1SGalaNAc residue of A. pleuropneumoniae (3.64, 3.66/64.1) that the 6-position of the serovar 8 1SGalaNAc was the likely attachment point of residue X (St Michael et al. 2004). A 31P-1H-HSQC-TOCSY experiment on the core oligosaccharide revealed correlations with the signals at 3.96 and 4.59 ppm, consistent with a phosphate moiety linking the 6-position of the 1SGalaNAc residue to the 2-position of the glyceric acid residue (Figure 2). Confirmation of the 3-position of Hep II as the location of PEtn substitution for serovar 8 was obtained from 31P-1H-HSQC and 31P-1H-HSQC-TOCSY experiments on the core oligosaccharide (Figure 2). Finally, a PEtn residue was also identified at the 6-position of the β-Gal residue by virtue of the correlation to the signal at 4.11 ppm, consistent with a phosphate moiety linking to the 6-position of the galactose residue. Comparison of the intensity of the signals consistent with the β-Gal residue elaborating or not elaborating a PEtn residue suggested that the PEtn residue was present ~50% of the time.

Genetic analyses
To identify the genes involved in serovar 8 and 13 LPS outer core assembly, we determined the sequence of the LPS outer core biosynthesis locus (located between the conserved genes pta4 and fpg) in each of the type strains, P1581 and P1591, respectively. Bioinformatic analysis of the serovar 8 and 13 LPS outer core biosynthesis loci revealed that they were highly conserved between the two strains, with over 99% identity at the nucleotide level. However, the serovar 8 locus contained eight open reading frames whereas the serovar 13 contained only seven open reading frames and one pseudogene (Figure 3B, Table III). Both loci encoded predicted glycosyltransferases with high levels of identity to the LPS outer core glycosyltransferases of the A. pleuropneumoniae serovar 1, strain 4074. This strain elaborates an LPS outer core structure that is identical to the P. multocida serovar 13 LPS outer core, with the exception that the fourth heptose is in an L,D configuration (Figure 3A) (St Michael et al. 2004; Ramjeet et al. 2005). The role of three of the four outer core glycosyltransferases expressed by A. pleuropneumoniae serotype 1 has been determined experimentally; AF143904_1 and AF143905_1 encode a galactose 1,3 transferase and a galactose 1,4 transferase, respectively and AF143905_2 encodes a n-glycero-n-manno-heptose-1,6 transferase (Galarneau et al. 2000; Ramjeet et al. 2005). Accordingly, we propose that the P. multocida GatE, which shares 74% similarity (52% identity) with the product of AF143904_1, is a galactose 1,3 transferase; GatC, which shares 89% similarity (74% identity) to the product of AF143905_1, is a galactose 1,4 transferase and HptE, which shares 70% similarity (52% identity) with the AF143905_2, is a LD-heptose1,6 transferase (Figure 3B, Table III) (Galarneau et al. 2000; Ramjeet et al. 2005). Supporting these predictions, HptE shares 94% similarity (90% identity) with the characterized P. multocida serovar 1 1,6-LD-heptosyltransferase and GatC shows 89% similarity (74% identity) with the P. multocida serovar 1 galactosyltransferase GatA (Harper et al. 2011). The fourth glycosyltransferase encoded within the P. multocida serovar 8 and 13 loci, designated NatA, shares 90% similarity (77% identity) with the product of the A. pleuropneumoniae appser1_10970 gene (Figure 3B). This enzyme belongs to the Group 1 family of glycosyltransferases, but its function has not been determined. As natA is the only glycosyltransferase gene within the LPS outer core biosynthesis locus that has not been assigned a role in the assembly of the...
LPS outer core, we predict that it encodes the glycosyltransferase required for 1SGal\textsubscript{a}NAc addition to Gal II. Supporting this prediction, the bacterial species \textit{Shewanella oneidensis} (strain MR-1) also elaborates an LPS structure with a 1SGal\textsubscript{a}NAc linked to Gal at the 4- and the 6-positions \cite{Vinogradov2003}. Bioinformatic analysis of the \textit{S. oneidensis} strain MR-1 genome, revealed a predicted glycosyltransferase (RefSeq. NP\_720196.1) that shared 76\% similarity (54\% identity) with

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
 & N & M & L & K & J & H & C \\
\hline
\textit{Z}= & & & & & & & \\
\hline
Hep I & 5.08–5.15 & 4.03–4.11 & 3.95–4.14 & 4.24 & 3.80 & 4.11 & nd \\
E & (95.5–102.8) & (71.6–72.6) & (72.0–74.9) & (75.1) & (73.1) & (80.6) & (nd) \\
\hline
Hep II\textsuperscript{a} & 5.75–5.86 & 4.31 & 4.40 & 4.08 & 3.57 & nd & nd \\
F & (99.7–100.0) & (80.3) & (76.6) & (66.5) & (74.0) & (nd) & (nd) \\
\hline
Hep III & 5.23 & 4.03 & 3.88 & 3.83 & 3.82 & nd & nd \\
E & (102.3) & (71.9) & (71.9) & (67.4) & (73.1) & (nd) & (nd) \\
\hline
\beta-Glc (Glc I) & 4.64 & 3.55 & 3.39 & 3.61 & 3.50 & 4.06 & 4.24 \\
J & (104.6) & (74.6) & (78.4) & (70.8) & (75.2) & (65.9) & (Hep I H-4) \\
\hline
\alpha-Glc (Glc II) & 5.20 & 3.59 & 3.82 & 3.59 & 3.92 & nd & 4.11 \\
H & (103.0) & (73.3) & (74.4) & (69.9) & (72.9) & (nd) & (Hep I H-6) \\
\hline
Hep IV & 5.09 & 4.00 & 4.09 & 4.04 & 3.83 & nd & 4.01, 3.71 \\
K & (100.7) & (70.5) & (70.4) & (78.3) & (71.3) & (Glc I H-6) & (nd) \\
\hline
\beta-Gal (Gal I) & 4.58 & 3.67 & 3.81 & 4.24 & 3.96 & 4.11 & 4.04 \\
L & (104.4) & (72.5) & (78.9) & (66.3) & (74.9) & (Hep IV H-4) & (11) \\
with 6-PEtn & & & & & & (Hep IV H-4) & (66.0) \\
\beta-Gal (Gal I) & 4.48 & 3.55 & 3.67 & 3.93 & 3.74 & nd & 4.04 \\
L & (104.1) & (72.3) & (73.8) & (69.9) & (76.6) & (Hep IV H-4) & (nd) \\
no PEtn & & & & & & (Hep IV H-4) & (nd) \\
\hline
\alpha-Gal (Gal II) & 5.19 & 3.98 & 4.07 & 4.26 & 4.15 & 4.13 & 3.81/3.67 \\
M & (97.9) & (69.3) & (69.3) & (77.2) & (64.6) & (Gal I H-3) & (70.0) \\
\hline
1SGalNAc\textsubscript{b,c} & 4.89 & 4.33 & 4.16 & 3.41 & 4.07 & 3.96 & 4.26 \\
N & (101.5) & (53.0) & (68.8) & (70.2) & (69.7) & (Gal II H-4) & (4.13, 4.01) \\
\hline
Z glyceric acid\textsuperscript{c} & 4.59 & 3.92 & & & & 3.96 & 4.26 \\
 & (173.3) & (173.3) & & & & (Gal II H-6) & (68.4) \\
\hline
Z diaminobutane\textsuperscript{c} & 3.33 & 1.64 & 1.70 & 3.02 & & & \\
 & (39.6) & (26.5) & (25.3) & (40.4) & & & \\
\hline
\end{tabular}
\caption{\textsuperscript{1}H- and \textsuperscript{13}C-NMR chemical shifts for the core OS from \textit{P. multocida} serovars 8 and 13}
\end{table}

Data were recorded at 25°C; referenced to internal acetone at 2.225/31.07 ppm.
\textsuperscript{a}Signals for ethanolamine of phosphoethanolamine at F-3 were observed at 4.19/63.1, 3.31/41.3.
\textsuperscript{b}Signals for acetate groups were observed at 2.08/23.4 ppm.
\textsuperscript{c}For serovar 13: H-6, (C-6) were 3.64, 3.66, (64.1) and residues labeled Z were absent.
the \textit{P. multocida} NatA and 77\% similarity (55\% identity) with the \textit{A. pleuropneumoniae} ppgC gene product.

Bioinformatic analysis of the unassigned genes within the \textit{P. multocida} locus revealed \textit{ppgA}, encoding a protein with shared identity with the cytidylyltransferase family of proteins, specifically glycerol-3-phosphate cytidylyltransferases, which transfer cytosine diphosphate (CDP) to glycerol-3-phosphate to form CDP-3-glycerol. The genes adjacent to \textit{ppgA} encode the proteins designated PpgB and PpgC, which share identity with asparagine synthase B (a member of the glutamine amidotransferase family) and LicD choline transferases, respectively. All three \textit{ppg} genes are completely absent from the genome of \textit{A. pleuropneumoniae} serovar 1, strain 4074, which elaborates an LPS outer core nearly identical in structure to the truncated outer core elaborated by the \textit{P. multocida} serovar 13. Therefore, we hypothesize that \textit{ppgA}, \textit{ppgB} and \textit{ppgC} are required for the biosynthesis of the terminal phospho-glycero moiety (identified as residue X in MS analysis). In support of this prediction, \textit{ppgC} in the serovar 13 type strain was shown to be a pseudogene, with a single deletion at nucleotide 290 resulting in a frame-shift. Thus, the absence of a functional copy of \textit{ppgC} in the serovar 13 outer core biosynthesis locus correlates with the lack of the terminal phospho-glycero moiety on the LPS outer core. Moreover, LPS compositional analysis of a serovar 13 field isolate, PM135, revealed that the terminal phospho-glycero moiety was absent from the LPS outer core. Genetic analysis revealed that the \textit{ppgA}, \textit{ppgB} and \textit{ppgC} genes were also absent from the LPS outer core biosynthesis locus because of a large deletion of 2210 nucleotides spanning the region from 18 nucleotides downstream of \textit{natA} to nucleotide 278 of \textit{ppgA}.

Fig. 2. NMR analyses of core OS from \textit{P. multocida} Heddleston serovar 8. Ring regions of $^3$P-$^1$H-HSQC-TOCSY (upper panel) and $^1$H-HSQC (lower panel) spectra of the core oligosaccharide. Phosphorylated residues are labeled as indicated in Table II.

**Discussion**

In this study we have determined the structure of the core oligosaccharides derived from the LPS of the type strains representing the Heddleston serovars, 8 and 13. The OS structure contains the conserved inner core structure that is present in LPS from all \textit{P. multocida} strains examined and is also consistently present in LPS from strains of \textit{A. pleuropneumoniae} (St Michael et al. 2004) and \textit{M. haemolytica} (Brisson et al. 2002; Logan et al. 2006). Both \textit{P. multocida} serovars 8 and 13 share the same LPS outer core structure; with the exception of a novel phospho-glycero moiety at the terminal end of the serovar 8 LPS. Thus, these studies have established that the LPS of serovars 8 and 13 are structurally related and clearly indicate that the terminal phospho-glycero moiety is serologically significant, allowing the two type strains to be differentiated using the Heddleston serotyping scheme.

The LPS outer core elaborated by the serovar 13 type strain (P1591) lacks the phospho-glycero moiety and is strikingly similar to the LPS outer core produced by the \textit{A. pleuropneumoniae} strain 4074. Moreover, bioinformatic analyses of the serovars 8 and 13 outer core biosynthesis locus revealed that three of the glycosyltransferases, HptE, GatC and GatE, are highly similar to the \textit{A. pleuropneumoniae} strain 4074 LPS glycosyltransferases whose functions have been determined (Galarneau et al. 2000; Ramjeet et al. 2005). We predict that the remaining LPS outer core glycosyltransferase, NatA, is a 1SGalNAc transferase, as homologs are present in \textit{A. pleuropneumoniae} strain 4074 and \textit{Shewanella oneidensis} (strain MR-1), both of which also elaborate an LPS with a 1SGalNAc residue linked to Gal at the 4- and the 6-positions (Vinogradov et al. 2003; St Michael et al. 2004).

We propose that the genes \textit{ppgA}, \textit{ppgB} and \textit{ppgC} are required for the biosynthesis and transfer of the novel phospho-glycero moiety to the terminal end of the serovar 8 LPS structure as the absence of this moiety in the serovar 13 type strain and strain PM135 correlates with an identified mutation in one or more of the \textit{ppg} genes. The corresponding LPS structure in \textit{A. pleuropneumoniae} strain 4074 also lacks this terminal moiety and there are no \textit{ppg} homologs on the \textit{A. pleuropneumoniae} genome. The biosynthetic steps required for synthesis of this product are unknown, but PpgC was identified as a putative glycerol-3-phosphate cytidylyltransferase, strongly suggesting that CDP-glycerol may be used in its synthesis (Table III).

In conclusion, the type strains representing the \textit{P. multocida} serovars 8 and 13 produce highly similar LPS structures and share the same outer core biosynthesis locus. However, the LPS produced by serovar 13 strains lack the terminal phospho-glycero moiety due to mutations within the \textit{ppg} biosynthesis genes. Bioinformatic analysis has allowed us to assign a predicted function to all of the glycosyltransferases encoded within the Heddleston 8/13 locus, including NatA, which we predict is a novel glycosyltransferase required for the addition of the open chain galactosamine, 1SGalNAc.

**Materials and methods**

\textit{Strain, media and growth conditions}  
The bacterial strains, plasmids and oligonucleotides used in this study are listed in Table IV. For LPS analysis,
\textit{P. multocida} type strains representing serovar 8 (P1581) and serovar 13 (P1591) were grown and killed as described previously (St Michael, Li, Cox 2005). For genetic manipulations, \textit{P. multocida} was routinely grown in brain heart infusion (BHI).

\textit{P. multocida} and \textit{Actinobacillus pleuropneumoniae} serovar 1 strain, 4074. The \textit{P. multocida} glycosyltransferase genes predicted to be required for the assembly of the outer core are shown below each linkage. Characterized \textit{A. pleuropneumoniae} glycosyltransferase genes are also shown. The \textit{P. multocida} serovar 13 LPS outer core structure is identical to that of serovar 8, but lacks the terminal phospho-glycero moiety (boxed). (B) Schematic representation of the genetic organization of the \textit{P. multocida} LPS outer core biosynthesis locus, common to both serovar 8 and 13, and the corresponding region in the \textit{A. pleuropneumoniae} serovar 1 genome (Genbank accession number ADOD01000015). The percentage identity between homologous proteins is shown beside the joining lines. LPS biosynthesis genes are shown in white, while genes unrelated to LPS biosynthesis are shown in gray. The boxed genes are predicted to be involved in the biosynthesis of the terminal phospho-glycero moiety and are absent in the field isolate PM135. The gene, ppgC, is nonfunctional in \textit{P. multocida} serovar 13 type strain P1591 because of a single frameshift mutation (asterisked). The \textit{A. pleuropneumoniae} gene numbers 10930-10890 are derived from the genome sequence NZ_ADOD01000015. The original glycosyltransferase gene names are also shown (AF143904_1, AF143905_1 and AF143905_2).

\textbf{Isolation and purification of LPS}

LPS was isolated and purified as described previously (St Michael, Li, Cox 2005). LPS was \textit{O}-deacylated, completely deacylated and core oligosaccharides (OS) were prepared as described previously (St Michael, Li, Cox 2005).

\textbf{Analytical methods, mass spectrometry and NMR spectroscopy}

Sugars were determined as their alditol acetate derivatives and linkage analysis determined following methylation analysis by GLC-MS as described previously (St Michael, Li, Cox 2005). ES-MS and nuclear magnetic resonance (NMR) experiments were performed as described previously (St Michael, Li, Cox 2005).

\textbf{Genetic analyses}

\textit{P. multocida} genomic DNA was prepared using the cetyltrimethyl ammonium bromide (CTAB) method (Ausubel et al.
Table III. Bioinformat-atic prediction of the role of each protein encoded within the P. multocida serovar 8/13 LPS outer core biosynthesis locus

<table>
<thead>
<tr>
<th>Gene</th>
<th>Predicted protein function</th>
<th>Predicted role in LPS biosynthesis/assembly</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpL31_2</td>
<td>50S ribosomal protein L31 α-1,3 galactosyltransferase</td>
<td>None</td>
</tr>
<tr>
<td>gatE</td>
<td>Family I glycosyltransferase</td>
<td>Galactosyltransferase, predicted to add Gal II to 3 position of Gal I</td>
</tr>
<tr>
<td>ppgC</td>
<td>LPS cholinephosphotransferase LiC family asparagine synthetase</td>
<td>Transfer of phospho-glycero moiety to LPS. Pseudogene in the serovar 13 strain, P1591.</td>
</tr>
<tr>
<td>ppgB</td>
<td>LPS glycerol-3-phosphate cytidylyltransferase</td>
<td>Involved in the biosynthesis of phospho-glycero moiety. Homologs catalyze the conversion of aspartate to asparagine.</td>
</tr>
<tr>
<td>ppgA</td>
<td>β-1,4 galactosyltransferase</td>
<td>Addition of Gal I to 4 position of Hep IV.</td>
</tr>
</tbody>
</table>

*1-((4-aminobutyl) amino)-3-hydroxy-1-oxopropan-2-yl hydrogen phosphate.

Table IV. Strains and oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Relevent description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
</tr>
<tr>
<td>P1581</td>
<td>Heddleston et al. (1972)</td>
</tr>
<tr>
<td>P1591</td>
<td>Heddleston et al. (1972)</td>
</tr>
<tr>
<td>Oligonucleotides</td>
<td></td>
</tr>
<tr>
<td>BAP3163</td>
<td>Reverse primer located at the 5’ end of rpL31_2. This study</td>
</tr>
<tr>
<td>BAP3166</td>
<td>GCAGGTCCAGTGCACGAGG Located within priA. Harper et al. (2011)</td>
</tr>
<tr>
<td>BAP3330</td>
<td>ACGAATATGTCATTGC Located within the 5’ end of fpg. Harper et al. (2011)</td>
</tr>
<tr>
<td>BAP4900</td>
<td>GGAAACGCCCATTGCTTTACG Forward primer located at the 3’ end of rpL31_2. This study</td>
</tr>
</tbody>
</table>

Genetic Analys. Sequencing chromatograms were analyzed and the LPS loci assembled using Vector NTI advance version 10. DNA and protein comparisons were made using the BLAST and ClustalW algorithms (Higgins et al. 1992; Thompson et al. 1994; Altschul et al. 1997). Sequence data for the P1581 and P1591 outer core biosynthesis loci have been deposited in GenBank under accession numbers JX987237 and JX987238, respectively.

Funding
This work was funded in part by the Australian Research Council, Canberra, Australia.

Acknowledgements
We thank our NRC-IBS colleagues, Jacek Stupak and Dr. Jianjun Li for mass spectrometry and Perry Fleming (core Biological Culture Facility) for large-scale biomass production. The P. multocida field isolate, PM135, was kindly provided by Dr Pat Blackall and Dr Conny Turni from the Queensland Alliance for Agricultural and Food Innovation.

Conflict of interest
None declared.

Abbreviations
1,5GalaNAc, (1,5)-2-acetamido-2-deoxy-o-galactose; BHI, brain heart infusion; CDP, cytosine diphosphate; CE-MS, capillary electrophoresis-electrospray mass spectrometry; CTAB, cetyltrimethyl ammonium bromide; Gal, galactose; Glc, glucose; GLC, gas-liquor chromatophathy; HSQC, heteronuclear single-quantum coherence; L-DHep, l-glycero-6-manno-heptose; LPS, lipopoly-saccharide; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; OH, O-deacylated; OS, oligosaccharides; PCR, Polymerase chain reaction; PEPN, phosphoethanolamine; SDS-PAGE, Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TOCSY, total correlation spectroscopy.

References

Polymerase chain reaction (PCR) amplification of DNA was performed using Taq DNA polymerase (Roche Diagnostics) and PCR products were purified using the Qiagen PCR Purification Kit. The DNA sequence of each LPS outer core locus was determined following PCR amplification, initially with oligonucleotides designed within the conserved flanking genes priA, rpL31_2 and fpg as listed in Table IV. Further primers for additional amplifications were designed within each newly sequenced region as required. The nucleotide sequence of the PCR fragments was determined for both strands using the Applied Biosystems 3730S Genetic Analys. Sequencing chromatograms were analyzed and the LPS loci assembled using Vector NTI advance version 10. DNA and protein comparisons were made using the BLAST and ClustalW algorithms (Higgins et al. 1992; Thompson et al. 1994; Altschul et al. 1997). Sequence data for the P1581 and P1591 outer core biosynthesis loci have been deposited in GenBank under accession numbers JX987237 and JX987238, respectively.


