Phosphoethanolamine substitution in the lipid A of *Escherichia coli* O157:H7 and its association with PmrC

Sang-Hyun Kim,1,2 Wenyi Jia,2 Valeria R. Parreira,1 Russell E. Bishop2† and Carlton L. Gyles1†

1Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada N1G 2W1
2Departments of Laboratory Medicine and Pathobiology and Biochemistry, University of Toronto, 6213 Medical Sciences Building, 1 King’s College Circle, Toronto, Ontario, Canada M5S 1A8

This study shows that lipid A of *Escherichia coli* O157:H7 differs from that of *E. coli* K-12 in that it has a phosphoform at the C-1 position, which is distinctively modified by a phosphoethanolamine (PEtN) moiety, in addition to the diphasphoryl form. The pmrC gene responsible for the addition of PEtN to the lipid A of *E. coli* O157:H7 was inactivated and the changes in lipid A profiles were assessed. The pmrC null mutant still produced PEtN-modified lipid A species, albeit in a reduced amount, indicating that PmrC was not the only enzyme that could be used to add PEtN to lipid A. Natural PEtN substitution was shown to be present in the lipid A of other serotypes of enterohaemorrhagic *E. coli* and absent from the lipid A of *E. coli* K-12. However, the cloned pmrC<sub>O157</sub> gene in a high-copy-number plasmid generated a large amount of PEtN-substituted lipid A species in *E. coli* K-12. The occurrence of PEtN-substituted lipid A species was associated with a slight increase in the MICs of cationic peptide antibiotics, suggesting that the lipid A modification with PEtN would be beneficial for survival of *E. coli* O157:H7 in certain environmental niches. However, PEtN substitution in the lipid A profiles was not detected when putative inner-membrane proteins (YhbX/YbiP/YijP/Ecf3) that show significant similarity with PmrC in amino acid sequence were expressed from high-copy-number plasmids in *E. coli* K-12. This suggests that these potential homologues are not responsible for the addition of PEtN to lipid A in the pmrC mutant of *E. coli* O157:H7. When cells were treated with EDTA, the amount of palmitoylated lipid A from the cells carrying a high-copy-number plasmid clone of pmrC<sub>O157</sub> that resulted in significant increase of PEtN substitution was unchanged compared with cells without PEtN substitution, suggesting that the PEtN moiety substituted in lipid A does not compensate for the loss of divalent cations required for bridging neighbouring lipid A molecules.

INTRODUCTION

Lipopolysaccharide (LPS) of Gram-negative bacteria is a complex amphipathic glycolipid constituting the outer leaflet of the outer membrane (OM) (Nikaido, 2003). Smooth LPS consists of a distal polysaccharide that confers serogroup-specific O-antigenicity, a proximal lipid A anchored to the OM, and a middle region called core oligosaccharide (Raetz & Whitfield, 2002). The structurally conserved lipid A molecule plays a central role as the permeability barrier to toxic substances in the growth environment (Nikaido & Vaara, 1985). The prototype structure of lipid A in *Escherichia coli* K-12 consists of a glucosamine disaccharide, which is bis-monophosphorylated at the 1 and 4′ positions, primarily acylated with R-3-hydroxymyraristate at the 2, 3, 2′ and 3′ positions, and secondarily acylated at the 2′ and 3′ positions that form two acyoxoyacyl linkages (Raetz, 2001). Lipid A is the endotoxic portion of LPS, the major factor involved in septic shock in patients with Gram-negative septicemia (Holst et al., 1996). The enzymic pathways of biosynthetic and structural modification of lipid A have been elucidated mostly in the past 15 years (Raetz & Whitfield, 2002). There is microheterogeneity in the structure of lipid A species. In *E. coli* K-12, approximately

### Abbreviations

CAMP, cationic antimicrobial peptide; CM, cytoplasmic membrane; EHEC, enterohaemorrhagic *E. coli*; L-Ara4N, 4-amino-4-deoxy-L-arabinose; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight; OM, outer membrane; PEtN, phosphoethanolamine; PMB, polymyxin B; PMBN, polymyxin B nonapeptide; STEC, Shiga toxin-producing *E. coli*; TM, transmembrane domain.

†Co-principal investigators.
two-thirds of the lipid A is a hexa-acylated disaccharide of glucosamine in which monophosphate groups are attached at positions 1 and 4′ (1-O-P lipid A) (Zhou et al., 1999). The remaining one-third of lipid A contains a diphosphate moiety at position 1 (1-O-PP lipid A, Fig. 1) (Zhou et al., 1999).

Polymyxin B (PMB) is a potent antibacterial lipopeptide characterized by a cationic cyclic peptide ring and a fatty-acid-containing N-terminal linear tail (Vaara, 1992). These unique features enable it to disorganize the bacterial OM, presumably by competitive displacement of divalent cations from the negatively charged LPS. Thus, it is a highly efficient OM-permeabilizing compound and it shows potent anti-endotoxin activity due to its high affinity for lipid A (Nikaido & Vaara, 1985; Vaara & Porro, 1996). The cytoplasmic membrane (CM) is believed to be the target for its lethal action, which is promoted by penetration through the CM by its hydrophobic N-terminal tail after ‘self-promoted’ uptake by its disruption of LPS packing (Hancock, 1997).

Resistance to PMB induced by the PmrA–PmrB regulatory system is well studied in Salmonella enterica serovar Typhimurium (Gunn & Miller, 1996; Groisman et al., 1997; Gunn et al., 1998; Zhou et al., 2001). This system is a part of the signal transduction network PhoP–PhoQ, which controls a plethora of virulence factors including modifications of LPS (Groisman, 2001). PmrA-induced PMB resistance takes place in response to environmental signals (Mg$^{2+}$ and pH), and involves modification of lipid A with 4-amino-4-deoxy-L-arabinose (1-Ara4N) and/or phosphoethanolamine (PEtN) in order to reduce the negative charge (Tamayo et al., 2002; Gibbons et al., 2005). The genes required for modification of lipid A with 1-Ara4N are upregulated by the PmrA–PmrB system and include the pmrCAB operon (at centisome 93·5), the pmrF operon (pmrHEIJKLM at centisome 51), and pmrE (also known as ugd at centisome 44) (Gunn et al., 1998). PmrC is responsible for the addition of PEtN to lipid A at the 1-phosphate residue, which may contribute to survival of S. Typhimurium after treatment with PMB (Lee et al., 2004). The pmrC gene is homologous to iptA (LPS phosphoethanolamine transferase for lipid A) of Neisseria meningitidis (Cox et al., 2003).

There are several hypothetical proteins homologous to PmrC in E. coli and S. Typhimurium (Trent & Raetz, 2002; Reynolds et al., 2005). E. coli O157:H7 possesses a 92 kb plasmid pO157 that carries the shf locus (also known as ecf) consisting of shf-wabB-ecf3-msbB2 (Boerlin et al., 1998; Kim et al., 2004; Yoon et al., 2004; Kaniuk et al., 2004). The ecf3 gene of this shf locus encodes a hypothetical protein highly homologous (83% identity) to YipP, which is one of the potential PmrC homologues. A recent report showed that a PmrA-regulated cpta gene (yipP orthologue in S. Typhimurium) is required for PEtN substitution in the LPS core (Tamayo et al., 2005). Therefore, Ecf3 is also predicted to be involved in the modification of PEtN in the first heptose residue of the LPS inner core. It would be interesting to determine if these homologous proteins are also functionally related to PmrC.

In this study, we analysed lipid A modifications that followed mutations in pmrC and/or ecf3 genes in E. coli O157:H7, and expression of pmrC0157 and its potential homologues in E. coli K-12 strain MC1061. We also determined that, in E. coli O157:H7 strains, there was a large amount of lipid A species containing the PEtN moiety (1-O-P-PEtN) (Fig. 1) and the presence of 1-O-P-PEtN lipid A conferred slight resistance to the cationic antimicrobial peptide PMBN. We employed E. coli’s rapid Pgp-mediated palmitoylation response to EDTA treatment (Jia et al., 2004) to assess whether the increase in PetN substitution in lipid A might be associated with the OM’s resistance to chelation of Mg$^{2+}$ ions that bridge neighbouring lipid A molecules.

![Fig. 1. Schematic illustration of structural microheterogeneity of lipid A species in E. coli O157:H7.](https://example.com/fig1.png)

**METHODS**

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this study are listed in Table 1, or are described in the text. The chromosomal pmrC gene was amplified from the purified genomic DNA of E. coli O157:H7 by a high-fidelity PCR with PEX1 (5’-ctttgcgaattctcgacttttgct-3’) and PEX2 (5’-tcagctacttgctgattagg-3’) primers. The 1·8 kb amplicon was ligated into pUC18 digested with EcoRI and HindIII, giving pMrC18 with an in-frame insertion under the Plac promoter. For a low-copy

---

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this study are listed in Table 1, or are described in the text. The chromosomal pmrC gene was amplified from the purified genomic DNA of E. coli O157:H7 by a high-fidelity PCR with PEX1 (5’-ctttgcgaattctcgacttttgct-3’) and PEX2 (5’-tcagctacttgctgattagg-3’) primers. The 1·8 kb amplicon was ligated into pUC18 digested with EcoRI and HindIII, giving pMrC18 with an in-frame insertion under the Plac promoter. For a low-copy
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli O157:H7</td>
<td>Wild-type strains 4304, EDL933, and Sakai</td>
<td>Lab collection</td>
</tr>
<tr>
<td>4304-PC</td>
<td>ΔpmrC::aacC1 mutant of strain 4304 (Gm')</td>
<td>This study</td>
</tr>
<tr>
<td>4304-PCE3</td>
<td>ecf3::aphA-3 mutant of 4304-PC (Gm'/Km')</td>
<td>This study</td>
</tr>
<tr>
<td>Sakai-28</td>
<td>pO157-cured derivative of O157Sakai (shf', Nah')</td>
<td>Tsutono et al. (2001)</td>
</tr>
<tr>
<td>S-28PC</td>
<td>ΔpmrC::aacC1 mutant of Sakai-28 (Gm'/Nah')</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli O111:NM</td>
<td>EHEC (eae' shf')</td>
<td>Lab collection</td>
</tr>
<tr>
<td>E. coli O145:H-</td>
<td>EHEC (eae' shf')</td>
<td>Lab collection</td>
</tr>
<tr>
<td>E. coli O26:H11</td>
<td>EHEC (eae' shf')</td>
<td>Lab collection</td>
</tr>
<tr>
<td>E. coli O121:H19</td>
<td>STEC (eae' shf')</td>
<td>Lab collection</td>
</tr>
<tr>
<td>E. coli O117:H4</td>
<td>EHEC (eae' shf')</td>
<td>Lab collection</td>
</tr>
<tr>
<td>MC1061</td>
<td>K-12 strain [F' ara Δ(lac-proAB) rpsL Δ(lacZ)M15]</td>
<td>Lab collection</td>
</tr>
<tr>
<td>BMS67C12</td>
<td>msbB::Tn5 mutant of JM83 (Km')</td>
<td>Somerville et al. (1999)</td>
</tr>
<tr>
<td>MC-msbB</td>
<td>msbB::Tn5 mutant of MC1061 (Km')</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMrC177</td>
<td>pACYC177 carrying pmrC\textsubscript{O157} gene (Ap')</td>
<td>This study</td>
</tr>
<tr>
<td>pMrC18</td>
<td>pUC18 carrying pmrC\textsubscript{O157} gene (Ap')</td>
<td>This study</td>
</tr>
<tr>
<td>pYijP18</td>
<td>pUC18 carrying yijP\textsubscript{O157} gene (Ap')</td>
<td>This study</td>
</tr>
<tr>
<td>pYbiP18</td>
<td>pUC18 carrying ybiP\textsubscript{O157} gene (Ap')</td>
<td>This study</td>
</tr>
<tr>
<td>pYbbX18</td>
<td>pUC18 carrying ybbX\textsubscript{O157} gene (Ap')</td>
<td>This study</td>
</tr>
<tr>
<td>pEc3</td>
<td>pUC18 carrying ecf3 gene (Ap')</td>
<td>This study</td>
</tr>
<tr>
<td>pR2-ΔpmrC::Gm</td>
<td>pRE112 carrying ΔpmrC::aacC1 allele (Gm'/Gm')</td>
<td>This study</td>
</tr>
<tr>
<td>pR7E3::Nm</td>
<td>pRE107 carrying ecf3::apha-3 allele (Ap'/Km')</td>
<td>This study</td>
</tr>
</tbody>
</table>

plasmid construct, primers PEtN-F (5'-tgctctggcgccatgctacttg-3'; XhoI) and PEX2 were used for cloning of pmrC\textsubscript{O157} into pACYC177, resulting in pMrC177 (Table 1). For pUC18-based expression of yijP, ybiP, and ybbX genes with in-frame insertions, the amplified high-fidelity PCR products were ligated into pUC18 digested with EcoRI and HindIII, generating pYijP18, pYbiP18, and pYbbX18, respectively (Table 1). The primers for cloning of those pmrC homologues are as follows; Yij-P-Eco forward primer (5'-gctttactatgtctgaaag-3'; EcoRI) and Yij-P-H3 reverse primer (5'-gctttactatgtctgaaag-3'; HindIII); Ybi-P-Eco forward primer (5'-gctttactatgtctgaaag-3'; EcoRI) and Ybi-P-H3 reverse primer (5'-gctttactatgtctgaaag-3'; HindIII); YbbX-Eco forward primer (5'-gctttactatgtctgaaag-3'; EcoRI) and YbbX-H3 reverse primer (5'-gctttactatgtctgaaag-3'; HindIII).

**Generation of non-polar mutants.** An internal deletion of a 600 bp DNA fragment from pMrC18 was achieved by using a modified inverse PCR method as described elsewhere (Kim et al., 2004). Phosphorylated primers EtN-F (5'-tacaccgtatccgcttttag-3') and EtN-R (5'-cagccgttaacctgagctgagac-3') were annealed to the cloned pmrC gene (pMrC18) to amplify a 4.5 kb DNA fragment, which was then blunt-end ligated and used as template for PCR amplification with EtN-Kpn (5'-tgctctggcgccatgctacttg-3'; KpnI) and EtN-Sac (5'-gctctggcgccatgctacttg-3'; SacI) primers in order to amplify a 1.3 kb insert containing the ΔpmrC allele. Then, the insert was cloned into the pRE112 vector (Edwards et al., 1998) digested with KpnI and SacI, resulting in pR2-ΔpmrC. Plasmid pR2-ΔpmrC was digested with Sall, which cuts at a single site located near the middle of the insert. A non-polar gentamicin (Gm) cassette (aacC1 gene) was purified from pUCGM (Schweizer, 1993) after digestion with Sall, and ligated into the Sall-digested pR2-ΔpmrC to create the ΔpmrC::aacC1 allele in the suicide vector. The resulting vector, designated pR2-ΔpmrC::Gm, was transformed into SM10 donor cells (Edwards et al., 1998) for conjugation. Two O157 strains (4304 and Sakai-28) were mated with SM10(pR2-ΔpmrC::Gm) in order to create ΔpmrC::aacC1 mutants (4304-PC and S-28PC) (Table 1).

For the ecf3 gene expression plasmid construction, the intact ecf3 gene was amplified from pO157 DNA by PCR with EcoF3-Kpn (5'-ggacctgctctttaaatctggg-3'; KpnI) and EcoH3 (5'-cagccgggagctctttaaatctggg-3'; HindIII) primers. The amplicon was cloned into the pUC18 vector digested with KpnI and HindIII, resulting in pEc3, with an in-frame insertion under the P1 promoter (Table 1). For ecf3::apha-3 mutation, plasmid pEc3 was digested with Sall for the insertion of a non-polar kanamycin (Km) cassette (apha-3 gene) near the middle of the cloned ecf3 gene. The apha-3 DNA fragment was purified after Sall digestion of pYA3265 (Menard et al., 1993) and was inserted into the Sall site of pEc3, resulting in pEc3::Km. Then, PCR with EcoF3-Kpn and EcoH3 primers amplified the ecf3::apha-3 allele for subcloning into the Small site of suicide vector pRE107 (Edwards et al., 1998). The resulting vector, designated pR7E3::Km, was transferred by transformation into SM10 donor cells, SM10(pR7E3::Km) was mated with 4304-PC, to create a double (ecf3::apha-3 and ΔpmrC::aacC1) mutant (4304-PCE3) of E. coli O157:H7. All mutants generated were verified by PCR.

**P1 transduction for transfer of the msbB::Tn5 allele to MC1061.** P1 transduction was used for introduction of an msbB mutation in E. coli MC1061. A temperature-sensitive P1 cmr-100 lysate of BMS67C12 (Somerville et al., 1999) was prepared as described elsewhere (Miller, 1992). E. coli MC1061 was then mixed with the P1 cmr-100 lysate prepared from BMS67C12 in order to transfer the msbB::Tn5 allele to MC1061. The resulting msbB::Tn5 mutant of MC1061 was verified by PCR and designated MC-msbB (Table 1).

**Analysis of lipid A by TLC.** Analysis of lipid A-compositional profiles was done by TLC separation of 32P-labelled lipid A species from E. coli O157:H7 Wild-type strains 4304, EDL933, and Sakai Lab collection.

http://mic.sgmjournals.org
released from a mild acid hydrolysis procedure, applied to bacteria cultured with or without EDTA treatment (Zhou et al., 1999; Jia et al., 2004). Briefly, the $^{32}$P-labelled bacteria were subjected to Bligh/Dyer extraction for the removal of membrane phospholipids (Bligh & Dyer, 1959). Then, the crude LPS precipitate was subjected to mild acid hydrolysis to cleave the ketosidic linkage between KdoI and the distal glucosamine sugar of lipid A. The lipid A fraction was then obtained from the lower phase of the Bligh/Dyer mixture consisting of chloroform/methanol/water (2:2:1 by vol.). The purified lipid A sample was dried under nitrogen gas and redissolved in solvent, consisting of chloroform/methanol (4:1, v/v). Approximately 1000 c.p.m. of the $^{32}$P-labelled sample was applied to the origin of a Silica Gel 60 TLC plate. The TLC plate was developed in a solvent system of chloroform/pyridine/88% formic acid/water (50:50:16:5 by vol.). The plate was dried and visualized with a PhosphorImager (Amersham Biosciences).

**Purification of lipid A species for mass spectrometry.** The lipid A species of wild-type *E. coli* O157:H7 strain 4304 were prepared from overnight culture with Luria–Bertani (LB) broth (200 ml) at 37°C. Cells were harvested, washed with PBS and extracted with a single-phase Bligh/Dyer mixture (Bligh & Dyer, 1959); the lipid A species were released from the cell residue by hydrolysis at pH 4.5 (100°C for 30 min) in the presence of SDS. The released lipid A species were subjected to two-phase partitioning and the lower phase was dried under a stream of nitrogen gas and redissolved in solvent, consisting of chloroform/methanol/water (2:2:1 by vol.).

**RESULTS AND DISCUSSION**

**Occurrence of PEN-substituted lipid A in *E. coli* O157 : H7**

We consistently observed that the lipid A profile of *E. coli* O157 : H7 differed from that of *E. coli* K-12 MC1061 in that it had a distinctive lipid A species (lane 2, Fig. 2a). We hypothesized that the spot in question might be a PEN-substituted lipid A (equivalent of EV4) which occurred in *E. coli* K-12 W3110 treated with the non-specific phosphatase inhibitor NH$_4$VO$_3$ (Zhou et al., 1999). Therefore, we followed the methods described by Zhou et al. (1999) to determine the molecular identity of this spot. The spectrum obtained from MALDI-TOF mass spectrometry (Fig. 2b) showed the same m/z (1918) as that obtained with EV4 (Zhou et al., 1999), demonstrating that the additional lipid A species that occurred in *E. coli* O157 : H7 is an equivalent PEN-containing moiety as depicted in Fig. 1. It is interesting that, unlike K-12 (W3110 and MC1061), *E. coli* O157 : H7 grown under laboratory culture conditions shows natural occurrence of an additional lipid A phosphofrom (1-O-P-PEN). The significance of the lipid A phosphoryl substitution in the pathobiology of *E. coli* O157 : H7 is unclear, but a recent report suggests that the occurrence of the 1-O-P-PEN form may contribute to survival of *S. Typhimurium* after PMB treatment (Lee et al., 2004).

The distinctive occurrence of the 1-O-P-PEN form raised questions regarding the possible regulatory and/or structural factors responsible for the lipid A modifications that may differ in *E. coli* O157 : H7 and other related pathogens. We demonstrated similar occurrences of the 1-O-P-PEN form of lipid A with representative serotypes of enterohaemorrhagic *E. coli* (EHEC) that possess the *shf* locus but not in a non-EHEC Shiga toxin-producing *E. coli* (STEC) *O117 : H4* that lacks this locus (Kumagai et al., 2001) (Fig. 3a). This led us to speculate that the *ecf3* gene of the *shf* locus that encodes a protein of unknown function may be associated with the occurrence of the 1-O-P-PEN lipid A, since Ecf3 and PmrC have considerable similarity (Kim et al., 2004). Therefore, we investigated lipid A profiles of the 

**Determination of MICs and the OM-permeabilizing activity of PMBN.** The microdilution method was used as recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2002). In brief, 100 μl of twofold serial dilutions of the antibiotics in double strength Mueller–Hinton broth (pH 7.2) were dispensed in 96-well microtitre plates. Bacteria (100 μl) at a final concentration of 5 × 10$^5$ c.f.u. ml$^{-1}$ in PBS were added to each well. The MICs were recorded as the lowest concentration of antibiotic that did not allow visible bacterial growth after 20 h incubation at 37°C. The OM-permeabilizing activity of PMBN was assessed by using a sublethal concentration of PMBN (10 μg ml$^{-1}$) together with twofold serial dilutions of the hydrophobic antibiotic erythromycin (Vaara & Porro, 1996; Tsunberg et al., 2001).
This suggests that the ecf3 gene product may not be involved in generation of 1-O-P-PEtN lipid A. To exclude the possibility of 4304-O157 strain specificity, we created the same DpmrC::aacC1 mutation in a pO157-cured strain of E. coli O157: H7 Sakai (Sakai-28) and showed that a weak 1-O-P-PEtN spot was also present among the lipid A species released from the DpmrC::aacC1 mutant (S-28PC) of Sakai-28 (lanes 2 and 3, Fig. 3c). Interestingly, the lipid A species of E. coli O157: H7 Sakai strain differed from those of the 4304-O157 strain in that two unidentified additional spots (A and B, lane 1 in Fig. 3c) were evident as well as the 1-O-P-PEtN spot. Lack of the shf operon due to absence of the pO157 plasmid in the cured Sakai-28 strain might be associated with the lower amount of 1-O-P-PEtN and the appearance of spots 'A' and 'B' in lane 2 (Fig. 3c). The weakened 1-O-PP spots were also evident in Sakai O157 derivatives, compared with MC1061 (lane 4, Fig. 3c).

A high-copy-number pmrC_{O157} clone generates 1-O-P-PEtN lipid A in MC1061

The difference in occurrence of the 1-O-P-PEtN lipid A in E. coli O157: H7 and K-12 might be related to conditions that increase expression of the pmrC gene. To test this possibility, MC1061 transformants carrying pmrC_{O157} in a low- (pMrC177) or a high- (pMrC18) copy plasmid (Table 1) were investigated by TLC experiments for possible lipid A modifications. As shown in Fig. 4(a), MC1061 carrying pMrC18 (lane 3) produced a large amount of PEtN-modified lipid A species (1-O-P-PEtN), whereas the pMrC177 transformant (lane 4, Fig. 4a) did not produce detectable quantities. The natural occurrence of 1-O-P-PEtN species in EHEC might therefore be associated with increased PmrC activity, since overproduction of PmrC in MC1061 generated similar amounts of 1-O-P-PEtN species without induction of further PmrA-mediated lipid A modification with L-Ara4N. Only a PMB-resistant mutant (Nummila et al., 1995) and NH4VO3-treated E. coli cells have previously been shown to be capable of modifying lipid A with PEtN, unlike the case with S. Typhimurium (Zhou et al., 1999). It is also known that the pmrCAB operon is activated in cultures grown under limited Mg2+ and a mild acid pH around 5.8–6.0 (Raetz, 2001; Gibbons et al., 2005). In this study, we discovered that pmrC_{O157} gene dosage affects the occurrence of 1-O-P-PEtN in E. coli K-12 strain MC1061 independent of the known PmrA-inducing signals. The natural occurrence of 1-O-P-PEtN in EHEC may also be independent of the activated PmrA-mediated lipid A modifications. The pMrC18 complementation of 4304-PC (lane 7, Fig. 4a) and no other distinctive occurrence of modified lipid A spots (except a small spot below the 1-O-P-PEtN, which appears to be
Fig. 3. [\(^{32}\)P]Lipid A profiles of representative EHEC strains and the pmrC mutants of O157:H7 strains 4304 and O157Sakai derivative. (a) Only the selected EHEC strains (lanes 1–4, and 6) produced 1-O-P-PEtN lipid A. Lanes: 1, O157:H7 strain EDL933; 2, O111:NM; 3, O145:H–; 4, O26:H11; 5, eae- and shf-negative STEC O117:H4; 6, O121:H19. (b) The 1-O-P-PEtN form of lipid A was less intense in the pmrC mutant 4304-PC (lane 2) than in wild-type 4304 (lane 1) but the intensity was slightly increased in 4304-PCE3 (lane 3). (c) The same ApmrC::aacC1 mutation was introduced into Sakai-28 and compared with wild-type O157Sakai. There was a weak 1-O-P-PEtN spot in the lipid A species released from Sakai-28 (lane 2) and the pmrC mutant S-28PC (lane 3). Interestingly, the lipid A species of O157Sakai (lane 1) were more diverse than those of MC1061 (lane 4) in that two additional (unidentified) spots (A and B, lane 1) were evident as well as the 1-O-P-PEtN spot, but the 1-O-PP spot was very weak compared with MC1061 (lane 4).

Fig. 4. Lipid A profiles of E. coli carrying low- and high-copy-number plasmid clones of the pmrC\(_{O157}\) gene and its potential homologues. (a) Consistently, a distinctive 1-O-P-PEtN species occurred in EHEC strain 4304 (lane 1), but not in the E. coli K-12 strain MC1061(pUC18) (lane 2). The 1-O-P-PEtN species was produced when pmrC was present in a high copy in MC1061(pMrC18) (lane 3) and in 4304-PC(pMrC18) (lane 7), but not when pmrC was present in low copy in MC1061(pMrC177) (lane 4) or 4304-PC(pMrC177). (b) Lipid A profiles of MC1061 expressing potential pmrC homologues cloned in pUC18. There was no detectable PEtN substitution in the lipid A profiles of any of the strains carrying clones of pmrC homologues. Lanes: 1, MC1061-pUC18; 2, MC1061-pYipP18; 3, MC1061-pEcf3; 4, MC1061-pYbX18; 5, MC1061-pYbiP18; 6, MC1061-pMrC18.
1-O-P-PEtN/4'-O-P-PEtN) in the TLC plate support this possibility.

Since a PmrC-related function of Ecf3 and other potential homologues (Table 2) of PmrC has not been defined, we examined lipid A profiles of MC1061 expressing those potential pmrC homologues cloned in pUC18. As shown in Fig. 4(b), there was no detectable PEtN substitution in the lipid A profiles of these cells (lanes: 1, MC1061-pUC18; 2, -pYijP18; 3, -pEcf3; 4, -pYhbX18; 5, -pYbiP18), suggesting that none of these potential homologues is responsible for the remaining PEtN-substituted lipid A in the pmrC mutant of E. coli O157:H7. Our expression strategy was designed to avoid toxicity associated with high-level overproduction of membrane proteins, with the consequence that we could not detect the expressed proteins using conventional SDS-PAGE. Nevertheless, the lipid A profiles clearly indicate expression of PmrC activity, and all other homologues were governed by the same transcriptional and translational start signals. We did not include yhjW (Table 2) because YhjW (EptB) was recently reported to be responsible for the addition of PEtN to the second Kdo residue, but not to lipid A (Reynolds et al., 2005). Interestingly, YijP (CptA) in S. Typhimurium mediates the addition of PEtN to the phosphorylated heptose-I residue (Tamayo et al., 2005). The Ecf3 of pO157 is highly homologous (83% identity) to YijP, suggesting a similar function to that of CptA of S. Typhimurium.

**Possible role of the PEtN moiety in lipid A–lipid A molecular interaction**

Since E. coli shows a rapid PagP-mediated palmitoylation response to EDTA treatment (Jia et al., 2004), we investigated whether the increase in PEtN substitution in lipid A contributes to the OM’s resistance to EDTA chelation of Mg$^{2+}$ ions that bridge neighbouring lipid A molecules. As shown in Fig. 5, the EDTA treatment markedly increased the amount of palmitoylated (hexa-acyl) 1-O-P species in the MC-msbB strain (lanes 1 and 2). MC-msbB carrying a low-copy-number plasmid clone of pmrC<sub>O157</sub> (pMrC177), which did not result in the PEtN substitution noticeably, showed similar palmitoylation (lane 4) to the case of MC-msbB (lane 2) when treated with EDTA. Although the EDTA-treated MC-msbB(pMrC18), having more PEtN-substituted lipid A, showed only a slight apparent increase in palmitoylation (lanes 5 and 6), the percentage of palmitoylation with respect to the new distribution of PEtN-modified species was unchanged. These results suggest that the PEtN moiety substituted in lipid A does not reduce the need for divalent cations. Since PagP-mediated palmitoylation is an instant indicator for OM disruption by Mg$^{2+}$-ion displacement (Bishop, 2005), we speculate that the PEtN moiety substituted in 1-O-P does not adequately replace divalent cations in bridging neighbouring lipid A molecules (1-O-P-PEtN).

### Table 2. E. coli inner-membrane protein – YhbX/YhjW/YijP/YjdB family

<table>
<thead>
<tr>
<th>Potential PmrC homologue</th>
<th>Percentage identity (and similarity)</th>
<th>No. of aa (and transmembrane domains)</th>
<th>Accession no.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>YhjW (EptB)</td>
<td>24 (44)</td>
<td>563 (5)</td>
<td>P37661</td>
<td>Reynolds et al. (2005)</td>
</tr>
<tr>
<td>YbiP</td>
<td>22 (37)</td>
<td>527 (4)</td>
<td>P75785</td>
<td>Tamayo et al. (2005)</td>
</tr>
<tr>
<td>YijP</td>
<td>23 (37)</td>
<td>577 (5)</td>
<td>P32678</td>
<td>Tamayo et al. (2005)</td>
</tr>
<tr>
<td>YhbX</td>
<td>22 (40)</td>
<td>541 (5)</td>
<td>P42640</td>
<td></td>
</tr>
<tr>
<td>Ecf3 (pO157)</td>
<td>23 (39)</td>
<td>573 (5)</td>
<td>L7028</td>
<td></td>
</tr>
</tbody>
</table>

http://mic.sgmjournals.org

Microheterogeneity of lipid A in E. coli O157:H7
The occurrence of 1-O-P-PetN lipid A confers resistance to OM-permeabilizing activity of PMBN

The possible contribution of the 1-O-P-PetN lipid A to PMB and PMBN resistance was assessed by determining MICs of these agents, and the OM-permeabilizing activity of PMBN on the E. coli strains was determined by the MIC of erythromycin in the presence of a sublethal concentration (10 μg ml⁻¹) of PMBN (Table 3). There was little difference in MICs of PMB between the strains compared; however, the occurrence of 1-O-P-PetN appeared to contribute slightly to resistance to PMBN. These results are consistent with the notion that PMB resistance is largely dependent on increased L-Ara4N substitution, which is mediated by PmrA activation, since PetN modification alone did not affect the MIC of PMB. Indeed, unlike L-Ara4N substitution of lipid A, the involvement of PetN substitution in resistance to cationic antimicrobial peptides (CAMPs) is still unclear (Trent, 2004). However, there was a fourfold increase in the MIC of erythromycin (Table 3) for E. coli containing 1-O-P-PetN lipid A, compared to those with unmodified (MC1061) or much less-modified (4304-PC) lipid A species. These results may suggest that PetN-substituted lipid A species contribute to a slight resistance of the E. coli to the OM-permeabilizing activity of CAMPs, which would be beneficial for survival of E. coli O157:H7 in some environmental niches. The four E. coli strains tested for MICs (Table 3) were all resistant to erythromycin alone (MIC > 100 μg ml⁻¹).

Resistance to PMB is primarily controlled by the PmrA-PmrB system, but cases of PmrA-independent PhoP-regulated resistance to PMB have also been reported in S. Typhimurium (Wosten et al., 2000; Shi et al., 2004). There are some differences between Salmonella and E. coli in

Table 3. MICs for E. coli K-12 strain MC1061 and O157:H7 strain 4304

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (μg ml⁻¹)*</th>
<th>PMB</th>
<th>PMBN</th>
<th>Erythromycin†</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC1061</td>
<td>1·5</td>
<td>31·25</td>
<td>1·25</td>
<td></td>
</tr>
<tr>
<td>MC1061(pMrC18)</td>
<td>1·5</td>
<td>62·5</td>
<td>5·0</td>
<td></td>
</tr>
<tr>
<td>4304 (wild-type)</td>
<td>1·0</td>
<td>62·5</td>
<td>5·0</td>
<td></td>
</tr>
<tr>
<td>4304-PC</td>
<td>0·75</td>
<td>31·25</td>
<td>1·25</td>
<td></td>
</tr>
</tbody>
</table>

*MICs were determined by the microdilution method (NCCLS, 2002) as described in Methods, and the pMrC18 transcomplemented strain was maintained by 50 μg ampicillin ml⁻¹.
†MICs of erythromycin were determined in the presence of a sublethal concentration (10 μg ml⁻¹) of PMBN to assess the OM-permeabilizing activity of PMBN on the E. coli strains. MC1061(pMrC18) is MC1061 with the pmrC gene in a high-copy plasmid. 4304-PC is 4304 with a deletion in the pmrC gene. The data were identical in two replications.

The occurrence of 1-O-P-PetN lipid A confers resistance to OM-permeabilizing activity of PMBN

The possible contribution of the 1-O-P-PetN lipid A to PMB and PMBN resistance was assessed by determining MICs of these agents, and the OM-permeabilizing activity of PMBN on the E. coli K-12 strain MC1061 and O157:H7 strain 4304. There was little difference in MICs of PMB between the strains compared; however, the occurrence of 1-O-P-PetN appeared to contribute slightly to resistance to PMBN. These results are consistent with the notion that PMB resistance is largely dependent on increased L-Ara4N substitution, which is mediated by PmrA activation, since PetN modification alone did not affect the MIC of PMB. Indeed, unlike L-Ara4N substitution of lipid A, the involvement of PetN substitution in resistance to cationic antimicrobial peptides (CAMPs) is still unclear (Trent, 2004). However, there was a fourfold increase in the MIC of erythromycin (Table 3) for E. coli containing 1-O-P-PetN lipid A, compared to those with unmodified (MC1061) or much less-modified (4304-PC) lipid A species. These results may suggest that PetN-substituted lipid A species contribute to a slight resistance of the E. coli to the OM-permeabilizing activity of CAMPs, which would be beneficial for survival of E. coli O157:H7 in some environmental niches. The four E. coli strains tested for MICs (Table 3) were all resistant to erythromycin alone (MIC > 100 μg ml⁻¹).

Resistance to PMB is primarily controlled by the PmrA-PmrB system, but cases of PmrA-independent PhoP-regulated resistance to PMB have also been reported in S. Typhimurium (Wosten et al., 2000; Shi et al., 2004). There are some differences between Salmonella and E. coli in...
the regulatory connections and/or structural genes involved in lipid A modifications. For example, Salmonella-specific UgtL promotes dephosphorylation of the 1-phosphate residue of lipid A, which contributes to PMB resistance (Shi et al., 2004), and the PmrD-mediated connection between PhoP–PhoQ and PmrA–PmrB systems is absent in E. coli (Winfield & Groisman, 2004). Given that E. coli pathogens like serotype O157:H7 have more complex structural and regulatory systems, compared to K-12, it is not surprising to see a difference in lipid A substitution with PETn between these E. coli. In this study, we have shown that a significant amount of PETn-modified lipid A species occurred in the EHEC without induction of the PmrA-mediated l-Ara4N substitution; this may provide some insights into the interplay of regulatory systems required for the periplasmic modifications of lipid A molecules during the course of biogenesis of the OM. This study may help us understand the possible contribution of the PETn modification of LPS to survival of serotype O157:H7 and other EHEC in environmental niches, considering the presence of the 1-O-P-PETn lipid A in EHEC but not in K-12 and an infrequently isolated STEC. Therefore, it is suggested that occurrence of the 1-O-P-PETn lipid A plays a protective role in the OM against CAMPs like PMBN.

Possible PETn substitutions in the lipid A core region of O157 LPS are depicted in Fig. 6. PmrC is proven to be responsible for the addition of PETn to the lipid A at the 1-phosphate residue (Lee et al., 2004; Reynolds et al., 2005). Our results have shown that none of the predicted PmrC homologues (Ybip/YhbX/YijP/Ecf3) was involved in the formation of the remaining 1-O-P-PETn lipid A species. However, YjhW (EptB) was recently reported to be responsible for the addition of PETn to the second Kdo residue (Reynolds et al., 2005), and YijP (CptA) in S. Typhimurium was involved in the addition of PETn to the phosphorylated heptose-I residue (Tamayo et al., 2005). E. coli O157:H7 possesses a plasmid-borne sfl locus consisting of sfl-wabB-ecf3-msbB2 (Kim et al., 2004; Kaniuk et al., 2004). The Ecf3 of pO157 is highly homologous (83% identity) to YijP, suggesting the same function as that of CptA of S. Typhimurium. In addition, WabB shows a UDP-N-acetylglucosamine: (heptose) LPS α-1,7-N-acetylglucosamine transferase that is responsible for a non-stoichiometric N-acetylglucosamine substitution in the heptose-III residue (Kaniuk et al., 2004). Therefore, the sfl locus seems to be a module involved in O157 LPS lipid A core modifications, and is also suggested to play a role in the survival and persistence of E. coli O157:H7 in cattle and in the environment (Yoon et al., 2005).

ACKNOWLEDGEMENTS

The authors thank Dr C. Sasakawa (University of Tokyo) for providing a pO157-cured strain of O157Sakai (Sakai-28), and Dr C. Whitfield (University of Guelph) for providing pYA3265. P1 cmr-100 phage lysogen (CmR) was kindly provided by Dr J. Wood (University of Guelph). S.H.K. was supported by the Canadian Institutes of Health Research (CIHR) training programme (Post-Doctoral) on the structure and function of membrane proteins linked to disease (University of Toronto) and by the Natural Sciences and Engineering Research Council (University of Guelph). Research in the laboratory of R. E. B. was supported by CIHR operating grant MOP-43886.

REFERENCES


