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. Bishop^{2†} and Carlton L. Gyles^{1†}

Correspondence

Carlton L. Gyles

cgyles@uoguelph.ca

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 diphosphoryl 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*_{O157} 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*_{O157} 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.

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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-hydroxymyristate at the 2, 3, 2' and 3' positions, and secondarily acylated at the 2' and 3' positions that form two acyoxacyl linkages (Raetz, 2001). Lipid A is the endotoxic portion of LPS, the major factor involved in septic shock in patients with Gram-negative septicaemia (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

†Co-principal investigators.

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.

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). PMB nonapeptide (PMBN), the deacylated PMB peptide derived from proteolytic cleavage of the N-terminal amino-acyl tail, is much less bactericidal than PMB, but still retains its OM-permeabilizing activity (Tsubery *et al.*, 2001).

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 (L-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 L-Ara4N are upregulated by the PmrA–PmrB system and include the *pmrCAB* operon (at centisome 93.5), the *pmrF* operon (*pmrHFIJKLM* 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 *lptA* (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 YijP, which is one of the potential PmrC homologues. A recent report showed that a PmrA-regulated *cptA* gene (*yijP* 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 *pmrC*_{O157} 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 PagP-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.

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'-ctttgcgagaattgcgcactttgttcg-3'; *EcoRI*) and PEX2 (5'-tgcagtcacgcttcggtgaacc-3'; *HindIII*) 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

Fig. 1. Schematic illustration of structural microheterogeneity of lipid A species in *E. coli* O157:H7. In *E. coli* K-12, approximately two-thirds of the lipid A is a hexa-acylated disaccharide of glucosamine with monophosphate groups at positions 1 and 4' (1-O-P lipid A). The remainder contains a diphosphate moiety at position 1 (1-O-PP lipid A) (Zhou *et al.*, 1999). A small amount of 1-dephosphorylated lipid A (identified herein as 1-OH) can be generated during the mild acid hydrolysis procedure. In *E. coli* O157:H7, another phosphoryl substitution occurs naturally at the phosphate of the proximal glucosamine residue, and this phosphoethanolamine (PEtN, dashed line) substituted lipid A species is designated 1-O-P-PEtN.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source
Strains		
<i>E. coli</i> O157:H7	Wild-type strains 4304, EDL933, and Sakai	Lab collection
4304-PC	$\Delta pmrC::aacC1$ mutant of strain 4304 (Gm ^r)	This study
4304-PCE3	<i>ecf3::aphA-3</i> mutant of 4304-PC (Gm ^r /Km ^r)	This study
Sakai-28	pO157-cured derivative of O157Sakai (<i>shf</i> ⁻ , Nal ^r)	Tatsuno <i>et al.</i> (2001)
S-28PC	$\Delta pmrC::aacC1$ mutant of Sakai-28 (Gm ^r /Nal ^r)	This study
<i>E. coli</i> O111:NM	EHEC (<i>eae</i> ⁺ <i>shf</i> ⁺)	Lab collection
<i>E. coli</i> O145:H-	EHEC (<i>eae</i> ⁺ <i>shf</i> ⁺)	Lab collection
<i>E. coli</i> O26:H11	EHEC (<i>eae</i> ⁺ <i>shf</i> ⁺)	Lab collection
<i>E. coli</i> O121:H19	STEC (<i>eae</i> ⁺ <i>shf</i> ⁺)	Lab collection
<i>E. coli</i> O117:H4	EHEC (<i>eae</i> ⁻ <i>shf</i> ⁻)	Lab collection
MC1061	K-12 strain [F ⁻ <i>ara</i> $\Delta(lac-proAB)$ <i>rpsL</i> $\Delta(lacZ)$ M15]	Lab collection
BMS67C12	<i>msbB::Tn5</i> mutant of JM83 (Km ^r)	Somerville <i>et al.</i> (1999)
MC- <i>msbB</i>	<i>msbB::Tn5</i> mutant of MC1061 (Km ^r)	This study
Plasmids		
pMrC177	pACYC177 carrying <i>pmrC</i> _{O157} gene (Ap ^r)	This study
pMrC18	pUC18 carrying <i>pmrC</i> _{O157} gene (Ap ^r)	This study
pYijP18	pUC18 carrying <i>yijP</i> _{O157} gene (Ap ^r)	This study
pYbiP18	pUC18 carrying <i>ybiP</i> _{O157} gene (Ap ^r)	This study
pYhbX18	pUC18 carrying <i>yhbX</i> _{O157} gene (Ap ^r)	This study
pEc3	pUC18 carrying <i>ecf3</i> gene (Ap ^r)	This study
pR2- $\Delta pmrC::Gm$	pRE112 carrying $\Delta pmrC::aacC1$ allele (Cm ^r /Gm ^r)	This study
pR7E3::Km	pRE107 carrying <i>ecf3::aphA-3</i> allele (Ap ^r /Km ^r)	This study

plasmid construct, primers PEtN-F (5'-tgcttctcgagcatatgcgcactttg-3'; *XhoI*) and PEX2 were used for cloning of *pmrC*_{O157} into pACYC177, resulting in pMrC177 (Table 1). For pUC18-based expression of *yijP*, *ybiP* and *yhbX* genes with in-frame insertions, the amplified high-fidelity PCR products were ligated into pUC18 digested with *EcoRI* and *HindIII*, giving pYijP18, pYbiP18 and pYhbX18, respectively (Table 1). The primers used for cloning of those *pmrC* homologues are as follows: YijP-Eco forward primer (5'-gttctactatgaattccacagaag-3'; *EcoRI*) and YijP-H3 reverse primer (5'-gcgtcgaagcttcgcatccggcaaaat-3'; *HindIII*); YbiP-Eco forward primer (5'-gtggcatatgaattcaaccctcaag-3'; *EcoRI*) and YbiP-H3 reverse primer (5'-tccgcctctcgggaagctttttttta-3'; *HindIII*); YhbX-Eco forward primer (5'-tggtgtgaattcgattctggatg-3'; *EcoRI*) and YhbX-H3 reverse primer (5'-gcagatggagaagcttgaacaaagg-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'-tacaaccgcatatccgcccagttc-3') and EtN-R (5'-caccagctctttgtgttgcggaac-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'-ttgttcgatggaggtaccgtgatgtgaag-3'; *KpnI*) and EtN-Sac (5'-gctgtaatgaccgagctcaagctttg-3'; *SacI*) primers in order to amplify a 1.3 kb insert containing the $\Delta pmrC$ allele. Then, the insert was cloned into the pRE112 vector (Edwards *et al.*, 1998) digested with *KpnI* and *SacI*, resulting in pR2- $\Delta pmrC$. Plasmid pR2- $\Delta pmrC$ 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- $\Delta pmrC$ to create the $\Delta pmrC::aacC1$ allele in the suicide vector. The resulting vector, designated pR2- $\Delta pmrC::Gm$, was transformed into SM10 donor

cells (Edwards *et al.*, 1998) for conjugation. Two O157 strains (4304 and Sakai-28) were mated with SM10(pR2- $\Delta pmrC::Gm$) in order to create $\Delta pmrC::aacC1$ 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 Ecf3-Kpn (5'-ggacggctacgtgcatttaataaccggacag-3'; *KpnI*) and Ecf-H3 (5'-cagaacggaaccgaagcttagaataacta-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 *Plac* 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 Ecf3-Kpn and Ecf-H3 primers amplified the *ecf3::aphA-3* allele for subcloning into the *SmaI* 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 $\Delta pmrC::aacC1$) 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

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 (2:2:1.8 by vol.). The purified lipid A sample was dried under a stream of 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 N_2 . The lipid A species were separated by anion-exchange chromatography on DEAE-cellulose (Zhou *et al.*, 1999). A 2 ml DEAE cellulose column (Whatman DE52) in the acetate form was prepared and equilibrated with the solvent of chloroform/methanol/water (2:3:1 by vol.). The total dried lipid A sample redissolved in 2 ml chloroform/methanol/water (2:3:1 by vol.) was loaded onto the column at its natural flow rate. The column was washed with 12 ml chloroform/methanol/water (2:3:1 by vol.) and 2 ml fractions were collected by sequential elution with 12 ml chloroform/methanol/60 mM aqueous ammonium acetate (2:3:1 by vol.), 12 ml chloroform/methanol/120 mM ammonium acetate (2:3:1 by vol.), 12 ml chloroform/methanol/240 mM ammonium acetate (2:3:1 by vol.) and 12 ml chloroform/methanol/480 mM ammonium acetate (2:3:1 by vol.). To locate the fractions containing the desired lipids, 20 μl of each fraction was spotted onto a 10 × 20 cm Silica Gel 60 TLC plate. The plate was developed in a solvent of chloroform/pyridine/88% formic acid/water (50:50:16:5 by vol.). The spots were visualized by charring on a hot plate after spraying the chromatogram with a mixture of ethanol/*p*-anisaldehyde/ H_2SO_4 /acetic acid (89:2.5:4:1 by vol.). The DEAE cellulose fractions containing the lipids of interest were then converted to neutral two-phase Bligh/Dyer mixtures by addition of the necessary amounts of chloroform and water. The lower phases were pooled and dried under N_2 and stored at -20 °C. For mass spectrometry, the purified lipid A sample was dissolved in chloroform/methanol (4:1, v/v) before being mixed (1:1, v/v) on a slide with a matrix of a saturated solution of 2,5-dihydroxybenzoic acid in 50% acetonitrile. The sample mixtures were allowed to dry at room temperature prior to mass analysis. The spectrum was acquired in the negative linear mode by using matrix-assisted laser desorption/ionization/time of flight (MALDI-TOF) mass spectrometry.

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 $\mu\text{g ml}^{-1}$) together with twofold serial dilutions of the hydrophobic antibiotic erythromycin (Vaara & Porro, 1996; Tsubery *et al.*, 2001).

RESULTS AND DISCUSSION

Occurrence of PEtN-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 PEtN-substituted lipid A (equivalent of EV4) which occurred in *E. coli* K-12 W3110 treated with the non-specific phosphatase inhibitor NH_4VO_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 PEtN-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 phosphoform (1-O-P-PEtN). 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-PEtN form may contribute to survival of *S. Typhimurium* after PMB treatment (Lee *et al.*, 2004).

The distinctive occurrence of the 1-O-P-PEtN 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-PEtN 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 (*eae*⁻ and *shf*⁻) (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-PEtN lipid A, since Ecf3 and PmrC have considerable similarity (Kim *et al.*, 2004). Therefore, we investigated lipid A profiles of the *pmrC* mutant (4304-PC) and a double (*pmrC* and *ecf3*) mutant (4304-PCE3) of *E. coli* O157:H7 to determine whether they lacked the 1-O-P-PEtN lipid A. As shown in Fig. 3(b), the intensity of the ^{32}P -labelled PEtN-modified lipid A spot was diminished in 4304-PC (lane 2, Fig. 3b), but some 1-O-P-PEtN lipid A was still present, suggesting that the *pmrC* gene is not the only structural gene encoding an enzyme responsible for the addition of PEtN to lipid A in the EHEC O157 strain. Interestingly, the non-polar double mutation ($\Delta\text{pmrC}::\text{aacC1}$ and *ecf3*::*aphA-3*) in 4304-PCE3 did not prevent the PEtN modification of lipid A (1-O-P-PEtN)

Fig. 2. Identification of PETN-modified lipid A species (1-O-P-PETN). (a) [³²P]Lipid A profiles of *E. coli* K-12 strain MC1061 (lane 1) and O157:H7 strain 4304 (lane 2). Note the spot (marked as ?) which is present in *E. coli* O157:H7 (lane 2) and absent in MC1061 (lane 1). (b) Negative-ion mode [M+H]⁻ MALDI-TOF mass spectrum of the unknown lipid A spot from *E. coli* O157:H7. The theoretical mass (*m/z*, 1918) of the 1-O-P-PETN lipid A species is calculated by addition of PETN moiety (PO₃-CH₂-CH₂-NH₂, *m/z* = 123) to the prototype *E. coli* lipid A (diphosphorylated, hexa-acylated 1-O-P form, *m/z* = 1795).

(lane 3, Fig. 3b). 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 $\Delta pmrC::aacC1$ 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 $\Delta pmrC::aacC1$ 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 (Nummala *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 $\Delta pmrC::aacC1$ 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-pYijP18; 3, MC1061-pEcf3; 4, MC1061-pYhbX18; 5, MC1061-pYbiP18; 6, MC1061-pMrC18.

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

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

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*_{O157} (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).

Fig. 5. Lipid A profiles of MC-*msbB* containing *pmrC*_{O157} clones (pMrC177 and pMrC18). To determine whether the increase of PeTn substitution in the lipid A contributes to the OM's resistance to chelation of Mg^{2+} ions, we examined responses to EDTA treatment (Jia *et al.*, 2004). Lanes 1 and 2 show lipid As of MC-*msbB*; lanes 3 and 4 show lipid As of MC-*msbB* carrying a low-copy-number plasmid clone of *pmrC*_{O157}(pMrC177) that did not result in detectable PeTn substitution; lanes 5 and 6 show lipid As of MC-*msbB*(pMrC18) having more PeTn substitution. In addition, there were two unidentified spots (A and B) that emerged from the pMrC18 transformants (lanes 5 and 6).

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

Strain	MIC (µg ml ⁻¹)*		
	PMB	PMBN	Erythromycin†
MC1061	1·5	31·25	1·25
MC1061(pMrC18)	1·5	62·5	5·0
4304 (wild-type)	1·0	62·5	5·0
4304-PC	0·75	31·25	1·25

*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* 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

Fig. 6. Proposed modifications of lipid A-core with phosphoethanolamine moieties in the LPS of *E. coli* O157:H7. Possible PETN substitutions are depicted.

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, YhjW (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 *shf* locus consisting of *shf-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 *shf* 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).

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