Modification of the 1-phosphate group during biosynthesis of Capnocytophaga canimorsus lipid A

Renzi, Francesco; Zaehringer, Ulrich; Chandler, C.E.; Ernst, R. K.; Cornelis, Guy; Ittig, Simon

Published in:
Infection and Immunity

DOI:
10.1128/IAI.01006-15
10.1128/IAI.01006-15

Publication date:
2015

IAI Accepted Manuscript Posted Online 7 December 2015
Infect. Immun. doi:10.1128/IAI.01006-15

Modification of the 1-phosphate group during biosynthesis of *Capnocytophaga canimorsus* lipid A

Francesco Renzi[1], Ulrich Zähringer[2], Courtney E. Chandler[3], Robert K. Ernst[3], Guy R. Cornelis[14], and Simon J. Ittig[4#]

[1]Université de Namur, Namur, Belgium

[2]Division of Immunochemistry, Research Center Borstel, Leibniz-Center for Medicine and Biosciences, Borstel, Germany

[3]Department of Microbial Pathogenesis, University of Maryland, Baltimore, Maryland, USA

[4]Biozentrum der Universität Basel, Basel, Switzerland

[#]Corresponding author:

Simon Ittig
Biozentrum
University of Basel
Klingelbergstr. 50-70
4056 Basel, Switzerland
Tel. +41 (0)61 267 22 00
E-mail:simon.ittig@unibas.ch

Running title: *C. canimorsus* lipid A modification

Key words: endotoxicity, Gram-negative bacteria, LPS, outer membrane, TLR4

Abstract

Capnocytophaga canimorsus, a commensal bacterium of dog's mouth flora causing severe infections in humans after dog bites or scratches, has a lipopolysaccharide (LPS, endotoxin) with low-inflammatory lipid A. In particular it contains a phosphoethanolamine (*P*-Etn) instead of a free phosphate group at the C-1 position of the lipid A backbone, usually present in highly toxic enterobacterial Gram-negative lipid A. Here we show that the *C. canimorsus* genome comprises a single operon encoding a lipid A 1-phosphatase (LpxE) and a lipid A 1 *P*-Etn transferase (EptA). This suggests that lipid A is modified during biosynthesis after completing acylation of the backbone by removal of the 1-phosphate and subsequent addition of a *P*-Etn group. As endotoxicity of lipid A is known to depend largely on the degree of unsubstituted or unmodified phosphate residues, deletion of *lpxE* or *eptA* led to mutants lacking the *P*-Etn group, with consequently increased endotoxicity and decreased resistance to cationic antimicrobial peptides (CAMP). Consistent with the proposed sequential biosynthetic mechanism, the endotoxicity and CAMP resistance of a double deletion mutant of *lpxE-eptA* was similar to that of a single *lpxE* mutant. Finally, the proposed enzymatic activities of LpxE and EptA based on sequence similarity could be successfully validated by MS-based analysis of lipid A isolated from corresponding deletion mutant strains.

212 words

Introduction

Some Gram-negative bacteria have evolved different modifications of their lipid A structure, leading to a reduced recognition by the host and sensitivity to CAMP (1-7). One of these modifications occurs on the 1- or on the 4'-phosphate of lipid A (1, 4, 7-10). 4'-phosphatases (LpxF) have been described in *Rhizobium leguminosarum*, *Rhizobium etli*, *Porphyromonas gingivalis, Francisella* species and *Helicobacter pylori* (1, 10-12). Deletion of *lpxF* and the resulting presence of the 4'-phosphate on lipid A leads to increased endotoxicity (1, 12) and decreased resistance to CAMP (10, 12). In the case of *Francisella* and *H. pylori* virulence is reduced (11, 12, 13). 1-phosphatases (LpxE) have been identified in *H. pylori*, *P. gingivalis*, *R. etli* and others (1, 6, 10, 12, 14-16). Deletion of *lpxE* and the resulting presence of the 1-phosphate on lipid A leads to a slightly increased endotoxicity (1) and CAMP sensitivity (10). In *H. pylori*, the 1 position is further modified by the addition of a *P*-Etn (15, 17, 18), a modification known from other bacteria (15, 17, 18). This happens via a two-step mechanism, which first involves dephosphorylation of one phosphate residue located at position C-1 of the lipid A backbone by LpxE and subsequent *P*-Etn transfer by a phosphoethanolamine transferase (EptA or PmrC) (15, 16). In *H. pylori lpxE* and *eptA* are encoded by one operon (Hp0021-Hp0022) (16).

We have previously characterized the lipid A structure of *C. canimorsus* (19), bacteria causing rare but severe sepsis or meningitis in humans after dog bites or scratches (20-24). *C. canimorsus* belongs to the family of *Flavobacteriaceae* in the phylum *Bacteroidetes* and are usual members of dog's mouth flora (21, 25-28). *C. canimorsus* lipid A consists of a 2,3-diamino-2,3-dideoxy-D-glucose (GlcN3N') β-(1'→6)-linked to

2-amino-2-deoxy-D-glucose (GlcN) [ß-D-Glc*p*N3N'-(1→6)-D-Glc*p*N lipid A hybrid backbone] containing a *P*-Etn group attached to the C-1 reducing end and lacking a 4'-phosphate (Fig. 1 A). 3-hydroxy-15-methylhexadecanoic acid [*i*17:0(3-OH)], 3-hydroxy-13-methyltetradecanoic acid (*i*15:0), 3-O-(13-methyltetradecanoyl)-15-methylhexadecanoic acid [*i*17:0[3-*O*(*i*15:0)]] and 3-hydroxyhexadecanoic acid [16:0(3-OH)] are attached to the backbone at positions 2, 3, 2', and 3', respectively (19). This structure differs from that of a potent Toll-like receptor 4 (TLR4) agonist like the *E. coli* lipid A (Fig. 1 B), consisting of a β-(1'→6)-linked GlcN disaccharide that is phosphorylated at positions 1 and 4' and carries four (*R*)-3-hydroxymyristate chains [14:0(3-OH)] (at positions 2', 3', 2 and 3). The 2' and 3' 3-hydroxylated acyl groups in GlcN(II) are further esterified with laurate and myristate, respectively (29).

We have identified genes *lpxE* and *eptA* in the genome of *C. canimorsus* and found the overlapping genes to be organized in one operon. We show that the deletion of *lpxE* or *eptA* leads to increased endotoxicity and decreased resistance to CAMP, where deletion of *lpxE* has a more severe effect. Interestingly, the endotoxicity and CAMP resistance of a double deletion mutant of *lpxE* and *eptA* was the same as that of a single *lpxE* mutant. This suggests that the *P*-Etn containing lipid A is synthesized by a similar two-step enzymatic process as in *H. pylori*, where dephosphorylation is necessary for substitution of 1-phosphate with *P*-Etn. Finally, we could successfully validate the proposed lipid A structures of the respective deletion mutants by MS-analysis, thus also further confirming, on a structural basis, the proposed enzymatic activities of LpxE and EptA as well as the two-step enzymatic mechanism in the lipid A biosynthesis.

Materials and Methods

Bacterial strains and growth conditions. The strains used in this study are listed in Table 1. *E. coli* strains were grown in LB broth at 37°C. *C. canimorsus* 5 (*Cc5*) (30) was routinely grown on Heart Infusion Agar (HIA; Difco) supplemented with 5% sheep blood (Oxoid) for 2 days at 37°C in presence of 5% CO_2. Bacteria were harvested by scraping colonies off the agar surface, washed and re-suspended in phosphate buffered saline (PBS). Selective agents were added at the following concentrations: erythromycin, 10 µg/ml; cefoxitin, 10 µg/ml; gentamicin, 20 µg/ml; ampicillin, 100 µg/ml; tetracycline, 10 µg/ml.

Genetic manipulations of *C. canimorsus*. Genetic manipulations of *Cc5* wt have been described (31). Briefly, replacement cassettes with flanking regions spanning approximately 500 bp homologous to direct *lpxE* or *eptA* framing regions (28) were constructed with a three-fragment overlapping-PCR strategy. As the ATG of *eptA* is within the coding region of *lpxE*, 106bp upstream of the *eptA* ATG were not deleted in *lpxE* single knockout (Δ 1833737 - 1833995). First, two PCRs were performed on 100 ng of *Cc5* genomic DNA with primers A and B (Table 2) for the upstream flanking regions and with primers E and F for the downstream regions. Primers B and E contained an additional 5' 20-nucleotide extension homologous to the *ermF* or *tetQ* insertion cassettes. The *ermF* and *tetQ* resistance cassettes were amplified from plasmids pMM13 and pMM104.A DNA respectively with primers C and D. All three PCR products were cleaned and then mixed in equal amounts for PCR using Phusion polymerase (Finnzymes). The initial denaturation was at 98°C for 2 min, followed by 12 cycles

without primers to allow annealing and elongation of the overlapping fragments (98°C for 30 s, 50°C for 40 s, and 72°C for 2 min). After the addition of external primers (A and F), the program was continued with 20 cycles (98°C for 30 s, 50°C for 40 s, and 72°C for 2 min 30 s) and finally 10 min at 72°C. Final PCR products consisting in *lpxE::ermF, eptA::ermF, lpxE/eptA/::ermF, lpxE::tetQ, eptA::tetQ, lpxE/eptA/::tetQ* insertion cassettes were then digested with *Pst*I and *Spe*I for cloning into the appropriate sites of the *C. canimorsus* suicide vector pMM25 (31). Resulting plasmids were transferred by RP4-mediated conjugative DNA transfer from *E. coli* S17-1 to *C. canimorsus* 5 or *C. canimorsus* 5 Y1C12 mutant to allow integration of the insertion cassette. Transconjugants were then selected for the presence of the *ermF* or *tetQ* cassette on erythromycin or tetracycline plates respectively and checked for sensitivity to cefoxitin. Deletion of the appropriate regions was verified by PCR.

Construction of complementation plasmids. Plasmid pMM47.A was used for expression of LpxE and EptA (31). Full length *lpxE, eptA* or *lpxE-eptA* genes were amplified with the specific primers listed in Table 2 and cloned into plasmid pMM47.A using *Nco*I and *Xba*I or *Nco*I and *Xho*I restriction sites leading to the insertion of a glycine at position 2. Ligated plasmids were cloned in *E. coli* top10.

Human TLR4 activation assay. HEK293 stably expressing human TLR4, myeloid differentiation factor-2 (MD-2), cluster of differentiation antigen 14 (CD14) and a NFκB dependent reporter (secreted embryonic alkaline phosphatase) were from InvivoGen (HEKBlue^{TM} hTLR4). Growth conditions and endotoxicity assay were performed as recommended by the supplier (InvivoGen). Briefly, desired amounts of heat-killed

bacteria were placed in a total volume of 20 µl (diluted in PBS) and distributed in a flat-bottom 96-well plate (BD Falcon). 25000 HEKBlue™ hTLR4 cells in 180 µl were then added and the plate was incubated for 20-24h at 37°C and 5% CO_2. Detection of the secreted phosphatase followed the QUANTI-Blue™ protocol (InvivoGen). 20 µl of challenged cells were incubated with 180 µl detection reagent (QUANTI-Blue™, InvivoGen). Plates were incubated at 37°C and 5% CO_2 and absorbance was measured at 655 nm using a spectrophotometer (BioRad).

Polymyxin B sensitivity assay. Polymyxin B sulphate was obtained from Sigma-Aldrich. The minimal inhibitory concentration (MIC) was determined by the agar dilution method based on the CLSI/NCCLS recommendations (32). Briefly, 10^4 bacteria contained in 2 µl PBS were spotted on HIA 5% sheep blood plates containing Polymyxin B ranging from 0.5 mg/L to 1024 mg/L (2-fold increase per step). Plates were incubated and examined for growth of visible colonies after 48h and 72h.

Genome annotation. *Blast*-p search tool (33) against the *C. canimorsus* 5 genome (28) was used. Search sequences were obtained from the National Center for Biotechnology Information. All available *Bacteroidetes*-group sequences were used as search, but also standard *E. coli* sequences have always been included. The highest scoring subjects over all the searches have been annotated as corresponding enzymes. Difficulties in annotation were only observed for *lpxE*. *lpxE* search was based on *lpxF* and/or *lpxE* protein sequences from *P. gingivalis (1)*, *F. novicida* (7), *R. etli* (10), *H. pylori* (12, 16) and on all available *Bacteroidetes*-group *pgpB* protein sequences.

Preparation of bacteria for LPS extraction. Compositional analysis of the LPS from the *C. canimorsus* 5 wt strain previously showed to be highly contaminated with glucose from amylopectin, flavolipin, and capnin, known to be present in *Capnocytophaga* spp. and *Flavobacteriaceae* (34). In contrast, the LPS from the *C. canimorsus* 5 Y1C12 mutant (35) was devoid of such contaminating material. Since the compositional analysis of the lipid A and LPS core obtained from the wt strain LPS and that of the Y1C12 mutant revealed no differences with respect to their sugars and fatty acids (19, 34), the Y1C12 mutant was chosen as background strain to isolate and analyse the lipid A of Δ*eptA*, Δ*lpxE* and Δ*lpxE-eptA* deletion mutants in detail by MS analysis. While the Y1C12 mutant was chosen as background strain for MS analysis, please note that human TLR4 activation assays and Polymyxin B sensitivity analysis are based on *C. canimorsus* 5 Δ*eptA*, Δ*lpxE* and Δ*lpxE-eptA* deletion mutants and complemented mutants based thereon. The *C. canimorsus* 5-based Y1C12 mutant has a transposon insertion within a predicted glycosyltranferase-encoding gene, probably the *N*-acetyl fucosamine transferase WbuB, necessary for the formation of the O-antigen (35). Endotoxicity of resulting *C. canimorsus* 5 Y1C12 Δ*eptA*, Δ*lpxE* and Δ*lpxE-eptA* deletion mutants was assessed and confirmed results obtained with *C. canimorsus* 5 Δ*eptA*, Δ*lpxE* and Δ*lpxE-eptA* deletion mutants (data not shown). *C. canimorsus* bacteria were harvested from 25 blood plates in PBS, followed by centrifugation at 18,000 x g for 30 min. Bacteria were resuspended in cold acetone, incubated with shaking, resuspended in PBS containing 0.5% phenol for killing, again harvested by centrifugation, washed with PBS and resuspended in 1ml water. 1/10 of the volume was plated on appropriate growth plates to ensure complete bacterial killing. Bacteria were air dried prior to LPS extraction.

Purification and isolation of free lipid A suitable for MS-analysis. Lipid A was isolated from lyophilized *C. canimorsus* cell pellets following the Caroff extraction method (36). Briefly, pellets were dissolved in 70% isobutyric acid and 1M ammonium hydroxide and incubated at 100°C for one hour. 400 ul of water was added, the samples were snap-frozen on dry ice and lyophilized overnight. Samples were then washed twice with 1ml methanol and reconstituted in 150ul chloroform:methanol:water (3:1.5:0.25, v:v:v).

MS based structural analysis. Lipid A structures were assessed by negative and positive-ion MALDI-TOF MS. Lyophilized lipid A was extracted in chloroform/methanol and then 1 µl was mixed with 1µl of norharmane MALDI matrix. All MALDI-TOF experiments were performed using a Bruker Microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA). Each spectrum was an average of 300 shots. ES tuning mix (Aligent, Palo Alto, CA) was used for calibration. Data were analyzed using Bruker Daltonik flex Analysis software.

Immunoblotting of proteinase K-resistant structures. Bacteria were harvested from blood-agar plates, washed once in 1ml of PBS and adjusted to an OD_{600} of 1.5 in PBS. 500µl bacterial suspension was pelleted and dissolved in 125µl loading buffer (1% sodium dodecyl sulfate [SDS], 10% glycerol, 50 mM dithiothreitol, 0.02% bromophenol blue, 45 mM Tris (pH 6.8) in ddH_2O). Samples were boiled at 99°C for 10 min. Proteinase K (50µg/ml final concentration) was added and samples were incubated at 37°C overnight. After incubation samples were boiled again for 10 min at 99°C and a

second volume of proteinase K (equal to the first) was added. Samples were incubated at 55°C for 3 h, boiled again for 5 minutes at 99°C and loaded on a 15% SDS-PAGE. Samples were analyzed by western blotting using polyclonal, *C. canimorsus* 5 Y1C12-absorbed, serum against *C. canimorsus* 5. This antibody was generated from rabbits by immunization with heat killed *C. canimorsus* 5 (Laboratoire d'Hormonologie, Marloie, Belgium). Further on, the *C. canimorsus* 5 Y1C12-absorbed serum was prepared by incubating twice an excess amount of Y1C12 mutant *C. canimorsus* 5 bacteria (harvested from blood plates and washed in PBS) with anti-*C. canimorsus* 5 serum at 4°C for 12 h. Bacteria were removed by repeated centrifugation. This results in an antiserum recognizing *C. canimorsus* 5 LPS (35).

Results and Discussion

Identification of enzymes leading to the presence of 1 *P*-Etn on lipid A. The genome of *C. canimorsus* 5 (28) (Embank: YP_004740918.1) was analyzed for proteins with high sequence similarity to the lipid A modifying enzymes, LpxE and EptA. Our search for a lipid A phosphatase was based on L*pxE* and/or LpxF sequences from *P. gingivalis* (1), *F. novicida* (11), *R. etli* (10) *H. pylori* (12, 16) and on all available *Bacteroidetes*-group *pgpB* sequences. Three *lpxE/F* candidates were found (*Ccan_16960*, *Ccan_14540* and *Ccan_06070*) and individually deleted. Interestingly, the gene downstream of *Ccan_16960* (Genbank: YP_004740919.1), *Ccan_16950* was found to have high sequence similarity to *eptA,* coding for a lipid A *P*-Etn transferase. *Ccan_16950* and *16960* form an operon and the two genes overlap by 20 base pairs (bp) (Fig. 2, lower panel). *Ccan_16960* has thus been annotated as *lpxE,* an annotation validated by mutagenesis, MS analysis and impact on endotoxicity and CAMP resistance, as described here below. The association of *lpxE* and *eptA* genes reinforces the idea that the two gene products acts in the same pathway and suggests that the modification of lipid A that they determine together is essential for survival in the dog's mouth environment.

Predicted lipid A structures in *eptA*, *lpxE* and *lpxE-eptA* deletion mutants. EptA has been proposed not to be active on lipid A, in case the 1-phosphate has not been removed before by LpxE (15, 16). Hence, deletion of the lipid A 1-phosphatase LpxE, the enzyme proposed to act first in this two-step mechanism, should result in a lipid A having a 1-phosphate (Fig. 2). Upon deletion of only the second enzyme acting in the

pathway, the *P*-Etn transferase EptA, a free hydroxy group at the "reducing end" of the lipid A backbone should result (Fig. 2). This would reflect that LpxE is still active even in the absence of EptA (15, 16). The resulting lipid A in the *eptA* deletion mutant is thus predicted to lack both the 4' and the 1-phosphate. In case of a double deletion mutant in *lpxE* and *eptA*, the same 1-phospho lipid A is predicted as for the single deletion in *lpxE* (Fig. 2).

The 1-phospho lipid A variant predicted for Δ*lpxE* and Δ*lpxE-eptA* should be the variant with the highest endotoxicity. In case of an *eptA* deletion mutant, the free (hydroxy) 4' and 1 position should result in a very low endotoxic lipid A as it is known from completely de-phosphorylated synthetic lipid A analogues (37).

LpxE and EptA impact on endotoxicity. To study the endotoxic activity after the removal of the 1-phosphate or the addition of a *P*-Etn to the free 1 position of lipid A, we engineered Δ*eptA* and Δ*lpxE* mutations and monitored endotoxicity using a HEK293 cell line overexpressing human TLR4/MD-2/CD14 and a secreted reporter protein (HEKBlue^{TM} human TLR4 cell line). Activation of this cell line essentially depends on TLR4 and other TLR stimuli may be neglected. Heat killed bacteria from both mutant strains showed increased endotoxicity compared to wt bacteria and mutation of *lpxE* had a more severe impact on endotoxicity (Fig. 3 A). Heat killed *C. canimorsus* Δ*lpxE-eptA* exhibited identical endotoxicity as *C. canimorsus* Δ*lpxE* (Fig. 3 A).

Complementation of the deleted genes with plasmid-borne genes expressed from the *ermF* promoter restored endotoxicity to the wt level indicating that none of the mutation was polar (Fig. 3 B, C and D). The Δ*lpxE* strain could be complemented in trans with *lpxE* or *lpxE-eptA*, but not with *eptA* alone (Fig. 3 B). A slight increase in TLR4 activation

of the Δ*lpxE* strain complemented with eptA compared to the Δ*lpxE* strain was observed (Fig. 3 B). This might be explained by transfer of *P*-Etn to other parts of the LPS molecule, as suggested by sequence similarity to the 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) *P*-Etn transferase EptB (38). Finally, the Δ*eptA* strain was complemented with *eptA* or *lpxE-eptA* (Fig. 3 C) and the *lpxE-eptA* deletion mutant was complemented with *lpxE-eptA* (Fig. 3 D). We conclude from these complementation experiments that the *eptA* and *lpxE* mutations were non-polar.

To exclude a strong impact on TLR4 activation upon mutation of *lpxE*, *eptA* or *lpxE* and *eptA* due to varying levels of LPS or LPS made accessible by heat killing, we determined the LPS amount in all strains by Western-blot experiments on equal amount of proteinase K treated bacterial lysates with a *C. canimorsus* 5 LPS specific antiserum (Fig. S1). We observed similar LPS band intensities for all strains tested, indicating that LPS amounts present in the bacteria and made accessible by heat treatment are not dramatically changed upon mutation of *lpxE*, *eptA* or *lpxE* and *eptA.* Notably, a slight size-shift of the LPS band was observed for all strains predicted not to exhibit a wt lipid A (Δ*lpxE*, Δ*eptA,* Δ*lpxE-eptA* and Δ*lpxE* + p-eptA). Migration pattern is altered for all strains predicted not to have the positively charged ethanolamine moiety present, which might explain this observation.

LpxE and EptA increase resistance to Polymyxin B. Lipid A modifications have been shown not only to affect endotoxicity, but also to alter resistance to CAMP such as Polymyxin B (10, 29, 39, 40). Hence, we monitored the MIC of Polymyxin B for *C. canimorsus* wt, Δ*lpxE*, Δ*eptA* and the double-knockout Δ*lpxE-eptA* strains. *C. canimorsus* wt was highly resistant to Polymyxin B, as it was still able to grow in the

presence of 512 mg/L Polymyxin B (MIC ≥1024 mg/L) (Fig. 4). The MIC decreased to 512 mg/L for Δ*eptA* mutant bacteria and to 128 mg/L for the Δ*lpxE* bacteria, showing an increased sensitivity to Polymyxin B (Fig. 4). The *C. canimorsus lpxE-eptA* double-mutant had the same MIC as the single Δ*lpxE* mutant (Fig. 4). The Δ*lpxE* strain could be complemented in trans with *lpxE*, but not with *eptA* alone (Fig. 4). The Δ*eptA* strain was complemented with *eptA* (Fig. 4) and the *lpxE-eptA* deletion mutant was complemented with *lpxE-eptA* (Fig. 4). We conclude from these complementation experiments that the *lpxE*, *eptA* and *lpxE-eptA* mutations were non-polar.

The *P*-Etn modification at position C-1 thus contributed to the low endotoxicity and Polymyxin B resistance of *C. canimorsus*, as was shown for *H. pylori* (12). The identical phenotype in endotoxicity and Polymyxin B sensitivity of the single Δ*lpxE* and the double Δ*lpxE-eptA* mutants suggests that the *P*-Etn containing lipid A is synthesized by a two-step enzymatic process similar to that described for *H. pylori* (15, 16). In *H. pylori*, lipid A also carries a *P*-Etn group at position C-1, generated in the course of the LPS biosynthesis by removal of the lipid A 1-phosphate by LpxE followed by transfer of a *P*-Etn residue by EptA from phosphatidylethanolamine to the free reducing end of GlcN(I), where dephosphorylation is necessary for substitution of 1-phosphate with *P*-Etn (12, 15, 16). The non-polar deletion of *lpxE* in *C. canimorsus* does not prevent the synthesis of EptA but likely leads to a lipid A with a 1-phosphate group, which would explain the high endotoxicity observed for this strain. Therefore, as in *H. pylori*, the *C. canimorsus* EptA seems to accept only the free reducing end of the lipid A backbone generated by the activity of LpxE as a substrate.

The Δ*lpxE* mutation had a more severe effect than the one of Δ*eptA*, both with respect to endotoxicity and Polymyxin B sensitivity. The difference between the two mutants can be explained by the fact that EptA adds a negative and a positive charge, whereas LpxE only removes a negative charge. In the two-step mechanism, the Δ*lpxE* mutation would lead to an increase of a negative charge (the unsubsituted 1-phosphate) compared to the wt, while the Δ*eptA* mutation would result in a free reducing end of lipid A as compared to the *P*-Etn in the wt. As net negative charges are important for interaction with CAMP as well as with TLR4/MD-2 (41) one would expect Δ*lpxE* to affect endotoxicity and CAMP sensitivity more than Δ*eptA*, which we found. This again supports the two-step enzymatic process of formation of the 1 *P*-Etn.

It is noteworthy that one would expect the mutation of *eptA* not to affect any charge dependent mechanisms, as no net charge change is expected. *C. canimorsus* Δ*eptA* bacteria showed increased endotoxicity and decreased CAMP resistance as the wt lipid A, while the lipid A variant predicted for a Δ*eptA* deletion mutant is lacking both the 1 and 4'-phosphate in the lipid A backbone. The *C. canimorsus* 1-dephospho lipid A in a Δ*eptA* mutant is not expected to be endotoxic at all, as this lipid A species lacks both phosphates, and thus the negative charges which are important for endotoxicity (41). Still the Δ*eptA* mutation had a small phenotype in Polymyxin B sensitivity and a more pronounced phenotype in endotoxicity. This hints at a heterogeneous lipid A population in the Δ*eptA* strain, which could result from a restricted activity of LpxE. Assuming a non-stoichiometric activity of LpxE in the Δ*eptA* strain, both the lipid A containing a free reducing end as well as the 1-phosphate at GlcN(I) should be present. In this case, the 1-phospho lipid A variant could account for the increase in endotoxicity, while its reduced amounts as compared to Δ*lpxE* would explain the higher endotoxicity of the

348 Δ*lpxE* over the Δ*eptA* deletion mutant strain. It might thus be that the accumulation of 1-
349 dephosphorylated lipid A exerts a feedback regulatory effect on the activity of LpxE,
350 preventing full dephosphorylation in the absence of EptA. The fraction of 1-phospho lipid
351 A would thus increase, which would then be responsible for the observed increase in
352 endotoxicity and sensitivity to Polymyxin B.
353
354 **MS-based structural analysis of *eptA*, *lpxE* and *lpxE-eptA* deletion mutants.** In
355 order to validate the enzymatic activities proposed for LpxE and EptA and the predicted
356 two-step enzymatic mechanism, we performed MS-based structural analysis of isolated
357 lipid A species of the corresponding deletion mutants. One of the predicted lipid A
358 structure, the 4'- and 1-hydroxy lipid A in case of the Δ*eptA* deletion mutant is devoid of
359 any negative charge and thus not accessible to be analyzed in the negative ion mode.
360 Therefore, negative and positive ion mode MS was run to determine all lipid A variants
361 expected based on the genetic analysis and endotoxic activity, respectively.
362
363 In the negative ion mode, MS analysis confirmed the wt lipid A (*m/z* calculated: 1716.3,
364 *m/z* found: 1717) (Table 3 and Fig. 5). Observed mass differences of 14 *m/z* units (*m/z*
365 1731 or 1703) were assigned to acyl chain heterogeneity. For all samples analyzed by
366 MS such peak "clusters" differing by Δ14 *m/z* units were found, suggesting that acyl
367 chain heterogeneity was independent of *lpxE* or *eptA* mutagenesis. This is in agreement
368 with our previous data on wt *C. canimorsus* lipid A (19, 34).
369 In the negative ion mode all deletion mutant strains (Δ*eptA*, Δ*lpxE* and Δ*lpxE-eptA*)
370 showed a main peak at *m/z* of 1674 (Table 3, Fig. 5). The 1-phospho lipid A variant
371 predicted for Δ*lpxE* and Δ*lpxE-eptA* has a calculated *m/z* of 1673.3. Hence, Δ*eptA*,

372 Δ*lpxE* and Δ*lpxE-eptA* deletion mutant strains feature 1-phospho lipid A. While this is the
373 variant expected to occur for Δ*lpxE* and Δ*lpxE-eptA* strains, Δ*eptA* had been predicted
374 to lack the 1-phosphate, thus having a free reducing end for GlcN(I) in the lipid A
375 backbone (*m/z* calculated 1575.3). However, due to the lack of a negative charged
376 group, this de-phospho lipid A variant can't be accessed by MS analysis in the negative
377 ion mode. Nevertheless, the detection of 1-phospho lipid A as well in the Δ*eptA* deletion
378 mutant strain is in perfect agreement with the intermediary phenotype observed in
379 endotoxicity and CAMP resistance (Figs. 3 and 4) and the proposed non-stoichiometric
380 activity of LpxE in the Δ*eptA* strain.
381
382 In order to further confirm the postulated enzymatic mechanisms, we performed
383 negative ion mode MS analysis also on complemented mutants (Fig. S2). The Δ*lpxE*
384 strain could be complemented in trans with *lpxE*, as we confirmed the wt lipid A for the
385 Δ*lpxE* strain complemented with p-*lpxE* (*m/z* calculated: 1716.3, found: 1717). The
386 Δ*lpxE* strain could not be complemented in trans with *eptA* alone (Fig. S2) and the
387 resulting strain showed a main peak at *m/z* of 1674 (Fig. S2), matching the 1-phospho
388 lipid A variant predicted for the Δ*lpxE* strain (*m/z* calculated: 1673.3). The Δ*eptA* strain
389 was complemented with *eptA* (Fig. S2) and the *lpxE-eptA* deletion mutant was
390 complemented with *lpxE-eptA* (Fig. S2), as in both cases the wt lipid A for these strains
391 was found (*m/z* calculated: 1716.3, found: 1717).
392 Additional peaks measured at *m/z* of 1755/1769 for the Δ*lpxE* + p-*lpxE* and the Δ*eptA* +
393 p-*eptA* strain are attributed to a minor lipid A variant with two phosphates present and
394 possibly with a classical GlcN-GlcN backbone, know to be present in *C. canimorsus 5*
395 (*m/z* calculated: 1755.209; peak shift of Δ14 *m/z* units due to acyl chain heterogeneity)

(19). The two phosphates might either be present as 1-phospho 4'-phospho lipid A or as 1-pyrophosphate lipid A. Based on 1-pyrophosphate species detected in various species (42) we would hypothesize, that the peaks measured at *m/z* of 1755/1769 correspond to a 1-pyrophosphate variant with a classical *E. coli*-type GlcN-GlcN lipid A backbone. The peak measured at *m/z* of 1725 for the Δ*lpxE* + p-eptA strain is similarly attributed to a bisphosphorylated species in combination with an exchange of 17:0(3-OH) by 15:0(3-OH) (*m/z* calculated: 1725.186). The detection of these peaks exclusively in complemented mutants and the resulting changes in acyl chain preference and backbone structure, however, deserves further clarification.

We conclude from these complementation experiments that the *eptA* and *lpxE* mutations were non-polar and that in the complemented mutants, Δ*lpxE* + p-lpxE, Δ*eptA* + p-eptA and Δ*lpxE-eptA* + p-lpxE-eptA, the wt lipid A is reconstituted.

In the positive ion mode, MS analysis confirmed the wt lipid A (*m/z* calculated: 1764.3, found: 1764) (Table 3 and Fig. 6). Again peak "clusters" differing by ∆14 *m/z* units were found for all samples. The 1-phospho lipid A variant has a calculated *m/z* of 1720.2 [M-H^++2Na^+] in the positive ion mode. The main peak for Δ*lpxE* and Δ*lpxE-eptA* deletion mutants was found at *m/z* of 1721.6 and 1721.0, respectively. This peak was absent in the Δ*eptA* deletion mutant strain. The Δ*eptA* deletion mutant's main peak present at *m/z* of 1604/1618 (peak shift of 14 *m/z* units due to acyl chain heterogeneity), corresponded to a free hydroxy group forms a reducing end in the lipid A backbone and has been calculated to *m/z* of 1603.3/1617.3. It is noteworthy that a peak at *m/z* of 1601 is found in all samples in the positive ion mode (and the corresponding peak shifted by 14 *m/z* units). The presence of 1-dephospho lipid A variants even in those mutants which

contain the 1-phospho group has been assigned as artefacts well known to appear from the wt strains due to the acid hydrolysis conditions necessary to liberate the lipid A from the phosphorylated Kdo found in the core of *C. canimorsus* LPS (19, 34). These conditions obviously lead to a partial de-phosphorylation at the 1 position of lipid A. Even more, the main peak from the 1-hydroxy lipid A cluster for Δ*lpxE* and Δ*lpxE-eptA* deletion mutants was found at *m/z* 1601 and not at *m/z* 1604/1618 as predicted. We assume that the peak at 1604 *m/z* found in the Δ*eptA* deletion mutant is identical to peaks at *m/z* of 1601 found in Δ*lpxE* and Δ*lpxE-eptA* deletion mutants.

According to the proposed model, the major and representative lipid A molecule of the Δ*eptA* mutant lacks any charged group and, therefore, its pseudomolecular ion [M+Na^+] can only be analyzed in the positive ion mode. In agreement with this, in the positive (but not the negative) ion mode of Δ*eptA* lipid A, the pseudomolecular ion [M+Na^+] was detected. The ion [M-H+] detected in the negative ion mode of Δ*eptA* lipid A was likely raised from incomplete de-phosphorylated lipid A, resulting in 1-phospho lipid A. Notably, the [M-H^+] ion was not detected for the Δ*eptA* mutant in the positive ion mode, possibly reflecting its small proportion.

We further performed positive ion mode MS analysis on complemented mutants (Fig. S3). The Δ*lpxE* strain could be complemented in trans with *lpxE*, as we confirmed the wt lipid A for this strain (*m/z* calculated: 1764.3, found: 1764). The Δ*lpxE* strain could not be complemented in trans with *eptA* alone (Fig. S3) and the resulting strain showed a main peak at *m/z* 1722 (Fig. S3), matching the 1-phospho lipid A variant predicted for Δ*lpxE* (*m/z* calculated: 1720.2). The Δ*eptA* strain was complemented with *eptA* (Fig. S3) and

444 the *lpxE-eptA* deletion mutant was complemented with *lpxE-eptA* (Fig. S3), as in both
445 cases the wt lipid A for these strains was found (*m/z* calculated: 1764.3, found: 1764).
446 Additional peaks measured at *m/z* of 1803/1817 for the Δ*lpxE* + p-*lpxE* and the Δ*eptA* +
447 p-*eptA* strain are attributed to a minor bisphosphorylated lipid A backbone variant,
448 probably with a *E. coli*-type classical GlcN-GlcN backbone know also to be present in
449 small amounts in *C. canimorsus 5* (*m/z* calculated: 1802.196; peak shift of 14 *m/z* units
450 due to acyl chain heterogeneity) (19) and correlate with additional peaks observed in the
451 negative ion mode MS analysis of these strains.
452 We conclude from these complementation experiments that the *eptA* and *lpxE* mutations
453 were non-polar and that in the complemented mutants, Δ*lpxE* + p-*lpxE*, Δ*eptA* + p-*eptA*
454 and Δ*lpxE-eptA* + p-*lpxE-eptA*, the wt lipid A is reconstituted.
455
456 Overall, 1-phospho lipid A was validated as main lipid A variant in Δ*lpxE* and Δ*lpxE-eptA*
457 deletion mutants. This confirms that LpxE acts as lipid A 1-phosphatase and further
458 corroborates the two step enzymatic mechanism, in which EptA is only active after LpxE
459 dependent removal of the 1-phosphate on lipid A. In agreement with endotoxicity and
460 CAMP resistance, both 1-phospho as well as 1-de-phospho lipid A variants were found
461 present in the Δ*eptA* deletion mutant. This validates the function of EptA as lipid A
462 phosphoethanolamine transferase and again supposes a two step enzymatic activity, in
463 which LpxE can dephosphorylate lipid A even in the absence of EptA. But LpxE seems
464 not to dehosphorylate every lipid A in the absence of EptA, which is reflected by the 1-
465 phospho lipid A species identified in the Δ*eptA* deletion mutant.
466

The lipid A modification described in this work represents a clear virulence factor since it dramatically reduces recognition and killing by the host's innate immune system. However, human infections are rare events and dead ends for *C. canimorsus*. Thus, we can envision that the lipid A modification most likely evolved as a factor favouring the adaptation of *C. canimorsus* to its natural niche, the dog's mouth.

Funding information

This work was supported by the Swiss National Science Foundation (grant 3100A0-128659) and the ERC (AdG grant 2011-293605-CAPCAN) to G. R. C.. F.R. is a postdoctoral research fellow (chargé de recherche) of the Belgian "Fonds National de la Recherche Scientifique" (FNRS). S.J.I. was supported by the Werner-Siemens Foundation. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

References

1. **Coats SR, Jones JW, Do CT, Braham PH, Bainbridge BW, To TT, Goodlett DR, Ernst RK, Darveau RP.** 2009. Human Toll-like receptor 4 responses to *P. gingivalis* are regulated by lipid A 1- and 4'-phosphatase activitie. Cell Microbiol **11:**1587-1599.
2. **Dixon DR, Darveau RP.** 2005. Lipopolysaccharide heterogeneity: innate host responses to bacterial modification of lipid a structure. J Dent Res **84:**584-595.
3. **Hajjar AM, Ernst RK, Tsai JH, Wilson CB, Miller SI.** 2002. Human Toll-like receptor 4 recognizes host-specific LPS modifications. Nat Immunol **3:**354-359.
4. **Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC.** 2007. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. Science **316:**1628-1632.
5. **Price NP, Jeyaretnam B, Carlson RW, Kadrmas JL, Raetz CR, Brozek KA.** 1995. Lipid A biosynthesis in *Rhizobium leguminosarum*: role of a 2-keto-3-deoxyoctulosonate-activated 4' phosphatase. Proc Natl Acad Sci U S A **92:**7352-7356.
6. **Wang X, Karbaz MJ, McGrath SC, Cotter RJ, Raetz CRH.** 2004. MsbA transporter-dependent lipid A 1-dephosphorylation on the periplasmic surface of the inner membrane: topography of francisella novicida LpxE expressed in *Escherichia coli* J Biol Chem **279:**49478.
7. **Wang X, McGrath SC, Cotter RJ, Raetz CRH.** 2006. Expression cloning and periplasmic orientation of the *Francisella novicida* lipid A 4'-phosphatase LpxF. J Biol Chem **281:**9321-9330.
8. **Curtis MA, Percival RS, Devine D, Darveau RP, Coats SR, Rangarajan M, Tarelli E, Marsh PD.** 2011. Temperature-dependent modulation of *Porphyromonas gingivalis* lipid A structure and interaction with the innate host defenses. Infect Immun **79:**1187-1193.

9. **Herrera CM, Hankins JV, Trent MS.** 2010. Activation of PmrA inhibits LpxT-dependent phosphorylation of lipid A promoting resistance to antimicrobial peptides. Mol Microbiol **76:**1444-1460.

10. **Ingram BO, Sohlenkamp C, Geiger O, Raetz CRH.** 2010. Altered lipid A structures and polymyxin hypersensitivity of *Rhizobium etli* mutants lacking the LpxE and LpxF phosphatases. Biochim Biophys Acta **1801:**593-604.

11. **Wang X, Ribeiro AA, Guan Z, Abraham SN, Raetz CRH.** 2007. Attenuated virulence of *Francisella* mutant lacking the lipid A 4'-phosphatase. Proc Natl Acad Sci U S A **104:**4136-4141.

12. **Cullen TW, Giles DK, Wolf LN, Ecobichon C, Boneca IG, Trent MS.** 2011. *Helicobacter pylori* versus the host: remodeling of the bacterial outer membrane is required for survival in the gastric mucosa. PLoS Pathog **7:**e1002454.

13. **Kanistanon D, Powell DA, Hajjar AM, Pelletier MR, Cohen IE, Way SS, Skerrett SJ, Wang X, Raetz CR, Ernst RK.** 2012. Role of *Francisella* lipid A phosphate modification in virulence and long-term protective immune responses. Infect Immun **80:**943-951.

14. **Ingram BO, Masoudi A, Raetz CRH.** 2010. *Escherichia coli* mutants that synthesize dephosphorylated lipid A molecules. Biochemistry (Mosc) **49:**8325-8337.

15. **Tran AX, Karbaz MJ, Wang X, Raetz CRH, McGrath SC, Cotter RJ, Trent MS.** 2004. Periplasmic cleavage and modification of the 1-phosphate group of *Helicobacter pylori* lipid A. J Biol Chem **279:**55791.

16. **Tran AX, Whittimore JD, Wyrick PB, McGrath SC, Cotter RJ, Trent MS.** 2006. The lipid A 1-phosphatase of *Helicobacter pylori* is required for resistance to the antimicrobial peptide Polymyxin. J Bacteriol **188:**4531-4541.

17. **Cox AD, Wright JC, Hood DW, Moxon ER, Richards JC.** 2003. Phosphorylation of the lipid A region of *meningococcal* lipopolysaccharide: identification of a family of

transferases that add phosphoethanolamine to lipopolysaccharide. J Bacteriol **185:**3270-3277.

18. **Kim SH, Parreira VR, Bishop RE, Gyles CL.** 2006. Phosphoethanolamine substitution in the lipid A of *Escherichia coli* O157 : H7 and its association with PmrC. Microbiology **152:**657-666.

19. **Ittig S, Lindner B, Stenta M, Manfredi P, Zdorovenko E, Knirel YA, dal Peraro M, Cornelis GR, Zähringer U.** 2012. The lipopolysaccharide from *Capnocytophaga canimorsus* reveals an unexpected role of the core-oligosaccharide in MD-2 binding. PLoS Pathog **8(5)** e1002667.

20. **Bobo RA, Newton EJ.** 1976. A previously undescribed gram-negative bacillus causing septicemia and meningitis. Am J Clin Pathol **65:**564-569.

21. **Brenner DJ, Hollis DG, Fanning GR, Weaver RE.** 1989. *Capnocytophaga canimorsus* sp. nov. (formerly CDC group DF-2), a cause of septicemia following dog bite, and *C. cynodegmi* sp. nov., a cause of localized wound infection following dog bite. J Clin Microbiol **27:**231-235.

22. **Butler T.** 2015. Capnocytophaga canimorsus: an emerging cause of sepsis, meningitis, and post-splenectomy infection after dog bites. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology **34:**1271-1280.

23. **Lion C, Escande F, Burdin JC.** 1996. *Capnocytophaga canimorsus* infections in human: review of the literature and cases report. Eur J Epidemiol **12:**521-533.

24. **Pers C, Gahrn-Hansen B, Frederiksen W.** 1996. *Capnocytophaga canimorsus* septicemia in Denmark, 1982-1995: review of 39 cases. Clin Infect Dis **23:**71-75.

25. **Bailie WE, Stowe EC, Schmitt AM.** 1978. Aerobic bacterial flora of oral and nasal fluids of canines with reference to bacteria associated with bites. J Clin Microbiol **7:**223-231.

26. **Blanche P, Bloch E, Sicard D.** 1998. *Capnocytophaga canimorsus* in the oral flora of dogs and cats. J Infect **36:**134.

27. **Mally M, Paroz C, Shin H, Meyer S, Soussoula LV, Schmiediger U, Saillen-Paroz C, Cornelis GR.** 2009. Prevalence of *Capnocytophaga canimorsus* in dogs and occurrence of potential virulence factors. Microbes Infect **11:**509-514.

28. **Manfredi P, Pagni M, Cornelis GR.** 2011. Complete genome sequence of the dog commensal and human pathogen *Capnocytophaga canimorsus* strain 5. J Bacteriol **193:**5558-5559.

29. **Raetz CRH.** 1990. Biochemistry of Endotoxins. Annu Rev Biochem **59:**129-170.

30. **Shin H, Mally M, Kuhn M, Paroz C, Cornelis GR.** 2007. Escape from immune surveillance by *Capnocytophaga canimorsus* J Infect Dis **195:**375-386.

31. **Mally M, Cornelis GR.** 2008. Genetic tools for studying *Capnocytophaga canimorsus*. Appl Environ Microbiol **74:**6369-6377.

32. **Ferraro MJ, NCCLS/CLSI.** 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard. NCCLS National Committee for Clinical Laboratory Standards CLSI Clinical Laboratory and Standards Institute.

33. **Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipdman DJ.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:**3389-3402.

34. **Zähringer U, Ittig S, Lindner B, Moll H, Schombel U, Gisch N, Cornelis GR.** 2014. NMR-based structural analysis of the complete rough-type lipopolysaccharide isolated from *Capnocytophaga canimorsus*. The Journal of biological chemistry **289:**23963-23976.

35. **Shin H, Mally M, Meyer S, Fiechter C, Paroz C, Zähringer U, Cornelis GR.** 2009. Resistance of *Capnocytophaga canimorsus* to killing by human complement and polymorphonuclear leukocytes. Infect Immun **77:**2262-2271.

36. **El Hamidi A, Tirsoaga A, Novikov A, Hussein A, Caroff M.** 2005. Microextraction of bacterial lipid A: easy and rapid method for mass spectrometric characterization. J Lipid Res **46:**1773-1778.

37. **Rietschel ET, Kirikae T, Schade FU, Mamat U, Schmidt G, Loppnow H, Ulmer AJ, Zahringer U, Seydel U, Di Padova F, Schreier M, Brade H.** 1994. Bacterial endotoxin: molecular relationships of structure to activity and function. FASEB journal : official publication of the Federation of American Societies for Experimental Biology **8:**217-225.

38. **Reynolds CM, Kalb SR, Cotter RJ, Raetz CR.** 2005. A phosphoethanolamine transferase specific for the outer 3-deoxy-D-manno-octulosonic acid residue of Escherichia coli lipopolysaccharide. Identification of the eptB gene and Ca2+ hypersensitivity of an eptB deletion mutant. The Journal of biological chemistry **280:**21202-21211.

39. **Lee H, Hsu FF, Turk J, Groisman EA.** 2004. The PmrA-regulated pmrC gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in *Salmonella enterica*. J Bacteriol **186:**4124-4133.

40. **Raetz CRH, Reynolds CM, Trent MS, Bishop RE.** 2007. Lipid A modification systems in Gram-negative bacteria. Annu Rev Biochem **76:**295-329.

41. **Park BS, Song DH, Kim HM, Choi B-S, Lee H, Lee J-O.** 2009. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature **458:**1191-1195.

42. **Jones JW, Shaffer SA, Ernst RK, Goodlett DR, Turecek F.** 2008. Determination of pyrophosphorylated forms of lipid A in Gram-negative bacteria using a multivaried mass spectrometric approach. Proc Natl Acad Sci U S A **105:**12742-12747.

607 **Figure legends**

608 **Figure 1.** Structures of *C. canimorsus* 5 and *E. coli* lipid A. (**A**) *C. canimorsus* 5 lipid A
609 consists of a β-(1'→6)-linked GlcN3N'-GlcN disaccharide, to which 3-hydroxy-15-
610 methylhexadecanoic acid, 3-hydroxy-13-methyltetradecanoic acid, 3-*O*-(13-
611 methyltetradecanoyl)-15-methylhexadecanoic acid, and 3-hydroxyhexadecanoic acid
612 are attached at positions 2, 3, 2', and 3', respectively. The disaccharide carries a
613 positively charged ethanolamine at the 1-phosphate and lacks a 4'-phosphate (from ref.
614 (19)) (**B**) *E. coli* hexa-acylated lipid A consisting of a β-(1'→6)-linked GlcN disaccharide
615 that is phosphorylated at positions 1 and 4' and carries four (*R*)-3-hydroxymyristate
616 chains (at positions 2', 3', 2 and 3). The 2' and 3' 3-hydroxylated acyl groups in GlcN'
617 are further esterified with laurate and myristate, respectively (from ref. (29)).
618
619 **Figure 2.** Schematic representation of the proposed enzymatic activity of LpxE, EptA
620 and LpxF in the biosynthesis of *C. canimorsus* lipid A (top) and illustration of the *lpxE-*
621 *eptA* operon (bottom, drawn to scale) corresponding to *Ccan_16960* and *Ccan_16950*,
622 respectively.
623
624 **Figure 3.** Effect of *lpxE* or *eptA* deletion on endotoxicity (A) Endotoxicity of heat-killed *C.*
625 *canimorsus* 5 wild-type (Cc5), Δ*lpxE*, Δ*eptA* or Δ*lpxE-eptA* bacteria. Indicated multiplicity
626 of infection (MOI) of heat-killed bacteria was assayed for TLR4 dependent NFκB
627 activation with HekBlue human TLR4 cells. Data were combined from n=3 independent
628 experiments, error bars indicated are standard error of the mean. (B-D) Same as A but
629 the mutations were complemented in trans by the indicated plasmids. All mutations

showed to be non-polar. Data were combined from n=3 independent experiments, error bars indicated are standard error of the mean.

Figure 4. Effect of *lpxE* or *eptA* deletion on resistance to Polymyxin B. MIC of Polymyxin B for wt *C. canimorsus* 5 (Cc5), Δ*lpxE*, Δ*eptA* or Δ*lpxE-eptA C. canimorsus* 5 as well as complemented mutants. Data were combined from n=3 or 4 independent experiments, where MIC measured were always identical.

Figure 5. Mass spectrometric analysis of lipid A of indicated strains as analyzed by MALDI-TOF MS in the negative ion mode.

Figure 6. Mass spectrometric analysis of lipid A of indicated strains as analyzed by MALDI-TOF MS in the positive ion mode.

644 **Table 1. Bacterial strains and plasmids used in this study**

Strains		
Cc5	Human fatal septicaemia after dog bite 1995	(30)
Cc5 ΔlpxE	Replacement of *Ccan_16960* by *ermF;*Em^r (primer 6493-6498) (Δ 1833737 - 1833995)	This study
Cc5 ΔeptA	Replacement of *Ccan_16950* by *ermF*; Em^r(primer 6499-6504) (Δ 1831370 - 1832888)	This study
Cc5 ΔlpxE-eptA	Replacement of *Ccan_16960-16950* by *ermF;*Em^r (primer 6493-6495 and 6502-6504) (Δ 1831370 - 1833995)	This study
Cc5 Y1C12	Tn4351 insertion in *Ccan_23370*, "*wbuB*" like glycosyltransferase	(35)
Cc5Y1C12 ΔlpxE	Replacement of *Ccan_16960* by *tetQ;*Tc^r (primer 7539-7544) (Δ 1833737 - 1833995)	This study
Cc5 Y1C12 ΔeptA	Replacement of *Ccan_16950* by *tetQ*; Tc^r (primer 7545-7550) (Δ 1831370 - 1832888)	This study
Cc5 Y1C12 ΔlpxE-eptA	Replacement of *Ccan_16960-16950* by *tetQ;*Tc (primers 7539; 7540;7543;7547;7548;7550) (Δ 1831370 - 1833995)	This study
Plasmids		
p-*lpxE*	pMM47.A*lpxE* (expression plasmid encoding complete *lpxE* gene from Cc5)	This study
p-*lpxE-eptA*	pMM47.A*lpxE-eptA* (expression plasmid encoding complete *lpxE-eptA* genes from Cc5)	This study
p-*eptA*	pMM47.A*eptA* (expression plasmid encoding complete *eptA* gene from *Cc5*)	This study
pMM13	ColE1 *ori*; Apr (Em^r); *ermF* from pEP4351	(31)
pMM25	ColE1 *ori*; Kmr(Cf^r); suicide vector for *C. canimorsus*	(31)
pMM47.A	ColE1 *ori* (pCC7 *ori*); Ap^r (Cf^r); *E. coli-C. canimorsus* expression shuttle plasmid, *C. canimorsus* expression is driven by a *ermF* promoter	(31)
pMM104.A	ColE1 *ori* (pCC7 *ori*); Ap^r (Tc^r); *E. coli-C. canimorsus* shuttle plasmid, RP4 oriT. *Pst*I fragment of pMM47.A containing repA inserted into *Pst*I site of pLYL001	(31)
pSI73	pMM25*lpxE::ermF* (suicide vector for deletion of *lpxE*, (Δ 1833737 - 1833995))	This study
pSI74	pMM25*eptA::ermF* (suicide vector for deletion of *eptA*, (Δ 1831370 - 1832888))	This study
pSI76	pMM25*lpxE-eptA::ermF* (suicide vector for deletion of *lpxE-eptA*, (Δ 1831370 - 1833995))	This study

pFR28	pMM25*lpxE::tetQ* (suicide vector for deletion of *lpxE*, (Δ 1833737 - 1833995))	This study
pFR29	pMM25*eptA::tetQ* (suicide vector for deletion of *eptA*, (Δ 1831370 - 1832888))	This study
pFR30	pMM25*lpxE-eptA::tetQ* (suicide vector for deletion of *lpxE-eptA*, (Δ 1831370 - 1833995))	This study

645

646

647 **Table 2. Oligonucleotides used in this study**

648

Ref.	Name	Sequence 5'-3'	Restriction site	Gene	PCR
6493	lpxE-A	CCCTGCAGGGCACGTTCGTACCAGTTA	*Pst*I	*lpxE*	A
6494	lpxE-B	GAGTAGATAAAAGCACTGTTATTTGCTTATTTTGAATATTTCGG		*lpxE*	B
6495	lpxE-C	CTTATATTTGCCGCCGAAATATTCAAAATAAGCAAATAACAGTGCTTTTATCTACTCCGATAGCTTC		*ermF*	C
6496	lpxE-D	CTTGCATTATCTTAACACTCATAAAAACAACACTCCCCTACGAAGGATGAAATTTTTCAGGGACAAC		*ermF*	D
6497	lpxE-E	AAAAATTTCATCCTTCGTAGGGGAGTGTTGTTTTTATGAGTGTT		*lpxE*	E
6498	lpxE-F	CAACTAGTAAACCGTTTCAGTTTGGGT	*Spe*I	*lpxE*	F
6499	eptA-A	CCCTGCAGTGTTCCTCGCCCTGTTAC	*Pst*I	*eptA*	A
6500	eptA-B	GAGTAGATAAAAGCACTGTTTTATTGATTTTTTTTAACATAAAATTTTATC		*eptA*	B
6501	eptA-C	GTTGTACTTAATGATAAAATTTTATGTTAAAAAAAATCAATAAAACAGTGCTTTTATCTACTCCGATAGCTTC		*ermF*	C
6502	eptA-D	ATCTTGTAAATTACGGATTGGTCATTCAATAATTCTACGAAGGATGAAATTTTTCAGGGACAAC		*ermF*	D
6503	eptA-E	AAAAATTTCATCCTTCGTAGAATTATTGAATGACCAATCCG		*eptA*	E
6504	eptA-F	CAACTAGTTCCACCTCATTGAGATTCAC	*Spe*I	*eptA*	F
6646	p-lpxE-fw	CGTACCATGGTTTTTAAAGAATCAGCAAATAACC	*Nco*I	*lpxE*	
6647	p-lpxE-rev	CAGTTCTAGATTATTGATTTTTTTTAACATAAAATTTTATC	*Xba*I	*lpxE*	
6648	p-eptA-fw	CGTACCATGGGATTAAAAAAAATCAATAAATGGACTAACA	*Nco*I	*eptA*	
6649	p-eptA_rev	GCTTCTCGAGTTAGTCAAAAATGCTCATTTGC	*Xho*I	*eptA*	
7539	lpxEtetKO-A	GGCTGCAGTTTCCATTCCTTTGGCACGTTCG	*Pst*I	*lpxE*	A
7540	lpxEtetKO-B	CAAAATCAAATGTTAAAAAAAAATTTGCTTATTTTGAATATTTCGGC		*lpxE*	B
7543	lpxEtetKO-C	GCCGAAATATTCAAAATAAGCAAATTTTTTTTTAACATTTGA		*tetQ*	C

		TTTTG			
7544	lpxEtetKO-D	GATTTTTTTTAACATAAAATTTTATCTTATTTTGATGACATTG ATTTTTGG		*tetQ*	D
7541	lpxEtetKO-E	CCAAAAATCAATGTCATCAAAATAAGATAAAATTTTATGTTA AAAAAAATC		*lpxE*	E
7542	eptAtetKO-F	GGACTAGTCAAGGTAAAGCCAATGTTAAGC 1.1.1	*Spe*I	*lpxE*	F
7545	eptAtetKO-A	GGCTGCAGTATGGGGAGGAAAGCGTCAATATTG	*Pst*I	*eptA*	A
7546	eptAtetKO-B	CAAAATCAAATGTTAAAAAAAAAGCGGTACATTGTTAGTCCA TTTATTG		*eptA*	B
7549	eptAtetKO-C	CAATAAATGGACTAACAATGTACCGCTTTTTTTTAACATTTG ATTTTG		*tetQ*	C
7550	eptAtetKO-D	CGGATTGGTCATTCAATAATTTTATTTTGATGACATTGATTT TTGG		*tetQ*	D
7547	lpxEtetKO-E	CCAAAAATCAATGTCATCAAAATAAAATTATTGAATGACCAA TCCG		*eptA*	E
7548	eptAtetKO-F	GGACTAGTCATTAAGTGCTACCCCTATCTTATC	*Spe*I	*eptA*	F

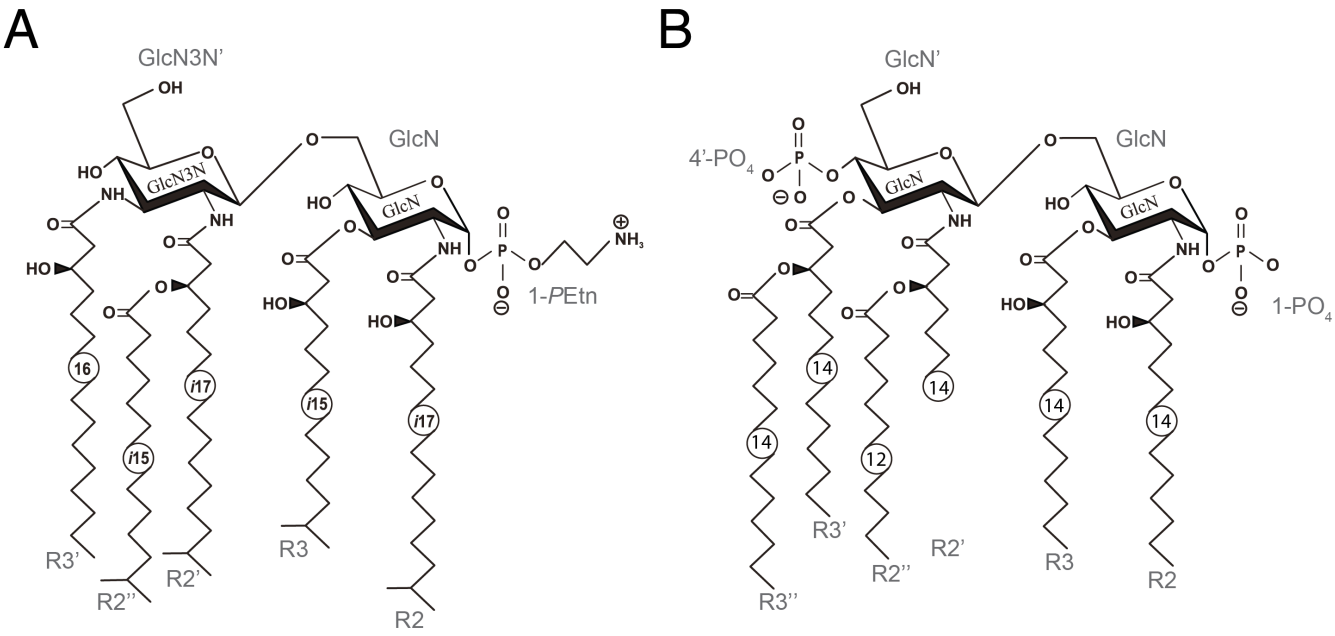
649

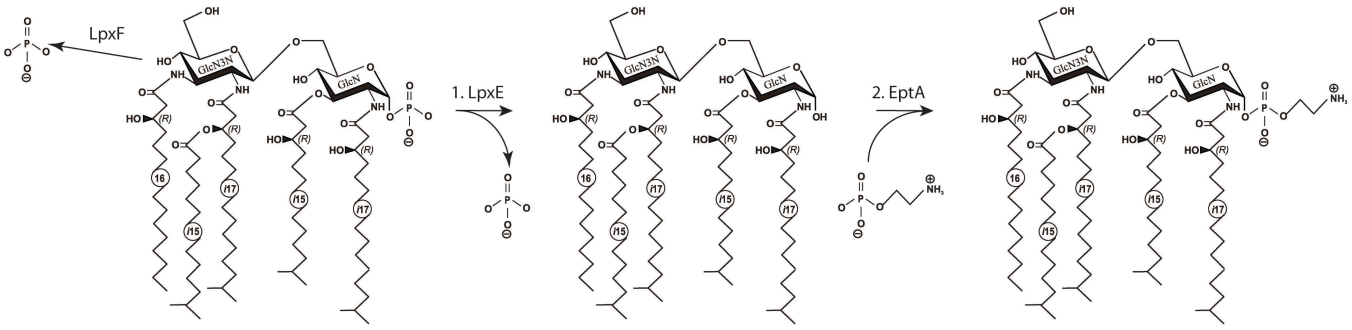
Infection and Immunity

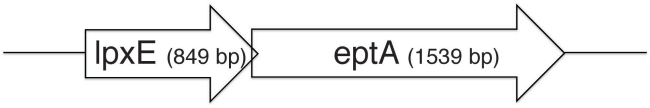
Table 3. MS analysis and interpretation of lipid A variants in wt, *lpxE*, *eptA*, and *lpxE-eptA* deletion mutant strains.

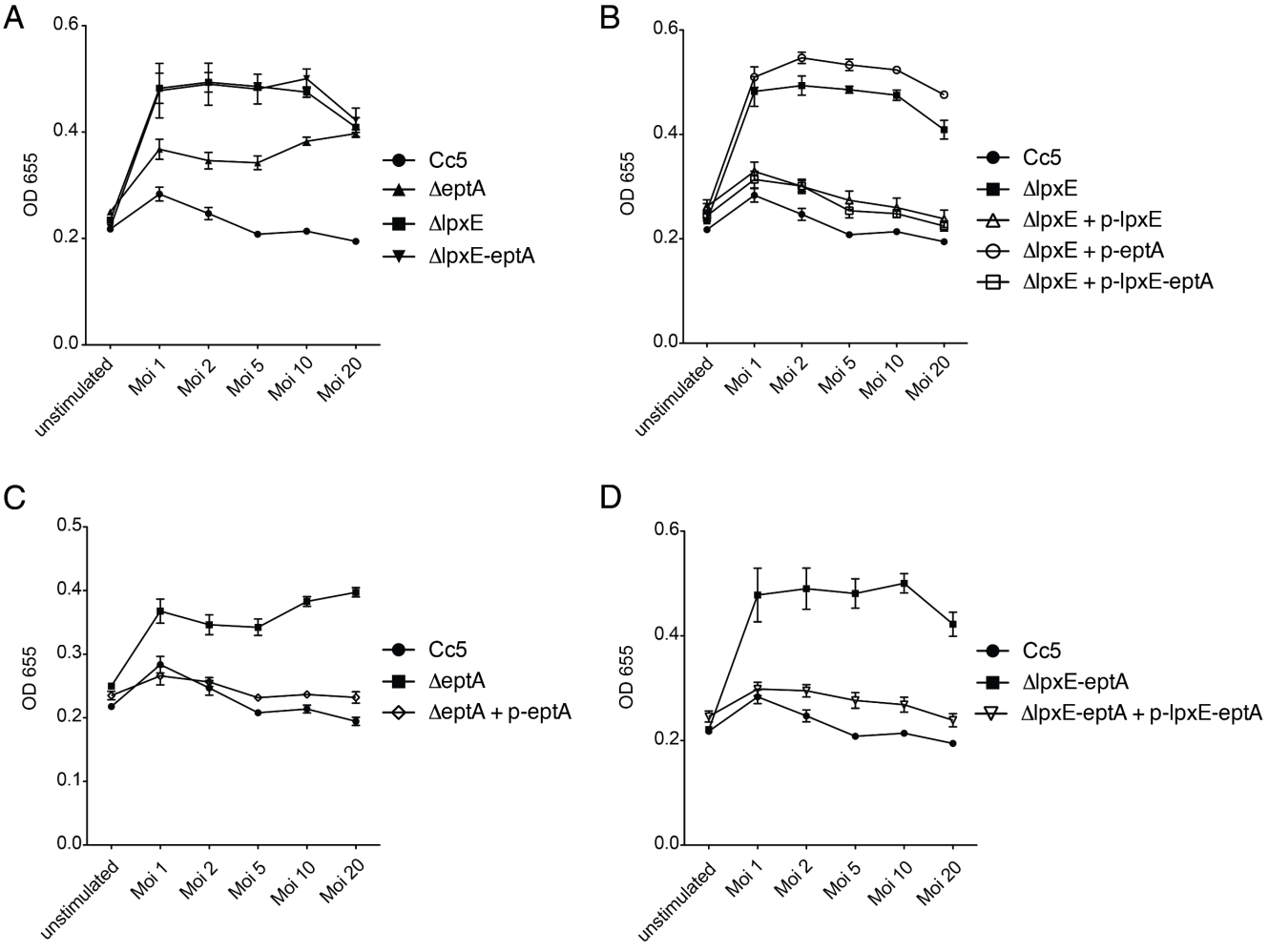
Component	wt	mutant		
		Δ*lpxE*	Δ*eptA*	Δ*lpxE-eptA*
GlcN	1	1	1	1
GlcN3N	1	1	1	1
P	1	1	0	1
Etn	1	0	0	0
*i*15:0	1	1	1	1
*i*15:0(3-OH)	1	1	1	1
16:0(3-OH)	1	1	1	1
*i*17:0(3-OH)	1	1	1	1
m/z calc. (neg. mode)	1716.3	1673.3	1575.3	1673.3
found for [M-H^+]	1716.8	1673.4	(1673.9)*	1673.1
m/z calc. (pos. mode)	1764.3	1720.2	1603.3	1720.2
found for	1764 [M-H^++2Na^+]	1722 [M-H^++2Na^+]	1604 [M+Na^+]	1722 [M-H^++2Na^+]

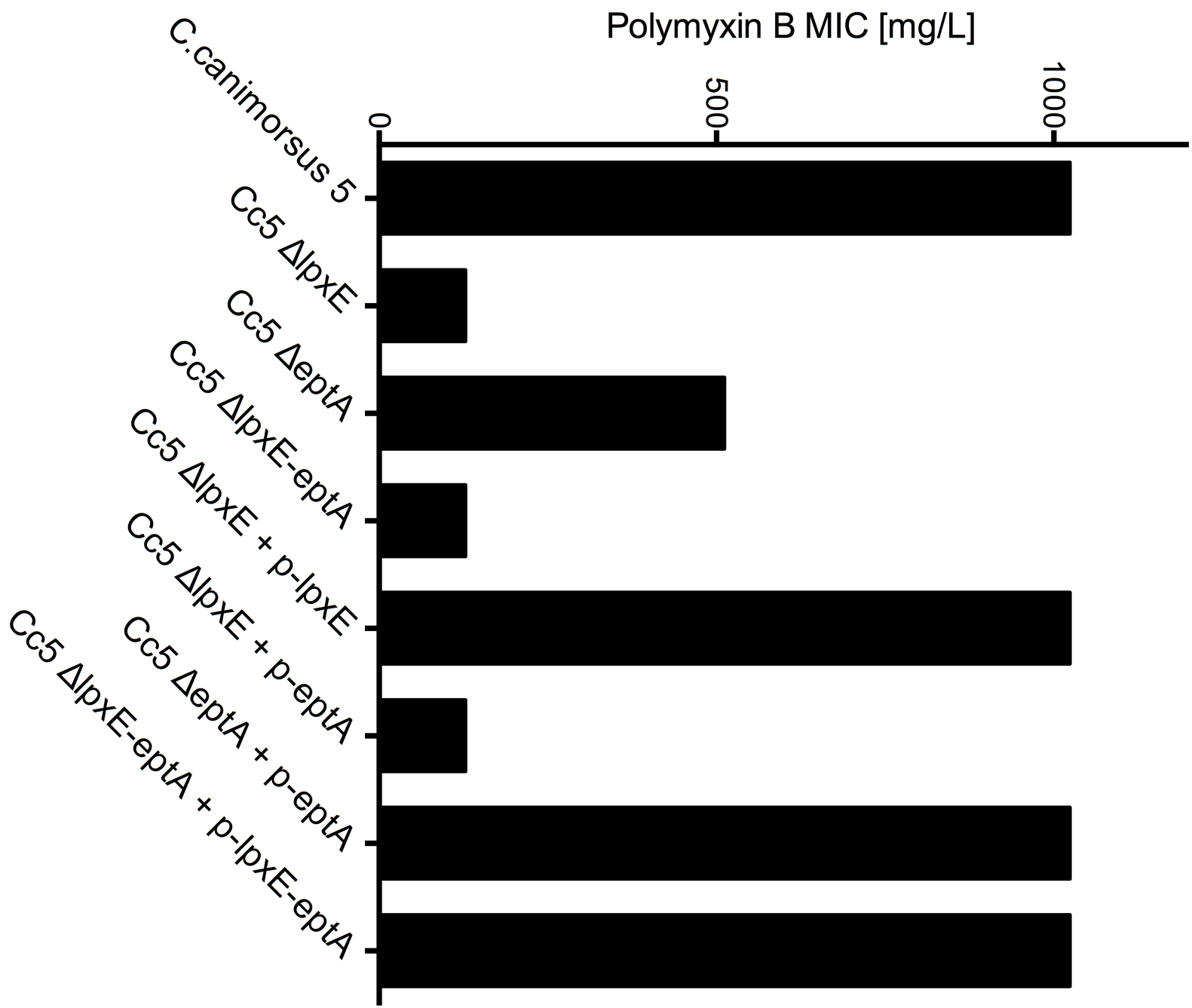
* Ion [M-H^+] detected in the neg. ion mode of Δ*eptA* lipid A was raised from incomplete de-phosphorylated lipid A. The major and representative lipid A molecule of this mutant lacks any charged group and, therefore, its pseudomolecular ion [M+Na^+] could only be analyzed in the positive ion mode.

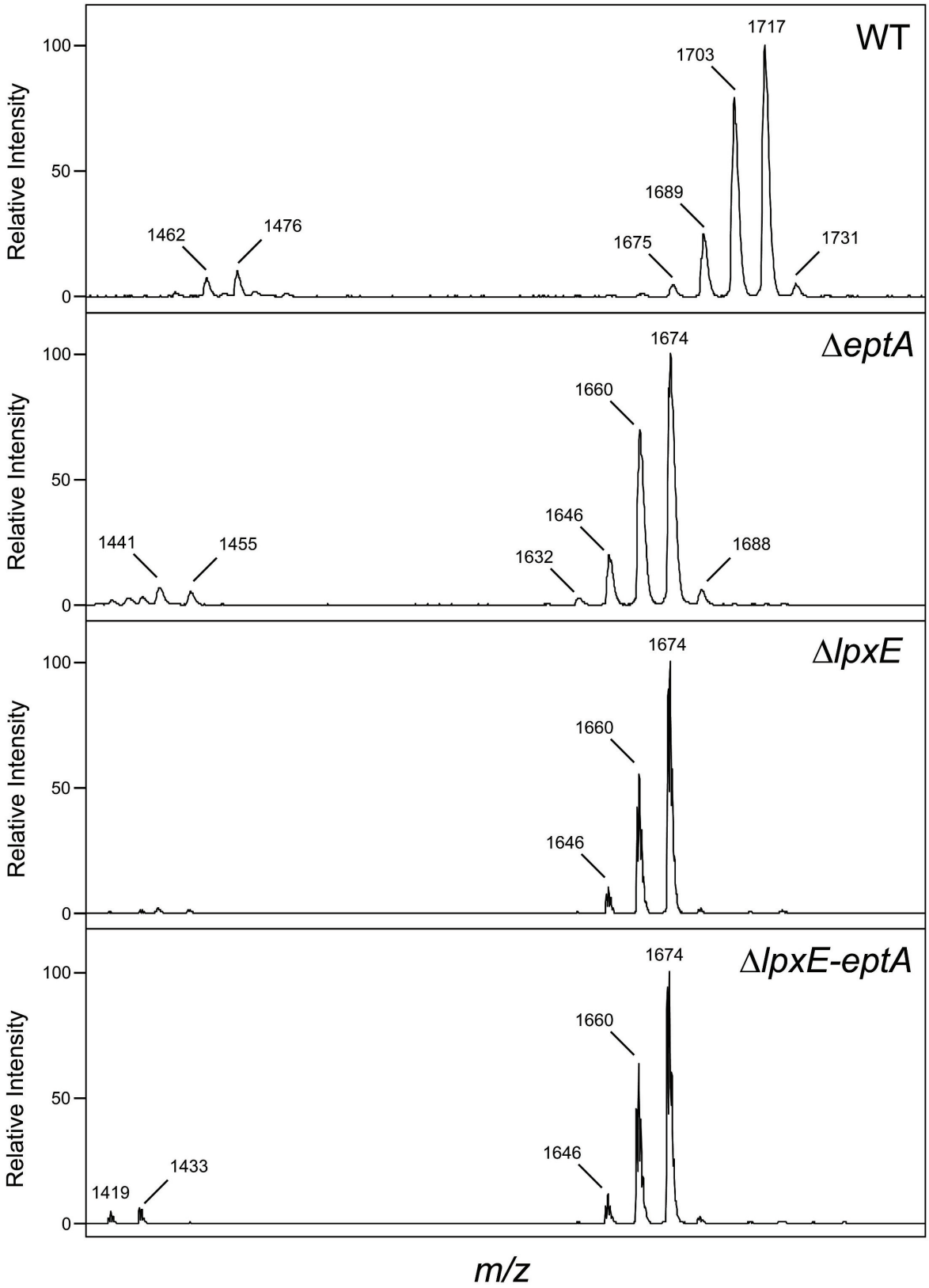




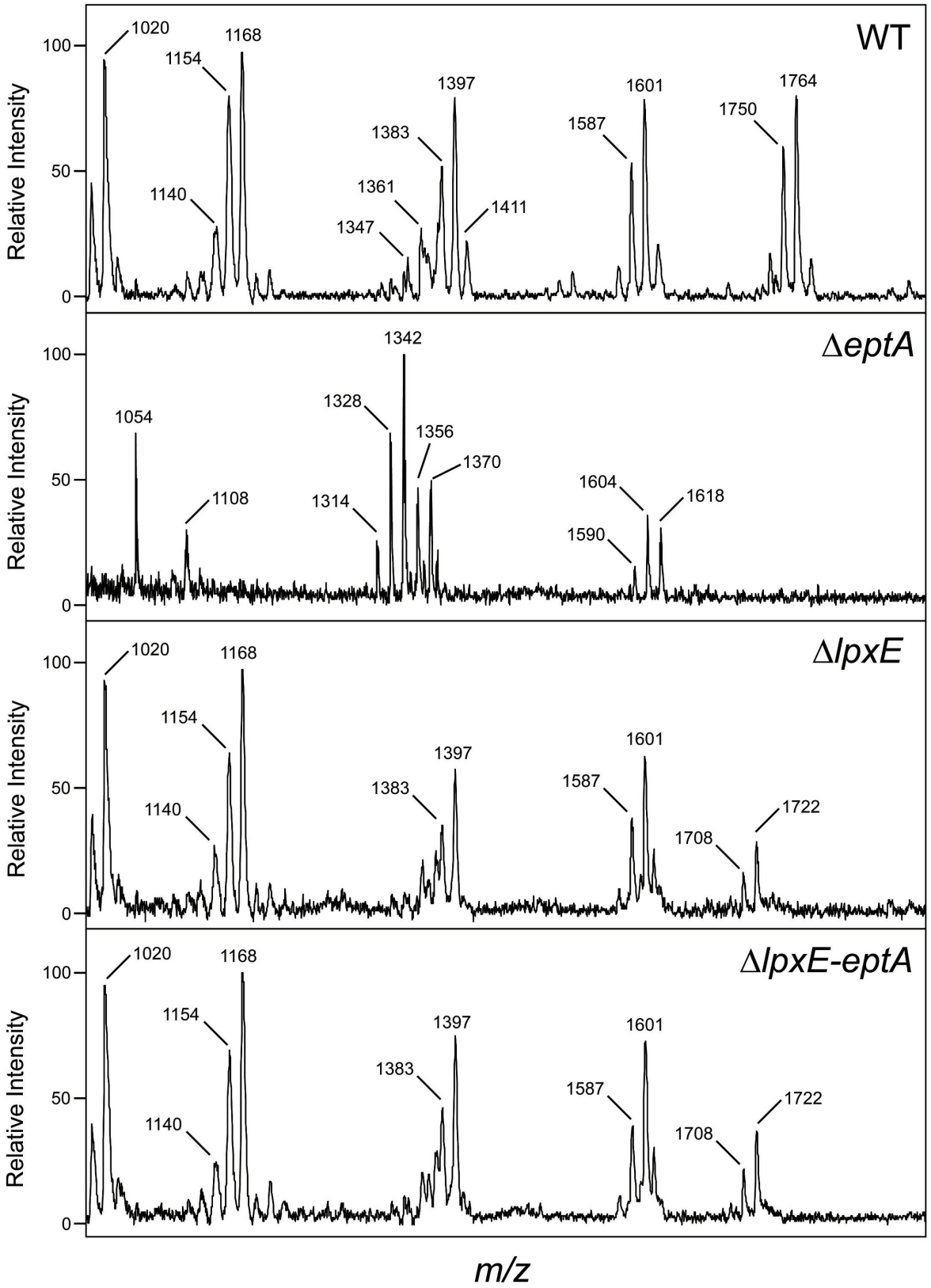





Polymyxin B MIC [mg/L]
0
500
1000
C.canimorsus 5
Cc5 ΔlpxE
Cc5 ΔeptA
Cc5 ΔlpxE-eptA
Cc5 ΔlpxE + p-lpxE
Cc5 ΔlpxE + p-eptA
Cc5 ΔeptA + p-eptA
Cc5 ΔlpxE-eptA + p-lpxE-eptA



Negative ion mode



Positive ion mode