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

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

3  
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24 Running title: *C. canimorsus* lipid A modification

25 Key words: endotoxicity, Gram-negative bacteria, LPS, outer membrane, TLR4  
26

27 **Abstract**

28

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

46

**212 words**

47

48 **Introduction**

49

50 Some Gram-negative bacteria have evolved different modifications of their lipid A  
51 structure, leading to a reduced recognition by the host and sensitivity to CAMP (1-7).

52 One of these modifications occurs on the 1- or on the 4'-phosphate of lipid A (1, 4, 7-10).

53 4'-phosphatases (LpxF) have been described in *Rhizobium leguminosarum*, *Rhizobium*  
54 *etli*, *Porphyromonas gingivalis*, *Francisella* species and *Helicobacter pylori* (1, 10-12).

55 Deletion of *lpxF* and the resulting presence of the 4'-phosphate on lipid A leads to  
56 increased endotoxicity (1, 12) and decreased resistance to CAMP (10, 12). In the case

57 of *Francisella* and *H. pylori* virulence is reduced (11, 12, 13 ). 1-phosphatases (LpxE)  
58 have been identified in *H. pylori*, *P. gingivalis*, *R. etli* and others (1, 6, 10, 12, 14-16).

59 Deletion of *lpxE* and the resulting presence of the 1-phosphate on lipid A leads to a  
60 slightly increased endotoxicity (1) and CAMP sensitivity (10). In *H. pylori*, the 1 position

61 is further modified by the addition of a *P*-Etn (15, 17, 18), a modification known from  
62 other bacteria (15, 17, 18). This happens via a two-step mechanism, which first involves

63 dephosphorylation of one phosphate residue located at position C-1 of the lipid A  
64 backbone by LpxE and subsequent *P*-Etn transfer by a phosphoethanolamine

65 transferase (EptA or PmrC) (15, 16). In *H. pylori* *lpxE* and *eptA* are encoded by one  
66 operon (Hp0021-Hp0022) (16).

67 We have previously characterized the lipid A structure of *C. canimorsus* (19), bacteria  
68 causing rare but severe sepsis or meningitis in humans after dog bites or scratches (20-

69 24). *C. canimorsus* belongs to the family of *Flavobacteriaceae* in the phylum  
70 *Bacteroidetes* and are usual members of dog's mouth flora (21, 25-28). *C. canimorsus*

71 lipid A consists of a 2,3-diamino-2,3-dideoxy-D-glucose (GlcN3N')  $\beta$ -(1'→6)-linked to

72 2-amino-2-deoxy-D-glucose (GlcN) [ $\beta$ -D-Glc<sub>p</sub>N<sub>3</sub>N'-(1→6)-D-Glc<sub>p</sub>N lipid A hybrid  
73 backbone] containing a *P*-Etn group attached to the C-1 reducing end and lacking a 4'-  
74 phosphate (Fig. 1 A). 3-hydroxy-15-methylhexadecanoic acid [*i*17:0(3-OH)], 3-hydroxy-  
75 13-methyltetradecanoic acid (*i*15:0), 3-O-(13-methyltetradecanoyl)-15-  
76 methylhexadecanoic acid [*i*17:0[3-O(*i*15:0)]] and 3-hydroxyhexadecanoic acid [16:0(3-  
77 OH)] are attached to the backbone at positions 2, 3, 2', and 3', respectively (19). This  
78 structure differs from that of a potent Toll-like receptor 4 (TLR4) agonist like the *E. coli*  
79 lipid A (Fig. 1 B), consisting of a  $\beta$ -(1'→6)-linked GlcN disaccharide that is  
80 phosphorylated at positions 1 and 4' and carries four (*R*)-3-hydroxymyristate chains  
81 [14:0(3-OH)] (at positions 2', 3', 2 and 3). The 2' and 3' 3-hydroxylated acyl groups in  
82 GlcN(II) are further esterified with laurate and myristate, respectively (29).

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

94

95

96 **Materials and Methods**

97

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

106

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

120 without primers to allow annealing and elongation of the overlapping fragments (98°C for  
121 30 s, 50°C for 40 s, and 72°C for 2 min). After the addition of external primers (A and F),  
122 the program was continued with 20 cycles (98°C for 30 s, 50°C for 40 s, and 72°C for 2  
123 min 30 s) and finally 10 min at 72°C. Final PCR products consisting in *lpxE::ermF*,  
124 *eptA::ermF*, *lpxE/eptA::ermF*, *lpxE::tetQ*, *eptA::tetQ*, *lpxE/eptA::tetQ* insertion cassettes  
125 were then digested with *PstI* and *SpeI* for cloning into the appropriate sites of the *C.*  
126 *canimorsus* suicide vector pMM25 (31). Resulting plasmids were transferred by RP4-  
127 mediated conjugative DNA transfer from *E. coli* S17-1 to *C. canimorsus* 5 or *C.*  
128 *canimorsus* 5 Y1C12 mutant to allow integration of the insertion cassette.  
129 Transconjugants were then selected for the presence of the *ermF* or *tetQ* cassette on  
130 erythromycin or tetracycline plates respectively and checked for sensitivity to cefoxitin.  
131 Deletion of the appropriate regions was verified by PCR.

132

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

138

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

144 bacteria were placed in a total volume of 20  $\mu$ l (diluted in PBS) and distributed in a flat-  
145 bottom 96-well plate (BD Falcon). 25000 HEKBlue<sup>TM</sup> hTLR4 cells in 180  $\mu$ l were then  
146 added and the plate was incubated for 20-24h at 37°C and 5% CO<sub>2</sub>. Detection of the  
147 secreted phosphatase followed the QUANTI-Blue<sup>TM</sup> protocol (InvivoGen). 20  $\mu$ l of  
148 challenged cells were incubated with 180  $\mu$ l detection reagent (QUANTI-Blue<sup>TM</sup>,  
149 InvivoGen). Plates were incubated at 37°C and 5% CO<sub>2</sub> and absorbance was measured  
150 at 655 nm using a spectrophotometer (BioRad).

151

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

158

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

167



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

192

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

200

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

208

209 **Immunoblotting of proteinase K-resistant structures.** Bacteria were harvested from  
210 blood-agar plates, washed once in 1ml of PBS and adjusted to an OD<sub>600</sub> of 1.5 in PBS.  
211 500 $\mu$ l bacterial suspension was pelleted and dissolved in 125 $\mu$ l loading buffer (1%  
212 sodium dodecyl sulfate [SDS], 10% glycerol, 50 mM dithiothreitol, 0.02% bromophenol  
213 blue, 45 mM Tris (pH 6.8) in ddH<sub>2</sub>O). Samples were boiled at 99°C for 10 min.  
214 Proteinase K (50 $\mu$ g/ml final concentration) was added and samples were incubated at  
215 37°C overnight. After incubation samples were boiled again for 10 min at 99°C and a

216 second volume of proteinase K (equal to the first) was added. Samples were incubated  
217 at 55°C for 3 h, boiled again for 5 minutes at 99°C and loaded on a 15% SDS-PAGE.  
218 Samples were analyzed by western blotting using polyclonal, *C. canimorsus* 5 Y1C12-  
219 absorbed, serum against *C. canimorsus* 5. This antibody was generated from rabbits by  
220 immunization with heat killed *C. canimorsus* 5 (Laboratoire d'Hygiène, Marloie,  
221 Belgium). Further on, the *C. canimorsus* 5 Y1C12-absorbed serum was prepared by  
222 incubating twice an excess amount of Y1C12 mutant *C. canimorsus* 5 bacteria  
223 (harvested from blood plates and washed in PBS) with anti-*C. canimorsus* 5 serum at  
224 4°C for 12 h. Bacteria were removed by repeated centrifugation. This results in an  
225 antiserum recognizing *C. canimorsus* 5 LPS (35).

226

227

228 **Results and Discussion**

229

230 **Identification of enzymes leading to the presence of 1 P-Etn on lipid A.** The  
231 genome of *C. canimorsus* 5 (28) (Embank: YP\_004740918.1) was analyzed for proteins  
232 with high sequence similarity to the lipid A modifying enzymes, LpxE and EptA. Our  
233 search for a lipid A phosphatase was based on *LpxE* and/or *LpxF* sequences from *P.*  
234 *gingivalis* (1), *F. novicida* (11), *R. etli* (10) *H. pylori* (12, 16) and on all available  
235 *Bacteroidetes*-group *pgpB* sequences. Three *lpxE/F* candidates were found  
236 (*Ccan\_16960*, *Ccan\_14540* and *Ccan\_06070*) and individually deleted. Interestingly, the  
237 gene downstream of *Ccan\_16960* (Genbank: YP\_004740919.1), *Ccan\_16950* was  
238 found to have high sequence similarity to *eptA*, coding for a lipid A P-Etn transferase.  
239 *Ccan\_16950* and *16960* form an operon and the two genes overlap by 20 base pairs  
240 (bp) (Fig. 2, lower panel). *Ccan\_16960* has thus been annotated as *lpxE*, an annotation  
241 validated by mutagenesis, MS analysis and impact on endotoxicity and CAMP  
242 resistance, as described here below. The association of *lpxE* and *eptA* genes reinforces  
243 the idea that the two gene products acts in the same pathway and suggests that the  
244 modification of lipid A that they determine together is essential for survival in the dog's  
245 mouth environment.

246

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

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

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

262

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

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

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

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

294

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

300 presence of 512 mg/L Polymyxin B (MIC  $\geq$ 1024 mg/L) (Fig. 4). The MIC decreased to  
301 512 mg/L for  $\Delta eptA$  mutant bacteria and to 128 mg/L for the  $\Delta lpxE$  bacteria, showing an  
302 increased sensitivity to Polymyxin B (Fig. 4). The *C. canimorsus* *lpxE-eptA* double-  
303 mutant had the same MIC as the single  $\Delta lpxE$  mutant (Fig. 4). The  $\Delta lpxE$  strain could be  
304 complemented in trans with *lpxE*, but not with *eptA* alone (Fig. 4). The  $\Delta eptA$  strain was  
305 complemented with *eptA* (Fig. 4) and the *lpxE-eptA* deletion mutant was complemented  
306 with *lpxE-eptA* (Fig. 4). We conclude from these complementation experiments that the  
307 *lpxE*, *eptA* and *lpxE-eptA* mutations were non-polar.

308

309 The *P*-Etn modification at position C-1 thus contributed to the low endotoxicity and  
310 Polymyxin B resistance of *C. canimorsus*, as was shown for *H. pylori* (12). The identical  
311 phenotype in endotoxicity and Polymyxin B sensitivity of the single  $\Delta lpxE$  and the double  
312  $\Delta lpxE-eptA$  mutants suggests that the *P*-Etn containing lipid A is synthesized by a two-  
313 step enzymatic process similar to that described for *H. pylori* (15, 16). In *H. pylori*, lipid A  
314 also carries a *P*-Etn group at position C-1, generated in the course of the LPS  
315 biosynthesis by removal of the lipid A 1-phosphate by LpxE followed by transfer of a *P*-  
316 Etn residue by EptA from phosphatidylethanolamine to the free reducing end of GlcN(I),  
317 where dephosphorylation is necessary for substitution of 1-phosphate with *P*-Etn (12,  
318 15, 16). The non-polar deletion of *lpxE* in *C. canimorsus* does not prevent the synthesis  
319 of EptA but likely leads to a lipid A with a 1-phosphate group, which would explain the  
320 high endotoxicity observed for this strain. Therefore, as in *H. pylori*, the *C. canimorsus*  
321 EptA seems to accept only the free reducing end of the lipid A backbone generated by  
322 the activity of LpxE as a substrate.

323

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

334 It is noteworthy that one would expect the mutation of *eptA* not to affect any charge  
335 dependent mechanisms, as no net charge change is expected. *C. canimorsus*  $\Delta eptA$   
336 bacteria showed increased endotoxicity and decreased CAMP resistance as the wt lipid  
337 A, while the lipid A variant predicted for a  $\Delta eptA$  deletion mutant is lacking both the 1  
338 and 4'-phosphate in the lipid A backbone. The *C. canimorsus* 1-dephospho lipid A in a  
339  $\Delta eptA$  mutant is not expected to be endotoxic at all, as this lipid A species lacks both  
340 phosphates, and thus the negative charges which are important for endotoxicity (41).  
341 Still the  $\Delta eptA$  mutation had a small phenotype in Polymyxin B sensitivity and a more  
342 pronounced phenotype in endotoxicity. This hints at a heterogeneous lipid A population  
343 in the  $\Delta eptA$  strain, which could result from a restricted activity of LpxE. Assuming a  
344 non-stoichiometric activity of LpxE in the  $\Delta eptA$  strain, both the lipid A containing a free  
345 reducing end as well as the 1-phosphate at GlcN(I) should be present. In this case, the  
346 1-phospho lipid A variant could account for the increase in endotoxicity, while its  
347 reduced amounts as compared to  $\Delta lpxE$  would explain the higher endotoxicity of the



348  $\Delta lpxE$  over the  $\Delta eptA$  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  $\Delta eptA$  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  $\Delta 14$   $m/z$  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 ( $\Delta eptA$ ,  $\Delta lpxE$  and  $\Delta lpxE-eptA$ )  
370 showed a main peak at  $m/z$  of 1674 (Table 3, Fig. 5). The 1-phospho lipid A variant  
371 predicted for  $\Delta lpxE$  and  $\Delta lpxE-eptA$  has a calculated  $m/z$  of 1673.3. Hence,  $\Delta eptA$ ,

372  $\Delta lpxE$  and  $\Delta lpxE-eptA$  deletion mutant strains feature 1-phospho lipid A. While this is the  
373 variant expected to occur for  $\Delta lpxE$  and  $\Delta lpxE-eptA$  strains,  $\Delta eptA$  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  $\Delta eptA$  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  $\Delta eptA$  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  $\Delta lpxE$   
384 strain could be complemented in trans with  $lpxE$ , as we confirmed the wt lipid A for the  
385  $\Delta lpxE$  strain complemented with  $p-lpxE$  ( $m/z$  calculated: 1716.3, found: 1717). The  
386  $\Delta lpxE$  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  $\Delta lpxE$  strain ( $m/z$  calculated: 1673.3). The  $\Delta eptA$  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  $\Delta lpxE + p-lpxE$  and the  $\Delta eptA +$   
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  $\Delta 14$   $m/z$  units due to acyl chain heterogeneity)

396 (19). The two phosphates might either be present as 1-phospho 4'-phospho lipid A or as  
397 1-pyrophosphate lipid A. Based on 1-pyrophosphate species detected in various species  
398 (42) we would hypothesize, that the peaks measured at  $m/z$  of 1755/1769 correspond to  
399 a 1-pyrophosphate variant with a classical *E. coli*-type GlcN-GlcN lipid A backbone. The  
400 peak measured at  $m/z$  of 1725 for the  $\Delta lpxE + p\text{-eptA}$  strain is similarly attributed to a  
401 bisphosphorylated species in combination with an exchange of 17:0(3-OH) by 15:0(3-  
402 OH) ( $m/z$  calculated: 1725.186). The detection of these peaks exclusively in  
403 complemented mutants and the resulting changes in acyl chain preference and  
404 backbone structure, however, deserves further clarification.

405 We conclude from these complementation experiments that the *eptA* and *lpxE* mutations  
406 were non-polar and that in the complemented mutants,  $\Delta lpxE + p\text{-lpxE}$ ,  $\Delta eptA + p\text{-eptA}$   
407 and  $\Delta lpxE\text{-eptA} + p\text{-lpxE-eptA}$ , the wt lipid A is reconstituted.

408

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

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

428

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

437

438 We further performed positive ion mode MS analysis on complemented mutants (Fig.  
439 S3). The  $\Delta lpxE$  strain could be complemented in trans with  $lpxE$ , as we confirmed the wt  
440 lipid A for this strain ( $m/z$  calculated: 1764.3, found: 1764). The  $\Delta lpxE$  strain could not be  
441 complemented in trans with  $eptA$  alone (Fig. S3) and the resulting strain showed a main  
442 peak at  $m/z$  1722 (Fig. S3), matching the 1-phospho lipid A variant predicted for  $\Delta lpxE$   
443 ( $m/z$  calculated: 1720.2). The  $\Delta eptA$  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  $\Delta lpxE + p-lpxE$  and the  $\Delta eptA +$   
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,  $\Delta lpxE + p-lpxE$ ,  $\Delta eptA + p-eptA$   
454 and  $\Delta lpxE-eptA + p-lpxE-eptA$ , the wt lipid A is reconstituted.

455

456 Overall, 1-phospho lipid A was validated as main lipid A variant in  $\Delta lpxE$  and  $\Delta lpxE-eptA$   
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  $\Delta eptA$  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 dephosphorylate every lipid A in the absence of EptA, which is reflected by the 1-  
465 phospho lipid A species identified in the  $\Delta eptA$  deletion mutant.

466

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

472

473

474

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476

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483

484 **References**

- 485 1. **Coats SR, Jones JW, Do CT, Braham PH, Bainbridge BW, To TT, Goodlett DR,**  
486 **Ernst RK, Darveau RP.** 2009. Human Toll-like receptor 4 responses to *P. gingivalis* are  
487 regulated by lipid A 1- and 4'-phosphatase activities. *Cell Microbiol* **11**:1587-1599.
- 488 2. **Dixon DR, Darveau RP.** 2005. Lipopolysaccharide heterogeneity: innate host responses  
489 to bacterial modification of lipid A structure. *J Dent Res* **84**:584-595.
- 490 3. **Hajjar AM, Ernst RK, Tsai JH, Wilson CB, Miller SI.** 2002. Human Toll-like receptor 4  
491 recognizes host-specific LPS modifications. *Nat Immunol* **3**:354-359.
- 492 4. **Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC.** 2007. The  
493 vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. *Science*  
494 **316**:1628-1632.
- 495 5. **Price NP, Jeyaretnam B, Carlson RW, Kadmas JL, Raetz CR, Brozek KA.** 1995.  
496 Lipid A biosynthesis in *Rhizobium leguminosarum*: role of a 2-keto-3-deoxyoctulosonate-  
497 activated 4' phosphatase. *Proc Natl Acad Sci U S A* **92**:7352-7356.
- 498 6. **Wang X, Karbaz MJ, McGrath SC, Cotter RJ, Raetz CRH.** 2004. MsbA transporter-  
499 dependent lipid A 1-dephosphorylation on the periplasmic surface of the inner  
500 membrane: topography of Francisella novicida LpxE expressed in *Escherichia coli* *J Biol*  
501 *Chem* **279**:49478.
- 502 7. **Wang X, McGrath SC, Cotter RJ, Raetz CRH.** 2006. Expression cloning and  
503 periplasmic orientation of the *Francisella novicida* lipid A 4'-phosphatase LpxF. *J Biol*  
504 *Chem* **281**:9321-9330.
- 505 8. **Curtis MA, Percival RS, Devine D, Darveau RP, Coats SR, Rangarajan M, Tarelli E,**  
506 **Marsh PD.** 2011. Temperature-dependent modulation of *Porphyromonas gingivalis* lipid  
507 A structure and interaction with the innate host defenses. *Infect Immun* **79**:1187-1193.

- 508 9. **Herrera CM, Hankins JV, Trent MS.** 2010. Activation of PmrA inhibits LpxT-dependent  
509 phosphorylation of lipid A promoting resistance to antimicrobial peptides. *Mol Microbiol*  
510 **76**:1444-1460.
- 511 10. **Ingram BO, Sohlenkamp C, Geiger O, Raetz CRH.** 2010. Altered lipid A structures and  
512 polymyxin hypersensitivity of *Rhizobium etli* mutants lacking the LpxE and LpxF  
513 phosphatases. *Biochim Biophys Acta* **1801**:593-604.
- 514 11. **Wang X, Ribeiro AA, Guan Z, Abraham SN, Raetz CRH.** 2007. Attenuated virulence of  
515 *Francisella* mutant lacking the lipid A 4'-phosphatase. *Proc Natl Acad Sci U S A*  
516 **104**:4136-4141.
- 517 12. **Cullen TW, Giles DK, Wolf LN, Ecobichon C, Boneca IG, Trent MS.** 2011.  
518 *Helicobacter pylori* versus the host: remodeling of the bacterial outer membrane is  
519 required for survival in the gastric mucosa. *PLoS Pathog* **7**:e1002454.
- 520 13. **Kanistanon D, Powell DA, Hajjar AM, Pelletier MR, Cohen IE, Way SS, Skerrett SJ,**  
521 **Wang X, Raetz CR, Ernst RK.** 2012. Role of *Francisella* lipid A phosphate modification  
522 in virulence and long-term protective immune responses. *Infect Immun* **80**:943-951.
- 523 14. **Ingram BO, Masoudi A, Raetz CRH.** 2010. *Escherichia coli* mutants that synthesize  
524 dephosphorylated lipid A molecules. *Biochemistry (Mosc)* **49**:8325-8337.
- 525 15. **Tran AX, Karbaz MJ, Wang X, Raetz CRH, McGrath SC, Cotter RJ, Trent MS.** 2004.  
526 Periplasmic cleavage and modification of the 1-phosphate group of *Helicobacter pylori*  
527 lipid A. *J Biol Chem* **279**:55791.
- 528 16. **Tran AX, Whittimore JD, Wyrick PB, McGrath SC, Cotter RJ, Trent MS.** 2006. The  
529 lipid A 1-phosphatase of *Helicobacter pylori* is required for resistance to the  
530 antimicrobial peptide Polymyxin. *J Bacteriol* **188**:4531-4541.
- 531 17. **Cox AD, Wright JC, Hood DW, Moxon ER, Richards JC.** 2003. Phosphorylation of the  
532 lipid A region of *meningococcal* lipopolysaccharide: identification of a family of



- 533 transferases that add phosphoethanolamine to lipopolysaccharide. J Bacteriol **185**:3270-  
534 3277.
- 535 18. **Kim SH, Parreira VR, Bishop RE, Gyles CL.** 2006. Phosphoethanolamine substitution  
536 in the lipid A of *Escherichia coli* O157 : H7 and its association with PmrC. Microbiology  
537 **152**:657-666.
- 538 19. **Ittig S, Lindner B, Stenta M, Manfredi P, Zdorovenko E, Knirel YA, dal Peraro M,**  
539 **Cornelis GR, Zähringer U.** 2012. The lipopolysaccharide from *Capnocytophaga*  
540 *canimorsus* reveals an unexpected role of the core-oligosaccharide in MD-2 binding.  
541 PLoS Pathog **8(5)** e1002667.
- 542 20. **Bobo RA, Newton EJ.** 1976. A previously undescribed gram-negative bacillus causing  
543 septicemia and meningitis. Am J Clin Pathol **65**:564-569.
- 544 21. **Brenner DJ, Hollis DG, Fanning GR, Weaver RE.** 1989. *Capnocytophaga canimorsus*  
545 sp. nov. (formerly CDC group DF-2), a cause of septicemia following dog bite, and *C.*  
546 *cynodegmi* sp. nov., a cause of localized wound infection following dog bite. J Clin  
547 Microbiol **27**:231-235.
- 548 22. **Butler T.** 2015. *Capnocytophaga canimorsus*: an emerging cause of sepsis, meningitis,  
549 and post-splenectomy infection after dog bites. European journal of clinical microbiology  
550 & infectious diseases : official publication of the European Society of Clinical  
551 Microbiology **34**:1271-1280.
- 552 23. **Lion C, Escande F, Burdin JC.** 1996. *Capnocytophaga canimorsus* infections in human:  
553 review of the literature and cases report. Eur J Epidemiol **12**:521-533.
- 554 24. **Pers C, Gahrn-Hansen B, Frederiksen W.** 1996. *Capnocytophaga canimorsus*  
555 septicemia in Denmark, 1982-1995: review of 39 cases. Clin Infect Dis **23**:71-75.
- 556 25. **Bailie WE, Stowe EC, Schmitt AM.** 1978. Aerobic bacterial flora of oral and nasal fluids  
557 of canines with reference to bacteria associated with bites. J Clin Microbiol **7**:223-231.

- 558 26. **Blanche P, Bloch E, Sicard D.** 1998. *Capnocytophaga canimorsus* in the oral flora of  
559 dogs and cats. J Infect **36**:134.
- 560 27. **Mally M, Paroz C, Shin H, Meyer S, Soussoula LV, Schmiediger U, Saillen-Paroz C,**  
561 **Cornelis GR.** 2009. Prevalence of *Capnocytophaga canimorsus* in dogs and  
562 occurrence of potential virulence factors. Microbes Infect **11**:509-514.
- 563 28. **Manfredi P, Pagni M, Cornelis GR.** 2011. Complete genome sequence of the dog  
564 commensal and human pathogen *Capnocytophaga canimorsus* strain 5. J Bacteriol  
565 **193**:5558-5559.
- 566 29. **Raetz CRH.** 1990. Biochemistry of Endotoxins. Annu Rev Biochem **59**:129-170.
- 567 30. **Shin H, Mally M, Kuhn M, Paroz C, Cornelis GR.** 2007. Escape from immune  
568 surveillance by *Capnocytophaga canimorsus* J Infect Dis **195**:375-386.
- 569 31. **Mally M, Cornelis GR.** 2008. Genetic tools for studying *Capnocytophaga canimorsus*.  
570 Appl Environ Microbiol **74**:6369-6377.
- 571 32. **Ferraro MJ, NCCLS/CLSI.** 2003. Methods for dilution antimicrobial susceptibility tests for  
572 bacteria that grow aerobically: approved standard. NCCLS National Committee for  
573 Clinical Laboratory Standards CLSI Clinical Laboratory and Standards Institute.
- 574 33. **Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipdman DJ.**  
575 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search  
576 programs. Nucleic Acids Res **25**:3389-3402.
- 577 34. **Zähringer U, Ittig S, Lindner B, Moll H, Schombel U, Gisch N, Cornelis GR.** 2014.  
578 NMR-based structural analysis of the complete rough-type lipopolysaccharide isolated  
579 from *Capnocytophaga canimorsus*. The Journal of biological chemistry **289**:23963-  
580 23976.
- 581 35. **Shin H, Mally M, Meyer S, Fiechter C, Paroz C, Zähringer U, Cornelis GR.** 2009.  
582 Resistance of *Capnocytophaga canimorsus* to killing by human complement and  
583 polymorphonuclear leukocytes. Infect Immun **77**:2262-2271.

- 584 36. **El Hamidi A, Tirsoaga A, Novikov A, Hussein A, Caroff M.** 2005. Microextraction of  
585 bacterial lipid A: easy and rapid method for mass spectrometric characterization. *J Lipid*  
586 *Res* **46**:1773-1778.
- 587 37. **Rietschel ET, Kirikae T, Schade FU, Mamat U, Schmidt G, Loppnow H, Ulmer AJ,**  
588 **Zahringer U, Seydel U, Di Padova F, Schreier M, Brade H.** 1994. Bacterial endotoxin:  
589 molecular relationships of structure to activity and function. *FASEB journal* : official  
590 publication of the Federation of American Societies for Experimental Biology **8**:217-225.
- 591 38. **Reynolds CM, Kalb SR, Cotter RJ, Raetz CR.** 2005. A phosphoethanolamine  
592 transferase specific for the outer 3-deoxy-D-manno-octulosonic acid residue of  
593 *Escherichia coli* lipopolysaccharide. Identification of the *eptB* gene and Ca<sup>2+</sup>  
594 hypersensitivity of an *eptB* deletion mutant. *The Journal of biological chemistry*  
595 **280**:21202-21211.
- 596 39. **Lee H, Hsu FF, Turk J, Groisman EA.** 2004. The PmrA-regulated *pmrC* gene mediates  
597 phosphoethanolamine modification of lipid A and polymyxin resistance in *Salmonella*  
598 *enterica*. *J Bacteriol* **186**:4124-4133.
- 599 40. **Raetz CRH, Reynolds CM, Trent MS, Bishop RE.** 2007. Lipid A modification systems in  
600 Gram-negative bacteria. *Annu Rev Biochem* **76**:295-329.
- 601 41. **Park BS, Song DH, Kim HM, Choi B-S, Lee H, Lee J-O.** 2009. The structural basis of  
602 lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* **458**:1191-1195.
- 603 42. **Jones JW, Shaffer SA, Ernst RK, Goodlett DR, Turecek F.** 2008. Determination of  
604 pyrophosphorylated forms of lipid A in Gram-negative bacteria using a multivariate mass  
605 spectrometric approach. *Proc Natl Acad Sci U S A* **105**:12742-12747.
- 606

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  $\beta$ -(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  $\beta$ -(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),  $\Delta$ *lpxE*,  $\Delta$ *eptA* or  $\Delta$ *lpxE-eptA* bacteria. Indicated multiplicity  
626 of infection (MOI) of heat-killed bacteria was assayed for TLR4 dependent NF $\kappa$ B  
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

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

632

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

637

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

640

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

643

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

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

pFR28	pMM25 <i>lpxE::tetQ</i> (suicide vector for deletion of <i>lpxE</i> , ( $\Delta$ 1833737 - 1833995))	This study
pFR29	pMM25 <i>eptA::tetQ</i> (suicide vector for deletion of <i>eptA</i> , ( $\Delta$ 1831370 - 1832888))	This study
pFR30	pMM25 <i>lpxE-eptA::tetQ</i> (suicide vector for deletion of <i>lpxE-eptA</i> , ( $\Delta$ 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	CCCTGCAGGGCAGTTTCGTACCAGTTA	<i>Pst</i> I	<i>lpxE</i>	A
6494	lpxE-B	GAGTAGATAAAAGCACTGTTATTTGCTTATTTTGAATATTTCCGG		<i>lpxE</i>	B
6495	lpxE-C	CTTATATTTGCCGCCGAAATATTCAAATAAGCAAATAACA GTGCTTTTATCTACTCCGATAGCTTC		<i>ermF</i>	C
6496	lpxE-D	CTTGCAATTATCTTAACACTCATAAAACAACACTCCCCTAC GAAGGATGAAATTTTTCAGGGACAAC		<i>ermF</i>	D
6497	lpxE-E	AAAAATTCATCCTTCGTAGGGAGTGTTGTTTTATGAGT GTT		<i>lpxE</i>	E
6498	lpxE-F	CAACTAGTAAACCGTTTCAGTTGGGT	<i>Spe</i> I	<i>lpxE</i>	F
6499	eptA-A	CCCTGCAGTGTTCCTCGCCCTGTTAC	<i>Pst</i> I	<i>eptA</i>	A
6500	eptA-B	GAGTAGATAAAAGCACTGTTTTATTGATTTTTTTAACATAA AATTTTATC		<i>eptA</i>	B
6501	eptA-C	GTTGTACTTAATGATAAAATTTTATGTTAAAAAATCAATAA AACAGTGCTTTTATCTACTCCGATAGCTTC		<i>ermF</i>	C
6502	eptA-D	ATCTTGTAATACGGATTGGTCATTCAATAATTCTACGAAG GATGAAATTTTTCAGGGACAAC		<i>ermF</i>	D
6503	eptA-E	AAAAATTCATCCTTCGTAGAATTATTGAATGACCAATCCG		<i>eptA</i>	E
6504	eptA-F	CAACTAGTCCACCTCATTGAGATTCAC	<i>Spe</i> I	<i>eptA</i>	F
6646	p-lpxE-fw	CGTACCATGGTTTTTAAAGAATCAGCAAATAACC	<i>Nco</i> I	<i>lpxE</i>	
6647	p-lpxE-rev	CAGTTCTAGATTATTGATTTTTTTAACATAAAATTTTATC	<i>Xba</i> I	<i>lpxE</i>	
6648	p-eptA-fw	CGTACCATGGGATTAATAAAATCAATAATGGACTAACA	<i>Nco</i> I	<i>eptA</i>	
6649	p-eptA_rev	GCTTCTCGAGTTAGTCAAAATGCTCATTTCG	<i>Xho</i> I	<i>eptA</i>	
7539	lpxEtetKO-A	GGCTGCAGTTCCATTCTTTGGCACGTTCCG	<i>Pst</i> I	<i>lpxE</i>	A
7540	lpxEtetKO-B	CAAAATCAATGTTAAAAAATTTGCTTATTTTGAATATTT CGGC		<i>lpxE</i>	B
7543	lpxEtetKO-C	GCCGAAATATTCAAATAAGCAAATTTTTTTTAAACATTGA		<i>tetQ</i>	C



		TTTTG			
7544	lpxEtetKO-D	GATTTTTTTTAAACATAAAATTTTATCTTATTTTGATGACATTG ATTTTTGG		<i>tetQ</i>	D
7541	lpxEtetKO-E	CCAAAAATCAATGTCATCAAATAAGATAAAATTTTATGTTA AAAAAATC		<i>lpxE</i>	E
7542	eptAtetKO-F	GGACTAGTCAAGGTAAAGCCAATGTTAAGC 1.1.1	<i>SpeI</i>	<i>lpxE</i>	F
7545	eptAtetKO-A	<u>GGCTGCAGTATGGGGAGGAAAGCGTCAATATTG</u>	<i>PstI</i>	<i>eptA</i>	A
7546	eptAtetKO-B	CAAAATCAAATGTTAAAAAAGCGGTACATTGTTAGTCCA TTTATTG		<i>eptA</i>	B
7549	eptAtetKO-C	CAATAAATGGACTAACAAATGTACCGCTTTTTTTAACATTG ATTTTG		<i>tetQ</i>	C
7550	eptAtetKO-D	CGGATTGGTCATTCAATAATTTTATTTTGATGACATTGATTT TTGG		<i>tetQ</i>	D
7547	lpxEtetKO-E	CCAAAAATCAATGTCATCAAATAAAATTTATTGAATGACCAA TCCG		<i>eptA</i>	E
7548	eptAtetKO-F	GGACTAGTCATTAAGTGCTACCCCTATCTTATC	<i>SpeI</i>	<i>eptA</i>	F

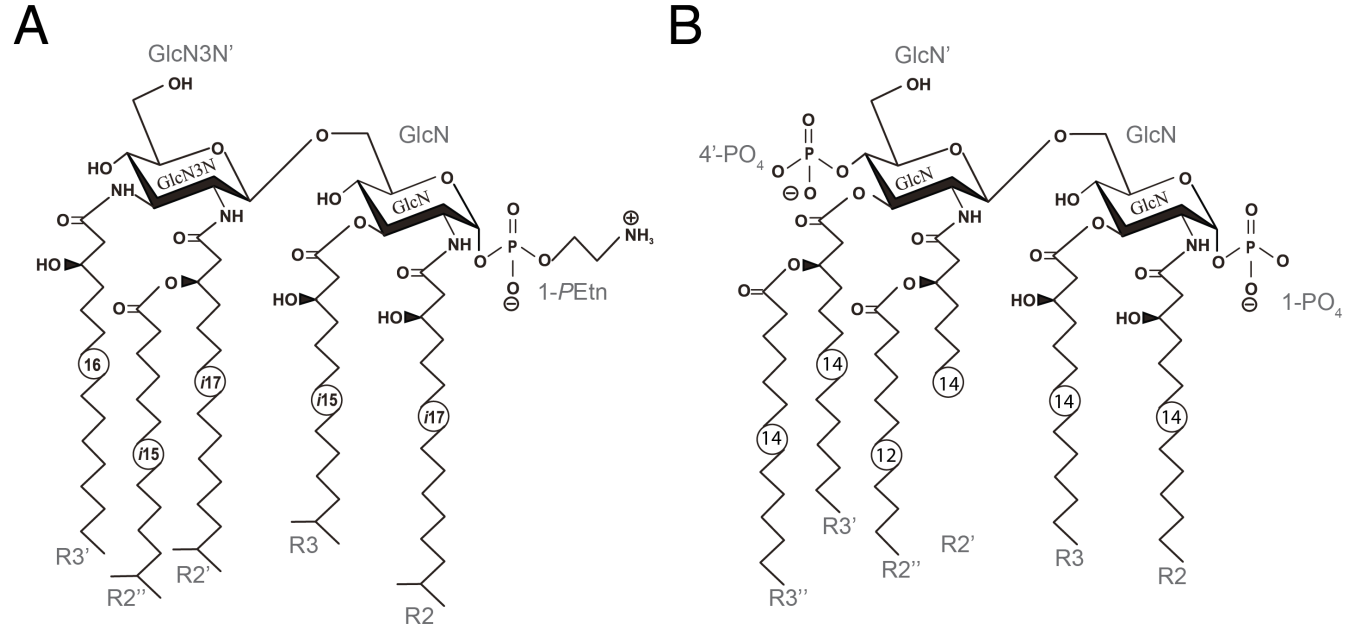
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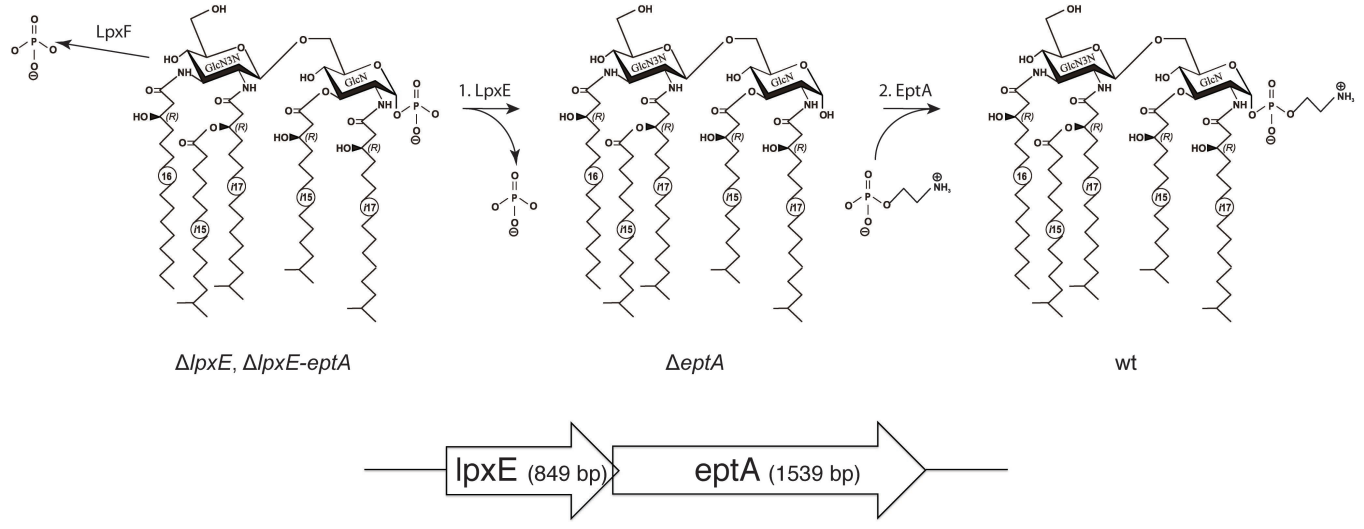
650 **Table 3. MS analysis and interpretation of lipid A variants in wt, *lpxE*, *eptA*, and**  
 651 ***lpxE-eptA* deletion mutant strains.**

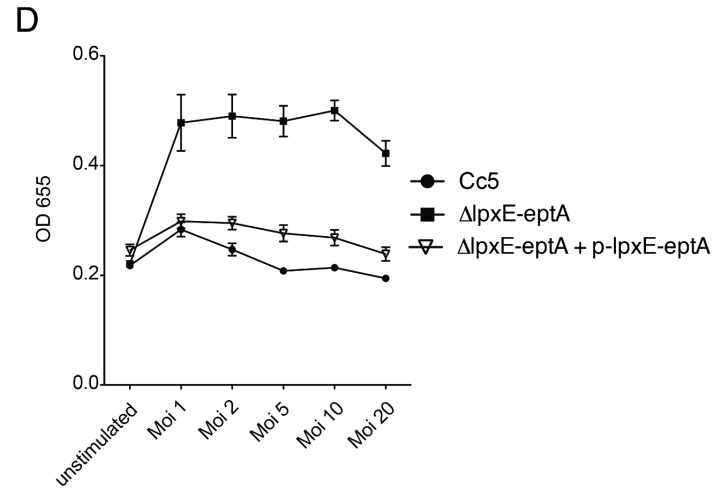
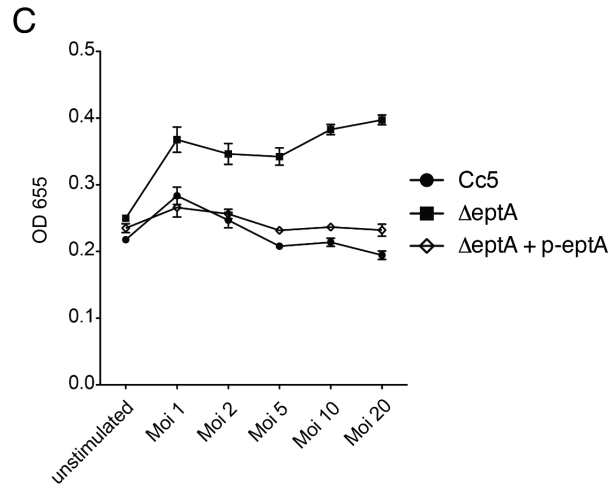
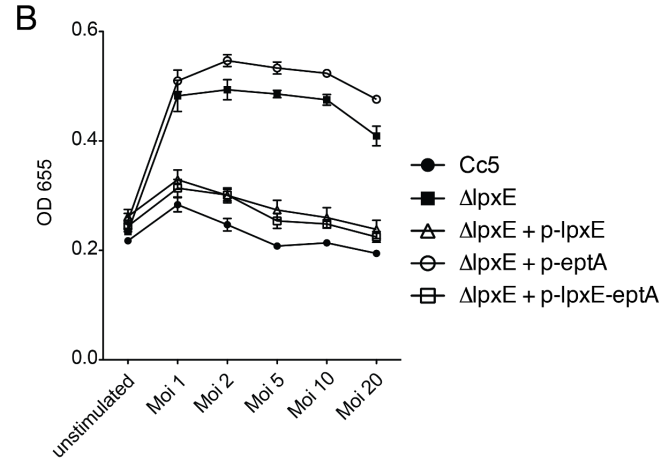
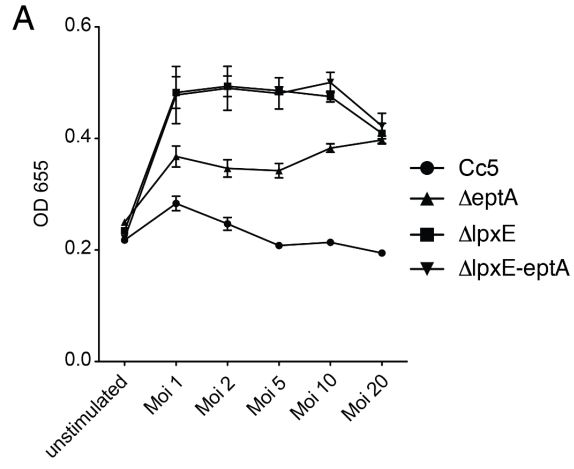
655 Component	653 wt		654 mutant	
		$\Delta lpxE$	$\Delta eptA$	$\Delta lpxE-eptA$
657 GlcN	1	1	1	1
658 GlcN3N	1	1	1	1
659 <i>P</i>	1	1	0	1
660 Etn	1	0	0	0
661 <i>i</i> 15:0	1	1	1	1
662 <i>i</i> 15:0(3-OH)	1	1	1	1
663 16:0(3-OH)	1	1	1	1
664 <i>i</i> 17:0(3-OH)	1	1	1	1
666 <i>m/z</i> calc. (neg. mode)	1716.3	1673.3	1575.3	1673.3
667 found for [M-H <sup>+</sup> ]	1716.8	1673.4	(1673.9)*	1673.1
669 <i>m/z</i> calc. (pos. mode)	1764.3	1720.2	1603.3	1720.2
670 found	1764	1722	1604	1722
671 for	[M-H <sup>+</sup> +2Na <sup>+</sup> ]	[M-H <sup>+</sup> +2Na <sup>+</sup> ]	[M+Na <sup>+</sup> ]	[M-H <sup>+</sup> +2Na <sup>+</sup> ]

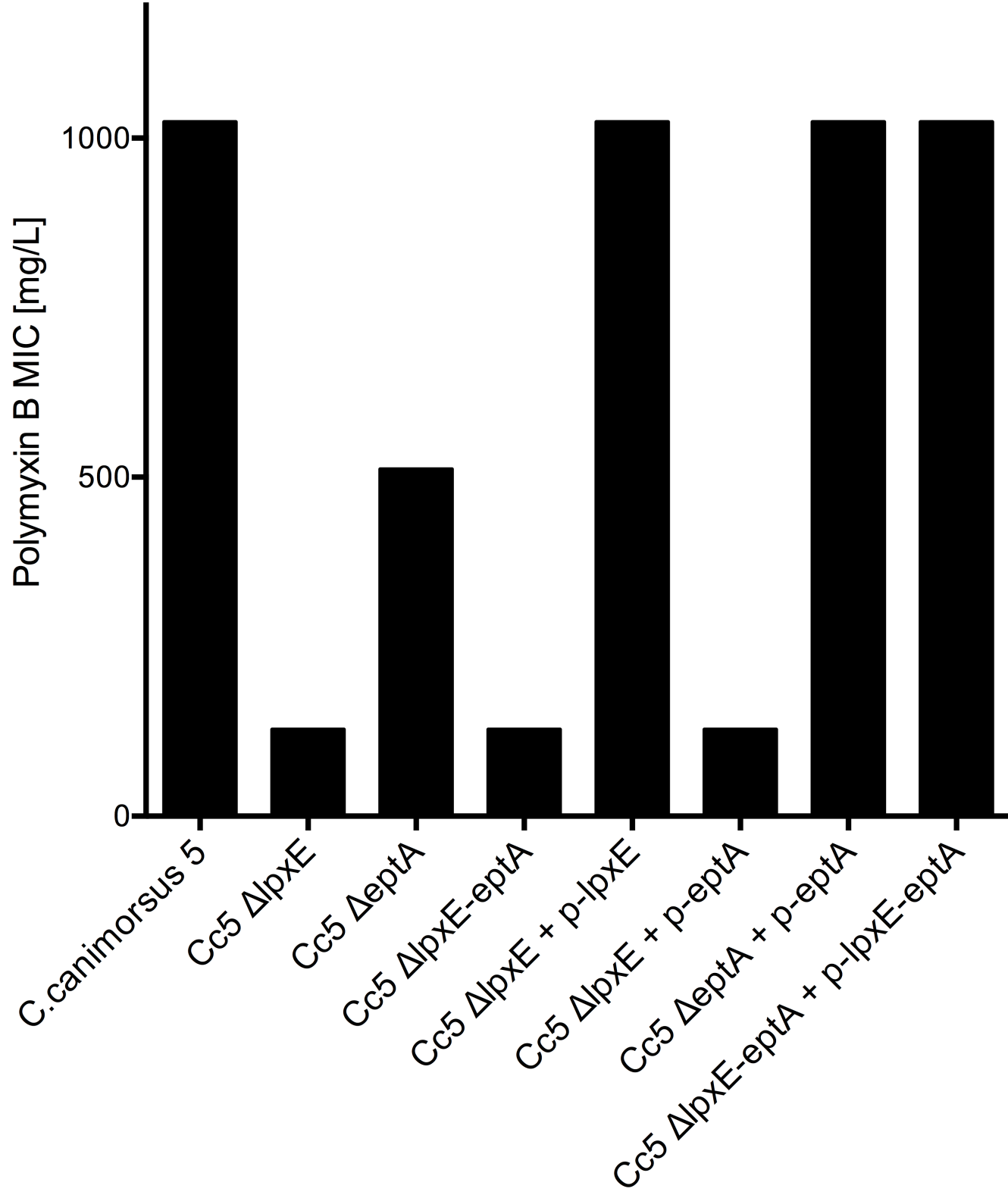
673 \* Ion [M-H<sup>+</sup>] detected in the neg. ion mode of  $\Delta eptA$  lipid A was raised from incomplete  
 674 de-phosphorylated lipid A. The major and representative lipid A molecule of this mutant  
 675 lacks any charged group and, therefore, its pseudomolecular ion [M+Na<sup>+</sup>] could only be  
 676 analyzed in the positive ion mode.

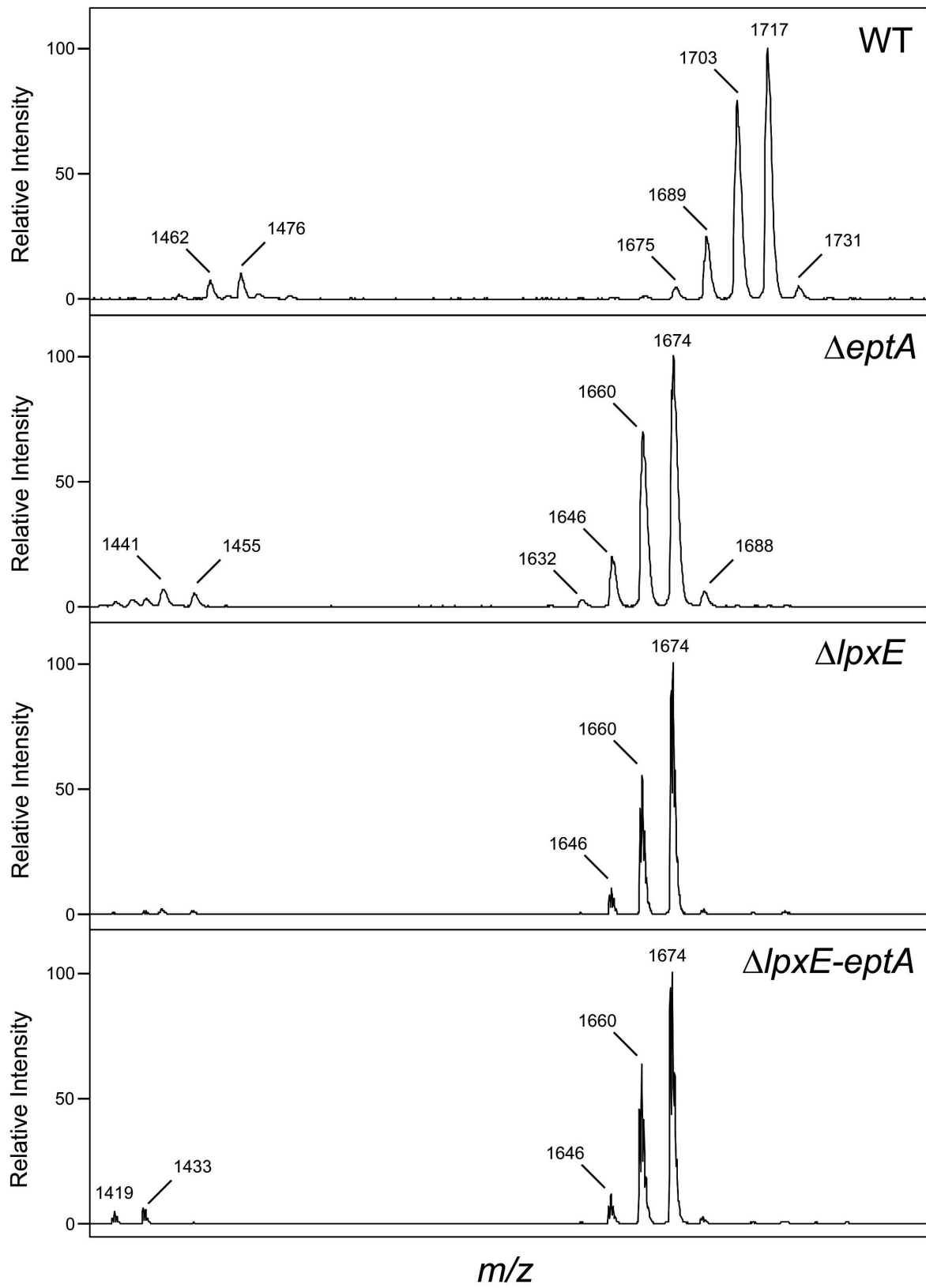
677



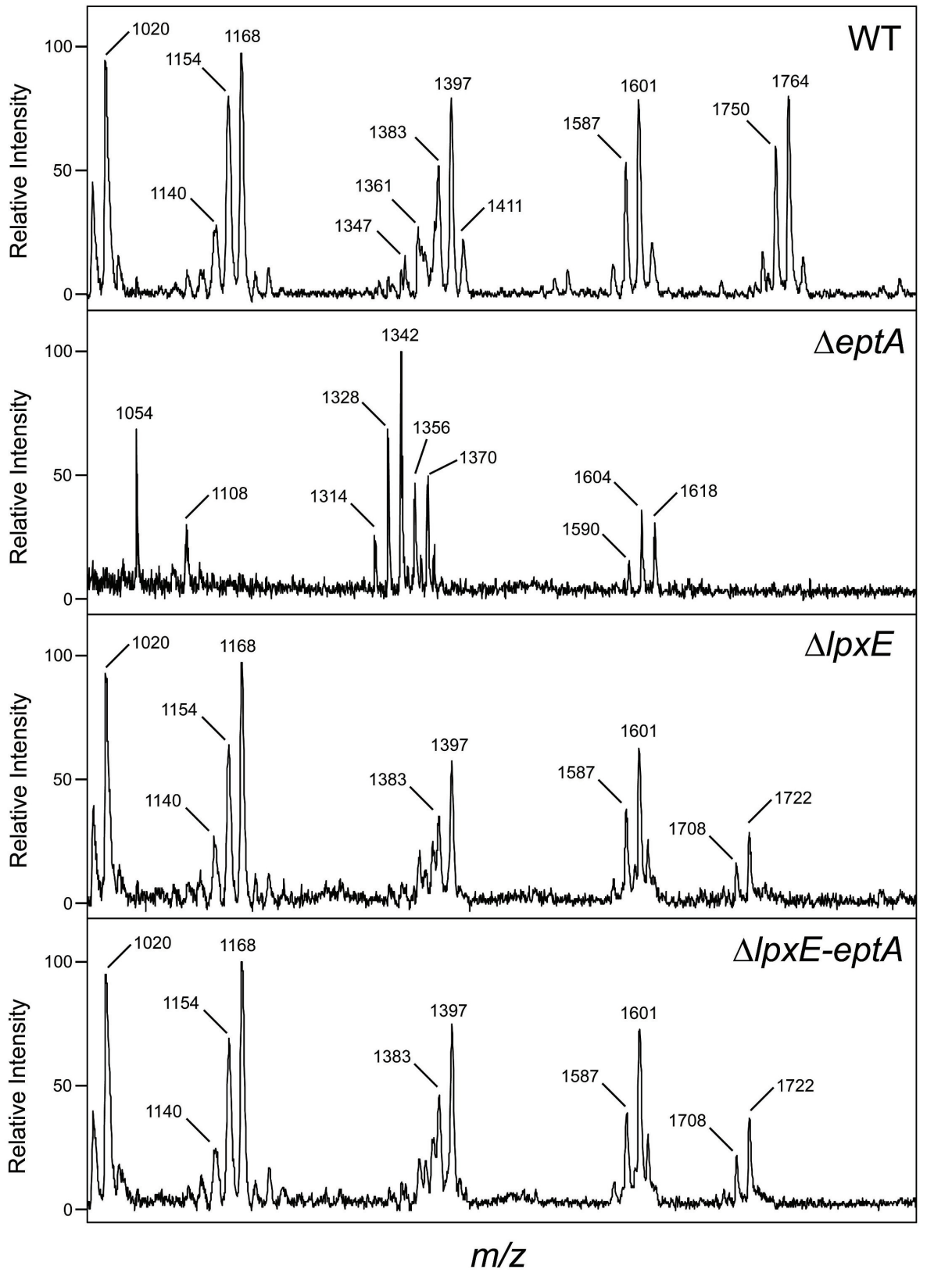








Negative ion mode



Positive ion mode