# The surface structures of *Capnocytophaga canimorsus* and their role in pathogenesis

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# 1 Introduction

## 1.1 Bacterial surface structures

### 1.1.1 Bacterial surface polysaccharides: structure and interaction with the innate immune system

The surface polysaccharides of commensal as well as pathogenic bacteria have to fulfil a multitude of functions to ensure viability (Bravo et al., 2008; Cardoso et al., 2006; Grossman et al., 1987; Lerouge and Vanderleyden, 2002; Murray et al., 2003; Nesper et al., 2002; Pluschke et al., 1983a; Porat et al., 1992; Raynaud et al., 2007; Ugalde et al., 2000). Dependent on the habitat of the bacterium the requirements for polysaccharides can vary largely, and even within a species or even an individual bacterium the conditions might change dramatically. Besides phospholipids the outer membrane of Gram-negative bacteria consists of a unique carbohydrate component, the lipopolysaccharide (LPS). The LPS of gram-negative bacteria consists of three regions: the lipid A, the core-oligosaccharide, and the O-antigen. Many Gram-negative (and Gram-positive) bacteria further have a polysaccharide capsule as outermost surface layer. Considering the functional diversity, not surprisingly a huge variety of surface polysaccharides exist (Bravo et al., 2008; Lerouge and Vanderleyden, 2002; Liu et al., 2008; Whitfield, 2006), including capsules or other exopolysaccharides as well as the LPS (Whitfield, 2006).

### 1.1.2 LPS part 1: lipid A – common structure and modification

The basic architecture of lipid A, the hydrophobic anchor of the LPS, is well conserved among all Gram-negative bacteria (Raetz, 1990a). It is composed of a disaccharide backbone to which a variable number of acyl chains of different length are attached at distinct positions. The backbone generally consists of GlcN, while some bacteria like

*Brevundimonas diminuta*, *Brevundimonas vesicularis*, *Legionella pneumophila*, *Campylobacter jejuni* or *Flavobacterium meningosepticum* (now *Elizabethkinga meningoseptica*) show a hybrid backbone including GlcN3N (Moran et al., 1991; Tanamoto et al., 2001). Often the lipid A further carries two phosphate groups, one on each sugar of the backbone, on the 1 or 4' position respectively.

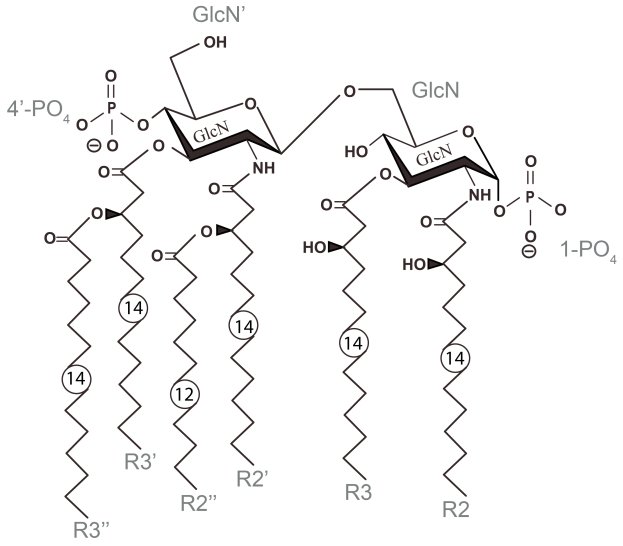


**Figure 1. Structure of *E. coli* hexa-acylated lipid A.** It consists of a β,1-6 linked glucosamine disaccharide that is phosphorylated at positions 1 and 4' and carries four R-3-hydroxymyristate chains (at positions 2, 3, 2' and 3'). The 2' and 3' acyl groups are further esterified with laurate or myristate, respectively (Raetz, 1990b).

The LPS represents the principal endotoxin of Gram-negative bacteria (Bryant et al., 2010) and since the main contribution generally comes from the lipid A part (Park et al., 2009; Raetz, 1990a) the terms lipid A and endotoxin have become synonyms. As a potent activator of the innate immune system, lipid A can induce endotoxic shock in patients suffering from bacterial septicemia. Recognition of lipid A by the host occurs via the TLR4/MD-2/CD14 receptor complex (Toll-like receptor 4 / myeloid differentiation factor 2 or Lymphocyte antigen 96 / cluster of differentiation antigen 14) (Beutler, 2000; Gioannini et al., 2004; Medzhitov et al., 1997; Shimazu et al., 1999; Ulevitch and Tobias, 1999). CD14, in its soluble and membrane-bound forms, as well as the LPS binding protein (LBP) have been shown to enhance the response to lipid A by capturing

and transporting single LPS or lipid A molecules out of a micellar state (Kirkland et al., 1993; Lee et al., 1992; Ulevitch, 1993; Wright et al., 1990; Yu et al., 1997). *E. coli* or *Salmonella typhymurium* lipid A are potent agonists of the TLR4/MD-2 receptor complex. They consist of a β,1-6 linked glucosamine disaccharide that is phosphorylated at positions 1 and 4' and carries four R-3-hydroxymyristate chains (at positions 2, 3, 2' and 3'). The 2' and 3' acyl groups are further esterified with laurate or myristate, respectively (Raetz, 1990b). The structural basis of lipid A recognition by the TLR4/MD-2 complex has been solved (Park et al., 2009). Key features for receptor binding, multimerization and therefore activation are the 1 and 4' phosphates, which form charge interactions with TLR4 and MD-2 (Park et al., 2009) (Fig. 2 B and C). In the process of TLR4 dimerization (formation of a TLR4/MD-2/lipid A/TLR4*/MD-2*/lipid A* – hexamer) *E. coli* lipid A can further interact with several amino acids of TLR4* (coloured green in Fig. 2B), which via its primary binding sites fixes the lipid A*. These lipid A – TLR4* interactions stabilize the multimer and might be crucial for endotoxic activity. For some of these ionic interactions, like the one involving K388 of TLR4* (see Fig. 2 B), contradictory results have been reported (Resman et al., 2009). It was further shown that the hydroxymyristate chain at position 2 forms hydrogen bonds and has hydrophobic interactions with TLR4. This chain is the only part of lipid A acyl chains that is not completely buried inside the MD-2 pocket and is partially exposed to the surface (Park et al., 2009). Beside the presence of the 1 and 4' phosphate groups, the number and length of the acyl chains seem thus to play a major role in determining the endotoxic behaviour of a lipid A.

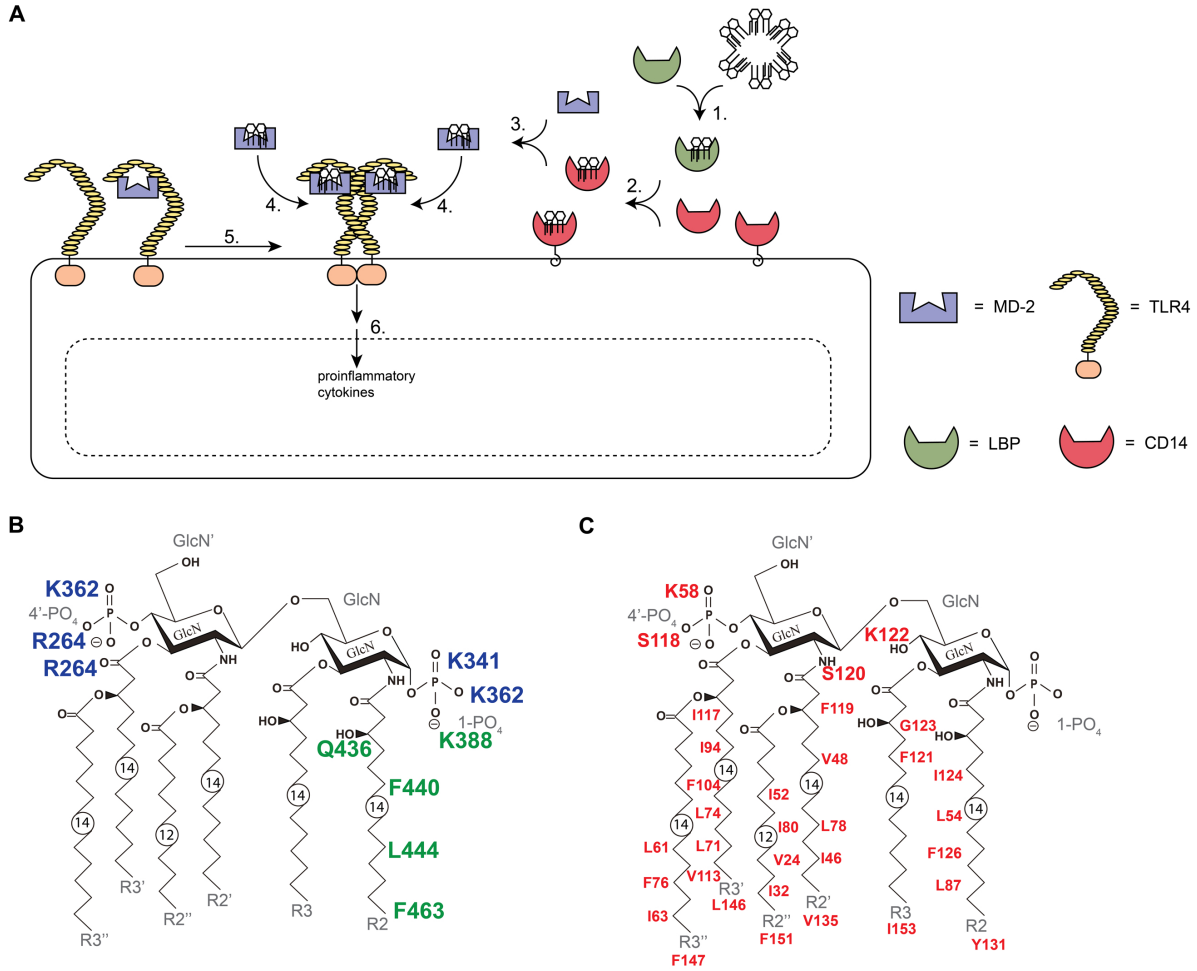


**Figure 2. LPS recognition by human TLR4/MD-2/CD14 receptor complex.** (A) LPS binding protein (LBP) binds a single LPS molecule out of the micellar state (1.), transports it to soluble or membrane bound CD14 (2.), from where the LPS is further passed on MD-2, which can be already bound to TLR4 (3. and 4.). LPS binding to MD-2 and TLR4 causes TLR4 dimerization (5.) which then leads to an intracellular signal cascade and finally to the release of proinflammatory cytokines (6.). (B) Proposed interactions of *E. coli* lipid A with indicated amino acids of human TLR4 (blue). Upon TLR4 dimerization (formation of a TLR4/MD-2/lipid A/TLR4*/MD-2*/lipid A* – hexamer) *E. coli* lipid A can interact with indicated amino acids of TLR4* (green), which via its primary binding sites fixes the lipid A*. (C) Proposed interactions of *E. coli* lipid A with indicated amino acids of human MD-2 (red). Data of B and C are based on the crystal structure of *E. coli* LPS bound to TLR4 and MD-2 (Park et al., 2009).

As the acyl chain length and number is crucial for endotoxicity, several bacteria have adapted their lipid A, e.g. by reducing the number of acyl chains attached to the lipid A (called underacylation). Underacylated lipid A have been studied in several gram-negative bacteria (Berezow et al., 2009; Hajjar et al., 2006; Kawasaki et al., 2004; Saitoh et al., 2004; Somerville et al., 1996; Teghanemt et al., 2005). Hyperacylation is less common and its contribution to pathogenesis is unclear, as was shown for *Salmonella* (Belden and Miller, 1994; Gibbons et al., 2005; Guo et al., 1997). Generally, underacylated lipid A structures have either four (tetra-acyl) or five acyl chain (penta-acyl). They are formed by either not adding further acyl chains (regulation of LpxL or LpxM) or by deacylation (*e.g.* by PagL in *Rhizobiae*). Tetra-acylated lipid A variants are antagonists of the action of hexa-acyl lipid A on human TLR4/MD-2, as has been shown in the peridontopathic bacterium *P. gingivalis* (Curtis et al., 2011; Kumada et al., 2008). Lipid IVa, a tetra-acylated precursor in *E. coli* lipid A biosynthesis, served as structural basis for the generation of the potent antagonist Eritoran that now is in clinical trials as anti-sepsis agent (E5564, Eisai research). X-ray crystallography studies of complexes between lipid IVa or Eritoran with MD-2 (Kim et al., 2007; Ohto et al., 2007; Park et al., 2009) have shown that the tetra-acylated lipid A variants are bound deeper into the MD-2 pocket and inverted by 180°C as compared to hexa-acylated lipid A. Due to the decrease in acyl chains the tetra-acylated variants can bind deeper into the MD-2 pocket (by 4-5 Angstrom), which leads to a repositioning of the phosphate groups. Therefore, although lipid IVa and Eritoran occupy the MD-2 binding sites, they do not lead to the human TLR4/MD-2 multimerization that is needed for signal transduction. Interestingly, lipid IVa was found to be an agonist or partial agonist of murine, cat and equine TLR4/MD-2. This was shown to result from few amino acids of MD-2 and TLR4, which alter multimerization potential upon lipid IVa addition (Meng et al., 2010; Walsh et

al., 2008). In case of the human TLR4, lipid IVa bound deep into the MD-2 pocket is no longer able to reach the binding sites of the phosphates (described in Fig. 2 B). And at the present position of the lipid IVa phosphates the human TLR4 has no adequate amino acids for proper interaction. This is in contrast to *e.g.* the murine TLR4, which harbours positively charged amino acids at the site of lipid IVa phosphates, thus enabling a stronger interaction with TLR4 and upon TLR4 dimerization (formation of a TLR4/MD-2/lipid A/TLR4*/MD-2*/lipid A* – hexamer) as well with amino acids of the TLR4*. The complexity of all this data implies a multistep activation mechanism, which remains to be elucidated in detail.

Penta-acylated lipid A are best described as „partial TLR4 agonists“ (Bryant et al., 2010). Upon binding to TLR4/MD-2, such partial agonists lead to some conformational changes, but they fail to induce a full activation. In presence of a potent stimulus like *E. coli* lipid A (an agonist), partial agonists lower the activation. Thus, they act as antagonist. Partial agonists compete for receptor/co-receptor binding sites with the agonist (Coats et al., 2007). Therefore a mixture of a potent agonist and a partial agonist can lead to a weaker stimulation of the receptor, compared to the activity of the agonist alone. Dependent on the weakness of its agonism, penta-acylated lipid A have been designated as weak agonists or as antagonists (Bäckhed et al., 2003; Berezow et al., 2009; Coats et al., 2003; Hajjar et al., 2006; Yoshimura et al., 2002; Zähringer et al., 2004). The notion of a partial agonist includes a weak agonsim as well as the antagonistic properties. It has been shown that the antagonistic effect of penta- and tetra-acyl lipid A is mainly based on direct competition between the antagonist and *E. coli* hexa-acyl lipid A for the identical binding site on human MD-2 (Coats et al., 2007). But the structure of a penta-acyl lipid A binding to human MD-2 remains to be solved. In

this thesis a model of a penta-acylated lipid A binding to MD-2 based on molecular mechanics calculations is presented.

Along with the number of acyl chains, the length of the chains has been shown to play an important role in determining the endotoxic potential (Bainbridge et al., 2006). It might be that longer acyl chain lead to a repositioning of the phosphate groups and thus alter the endotoxicity. But one has to remind that acyl chains should not be seen as stiff sticks but rather as flexible. Hence, they might be bent to fit into the MD-2 pocket.

Besides underacylation, Gram-negative bacteria have evolved different strategies to modify the lipid A structure (Coats et al., 2009; Dixon and Darveau, 2005; Hajjar et al., 2006; Mata-Haro et al., 2007; Price et al., 1995; Wang et al., 2004; Wang et al., 2006). A complete overview on possible modifications has been published by Raetz et al. (Raetz et al., 2007). Modification in the 1 or 4' phosphates of lipid A have been reported to alter endotoxicity and/or resistance to cationic antimicrobial peptides (CAMPs) (Coats et al., 2009; Curtis et al., 2011; Herrera et al., 2010; Ingram et al., 2010; Mata-Haro et al., 2007; Wang et al., 2006). 4' phosphatases (LpxF) have been described in *R. leguminosarum*, *R. etli*, *P. gingivalis, H. pylori* and *Francisella* species. Removal of the 4' phosphate leads to increased endotoxicity (Coats et al., 2009; Cullen et al., 2011), decreased resistance to CAMPs (Cullen et al., 2011; Ingram et al., 2010) and in case of *Francisella* and *H. pylori* to reduced virulence (Cullen et al., 2011; Kanistanon et al., 2011; Wang et al., 2006). 1 phosphatases (LpxE) have been identified in *H. pylori*, *P. gingivalis*, *R. etli* and others (Coats et al., 2009; Cullen et al., 2011; Ingram et al., 2010; Tran et al., 2004; Tran et al., 2006; Wang et al., 2004). Removal of the 1 phosphate leads to a slightly increased endotoxicity (Coats et al., 2009) and CAMP sensitivity (Ingram et al., 2010). After dephosphorylation, the 1 and 4' positions can be further modified. *H. pylori* is known to add a phosphoethanolamine (*P*-Etn) to the 1 position of

lipid A (Cox et al., 2003; Kim et al., 2006; Tran et al., 2004). This happens via a two-step mechanism, which first involves 1 dephosphorylation by LpxE and subsequent *P*-Etn transfer by a phosphoethanolamine transferase (EptA) (Tran et al., 2004).

The variety of lipid A modifications reflects the different niches colonized by bacteria. Still, most lipid A modifications might reduce endotoxicity, but other examples have been described as well (Dixon and Darveau, 2005). The tight regulation of some enzymes leading to changes in lipid A in function of pH, temperature or other host related parameters highlights the role of lipid A modifications in pathogenesis (Dentovskaya et al., 2008; Dixon and Darveau, 2005; Suomalainen et al., 2010). In the bacterial membrane, phosphates of neighbouring lipid A are bridged by divalent cations (likely $Mg^{2+}$), leading to increased membrane stability (Kim et al., 2006). The lateral stability of the outer membrane hence is largely dependent on the presence of lipid A phosphate that can be glued with a divalent cation. It can be guessed that some modifications on the lipid A phosphates might thus influence membrane stability and will only be induced in favourable conditions.

### 1.1.3 LPS part 2: the core-oligosaccharide

The core-oligosaccharide structure is divided into two regions, the inner core (adjacent to the lipid A) and the outer core, to which the O-antigen is attached. Mucosal pathogens often lack the O-antigen, and produce instead lipooligosaccharides (LOS), which consist of mono- or oligosaccharide branches attached to the inner core (Raetz and Whitfield, 2002a).

The inner part of the core-oligosaccharide is rather well conserved within a genus or a family. The only sugar present in all known inner-core structures is the Kdo (3-deoxy-D-*manno*-oct-2-ulosonic acid), which is linked to the lipid A backbone (5' position). In very few bacteria Kdo is replaced by a derivative (Ko, D-*glycero*-D-*talo*-oct-ulosonic acid) (Brade, 1999). In most cases a second Kdo is linked to the first, which is then often followed by L-*glycero*-D-*manno*heptose residue(s) (Brade, 1999; Raetz and Whitfield, 2002a).This basic structure is modified by addition of other sugars, with phosphates, pyrophosphoethanolamines or phosphorylcholines attached. These various possibilities lead to heterogeneity in inner core structures, while within a genus or family the structure of the inner core tends to be pretty much conserved (Raetz and Whitfield, 2002a).

The outer core is structurally more diverse, following the trend that structures more exposed to the environment tend to be less conserved. But within a genus only limited structural variation exists (Brade, 1999; Raetz and Whitfield, 2002a). In *E. coli* five core-types are known. They vary not exclusively but predominantly in the outer core (Raetz and Whitfield, 2002a).

The role of the core in membrane stability was highlighted by studies of *E. coli* and *S. enterica* deep-rough mutants lacking the core (except for the Kdo which is vital and added to the lipid A before completion of its biosynthesis) (Nikaido and Vaara, 1985;

Schnaitman and Klena, 1993). The membrane stability was shown to be dependent on phosphate groups of the core-oligosaccharide, which are used to cross-link adjacent LPS molecules via divalent cations (just like the lipid A phosphates) (Nikaido and Vaara, 1985).

The (inner) core of *E. coli* was recently shown to be sufficiently close to residues of TLR4 and MD-2 to establish interactions with TLR4 and MD-2 (Park et al., 2009). As it is generally reported that the lipid A is sufficient for endotoxicity (Rietschel et al., 1994), the importance of inner core interactions with TLR4 in *E. coli* lipid A binding to TLR4 seems thus not obvious. The reported differences in endotoxicity of the lipid A and LPS for some bacteria (Kumada et al., 2008; Swierzko et al., 2000) were so far contributed only to changes in solubility in water. Even if the LPS-core has so far never been shown to play a major role in TLR4 binding of a specific lipid A, there is some evidence that the core is directly involved in the multistep process involving LBP, CD14, MD-2 and TLR4 (Gomery et al., submitted; Muller-Loennies et al., 2003).

### 1.1.4 LPS part 3: O-antigen structures

LPS structures containing an O-antigen, called smooth LPS (S-LPS), are typically produced by *Enterobacteriaceae*, *Pseudomonaceae*, *Pasteurellaceae*, *Vibrionaceae* and many other Gram-negative bacteria (Raetz and Whitfield, 2002b). A tremendous diversity of O-antigens exists, which is based on more than 60 different sugars and 30 different non-sugar compounds (Brade, 1999; Knirel and Kochetkov, 1994). The O antigen is synthesized independently of the lipid A-core (Raetz, 1990b). Prior to transport to the surface the O-antigen and the lipid A-core part are ligated in the periplasm. Three pathways have been described for LPS biosynthesis and translocation (see Fig. 3). They are distinguished by the export mechanism and are

called Wzy-dependent (Fig. 3 A), ATP-binding cassette (ABC)-transporter dependent (Fig. 3 B) and synthase dependent (Fig. 3 C) (Raetz and Whitfield, 2002b). The Wzy-dependent export pathway is characterized by the formation of undecaprenyl pyrophosphate-linked repeat units in the cytoplasm. These units are then further polymerized block-wise at the periplasmic face of the inner membrane resulting in a polymer, which is transported across the outer membrane through a protein-machinery likely resembling the capsular export system formed by Wza in *E. coli* (Paulsen et al., 1997; Raetz and Whitfield, 2002b). In the ABC-transporter dependent pathway the complete O-chain is assembled in the cytoplasm. Such linear O-polysaccharide chains that are linked as well to undecaprenyl pyrophosphate grow by stepwise addition of single glycosyl residues to the non-reducing end. This process seems to be independent of a specific polymerase (Raetz and Whitfield, 2002b). Finally, the polymer is transported across the inner membrane dependent on the ABC-transporter (formed of WecA, Wzm and Wzt in *E. coli*) and subsequently across the outer membrane, presumably involving a similar pathway as in the Wzy-dependent export. The synthase-dependent export is branded by a single enzyme, the integral membrane protein synthase that performs synthesis and export across the inner membrane (Raetz and Whitfield, 2002b). The transport across the outer membrane likely involves the same machinery as in the other pathways.

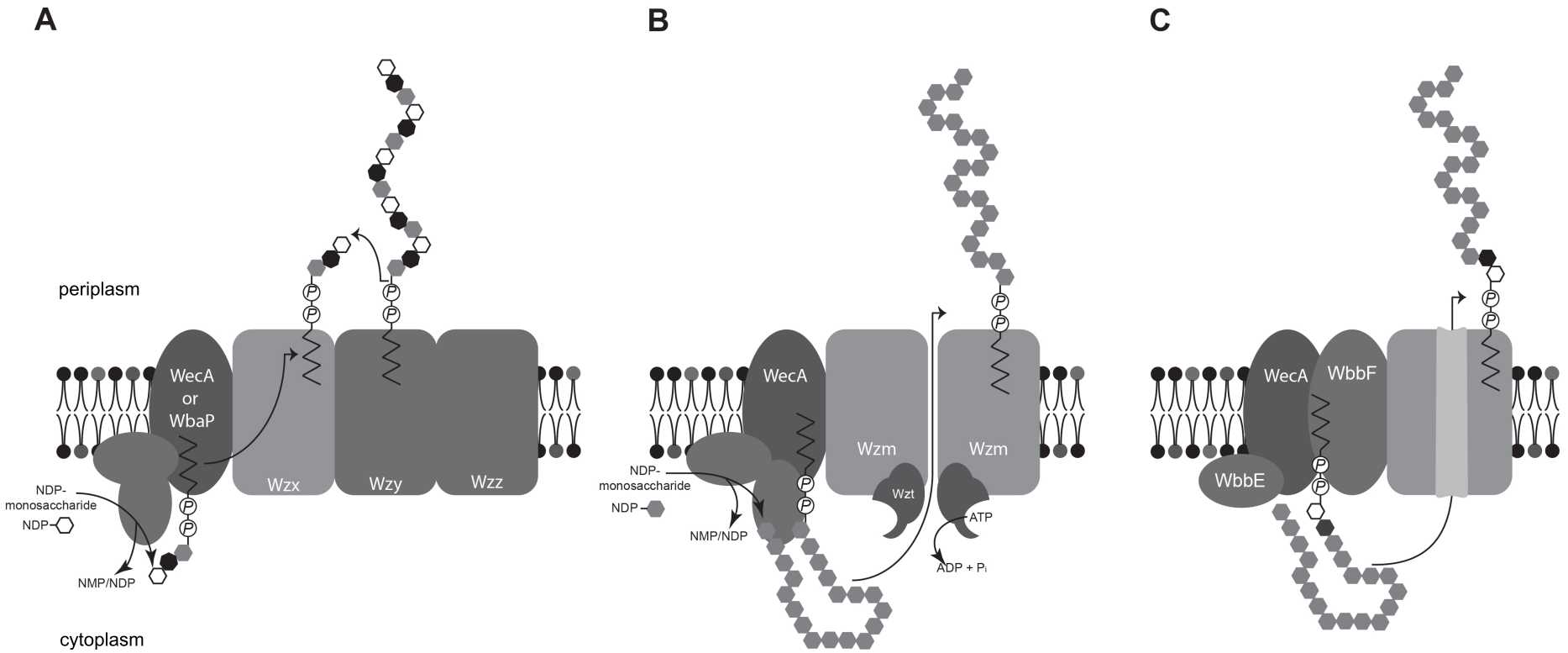


**Figure 3. LPS O–antigen biosynthesis and assembly pathways.** Three pathways have been described for LPS biosynthesis and translocation. They are distinguished by the export mechanism and are called Wzy-dependent (A), ATP-binding cassette (ABC)-transporter dependent (B) and synthase dependent (C) (Raetz and Whitfield, 2002b). In the Wzy-dependent pathway (A) undecaprenyl pyrophosphate-linked repeat units are formed in the cytoplasm. These units are polymerized block-wise at the periplasmic face of the inner membrane (involving Wzx, Wzy and Wzz) resulting in a polymer. In the ABC-transporter dependent pathway (B) the complete O-chain is assembled in the cytoplasm. These linear O-polysaccharide chains are linked to undecaprenyl pyrophosphate and grow by stepwise addition of single glycosyl residues to the non-reducing end. Finally, the polymer is transported across the inner membrane dependent on the ABC-transporter (formed of WecA, Wzm and Wzt in *E. coli*). The synthase-dependent export (C) is characterized by a single enzyme, the integral membrane protein synthase, which performs synthesis and export across the inner membrane. Based on figures in (Raetz and Whitfield, 2002b).

The O-antigen generally consists of several repeats of an oligosaccharide called the O repeat unit or O-unit. The O-units vary in monomer content, the position and stereochemistry of the linkages and presence of modifications. The O-unit can be linear or branched, composed of only one sugar (homopolymer) or more frequently of several sugars (heteropolymer). The O-antigen variation within species provides the main basis for serotyping. In *E. coli* around 170 different serotypes have been identified, in *S. enterica* 46 serogroups have been described (Raetz and Whitfield, 2002b). While most bacterial isolates express only one O-antigen, for some bacteria more than one LPS type has been identified (Lam et al., 2011; Raetz and Whitfield, 2002a). Some bacteria further contain lipid A-core linked polysaccharides, which are not called LPS, but are referred as capsules. In which case the lipid A-core attached form is referred as capsule or LPS does not follow a strict rule but rather depends on the size and likely on the history of identification. In *E. coli*, lipid A-core has been shown to anchor O-antigens, one form of the *Enterobacterial* common antigen polymer as well as some of the group 1 and 4 capsules (Kuhn et al., 1988; Whitfield, 2006).

The O-polysaccharide can be a virulence factor contributing to serum resistance (Murray et al., 2003; Murray et al., 2005; Murray et al., 2006; Nesper et al., 2001; Nesper et al., 2002; Pluschke et al., 1983a; Pluschke et al., 1983b; Raynaud et al., 2007; Slaney et al., 2006; Ugalde et al., 2000; West et al., 2005). O-antigen deficient strains of different bacteria have generally reduced virulence compared to the wt strains producing the complete LPS (Raynaud et al., 2007; Ugalde et al., 2000). Studies in *E. coli* and *S. enterica* reported a correlation between O-antigen length and resistance to complement (Bravo et al., 2008; Burns and Hull, 1998; Murray et al., 2003; Murray et al., 2005; Porat et al., 1992). Long chain S-LPS seems to prevent assembly of the membrane attack complex and thus protects from complement dependent killing.

### 1.1.5 Capsular polysaccharides and exopolysaccharides

The outermost layer of many Gram-negative (and positive) bacteria is formed by capsules or exopolysaccharides. Capsules are exported across the inner membrane by one of the three pathways identified in LPS transport (Wzy-, ABC- or synthase-dependent). Capsules may share the same repeat units as the O-antigen. In *E. coli* these O-antigens are classified as type-1 and 4 capsules. While these capsules probably share common repeat-unit donors and polymerization machinery (like the Wzx/Wzy/Wzz system) with the LPS O-antigen, they use a separate translocation system across the outer membrane. In *E. coli* this system involves Wza, which forms a multimeric putative translocation channel (Whitfield, 2006). Other capsules are made out of sugars not related to the O-antigen. In *E. coli* they are classified as type-2 and 3 capsules (Whitfield, 2006). Capsular polysaccharides not made out of O-antigen repeat units require a different polymerization and outer membrane translocation machinery (Whitfield, 2006). A well-studied example of a *E. coli* type-2 capsule is the K1 antigen, a polysialic acid capsule, which was shown to contribute to neonatal meningitis (Bonacorsi et al., 2001; Mushtaq et al., 2004). As for the O-antigens a huge diversity of capsular polysaccharides exists. Single strains of *Bacteroides fragilis* have been reported to produce 2 or even three different capsular polysaccharides, which contribute to formation of intra-abdominal infections (Baumann et al., 1992; Kalka-Moll et al., 2001; Tzianabos et al., 1992).

Capsules can be anchored into the outer membrane by diacylglycerol or by the lipid A-core, as explained before (Gotschlich et al., 1981; Raetz and Whitfield, 2002a). For many capsules the anchor has not been identified. In which case the lipid A-core attached form is called capsule or LPS depends on the size and likely on the history of

identification. Lipid A linked polysaccharides that have been identified by a capsular staining technique (like China ink) might be referred to as capsule, even if the later identified lipid A linker allows its classification as a LPS.

Exopolysaccharides are glycan polymers that are not anchored in the (outer) membrane. In *P. aeruginosa* an extracellular polymer substance containing a polysaccharide has been reported to be important in biofilm formation and thus pathogenesis (Ryder et al., 2007). Several exopolysaccharide structures of *E. coli* play as well a role in biofilm formation (Branda et al., 2005).

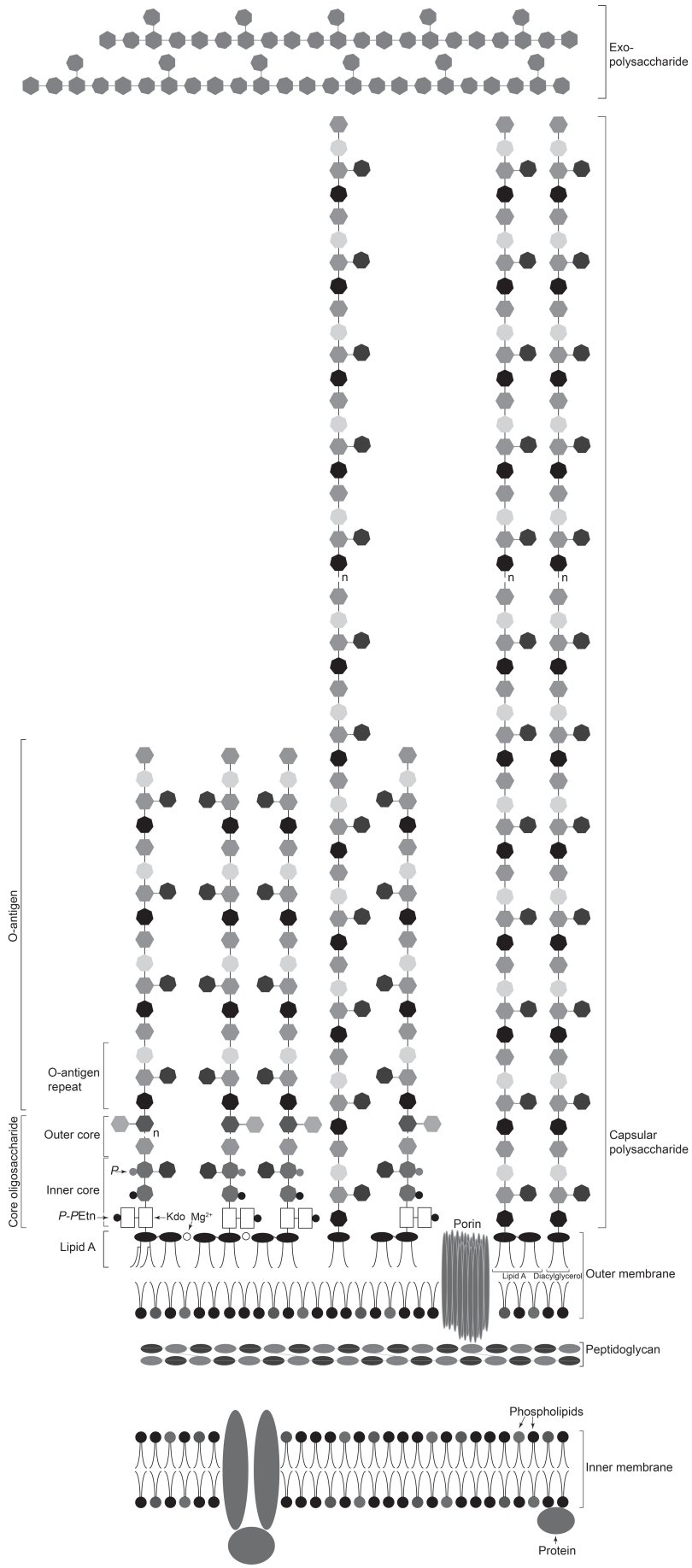


**Figure 4. Schematic model of the inner and outer membrane of a Gram-negative bacterium like *E. coli*.** Adapted from (Raetz and Whitfield, 2002a) where *P* is phosphate, *P*-*P*Etn is Pyrophosphoethanolamine and Kdo is 3-deoxy-D-*manno*-oct-2-ulosonic acid.

### 1.1.6 Known *Capnocytophaga* surface polysaccharide structures

The structure of lipid A of *Capnocytophaga spp* bacteria has so far not been investigated in detail. Only fatty acids present in the LPS of some *Cytophaga* strains have been identified as [13-Me-14:0 (i15:0), 13-Me-14:0(3-OH)(*i*15:0(3-OH), 16:0(3-OH) and 15-Me-16:0(3-OH) (i17:0(3-OH) (Ratledge and Wilkinson, 1988; Rosenfelder et al., 1974). The phylogenetically closest bacterium, in which the lipid A was characterized in detail, is *Elizabethkinga meningoseptica (former Flavobacterium meningosepticum),* which belongs to the *Flavobacteriaceae* (Tanamoto et al., 2001). The phylogenetic relatedness is in agreement with structural similarities between lipid A's of *E. meningoseptica, P. gingivalis (Kumada et al., 1995), Bacteroides fragilis (Weintraub et al., 1989),* and, as it will be shown here, also of *C. canimorsus*.

*C. ochracea* was shown to possess an immunosuppressive exopolysaccharide containing large amounts of mannose with lesser quantities of glucose, galactose, glucuronic acid, and glucosamine (Bolton et al., 1985; Dyer and Bolton, 1985). This exopolysaccharide was found free of protein, nucleic acid, and lipopolysaccharide.

## 1.2 Pathogenesis of *Capnocytophaga canimorsus*

Since 1976 there have been numerous case reports about severe sepsis or meningitis in humans after dog bites or scratches (Bobo and Newton, 1976; Brenner et al., 1989; Lion et al., 1996; Pers et al., 1996). The bacterium causing these dramatic infections was identified as *Capnocytophaga canimorsus* (former dysgenic fermenter 2, DF-2) (Bobo and Newton, 1976). *C. canimorsus* belong to the family of *Flavobacteriaceae* in the phylum *Bacteroidetes* and are usual members of dog's mouth flora (Bailie et al., 1978; Blanche et al., 1998; Brenner et al., 1989; Mally et al., 2009; Manfredi et al., 2011a)}. Human infections are rare and occur with an approximate frequency of one case per million inhabitants and year (Pers et al., 1996).

Previous studies have shown that macrophages infected with *C. canimorsus* 5, a strain isolated from a patient with fatal septicemia (Shin et al., 2007), fail to release proinflammatory cytokines (Shin et al., 2007). By virtue of the LPS and/or a capsule, *C. canimorsus* are also able to escape complement killing (Shin et al., 2009). They further resist killing by human polymorphonuclear leukocytes and macrophages (Meyer et al., 2008). Besides this passive evasion strategy, live but not heat-treated *C. canimorsus* bacteria have been shown to inhibit nitric oxide (NO) and TNFα release by LPS stimulated murine macrophages (Shin et al., 2007). Further, *C. canimorsus* has been reported to feed on eukaryotic glycoproteins at the surface of animal cells, including phagocytes (Mally et al., 2008; Manfredi et al., 2011b; Renzi et al., 2011). This deglycosylation is achieved by a multi-protein complex encoded by a polysaccharide utilization locus (PUL) (Renzi et al., 2011). PULs are a hallmark of the *Cytophaga-Flavobacteria-Bacteroides* group (Martens et al., 2008; Martens et al., 2009) and the

archetype of the feeding system they encode is the *Bacteroides thetaiotaomicron* starch utilization system (Sus). Sus consists of a complex of surface-exposed and periplasmic proteins and lipoproteins devoted to starch foraging. SusG, an $\alpha$-amylase, hydrolyses starch bound by SusC and SusD. The so generated starch-oligosaccharides are then transported via the TonB dependent transporter SusC into the periplasm for further breakdown (Anderson and Salyers, 1989; Reeves et al., 1997; Shipman et al., 2000). PULs characteristically encode a complex of surface exposed lipoproteins, a TonB-dependent transporter and further lipoproteins oriented towards the periplasm. Some of the periplasm- and/or surface-facing lipoproteins are glycosyl hydrolases, while others play a role in binding of a specific substrate. The genome of *C. canimorsus* 5 (Cc5) contains 13 PULs (Manfredi et al., 2011a; Manfredi et al., 2011b). Some have been studied in detail. PUL5 was shown to encode a system devoted to deglycosylation of N-linked glycan glycoproteins and called Gpd (Renzi et al., 2011). Gpd proteins have been shown to be surface-exposed and to co-purify as a group, together with sialidase SiaC (Manfredi et al., 2011b). For other PULs-encoded feeding systems, the enzymatic activity remains to be found.

## 1.3 References

### 1.3.1 References: Bacterial surface structure


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# 2 Lipid A of *Capnocytophaga canimorsus*

## 2.1 Manuscript submitted: The Lipopolysaccharide from *Capnocytophaga canimorsus* Reveals an Unexpected Role of the Core-Oligosaccharide in MD-2 Binding

**Author contributions:** SI, MS, YK, MdP, GC and UZ conceived and designed the experiments. SI, BL, MS, EZ, YK and UZ performed the experiments. SI, BL, MS, EZ, YK and UZ analyzed the data. SI, MS, GC, UZ wrote the paper.

**Statement of my work:** My contribution was the data of figures 3, 4, 5, 7 and S5. UZ, BL, YK and EZ performed all chemical analysis. MS and MdP performed molecular modeling.

# The Lipopolysaccharide from *Capnocytophaga canimorsus* Reveals an Unexpected Role of the Core-Oligosaccharide in MD-2 Binding

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Running head: *C. canimorsus* lipid A structure and function

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**Abstract**

*Capnocytophaga canimorsus* is a usual member of dog's mouths flora that causes rare but dramatic human infections after dog bites. We determined the structure of *C. canimorsus* lipid A. The main features are that it is penta-acylated and composed of a ”hybrid backbone“ lacking the 4’ phosphate and having a 1-*P*-Etn at GlcN. *C. canimorsus* LPS was 100 fold less endotoxic than *Escherichia coli* LPS. Surprisingly, *C. canimorsus* lipid A was 20,000 fold less endotoxic than the *C. canimorsus* lipid A-core. This represents the first example in which the core-oligosaccharide dramatically increases endotoxicity of a low endotoxic lipid A. The binding to human MD-2 was dramatically increased upon presence of the LPS core on the lipid A, explaining the difference in endotoxicity. Interaction of MD-2 or LBP/CD14 with the negative charge in the Kdo of the core might be needed to form the MD-2 – lipid A complex in case the 4’ phosphate is not present. Overall the properties of the lipid A-core may explain how this bacterium first escapes recognition by receptors of the innate immune system, but nevertheless is able to provoke a shock at the septic stage.

**Author summary**

*Capnocytophaga canimorsus*, a commensal bacterium in dogs mouths, causes rare but dramatic infections in humans that have been bitten by dogs. The disease often begins with mild symptoms but progresses to severe septisemia. The lipopolysaccharide (LPS), composed of lipid A, core and O-antigen, is one of the most pro-inflammatory

bacterial compounds. The activity of the LPS has so far been attributed to the lipid A moiety. We present here the structure of *C. canimorsus* lipid A, which shows several features typical for low-inflammatory lipid A. Surprisingly, this lipid A, when attached to the core-oligosaccharide was far more pro-inflammatory than lipid A alone, indicating that in this case the core-oligosaccharide is able to contribute significantly to endotoxicity. Our further work suggests that a negative charge in the LPS-core can compensate the lack of such a charge in the lipid A and that this charge is needed not for stabilization of the final complex with its receptor but in the process of forming it. Overall the properties of the lipid A-core may explain how this bacterium first escapes the innate immune system, but nevertheless can cause a shock at the septic stage.

## Introduction

*Capnocytophaga canimorsus*, a usual member of dog's mouths flora [1] was discovered in 1976 [2] in patients who underwent dramatic infections after having been bitten, scratched or simply licked by a dog. The most common syndrome is sepsis, sometimes accompanied by peripheral intravascular coagulation and septic shock [3]. *C. canimorsus* is a Gram-negative rod belonging to the family of *Flavobacteriaceae* in the phylum *Bacteroidetes* [4, 5]. Human infections occur, worldwide, with an approximate frequency of one per million inhabitants per year [6].

*C. canimorsus* are able to escape complement killing and phagocytosis by human polymorphonuclear leukocytes and macrophages [7, 8]. Whole bacteria are also poor agonists of Toll-like receptor (TLR) 4, which results in a lack of release of pro-

inflammatory cytokines by macrophages [9]. In addition to these “passive” features, *C. canimorsus* have been shown to harvest glycan moieties from glycoproteins at the surface of animal cells, including phagocytes [10-12], in addition they also deglycosylate human IgG [12].

One of the most pro-inflammatory bacterial compounds is the lipopolysaccharide (LPS, endotoxin) [13], consisting of three domains: lipid A, the core oligosaccharide and the O-polysaccharide (O-antigen). As a potent activator of the innate immune system, LPS can induce endotoxic shock in patients suffering from septicemia. Recognition of LPS by the host occurs via the TLR4/MD-2/CD14 receptor complex [14-16], at which two proteins, cluster of differentiation antigen 14 (CD14) and LPS-binding protein (LBP), have been shown to enhance the response to LPS by transporting single LPS molecules [17-20]. It has been shown that the lipid A moiety of the LPS is sufficient for TLR4 binding and stimulation [21, 22]. The interaction of lipid A and its receptor was unravelled by x-ray crystallography pioneering studies of complexes between myeloid differentiation factor 2 (MD-2) and the lipid A analog Eritoran [23] or lipid $IV_A$ [24]. The identification of the binding sites of lipid A to MD-2 and also to the LRR-domains of TLR4 [21] is a landmark achievement that enables a deeper understanding of the structure-function relationship between LPS/lipid A and its receptors. According to these data, the 1 and 4’ phosphates of the lipid A backbone, which form charge interactions with TLR4 and MD-2, are the key elements for receptor activation [21, 25], even though for some of the interactions conflicting data have been reported [26]. It was further shown that the β-hydroxymyristate chain at position 2 forms hydrogen bonds and hydrophobic interactions with TLR4. At present, there is no evidence that the LPS-core plays any major role in binding to TLR4; only a 10- to 100-fold difference in endotoxicity of lipid A and LPS has been reported for *E. coli*, *Porphyromonas gingivalis*

or *Proteus mirabilis* [27, 28], but these small differences could be attributed to differences in solubility. The core-oligosaccharide has so far never been shown to alter TLR4/MD-2 binding of a specific lipid A, only slight changes in MD-2 binding have been reported [29].

In this work, we investigated the lipid A structure of *C. canimorsus* in order to clarify its contribution to the septicemia and shock provoked by these bacteria. Very few lipid A structures have actually been solved in the *Cytophaga/Flavobacterium* group, with the exception of the lipid A from *Elizabethkingia meningoseptica* (former *Flavobacterium meningosepticum*) [30]. Already some time ago, the fatty acids present in the LPS of *Cytophaga* bacteria have been identified as [13-Me-14:0 (*i*15:0), 13-Me-14:0(3-OH)(*i*15:0(3-OH), 16:0(3-OH) and 15-Me-16:0(3-OH) (*i*17:0(3-OH)] [31]. Here we show that lipid A of *C. canimorsus* consists of the penta-acylated hybrid backbone β-D-GlcN3N'-(1'→6)-α-D-GlcN where the 4' phosphate group is missing and the 1 phosphate is linked to a ethanolamine group, forming a phosphoethanolamine (*P*-Etn). Not unexpectedly, this lipid A was of very low endotoxicity but, surprisingly, when bound to the core (lipid A-core, LA-core) it became 20,000 fold more endotoxic. In agreement with this observation, we show that the LPS core promotes the binding of *C. canimorsus* lipid A to MD-2. This is the first example of a core-oligosaccharide dramatically changing the endotoxicity of lipid A, in which the carboxy group of Kdo probably takes over the function of ionic binding of the missing 4' phosphate.

## Results

**Compositional analyses of lipid A.** 2-Amino-2-deoxy-D-glucose (GlcN) and 2,3-diamino-2,3-dideoxy-D-glucose (GlcN3N) were found in a ratio of approx. 2:1 (table I). Based on the notion that by GLC analysis synthetic GlcN3N expressed a response factor of about 50% when compared with GlcN (or GalN as internal standard), it was inferred that GlcN and GlcN3N are present in equimolar amounts in the lipid A backbone, suggesting the presence of a “hybrid backbone” in *C. canimorsus* lipid A (table I). Total fatty acid analysis revealed *i*15:0, *i*15:0(3-OH), 16:0(3-OH), and *i*17:0(3-OH) in a molar ratio of 1:1:1:2 in lipid A preparations. Analysis of ester-bound fatty acids indicated the presence of *i*15:0 and *i*15:0(3-OH) in approximately equimolar amounts, indicating that one 16:0(3-OH) and two *i*17:0(3-OH) residues are primary fatty acids *N*-linked to the lipid A backbone (table I). This result suggests a penta-acylated lipid A species.

**HPLC and MS analyses of lipid A.** The reversed phase HPLC profile of the lipid A sample is shown in Figure S1. Peak 2 expressed a molecular ion at *m/z* 1716.30, which is in excellent agreement ($\Delta m$ = 0.3 ppm) with a lipid A containing *i*15:0, *i*15:0(3-OH), 16:0(3-OH), and two moles of *i*17:0(3-OH) attached to the lipid A backbone (GlcN3N-GlcN), which also carries one *P*-Etn residue. The second major fraction (peak 5) at *m/z* 1594.29 was compatible with lipid A lacking the *P*-Etn. Based on peak intensities (peaks 2 and 5) about 40% of the *P*-Etn was liberated, most likely from the lipid A under the hydrolysis conditions used (Fig. S1).

All lipid A fractions investigated expressed a certain heterogeneity with respect the chain length of fatty acids (-$CH_2$- groups), as all mass spectra (MS) showed peak

“clusters” differing by 14 u, thus suggesting fatty acid heterogeneity (table II, Fig. S2). GLC-MS analysis of the fatty acid revealed that the mass difference of Δ*m* = 14 u was not due to the exchange of one single, prominent shorter fatty acid [e.g. 16:0(3-OH) → *i*15:0(3-OH)]. Instead, the lipid A showed a certain structural “fuzziness” with respect to the size and position of the individual fatty acids, which, according to this finding, appeared to be statistically distributed over all positions with no specific structural variation.

The ESI-MS data of the wt strain shown in table II indicated identical mass at *m/z* 1716.30 for peaks 2 and 3. As these lipid A fractions differed in their retention time, we conclude that they represent different structural isomers as they could be baseline-separated by HPLC. This HPLC analysis in combination with ESI-MS data thus shows that structural heterogeneity might not be solely related to the chain length of one fatty acid, but also to its position within the lipid A backbone.

In order to allocate the type of the hybrid lipid A backbone, the fatty acid distribution over the lipid A backbone, and the attachment side of the *P*-Etn, electrospray-ionization Fourier transform ion-cyclotron resonance (ESI FT-ICR) MS/MS mass spectrum in the positive mode was run [32]. The triethylammonium (TEN) salt of HPLC purified lipid A at *m/z* 1820.40 was selected as precursor ion (Fig. S3). Infrared multiphoton dissociation (IRMPD)-MS/MS generated one abundant characteristic B-fragment oxonium-ion of the non-reducing end at *m/z* 907.77 which is in excellent agreement with the mass value calculated for GlcN3N with *i*15:0, 16:0(3-OH), and *i*17:0(3-OH) attached (*m/z* 907.77). This fragmentation pattern also showed that *P*-Etn is attached at the reducing end - most likely at position C-1. Thus the lipid A in *C. canimorsus* is penta-acylated with an acylation pattern of three being attached to the

"non-reducing" GlcN3N' and two to the reducing GlcN sugar (3+2) in the lipid A hybrid backbone.

**NMR analysis of lipid A.** The lipid A was studied further by high-field nuclear magnetic resonance (NMR) spectroscopy using correlation spectroscopy (COSY), TOCSY, ROESY $^{1}H,^{13}C$-HSQC, $^{1}H,^{31}P$-HMQC, and $^{1}H,^{31}P$-HMQC-TOCSY experiments. The results are depicted in the supplement (supplementary table I). The $^{1}H,^{13}C$-HSQC spectrum (Fig. 1) showed two H-1,C-1 cross-peaks at δ 4.28/103.4 and 5.29/92.8 for GlcN3N' and GlcN, which were distinguished by correlations between protons at nitrogen-bearing carbons and the corresponding carbons (C-2' and C-3' of GlcN3N' and C-2 of GlcN, at δ 52.9, 54.6, and 51.4, respectively). $^{3}J_{1,2}$ coupling constants of 8.0 and 2.9 Hz for the H-1 signals at δ 4.28 and 5.29, were determined from the $^{1}H$ NMR spectrum and showed that GlcN3N is β- and GlcN α-linked. The H-1 signal of α-GlcN was additionally split due to coupling to phosphorus ($^{2}J_{1,P}$ 7.9 Hz), thus indicating that α-GlcN is phosphorylated with *P*-Etn and β-GlcN3N' occupies the "non-reducing" end of the lipid A backbone. The β1'→6-linkage between the two amino sugars was evident from strong cross-peaks of H-1' of GlcN3N' with protons H-6a',6b' of GlcN at δ 3.64 and 3.87 in the ROESY spectrum. The location of the *P*-Etn residue at position 1 of α-GlcN was further confirmed by $^{1}H,^{31}P$-HMQC and $^{1}H,^{31}P$-HMQC-TOCSY (Fig. S4) as well as ROESY experiments, which showed correlations between H-1 of GlcN at δ 5.29 and H-1a,1b of Etn at δ 3.91 and 3.98. In accordance with the 1'→6 linkage and the position of GlcN3N at the "non-reducing end", the $^{13}C$ NMR spectrum (supplementary table I) displayed a typical down-field displacement by ~10 ppm for C-6 of the 6-substituted GlcN (δ 71.0; compared with δ 60.0 for C-6 of GlcN3N', which is non-substituted in the free lipid A). The acylation pattern was confirmed by $^{1}H,^{13}C$-HSQC spectroscopy (Fig.

1), which showed only one characteristic downfield shift due to a deshielding effect for the *i*17:0[3-*O*(*i*15:0)]R2' i.e. the H-3R2'/C-3R2' cross-peak at δ 4.95/70.7. This finding indicated that only the OH-group of *i*17-0(3-OH) is acylated giving rise to an acyloxyacyl residue [*i*17:0-3-*O*(*i*15:0)] and that the 3+2 type fatty acids distribution in the penta-acylated lipid A, which is in good agreement with the MS data (Figs. S2 and S3). Taking together the data of the chemical studies defines the structure of the lipid A of *C. canimorsus* shown in Figure 2 A. The structure of *E. coli* hexa-acylated lipid A is depicted for comparison (Fig. 2 B). The *E. coli* lipid A consists of a β-(1'→6)-linked GlcN disaccharide that is phosphorylated at positions 1 and 4' and carries four (*R*)-3-hydroxymyristate chains (at positions 2', 3', 2 and 3). The 2' and 3' 3-hydroxylated acyl groups in GlcN' are further esterified with laurate and myristate, respectively [22].

***C. canimorsus* LPS core features only one Kdo.** The structure of *C. canimorsus* LA-core is depicted in Figure 2 C and its structural analysis will be described elsewhere (Zähringer et al., manuscript in preparation). The *C. canimorsus* LPS core features only one Kdo, to which a phosphoethanolamine (*P*-Etn) is attached in position 4. Usually, mono-Kdo LPS-core have a phosphate attached to the Kdo at that position. Thus, the only net negative charge in the core oligosaccharide originates from the carboxy-group of the Kdo. The inner core continues with two mannoses (Man) to which another *P*-Etn is attached in position 6 of Man$^{I}$ residue in the core oligosaccharide. The outer core consists of Galactose (Gal) and L-Rhamnose [to which the O-antigen is attached (U. Zähringer, unpublished results)]. A positively charged Galactosamine (GalN) residue is linked to the second Man residue in position 6.

**The structure identified matches the *C. canimorsus* genome.** *E. coli* lipid A biosynthesis has been unravelled in detail [22, 33]. Analyzing the genome of *C. canimorsus* 5 [5], we identified the genes required for the synthesis of lipid A-Kdo [33]. Only *lpxA*, *lpxA'*, *lpxC* and *lpxD* seem to cluster in one operon, the other genes are dispersed (Fig. 3 A). The difference in acylation of the 3' and 3 position and the hybrid backbone of the lipid A consisting of a β-1',6-linked GlcN3N'-GlcN disaccharide, suggests that two *lpxA* genes might be present in *C. canimorsus* and indeed two *lpxA* genes were identified (termed *lpxA* and *lpxA'*) in the *C. canimorsus* 5 genome (Fig. 3 A). In *Acidithiobacillus ferrooxidans* GnnA and GnnB are responsible for the biosynthesis of GlcN3N [34]. Based on the sequences of *A. ferrooxidans*, *gnnA* and *gnnB* could be identified in the genome of *C. canimorsus* (Fig. 3 A). In the biosynthetic pathway of *E. coli* lipid A, enzyme LpxM adds the acyloxyacyl-residue [14:0-3-*O*(14:0)] representing the 6$^{th}$ acyl chain [22]. In good agreement with the penta-acylation of lipid A in *C. canimorsus* 5 was our finding that *lpxM* could not be identified in the genome (Fig. 3 A). *C. canimorsus* LPS core features only one Kdo, suggesting a mono-functional Kdo transferase (WaaA/KdtA) or a Kdo hydrolase two-protein complex (KdoH1/2) as in *Helicobacter pylori* or *Francisella novicida* [35, 36]. Searches with KdoH1/2 did not hit any gene in the *C. canimorsus* 5 genome. Therefore, *C. canimorsus* possesses either a mono-functional WaaA or a KdoH1/2 complex without significant sequence similarity to known Kdo hydrolases. We have further investigated the enzymes leading to the addition of an ethanolamine (Etn) at the 1 phosphate of lipid A. In *H. pylori*, the addition of a *P*-Etn at 1 position has been proposed to result from a two-step mechanism [37]. In a first step the 1 phosphate is removed by a phosphatase (LpxE), and subsequently a *P*-Etn-transferase (EptA or PmrC, YjdB) adds a *P*-Etn to the 1 position of lipid A [37] (Fig. 3 B). In *H. pylori lpxE* and *eptA* are encoded by one

operon (Hp0021-Hp0022). *C. canimorsus eptA* was annotated as *Ccan* 16950. Search for a lipid A phosphatase were based on *lpxE* and/or *lpxF* sequences from *P. gingivalis [38]*, *F. novicida* [39], *Rhizobium etli* [40] *H. pylori* [37] and on all available *Bacteroidetes*-group *pgpB* sequences. Three *lpxE/F* candidates have been found in the *C. canimorsus 5* genome (*Ccan*16960, *Ccan*14540 and *Ccan*6070). All candidates were deleted and the mutated bacteria were tested for endotoxicity. Only deletion of *Ccan16960* affected endotoxicity (data not shown). Interestingly, *Ccan16960* is located within the same operon as *eptA* and the two genes overlap by 20 bp. Following the operon organisation of *H. pylori*, *Ccan*16960 has been annotated as *lpxE*. The predicted function of *lpxE* and *eptA* was validated by KO and analysis of the resulting phenotype (Ittig et al., manuscript in preparation).

The presence of the 4' kinase LpxK and the absence of a 4' phosphate leads to the assumption of the presence of a 4' phosphatase, LpxF. Several candidate genes were identified (besides *lpxE*: *Ccan 14540* and *Ccan6070*) and deleted but they had to be ruled out, as no deletion did affect the endotoxic activity (data not shown), thus, we lack annotation of *lpxF*. The proposed complete biosynthesis of *C. canimorsus* lipid A-Kdo is depicted in Figure 3 C, starting from UDP-N-acetyl-D-glucosamine and Ribulose-5 phosphate.

***C. canimorsus* LPS is 100-fold less endotoxic than *E. coli* O111 LPS.** The endotoxic activity of wt *C. canimorsus* 5 LPS (S-form) was compared to the endotoxic activity of *E. coli* O111 LPS using two different approaches: (i) Purified LPS samples were assayed for TLR4 dependent NFκB activation with HEK293 cells overexpressing human TLR4/MD-2/CD14 and a secreted reporter protein (HEKBlue human TLR4 cell line), (ii) purified LPS samples were assayed for induction of TNFα release by human

THP-1 macrophages. In both assays (Fig. 4 A and Fig. 4 C) *C. canimorsus* LPS appeared to be about 100 fold less endotoxic than *E. coli* O111 LPS (both S-form LPS).

***C. canimorsus* lipid A and LA-core exhibit striking difference in endotoxicity.** Generally, the lipid A part of a LPS is considered as sufficient to trigger full TLR4 activation. Minor differences to the LPS or LA-core might be explained by differential bioavailability/solubility in water. We have, therefore, examined the endotoxic activity of *C. canimorsus* lipid A, LA-core and LPS using the HEKBlue hTLR4 cell line and the TNFα release by human THP-1 macrophages. LPS and LA-core exhibited an endotoxicity in the same range, whereas the LPS was less than 10-fold more endotoxic than the LA-core (Fig. 4 B and Fig. 4 D). In contrast, *C. canimorsus* lipid A appeared to be absolutely non-stimulatory up to 5 μg/ml (Fig. 4 B and Fig. 4 D), around 20,000-fold less active than the LA-core and 200,000-fold less active than LPS on a weight basis (ng/ml) indicating a even higher difference on a molar basis. As the *C. canimorsus* LPS and the LA-core showed similar endotoxicity, the increase in endotoxicity in comparison to the lipid A must have been raised by the contribution of the core oligosaccharide. Minor differences in endotoxicity between LPS and LA-core as the 10- to 100-fold difference observed between *E. coli* lipid A and *E. coli* O111 LPS (Fig. 4 B and Fig. 4 D) might be explained by differential bioavailability/solubility in water/buffer. However, differential bioavailability cannot account for the huge difference observed here for *C. canimorsus* lipid A and LA-core.

***C. canimorsus* LPS core is essential for proper MD-2 binding of the lipid A.** The increase in endotoxicity of the *C. canimorsus* LA-core in comparison to the lipid A must have been raised by the contribution of the core oligosaccharide (Fig. 4). The 4’

phosphate of *E. coli* lipid A is known to interact with $Arg_{264}$ and $Lys_{362}$ of TLR4 and $Lys_{58}$ and $Ser_{118}$ of MD-2 [21]. *C. canimorsus* lipid A lacks the 4' phosphate and features only one net negative charge in the LPS core, namely the carboxylic oxygen of Kdo. Based on the known structure of *E. coli* LPS bound to TLR4/MD-2 [3FXI, [21]] we measured the interaction distances from the carboxylic oxygen of Kdo to $Arg_{264}$ and $Lys_{362}$ of TLR4 and to $Lys_{58}$ and $Ser_{118}$ of MD-2. The carboxylic oxygen of Kdo is within close distance to $Arg_{264}$ and $Lys_{362}$ of TLR4 and $Lys_{58}$ and $Ser_{118}$ of MD-2 and hence could contribute to binding to MD-2 or TLR4.

To assess the ability of *C. canimorsus* lipid A or LA-core to interact with human MD-2, we monitored their ability to compete with the binding of *E. coli* LPS-Biotin to MD-2. Culture supernatants of cells producing human MD-2 were incubated with biotinylated *E. coli* O111 LPS, either alone or in combination with different concentrations of a competitor. As a source of LBP and soluble CD14, 7.5% FCS (v/v) was added. After purification of LPS based on biotin, co-purification of MD-2 was monitored by Western blotting. *C. canimorsus* LA-core abolished the copurification of MD-2 with the *E. coli* LPS-Biotin at higher concentration than the positive controls, *E. coli* O111 LPS and lipid $IV_A$ but at lower concentration than unbiotinylated *E. coli* penta-acyl lipid A (Fig. 5 A and B). These results indicate that *C. canimorsus* LA-core binds to human MD-2, likely in the same pocket as *E. coli* LPS. This experiment does not reflect the antagonistic capacity of *C. canimorsus* LA-core as even native *E. coli* O111 LPS could prevent the co-purification of human MD-2 (Fig. 5 A and B). In contrast to the LA-core, *C. canimorsus* lipid A did not significantly affect the copurification of MD-2 with *E. coli* LPS-Biotin even at high concentration (Fig. 5 A and B). Thus, *C. canimorsus* lipid A seems not to bind to human MD-2 at all or to bind to MD-2 only very weakly, in contrast to the LA-core. We conclude from this experiment that the *C. canimorsus* LPS core promotes

the interaction and binding of the lipid A to MD-2 either via direct interaction with MD-2 or via binding to LBP or CD14.

**The final complex of human MD-2 and lipid A of *C. canimorsus* would be as stable as MD-2 and lipid A of *E. coli*.** In order to assess the contribution of the *C. canimorsus* LPS core in binding of the lipid A to MD-2, we modelled the binding of *C. canimorsus* lipid A to human MD-2 (Fig. 6 A) and compared it to the binding of *E. coli* lipid A. Some differences between the two complexes could be observed at the level of the lipid chains after just few ns of simulation (Fig. 6 A). In both cases the R3' and R3 chains (see Fig. 2 for nomenclature) were fully stretched and interacted with the same residues. No empty space was left by R3'' (missing in *C. canimorsus*) because the longer R2' and R2'' chains filled the void. While in *E. coli* the R2 chain is stretched toward the inner side of the pocket, in *C. canimorsus* it was projected toward the pocket exterior, due to both i) its longer size and ii) to the presence of the bifurcated terminus of the close R2''. The R2 chain of *C. canimorsus* lipid A was thus not completely buried inside the MD-2 pocket and it was even more exposed to the surface than the hydroxymyristate chain at position 2 in *E. coli*. This probably enables the *i*17:0(3-OH) chain at position 2 to interact with TLR4, as has been reported for the R2 chain of hexa-acylated *E. coli* LPS [21]. It should be mentioned here that penta-acylated *E. coli* lipid A is endotoxically almost inactive [13], and the acyl chains might be completely buried inside MD-2. Thus *C. canimorsus* penta-acylated lipid A is expected to behave differently from penta-acylated *E. coli* lipid A due to the extended length of the acyl chains and the bulky iso-groups. Overall the arrangement of the sugar moieties with respect to the MD-2 was similar for both complexes, the only major discrepancies being the orientation of the 1-phosphoryl group (1 phosphate in *E. coli*, 1 *P*-Etn in *C.*

*canimorsus*). The calculated binding energy for the two complexes was very similar when calculated at both MM-GBSA (molecular mechanics, the generalized Born model and solvent accessibility) and MM-PBSA (molecular mechanics, Poisson-Boltzmann solvent accessible surface area) level, being in both cases the MD-2 – *E. coli* lipid A complex slightly more stable (Fig. 6 C). To understand this trend the total binding free energy was fractionated into a list of interactions energies between each residue of MD-2 and each fragment of lipid A (Fig. 6 B), as coded in Figure 2. Each pairwise binding free energy value has been further fractioned into its electrostatic, steric (Van der Waals), and solvation (polar and cavitation) components. For each term contributions arising from backbone and sidechain have been singled out. In both cases the GlcN' (*E. coli*) or the GlcN3N' (*C. canimorsus*) moieties (R2' NH group) interacted with the backbone carbonyl of $Ser_{120}$ establishing a strong (about 4-5 kcal/mol) and persistent interaction. Favourable interactions were also observed between GlcN and residues $Phe_{121}$ and $Lys_{122}$. The side chain of $Phe_{121}$ established a strong apolar interaction (Van der Waals, non-polar solvation) with the extended R3 acyl chain in both complexes. The hydrogen bond between the NH group of $Ser_{120}$ and the carbonyl of the R3' chain was found to be strong and persistent in both cases. Neither the 1 phosphate group (*E. coli*) nor the 1 *P*-Etn (*C. canimorsus*) established favourable interactions with MD-2, whereas the 4' phosphate group (missing in *C. canimorsus*) could be accounted for the slightly greater stability of the MD-2 *E. coli* lipid A, due to the strong (about 7.5 kcal/mol) interaction established with both the backbone and the sidechain of $Ser_{118}$ (see Fig. 6 B). In summary, we found that in the final complex the arrangement of the sugar moieties with respect to the MD-2 and the calculated binding energy for the two complexes was very similar for *E. coli* lipid A and *C. canimorsus* lipid A.

***C. canimorsus* lipid A is no antagonist of TLR4.** *C. canimorsus* LPS, lipid A or LA-core were further tested for a possible antagonistic activity on the action of *E. coli* O111 LPS using HEKBlue human TLR4 cells. The cells were preincubated for 3h with various concentrations of purified *C. canimorsus* lipid A, LA-core or LPS samples, then stimulated with 5 ng/ml *E. coli* O111 LPS for further 20-24h and the TLR4 dependent NFκB activation was measured. *C. canimorsus* LPS, LA-core and lipid A appeared to be no antagonist of *E. coli* O111 LPS binding to human TLR4, in contrast to the tetra-acylated antagonist lipid $IV_A$ (Fig. 7 A and B). In a second assay, human THP-1 macrophages were preincubated for 3h with purified *C. canimorsus* lipid A, LA-core or LPS samples at the concentration indicated. Then the THP-1 cells were stimulated with 1 ng/ml *E. coli* O111 LPS for further 20 h and TNFα release was measured. *C. canimorsus* lipid A exhibited no antagonism to *E. coli* O111 LPS binding to human TLR4 (Fig. 7 D). Again lipid $IV_A$ showed the expected antagonism (Fig. 7 C and D). Dependent on the assay no antagonism or a very weak antagonism of *C. canimorsus* LPS was observed. This is in agreement with the notion of a partial agonist [41], which includes a certain degree of antagonism at sub-agonist concentration.

All tested lipid A and LA-core fractions exhibited no activity towards human TLR2, as tested by HEK293 cells overexpressing human TLR2/MD-2 and a secreted reporter (Fig. S5).

## DISCUSSION

We showed here that *C. canimorsus* has a penta-acylated lipid A, a feature often correlated to low endotoxicity [13, 25]. In addition, the ester-bound 4' phosphate is lacking. This structural feature is known to reduce the endotoxic activity by a factor of ~100 [13], which can now be better explained based on the recent data obtained with x-ray crystallography on the TLR4/MD-2/LPS complex [21]. In this complex, phosphate groups of lipid A play a crucial role. The 4' phosphate is thought to bind to positively charged amino acids in the LRR of TLR4 ($Arg_{264}$, $Lys_{362}$) as well as to MD-2 ($Ser_{118}$ and $Lys_{58}$) in a well-defined manner. This ionic interaction seems to be critical for the ligand affinity of lipid A, enabling formation of a hexameric $(TLR4/MD\text{-}2/LPS)_2$ complex necessary for signalling [21]. In the endotoxic lipid A, there is another negatively charged group, 1 phosphate, which binds to positively charged amino acids in the complex, especially in the LRR of both TLR4 and the counter TLR4, called TLR4* ($Lys_{388}$* of TLR4*, $Lys_{341}$, $Lys_{362}$ of TLR4) and also to $Arg_{122}$ of MD-2. In contrast to the 4' phosphate which binds to two proteins (TLR4 and MD-2), the 1 phosphate is involved in binding to three proteins in the complex (TLR4, TLR4*, and MD-2), suggesting that this group might be even more important for the formation of a stable hexameric $(LPS/TLR4/MD\text{-}2)_2$ complex, as has been reported [42]. We showed in this work that the lipid A of *C. canimorsus* contains a *P*-Etn group at position 1, thus neutralizing the negative charge of the 1 phosphate group. Therefore, we propose that such modified phosphorylation may exert a "shielding effect" on the negative charge of the phosphate and, hence, can explain why the lipid A of *C. canimorsus* is significantly reduced in its endotoxic activity.

The lipid A structure of *C. canimorsus* is similar to that of the closely genetically related *E. meningoseptica* with respect to the nature and position of the fatty acids [30]. As reported for *E. meningoseptica,* we also found some heterogeneity with respect to the nature of the amino sugar at the non-reducing end in the lipid A backbone, but it was significantly lower (2-5% in *C. canimorsus* compared to ~ 30% in *E. meningoseptica*) [30]. It has to be pointed out that this structural modification has no influence on the biological activity of lipid A, as it was shown for *Campylobacter jejuni* [43]. The Etn substitution at position 1 of *C. canimorsus* lipid A is however not present in *E. meningoseptica* [30]. One might thus expect that the lipid A of *C. canimorsus* is less endotoxic than that of *E. meningoseptica*. To confirm this suggestion a comparative study of lipid A of both species must be carried out. Since the genus *Capnocytophaga* belongs to the *Bacteroidetes* phylum [44], it is also not surprising that the structure of lipid A from *C. canimorsus* shares some important traits involved in specific TLR4 and MD-2 binding with the structure of *Bacteroides fragilis* lipid A*,* which we determined earlier [45]. In particular, the lipid A's from both bacteria are (3+2) penta-acylated, lack the 4' phosphate and share *iso*-branched fatty acids, including *i*15:0, 16:0(3-OH), and *i*17:0(3-OH).

In agreement with its structural traits, *C. canimorsus* lipid A was shown here to exhibit a very low activity towards human TLR4. *C. canimorsus* LPS and LA-core are 100- respectively 1000- fold less endotoxic than *E. coli* O111 LPS, which reminds the activity of the closely related lipid A of *E. meningospetica* [30]. However, in contrast to what was shown in *Capnocytophaga ochracea* [46], *C. canimorsus* LPS and lipid A were found not to antagonize the action of *E. coli* LPS on human TLR4.

The endotoxicity of the *C. canimorsus* LPS is probably reduced to the level, which is tolerable in the dog mouth. This reduced endotoxicity may probably explain

why the disease in humans often begins with mild symptoms [2, 6, 47] and finally progresses to severe septicaemia with shock and intravascular coagulation. Features of the LPS could therefore account for initial evasion of *C. canimorsus* from the host immune system, while the same LPS might later on induce the endotoxic shock.

*E. coli* lipid A and O111 LPS exhibit a 10- to 100-fold difference in endotoxicity and similar findings were made for *P. gingivalis* or *Proteus mirabilis* [27, 28]. The lipid A from *E. meningoseptica* also shows only minor differences in TLR4 activation to its LPS [30]. In contrast, we found that *C. canimorsus* lipid A was around 20,000 fold less endotoxic than the LA-core, even higher when compared on a molar basis, suggesting an important role of the core-oligosaccharide in TLR4/MD-2 binding and activation. This indicates the importance of the LPS core for TLR4 activation in the case of *C. canimorsus*, which has a lipid A devoid of a net negative charge. The *C. canimorsus* LPS core exhibits only one unshielded negative charge, on the carboxylic oxygen of Kdo. The negative charged carboxyl-group of Kdo in the *C. canimorsus* core could therefore directly participate in TLR4 or MD-2 binding, besides the reported inner core interactions with TLR4/MD-2 [21]. We found that the MD-2 binding ability of *C. canimorsus* lipid A is strongly reduced compared to the LA-core. This finding could explain the difference in endotoxicity, as a lipid A not properly bound to MD-2 cannot activate TLR4. It seems as if the *C. canimorsus* LPS core interacts with CD14, LBP or MD-2 and thus enables the binding to MD-2. By molecular modeling *C. canimorsus* lipid A was predicted to bind MD-2 in a very similar way as *E. coli* lipid A and the calculated binding energy for the two complexes was similar. As the energetic state of the final complex would therefore be stable and favourable in the case of *C. canimorsus* lipid A, we propose that the interactions of the LPS core with MD-2 (or LBP/CD14) preceed the

final lipid A – MD-2 binding, rather than only stabilizing it. In our model, summarized in Fig. 8, we suggest an intermediate state in which the lipid A in the case of *E. coli* or the core in the case of *C. canimorsus* form ionic interactions or hydrogen bonds with MD-2 allowing the lipid A – MD-2 complex to form at all. However, we could not rule out a direct role of the LPS-core in binding to CD14 or LBP. To our knowledge, this is the first reported example of the core-oligosaccharide changing dramatically the endotoxicity of lipid A.

## Materials and Methods

**Chemicals.** 13:0(3-OH) was purchased from Larodan, Malmö, Sveden and 2,3 diamino-2,3-dideoxy-D-glucose (2 x HCl) from United States Biochemical Corporation, Cleveland, OH, USA. All other chemicals, solvents and reagents were of highest purity commercially available. *E. coli* O111 LPS was purchased from Sigma-Aldrich, lipid $IV_A$ from PeptaNova. *E. coli* F515 lipid A (hexa- and penta-acyl) was purified as described [48, 49]. The analysis and isolation of *C. canimorsus* LA-core will be described elsewhere (Zähringer et al., manuscript in preparation). Purchased reagents were resolved according to manufacturer's instructions. Aliquots of lipid $IV_A$ were kept at -80°C.

**Isolation of LPS.** *C. canimorsus* bacteria were harvested from 600 blood plates in phosphate buffered saline (PBS) and washed with distilled water, ethanol (300ml) and acetone (300ml), followed each time by centrifugation at 18,000 x *g* for 30 min. Bacteria were air dried and resuspended in PBS containing 1% phenol for killing and storage in the deep freezer prior to LPS extraction. Cells were washed with ethanol, acetone and diethyl ether (each 1 L) under stirring (1 h, room temperature). After centrifugation cells were dried on air to give 11.2 g. For the isolation of LPS, *C. canimorsus* 5 bacteria were extracted by phenol-water [50]. The LPS was identified in the water phase, which also contained a large amount of an unknown glucan polymer separated by repeated ultracentrifugation (100,000 x *g*, 4h, 4°C, 3 times). The glucan was further analyzed (U. Zähringer and S. Ittig, manuscript in preparation) and the LPS identified in the sediment. The crude LPS preparation was further subjected to RNAse/DNAse treatment (30 mg, Sigma) for 24 h at room temperature followed by

Proteinase K digestion (30 mg, 16 h, room temp.) and dialysis (2 days, 4°C), and lyophilization. The yield of enzyme-treated LPS related to bacterial dry mass was 70mg (0.6%).

**Isolation of lipid A**. Lipid A was prepared from *C. canimorsus* 5 (25 mg) LPS by hydrolysis with 2% AcOH (4 ml) at 100°C until precipitation of lipid A (2-8 h). The sediment was extracted three times with a water-chloroform mixture (10 ml) and the organic phase was concentrated to dryness under a stream of nitrogen to give 17.7 mg of crude lipid A. The lipid A was purified by reversed phase HPLC as described elsewhere [51] with the following modifications: an Abimed-Gilson HPLC system equipped with a Kromasil C18 column (5µm, 100 Å, 10 x 250 mm, MZ-Analysentechnik) was used. Crude lipid A samples (2–5 mg) were suspended in 0.4 mL solvent A and the mixture was sonicated. A 0.1 M EDTA-sodium salt solution (100 µl, pH 7.0) was added forming a bi-phasic mixture, which was vortexed and injected directly onto the column. Samples were eluted using a gradient that consisted of methanol-chloroform-water (57:12:31, v/v/v) with 10 mM NaOAc as mobile phase A and chloroform-methanol (70.2:29.8, v/v) with 50 mM NaOAc as mobile phase B. The initial solvent consisted of 2% B which was maintained for 20 min after injection, followed by a linear three step gradient raising from 2 to 17% B (20-50 min), 17 to 27% B (50-85 min), and 27 to 100% B (85-165 min). The solvent was held at 100% B for 12 min and re-equilibrated 10 min with 2% B and hold for additional 20 min before the next injection. The flow rate for preparative runs was 2 ml/min (~80 bar) using a splitter (~1:35) between the evaporative light-scattering detector (ELSD) and fraction collector. The smaller part of the eluate was split to a Sedex model 75C ELSD (S.E.D.E.R.E., France) equipped with a low-flow nebulizer. The major part was collected by a fraction collector in 1 min

intervals (~2 ml each). Nitrogen (purity 99.996%) was used as gas to nebulize the post column flow stream at 3.5 bar into the detector at 50°C setting the photomultiplier gain to 9. The detector signal was transferred to the Gilson HPLC Chemstation (Trilution LC, version 2.1, Gilson) for detection and integration of the ELSD signal.

**GLC and GLC-MS analyses.** Sugar and fatty acid derivatives were analysed by gas-liquid chromatography (GLC) on a Hewlett-Packard HP 5890 Series chromatograph equipped with a 30-m fused-silica SPB-5 column (Supelco) using a temperature gradient 150 °C (3 min) → 320 °C at 5°/min. GLC-MS was performed on a 5975 inert XL Mass Selective Detector (Agilent Technologies) equipped with a 30-m HP-5MS column (Hewlett-Packard) under the same chromatographic conditions as in GLC.

**ESI-MS Analysis.** Analyses of lipid A were performed in negative and positive ion modes on a high resolution Fourier transform ion cyclotron resonance mass spectrometer, FT ICR-MS (Apex Qe, Bruker Daltonics, Billerica, MA, USA), equipped with a 7 T superconducting magnet and an Apollo dual electrospray-ionization (ESI) / Matrix-assisted laser desorption ionization (MALDI) ion source. Data were recorded in broadband mode with 512K data sampling rate. The mass scale was calibrated externally by using compounds of known structure. For the negative ion mode samples (ca. 10 ng/µl) were dissolved in a 50:50:0.001 (v/v/v) mixture of 2-propanol/water/triethylamine (pH ~ 8.5). For the positive ion mode samples, a 50:50:0.03 (v/v/v) mixture of 2-propanol/water/30 mM ammonium acetate adjusted with acetic acid to pH 4.5 was used. The samples were sprayed at a flow rate of 2 µL/min. The capillary entrance voltage was set to 3.8 kV and the drying gas temperature to 150 °C. The mass numbers given refer to that of the monoisotopic ion peak. For MS/MS in

the positive ion-mode small amounts of TEN were added to the sample preparation to obtain the $[M+TEN+H]^+$ adduct ions [32] which were selected for collision induced decay (CID) in the collision cell infrared multiphoton dissociation (IRMPD) within the ion cyclotron resonance (ICR) cell.

**NMR spectroscopy.** Lipid A samples (1-3 mg) were exchanged twice with deuterated solvents [chloroform-$d_1$/methanol-$d_4$ 1:1 (v/v), Deutero GmbH, Kastellaun, Germany] and evaporated to dryness under a stream of nitrogen. Samples were dissolved in 180 µl chloroform-$d_1$/methanol-$d_4$/$D_2O$ 40:10:1 (v/v/v, 99.96%) and analyzed in 3 mm NMR tubes (Deutero). $^1$H-, $^{13}$C-, and $^{31}$P-NMR spectra were recorded at 700.7 MHz ($^1$H) on an Avance III spectrometer equipped with a QXI-cryoprobe (Bruker, Germany) at 300K. Determination of NH-proton signals was performed in chloroform-$d_1$(99.96%)/methanol/$H_2O$ 40:10:1 without exchange in deuterated solvents. Chemical shifts were referenced to internal chloroform ($\delta_H$ 7.260, $\delta_C$ 77.0). $^{31}$P NMR spectra were referenced to external aq. 85% $H_3PO_4$ ($\delta_P$ 0.0). Bruker software Topspin 3.0 was used to acquire and process the NMR data. A mixing time of 100 ms and 200 ms was used in TOCSY and ROESY experiments, respectively.

**Compositional analyses**. Quantification of GlcN, GalN (internal standard) and GlcN3N by GLC and GLC-MS was done after strong acid hydrolysis of 0.5 mg lipid A in 4 M HCl (16 h, 100 °C), followed by acetylation (*N*-acetylation) in pyridine/acetic acid anhydride (10 min, 85°C), reduction ($NaBH_4$) and per-*O*-acetylation. The response factor of the per-*O*-acetylated GlcNAc-ol, GalNAc-ol, and GlcNAc3NAc-ol derivatives, necessary for the quantification of GlcN3N by GLC, was determined in addition by external calibration with synthetic reference sugars. Etn, GlcN, GlcN3N and their corresponding

phosphates (GlcN-*P* and Etn-*P*), were determined from the hydrolysate by reversed phase HPLC using the Pico-tag method and pre-column derivatization with phenylisothiocyanate according to the supplier's instructions (Waters, USA). Quantification of total phosphate was carried out by the ascorbic acid method [52]. For analysis of ester- and amide-linked fatty acids, the lipid A was isolated from LPS (1 mg) by mild acid hydrolysis (0.5 mL, 1% AcOH, 100°C, 2 h), centrifuged and the lipid A sediment was separated into two aliquots and lyophilized. Ester-linked fatty acids were liberated from the first aliquot by treatment with 0.05 M NaOMe in water-free methanol (0.5 mL) at 37°C for 1 h. The mixture was dried under a stream of nitrogen and acidified (M HCl) prior to extraction with chloroform. The free fatty acids were converted into methyl esters by treatment with diazomethane and hydroxylated fatty acids were trimethylsilylated with *N,O*-bis(trimethylsilyl)trifluoroacetamide for 4 h at 65°C [53]. The fatty acids derivatives were quantified by GLC-MS using the corresponding derivatives of 17:0 (50 µg) and 13:0(3-OH) (50 µg, Larodan, Malmö, Sweden) as internal standards for the calibration of the response factor of non-hydroxylated and hydroxylated fatty acids, respectively. For analysis of total fatty acids, the second aliquot was subjected to a combined acid/alkaline hydrolysis as described [54]. Briefly, fatty acids were liberated from the lipid A by strong acid hydrolysis (4 M HCl, 100 °C, 21 h) and extracted three times with water/chloroform (0.5 mL each). The organic phase containing the *N*- and *O*-linked fatty acids was treated with diazomethane, trimethylsilylated and quantified as described above.

**Bacterial strains and growth conditions**. The strains used in this study are listed in Supplementary Table II. *E. coli* strains were grown in LB broth at 37°C. *C. canimorsus* 5 [9] 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 resuspended in PBS. Selective agents were added at the following concentrations: erythromycin, 10 mg/ml; cefoxitin, 10 mg/ml; gentamicin, 20 mg/ml; ampicillin, 100 mg/ml.

**Human TLR4 activation assay.** HEK293 stably expressing human TLR4, MD-2, CD14 and a secreted NFκB dependent reporter were purchased from InvivoGen (HEKBlue™ hTLR4). Growth conditions and endotoxicity assay were as recommended by InvivoGen. Briefly, desired amount of LPS or lipid A were placed in a total volume of 20 µl (diluted in PBS) an added 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% $CO_2$. If the antagonistic activity of a compound on the action of *E. coli* O111 LPS was assayed, the compound was added in a total volume of 10 µl (diluted in PBS), 25000 HEKBlue™ hTLR4 cells in 180 µl were added and the plate was incubated for 3h at 37°C and 5% $CO_2$. Then the cells were stimulated with 5 ng/ml *E. coli* O111 LPS and the plate was incubated as above. Detection 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 colour developed was measured at 655nm using a spectrophotometer (BioRad).

**TNFα release by human THP-1 cells.** Human THP-1 monocytes (ATCC TIB-202™) were cultured as recommended by the American Type Culture Collection (RPMI 1640 medium complemented with 10% v/v heat-inactivated fetal bovine serum, 2mM L-Glutamine). Monocytes were seeded at $1.5\times10^5$ cells/ml in 24 well-plates (BD Falcon) in growth medium containing $10^{-7}$M PMA (Sigma-Aldrich). For differentiation and

attachment the cells were incubated for 48h at 37°C and 5% $CO_2$ and then washed with growth medium and fresh PMA-free medium was added. After further incubation for >1h the cells were challenged with the indicated amount of LPS or lipid A in a total volume of 20 µl (diluted in PBS). After 20h of incubation the supernatants were harvested and immediately analyzed for TNFα by an ELISA. ELISA was performed in accordance with the manufacturers instructions (BD OptEIA$^{TM}$). If an antagonist of *E. coli* O111 LPS was assayed, the compound was added in a total volume of 10 µl (diluted in PBS) to the THP-1 cells and the plates were incubate for 3h at 37°C and 5% $CO_2$. Then the cells were stimulated with 1 ng/ml *E. coli* O111 LPS and the plate was incubated for 20h at 37°C and 5% $CO_2$.

**LPS Biotinylation.** Biotinylation of *E. coli* O111 LPS (Sigma-Aldrich) was performed as described previously [55] using biotin-LC-hydrazide (Pierce, Rockford, IL). To verify that the biotinylation did not affect the functionality of the LPS, *E. coli* LPS-Biotin was assayed for endotoxicity with the HEKBlue human TLR4 cell line (Data not shown). Biotinylation reduced the endotoxic potential at low concentrations, but only slightly at concentrations used in the MD-2 binding assay.

**Human MD-2 binding assay.** MD-2 binding assays was performed as described [55, 56]. HEK293 cells were transfected using Fugene6 (Roche, 3:2 protocol) with a plasmid (kind gift of K. Miyake and C. Kirschning) encoding human MD-2 with a C-terminal Flag-His-tag (pEFBOS-hMD2-Flag-His) [15]. The medium was exchanged 3-8 h post transfection with fresh growth medium. The cells were incubated for 48h and the supernatant was harvested and pooled. Fresh FCS was added to the hMD-2 supernatant (7.5% v/v). For each binding reaction, 4 ml of hMD-2 supernatant were

combined with 250 ng, 500 ng, 1 µg, 2 µg, 5 µg or 10 µg of the competitor, incubated at room temperature and gently rocked for 30 min. 1 µg of biotinylated *E. coli* O111 LPS was added and the supernatant was further incubated for 3-4 h at room temperature. Biotinylated LPS–hMD-2 complexes or single biotinylated LPS were captured by addition of 120 µl (total volume) streptavidin-agarose beads (IBA) per sample. The beads were previously prepared by washing them three times with a buffer (100 mM Tris, 150 mM NaCl, pH 8.0). For binding, the supernatants containing the beads were incubated overnight on a rotator at 4°C. Agarose beads were pelleted by centrifuging for 30 s at 5000 × *g* and 4°C and washed three times with PBS containing 0.5% Tween 20. The beads were finally resuspended in 60 µl SDS-loading dye (without dithiothreitol) and boiled for 5min at 95°C. The protein content in the sample was analyzed by non-reducing, denaturing 4–12% Tris-glycine Polyacrylamide gels (Invitrogen) or 4-15% Tris-glycine Polyacrylamide gels (BioRad) and then transferred to polyvinylidene fluorid (PVDF) membrane (ImmobilonP, Millipore). Membranes were probed using monoclonal anti-Flag antibody (Sigma-Aldrich) according to the manufacturer's instructions using ECL-Plus reagent (GE Healthcare).

**Genome annotation.** *Blast*-p search tool [57] against the *C. canimorsus* 5 genome [5] was used. Search sequences were obtained from the National Center for Biotechnology Information. All available *Bacteroidetes*-group sequences were used as search if available, but 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* sequences from *P. gingivalis [38]*, *F. novicida* [58], *R. etli* [40], *H. pylori* [37] and on all available *Bacteroidetes*-group *pgpB* sequences. Three *lpxE/F*

candidates have been found in the *C. canimorsus 5* genome (*Ccan* 16960, *Ccan* 14540 and *Ccan* 6070). All candidates have been deleted and only deletion of *Ccan* 16960 affected endotoxicity (data not shown). Since this gene is encoded in an operon with the predicted *eptA* and since the same operon structure (*lpxE-eptA*) has been identified in *H. pylori* [37] *Ccan*16960 was annotated as *lpxE*.

**Molecular modeling.** The MD-2 - *E. coli* LPS complex (PDB code 3FXI) [21] was used to construct models for the MD-2 - *E. coli* lipid A and for the MD-2 – *C. canimorsus* Lipid A. The modeling of the lipid A moieties was performed using the VMD [59] program and the *leap* module of the AMBER11 [60] suite of programs. To investigate the time-dependent properties of the two MD-2 – lipid A complexes, the constructed systems were subjected to molecular dynamics simulations [61] in the framework of a classical molecular mechanics [62] (MM) description. MM parameters from the Glycam06 [63, 64] force field were adapted to describe the acyl chains and the sugar moieties, while the Amber99SB [65, 66] force field was employed for the MD-2 protein. Advanced methods based on quantum chemistry were employed to obtain the missing parameters of the ester linkages and hydroxyl groups on the acyl chain C2 atoms, the branching at the bottom of the *C. canimorsus* acyls, the phosphate/*P*-Etn groups and the GlcN3N' moiety. Bonding parameters were obtained by performing relaxed potential energy scans [67] (bonds, angles, dihedrals), while charges were calculated on the optimized geometries of selected capped fragments. All the scan and geometry optimizations were conducted at the RI-MP2/def2-TZVP [68-70] level using the COBRAMM [71] suite of programs efficiently linking the ORCA2.8 [72] (wave-function calculation) and the GAUSSIAN09 [73] (optimization/scan driver) programs. Charges were calculated according to the RESP procedure at the HF/6-31G*//MP2/def2-TZVP. Both MD-2 – lipid

A complexes were embedded in a 6.5 × 6.5 × 6.5 $nm^3$ box of TIP3P [74] water molecules and the appropriate number of $Na^+$ and $Cl^-$ ions were added to neutralize the systems charge. The systems were relaxed (conjugate gradient geometry optimization) to remove clashes before stating molecular dynamics simulations. The systems were both heated to 300 K in the NVT (constant particle number, volume, temperature) ensemble for 500ps and then equilibrated in the NPT (constant particle number, pressure, temperature) until relevant structural parameters (density, RMSD on the protein Cα) were found to be stable (1 ns). Statistics were then performed on trajectories collected from 10 ns long simulations of the equilibrated systems. All molecular dynamics calculations were performed with the sander module of the AMBER11 package; bonds involving H atoms were constrained using the SHAKE algorithm [75] to allow for using a time step of 2 fs. Pressure was controlled *via* a simple Berendsen weak coupling approach [76], while a Langevin thermostat (collision frequency set to 3 $ps^{-1}$) was used to enforce the desired temperature. Molecular dynamics trajectories were analyzed using the VMD software, the *ptraj* module of the AMBER11 suite and the ProDy [77] package. A set of 300 snapshots of the equilibrated trajectories was subjected to further analysis to quantify the binding energy between MD-2 and each of the two lipid A moieties. Both the MM-PBSA and MM-GBSA approaches [78] were used to calculate the MD-2 – lipid A binding energy, while a full interaction energy decomposition [79, 80] was performed using the cheaper MM-GBSA method; the *MMPBSA.MPI* module of AMBER11 was used to perform the binding free energy calculations, while a locally developed software was used to process, analyze and plot the results.

**Western-blot quantification.** Quantification was performed using MultiGauge software (Fujifilm).

**Online supplemental material.** Fig. S1 shows a HPLC elution profile of the lipid A from *C. canimorsus*. Fig. S2 shows a negative mode ESI mass spectrum of lipid A from *C. canimorsus*. Fig. S3 shows a CID-MS/MS (positive mode) of lipid A from *C. canimorsus*. Fig. S4 shows a $^{1}H,^{31}P$-HMQC and $^{1}H,^{31}P$-HMQC-TOCSY spectra of lipid A from *C. canimorsus*. Fig. S5 shows the activation of human TLR2 with *C. canimorsus* and *E. coli* lipid A or LA-core preparations.

**Footnotes**

Abbreviations used:

CD14, cluster of differentiation antigen 14; ELSD, evaporative light-scattering detector; Etn, ethanolamine; Gal, Galactose; GalN, Galactosamine; GLC, gas-liquid chromatography; GLC-MS, combined GLC/mass spectrometry; GlcN, 2-amino-2-deoxy-D-glucose; GlcN3N, 2,3-diamino-2,3-dideoxy-D-glucose; HPLC, high-performance liquid chromatography; HMQC, heteronuclear multiple-quantum coherence; HSQC, heteronuclear single-quantum coherence; *i*15:0, *iso*-pentadecanoic acid (13-methyltetradecanoic acid, 13Me-14:0); *i*15:0(3-OH), *iso*-(*R*)-3-hydroxypentadecanoic acid [(*R*)-3-hydroxy-13-methyltetradecanoic acid, 13Me-14:0(3-OH)]; 16:0(3-OH), (*R*)-3-hydroxyhexadecanoic acid; *i*17:0(3-OH), *iso*-(*R*)-3-hydroxyheptanoic acid [(*R*)-3-hydroxy-15-methylhexanoic acid, 15-Me-16:0(3-OH)]; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; LA-core, lipid A-core; LBP, LPS-binding protein; LRR, Leucine-riche repeat; Man, Mannose; MD-2, myeloid differentiation factor 2 or Lymphocyte antigen 96; MM, molecular mechanics; MM-GBSA, molecular mechanics, the generalized Born model and solvent accessibility; MM-PBSA, molecular mechanics, Poisson-Boltzmann solvent accessible surface area; MS, mass spectrometry; NMR, nuclear magnetic resonance; *P*, phosphate; PBS, phosphate buffered saline; *P*-Etn, phosphoethanolamine; ROESY, rotating-frame nuclear Overhauser effect spectroscopy; TEN, triethylamine; TLR2 and TLR4, toll-like receptor 2 and 4; TOCSY, total correlation spectroscopy.

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## Figures

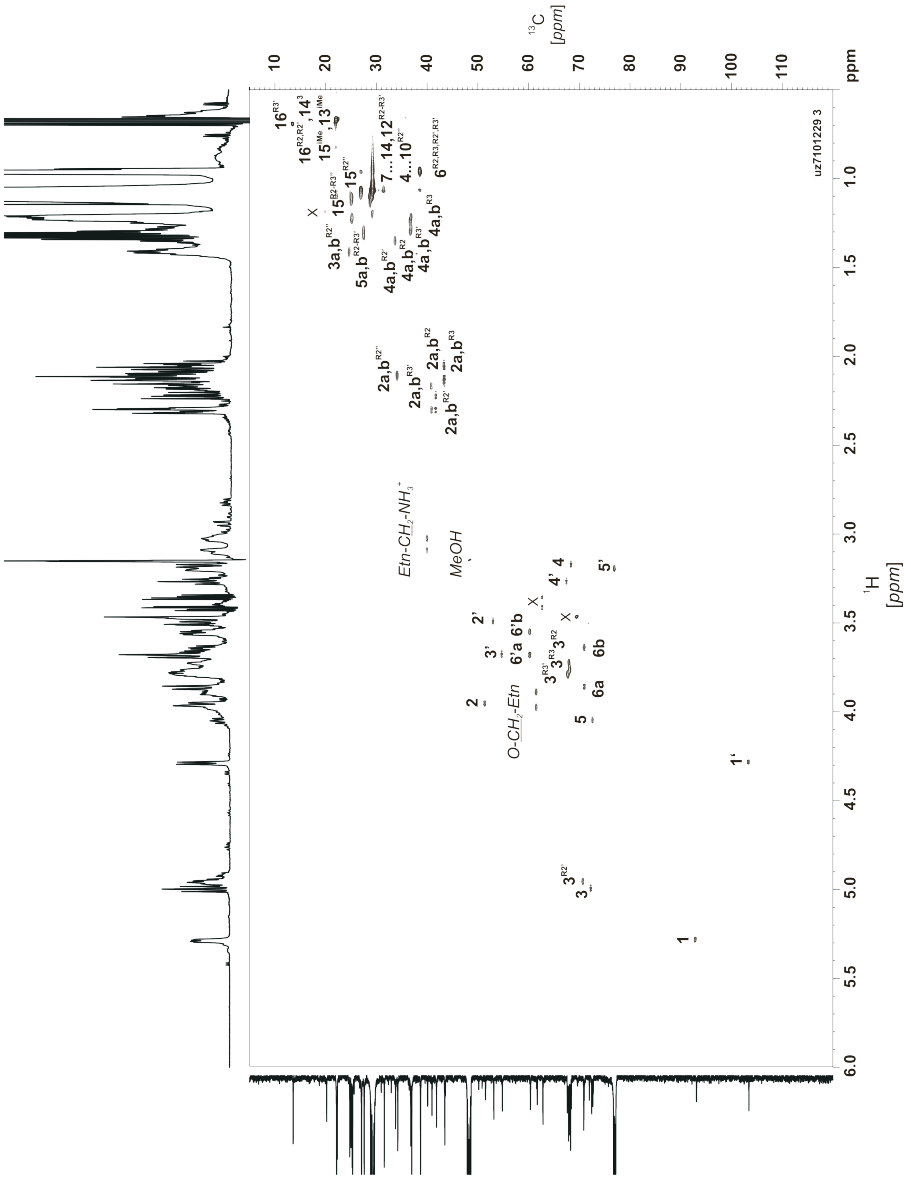


**Figure 1. NMR analysis of the lipid A from *C. canimorsus* wild type.** $^{1}H,^{13}C$-HSQC spectrum (700 MHz) of lipid A in chloroform-methanol-water (20:10:1, v/v/v) at 27°C. The corresponding parts of the $^{13}C$ and $^{1}H$ NMR spectra are displayed along the F1 and F2 axes, respectively. Numerals refer to atoms in sugar and fatty acid residues denoted by letters as shown in Supplementary table I and Figure S2. Signals from an unidentified contaminating lipid are indicated by X.

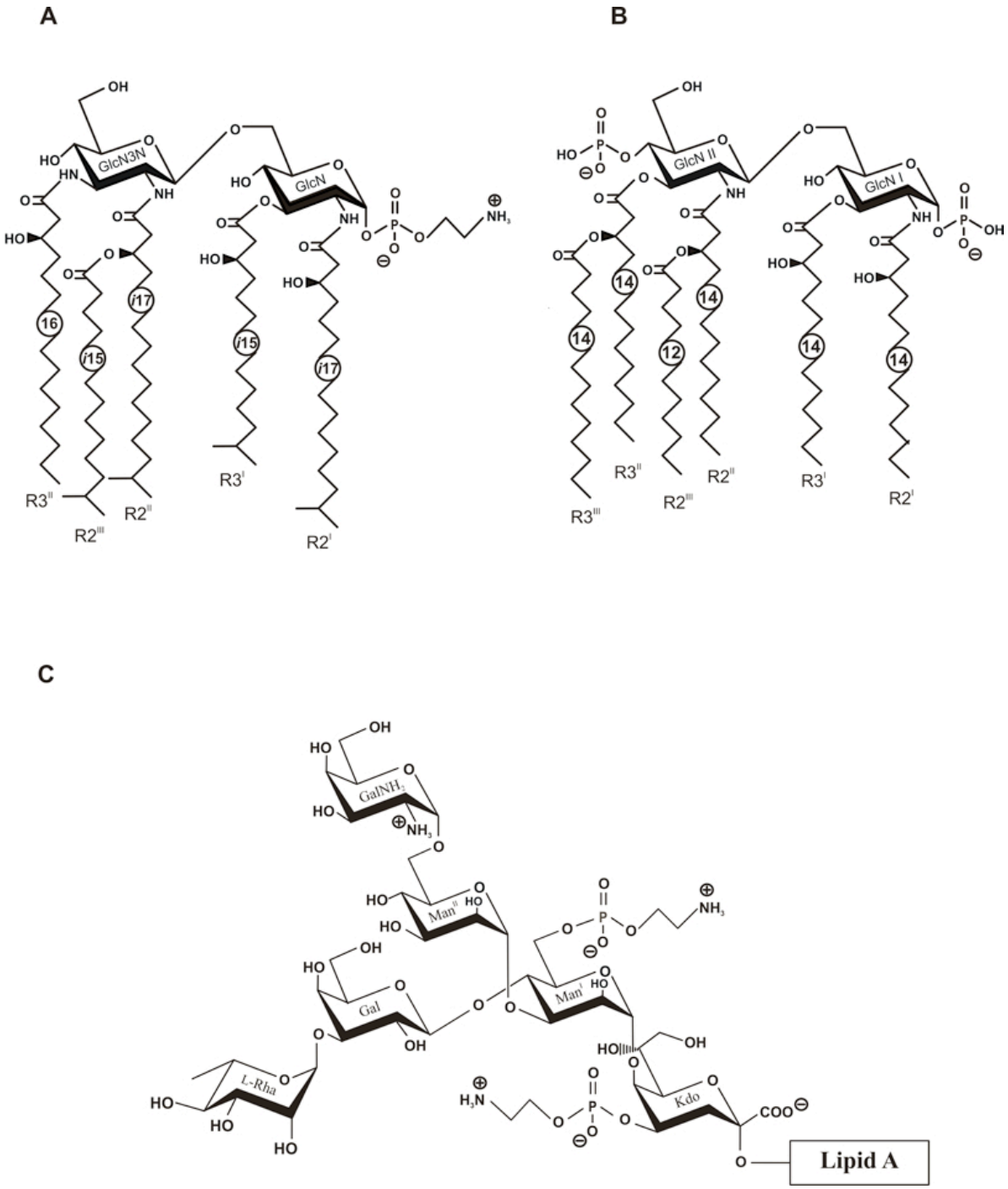


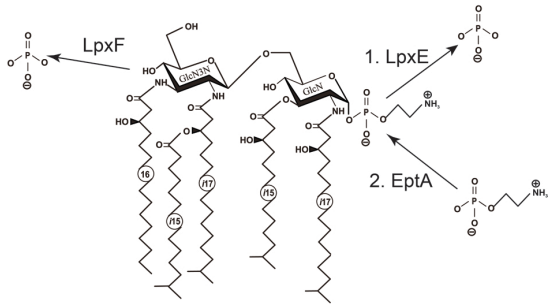

**Figure 2. Structures of *C. canimorsus* lipid A (A), *E. coli* lipid A (B) and core-oligosaccharide of *C. canimorsus* attached to the lipid A (C).** (A) *C. canimorsus* lipid A consists of a β-(1'→6)-linked GlcN3N'-GlcN disaccharide, to which 3-hydroxy-15-methylhexadecanoic acid, 3-hydroxy-13-methyltetradecanoic acid, 3-*O*-(13-methyltetradecanoyl)-15-methylhexadecanoic acid, and 3-hydroxyhexadecanoic acid

are attached at positions 2, 3, 2', and 3', respectively. The disscharide carries a positively charged ethanolamine at the 1 phosphate and lacks a 4' phosphate. (B) Structure of *E. coli* hexa-acylated lipid A. (C) *C. canimorsus* LPS core features only one Kdo, to which a phosphoethanolamine (*P*-Etn) is attached. The only net negative charge present is from the carboxy group of the Kdo. The inner core continues with Man to which another a *P*-Etn is attached. The outer core consists of Gal and L-Rhamnose (L-Rha), to which the O-antigen is attached (U. Zähringer, unpublished results).

**A**

| *Ccan Nr.* | Predicted function as |
|---|---|
| 16950 | eptA |
| 14390 | gnnA |
| 18700 | gnnB |
| 19830 | kdsA |
| 3250 | kdsB |
| 10150 | kdsC |
| 12170 | kdsD |
| 13790/13830 | lpxA/lpxA' |
| 9600 | lpxB |
| 13780 | lpxC |
| 13770 | lpxD |
| 16960 | lpxE |
| ? | lpxF |
| 3950 | lpxH |
| 17380 | lpxK |
| 6750 | lpxL |
| ND | lpxM |
| 630 | waaA |

**B**




**C**

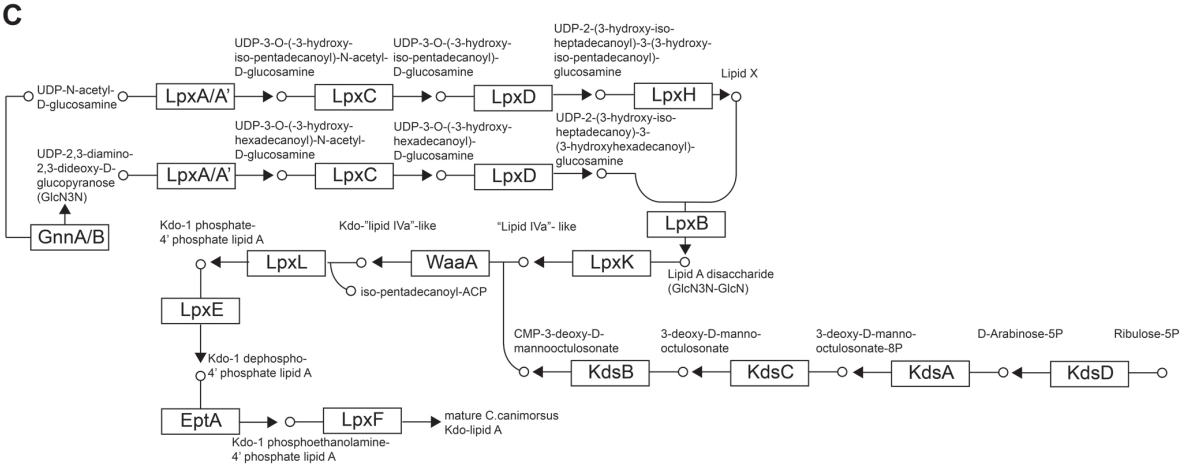



**Figure 3. Biosynthesis of *C. canimorsus* lipid A-Kdo.** (A) Alphabetic list of enzymes required and the corresponding gene codes in the *C. canimorsus* 5 genome are listed. (B) Proposed enzymatic modification on lipid A by LpxF, LpxE and EptA. (C) Single steps in the biosynthesis of *C. canimorsus* lipid A-Kdo (adapted from KEGG map00540).

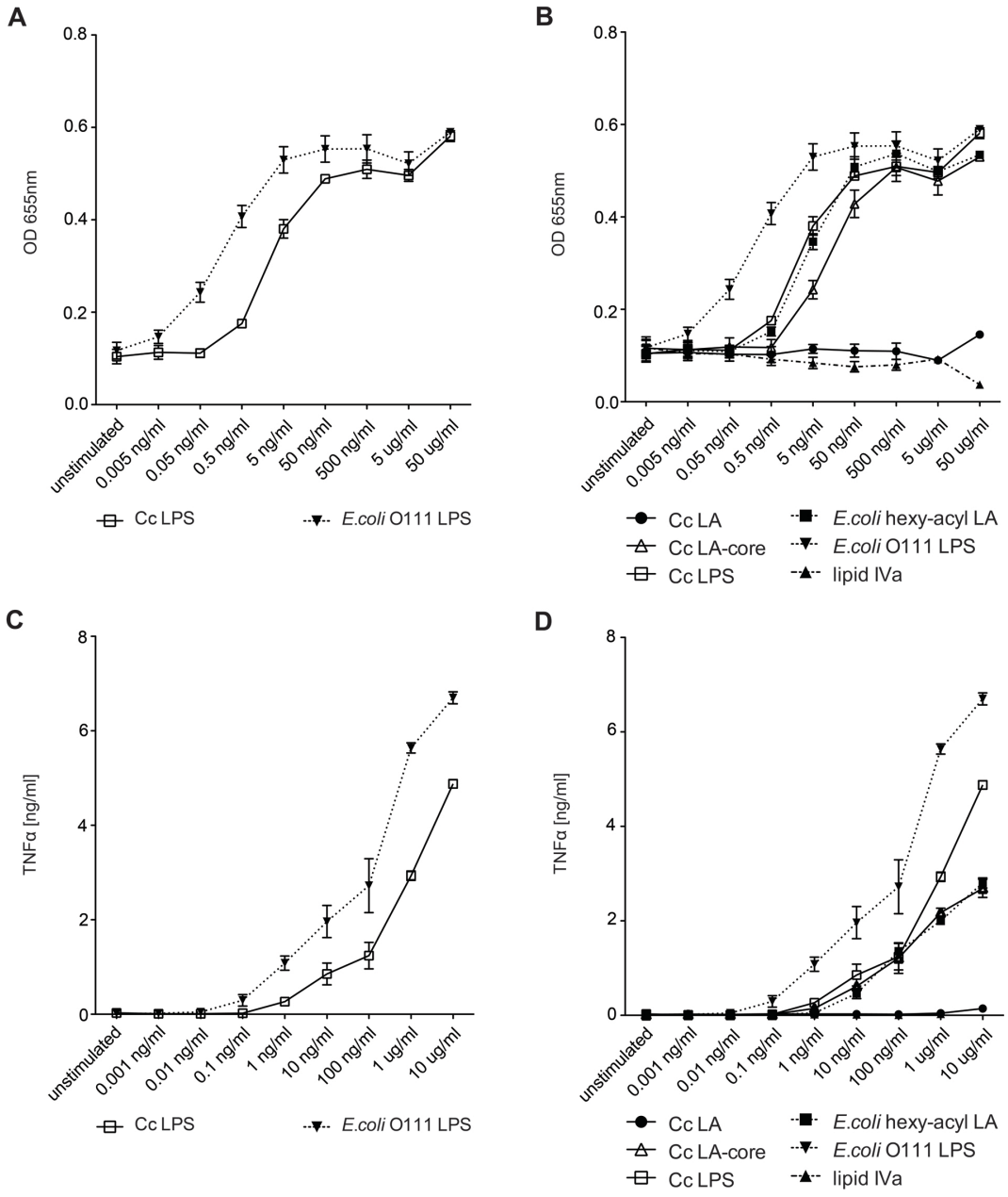



**Figure 4. Endotoxic activity of *C. canimorsus* (Cc) LPS, lipid A (LA) or lipid A-core (LA-core) and contribution of the LPS core to endotoxicity.** (A-B) Dose-response curve of purified lipid A, LA-core or LPS samples were assayed for TLR4 dependent NFκB activation with HEKBlue human TLR4 cells. (C-D) Purified lipid A, LA-core or LPS samples were assayed for induction of TNFα release by human THP-1 macrophages. Data were combined from n=3 independent experiments, error bars indicated are standard error of the mean.

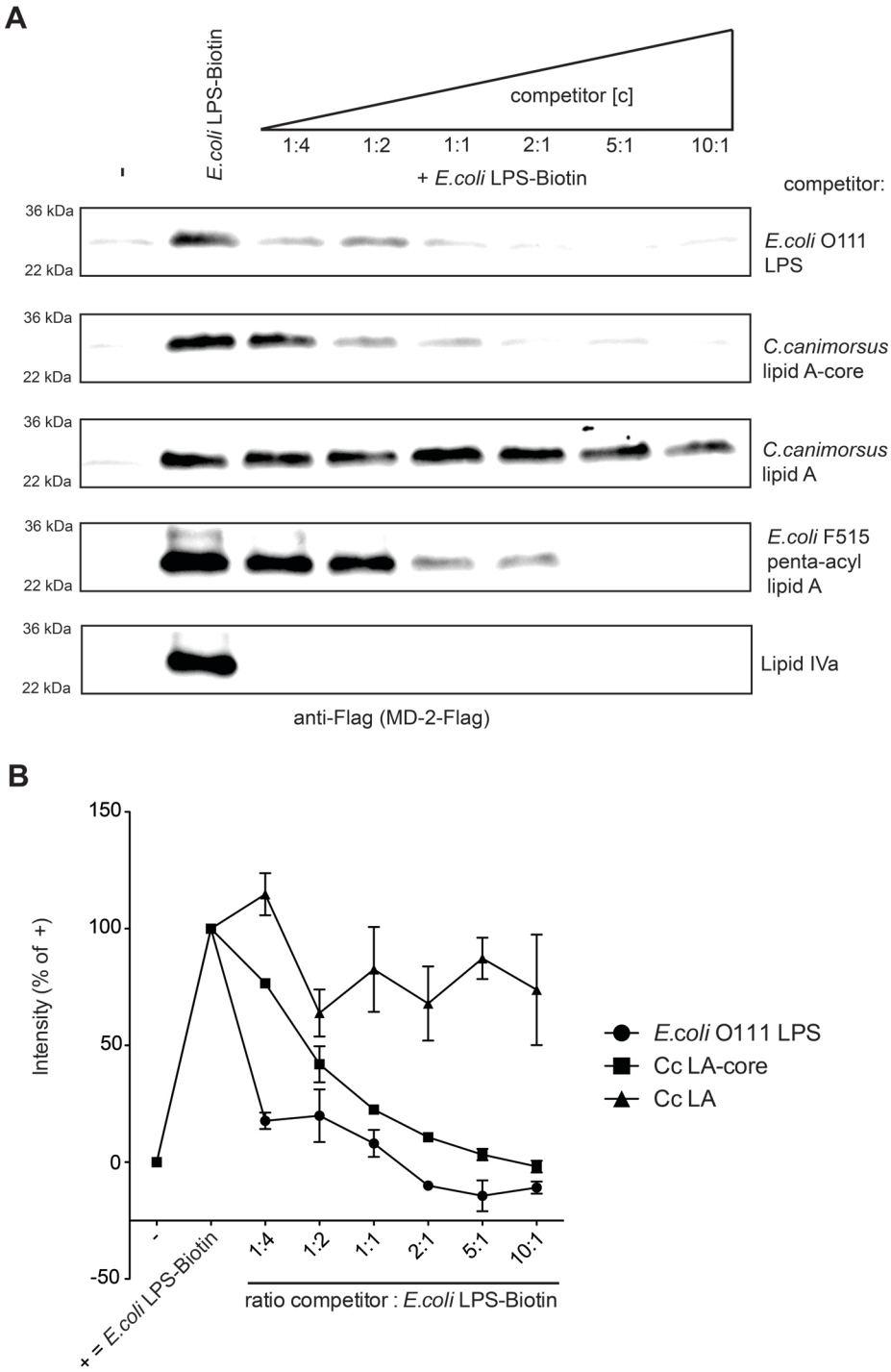
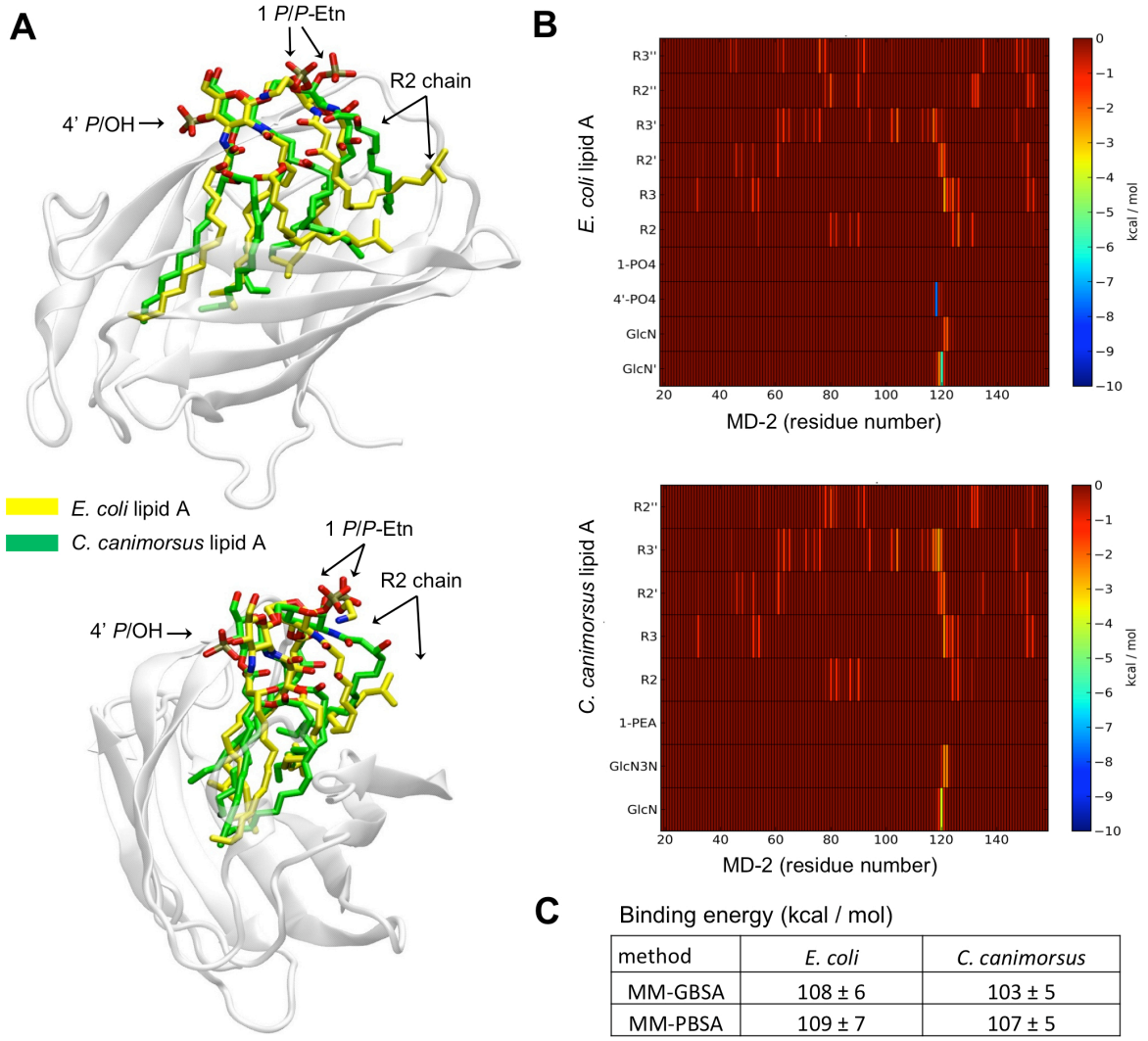



**Figure 5. Binding to human MD-2 of *C. canimorsus* lipid A depends on the core-oligosaccharide.** Soluble human MD-2 from cell culture supernatant was combined with the indicated mixture of *E. coli* LPS-Biotin and a competitor (either *C. canimorsus* lipid A, lipid A-core, *E. coli* O111 LPS, penta-acyl *E. coli* lipid A or lipid $IV_A$). Biotinylated

*E. coli* LPS-MD-2 complexes were purified and analysed by non-reducing, denaturing Western blotting for presence of MD-2. (A) Untreated human MD-2 did not bind to the Strep-column (lane 1), addition of *E. coli* LPS-biotin lead to co-purification of human MD-2 (lane 2). Results shown are representative of three independent determinations. (B) Quantification of Western-blots as shown in A. Values are shown as percentage of the corresponding positive control. Data were combined from n=3 independent experiments, error bars indicated are standard error of the mean.




**C**

Binding energy (kcal / mol)

| method | *E. coli* | *C. canimorsus* |
|---|---|---|
| MM-GBSA | 108 ± 6 | 103 ± 5 |
| MM-PBSA | 109 ± 7 | 107 ± 5 |

**Figure 6. Modeled binding of *C. canimorsus* lipid A to human MD-2.** (A) Front and side view of the equilibrated complexes between MD-2 (gray) and *C. canimorsus* (yellow) and *E. coli* (green) lipid A. (B) Pairwise decomposition of the global total (Van der Waals + electrostatic + solvation) binding free energy calculated at MM-GBSA level. (C) Binding energy between MD-2 and the two lipid A molecules calculated using the MM-GBSA and MM-PBSA methods on 300 snapshots extracted from two 10 ns long equilibrated NPT molecular dynamics simulations.

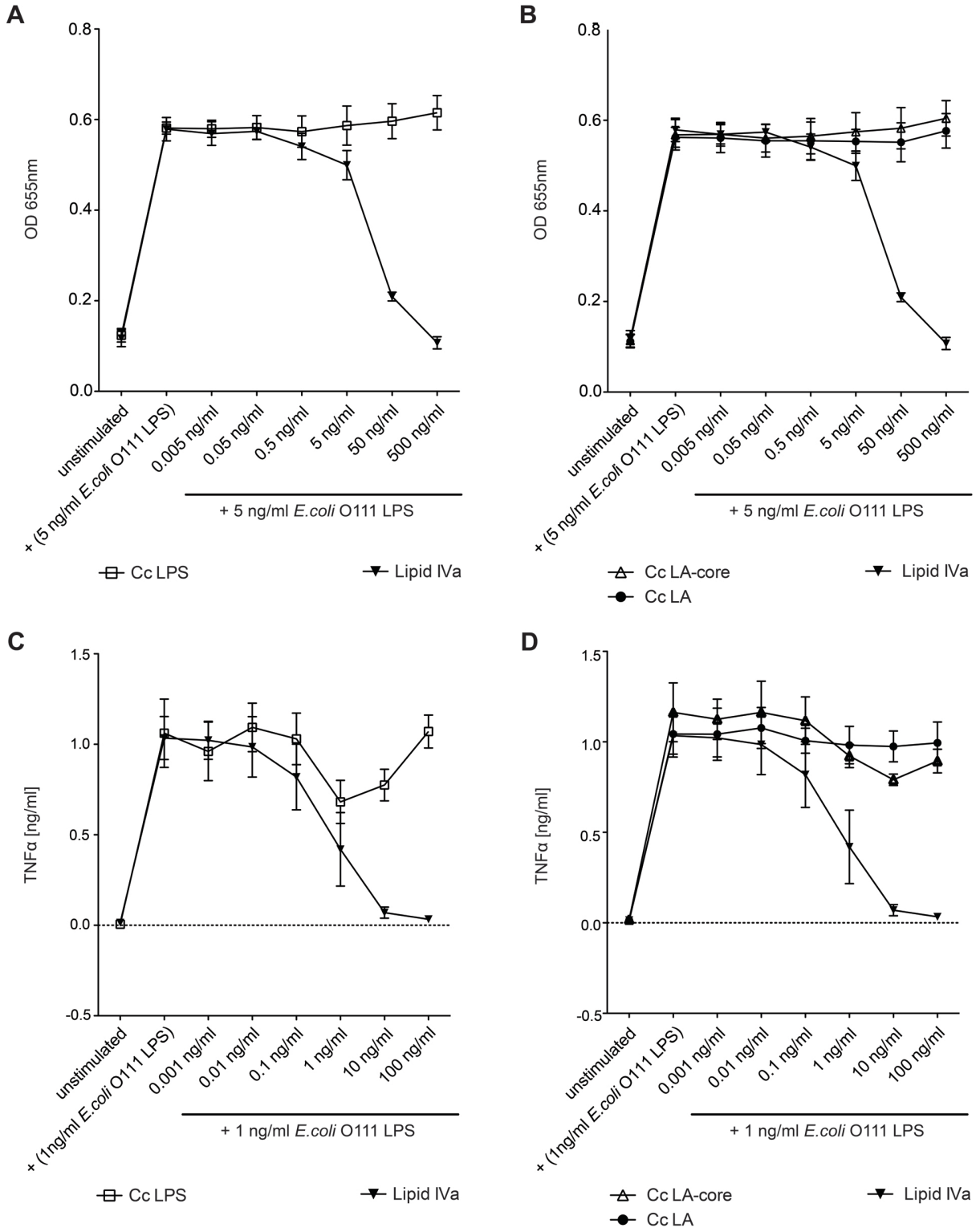



**Figure 7. Antagonistic activity of *C. canimorsus* (Cc) LPS, lipid A (LA) or LA-core on the action of *E. coli* O111 LPS.** (A-B) HEKBlue human TLR4 cells were preincubated for 3h with purified lipid A, LA-core or LPS samples at the concentration

indicated. Then the cells were stimulated with 5 ng/ml *E. coli* O111 LPS for further 20-24h and TLR4 dependent NFκB activation was measured. (C-D) Human THP-1 macrophages were preincubated for 3h with purified lipid A, LA-core or LPS samples at the concentration indicated. Then the cells were stimulated with 1ng/ml *E. coli* O111 LPS for further 20h and TNFα release was measured. Data were combined from n=3 independent experiments, error bars indicated are standard error of the mean.

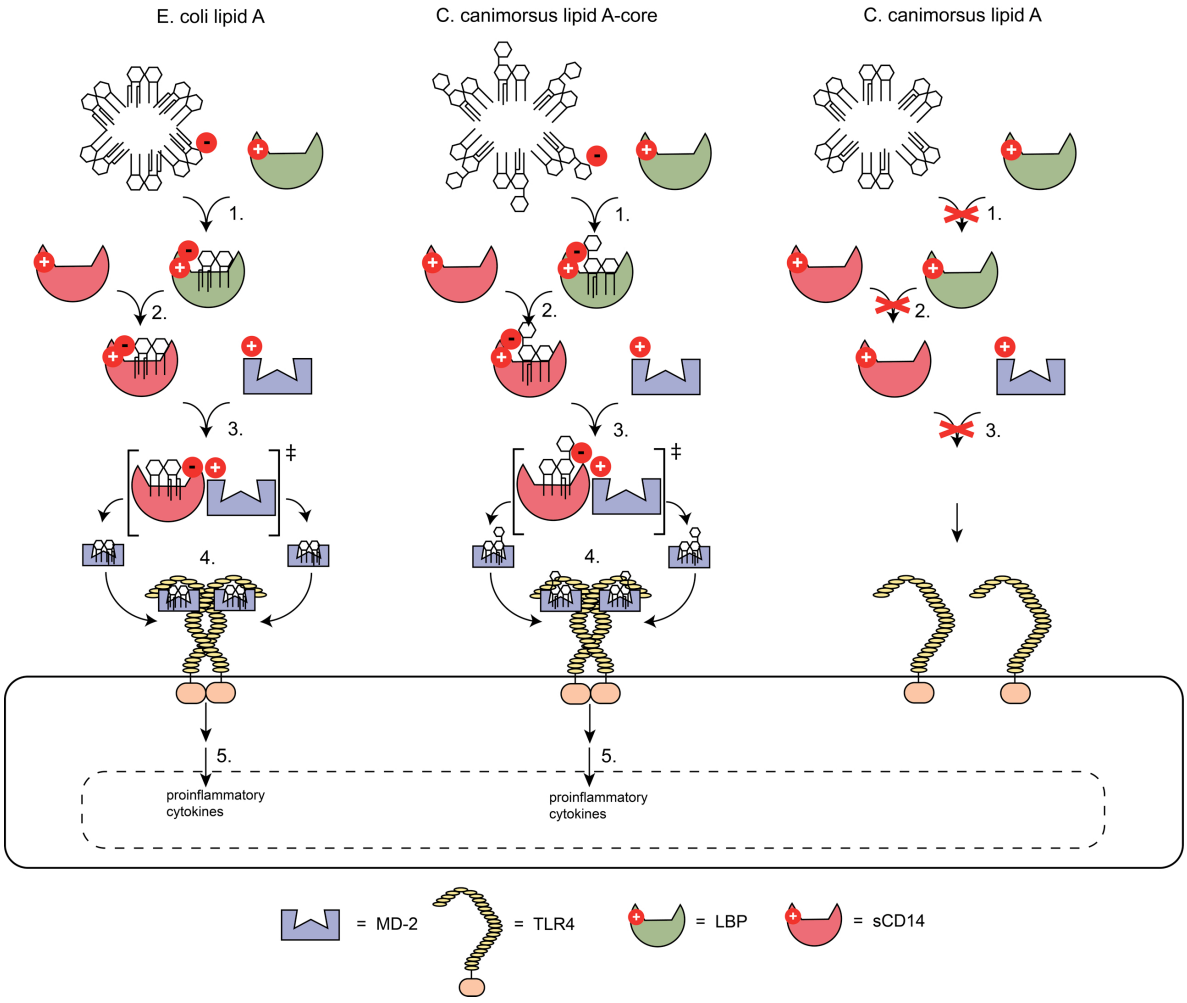



**Figure 8. Proposed model for the implication of the LPS core or the 4' phosphate in enabling the binding to MD-2.** Ionic interactions or hydrogen bonds involving the 4' phosphate or the Kdo carboxy group in LPS lacking a 4' phosphate enable the binding of lipid A to either LBP (1.), soluble CD14 (sCD14) (2.) or via an intermediate state to MD-2 (3.). Dependent on the type of lipid A bound to MD-2 this leads to TLR4 mutimerization (4.), a downstream signaling cascade and finally release of proinflammatory cytokines (5.).

## Tables

**Table I**. Compositional analysis data of the purified lipid A of *C. canimorsus* 5 wild type.

| Component | nmol/mg | mol/mol GlcN |
|---|---|---|
| **Sugars** | | |
| GlcN3N[a,c,*] | 167 | 0.5 |
| GlcN[a,c] | 358 | 1.0 |
| **Polar substituents** | | |
| *P*[b] | 468 | 1.3 |
| Etn-*P*[c] | ND | - |
| Etn[c] | ND | - |
| **Fatty acids**[a] | | |
| *i*15:0 | 278 | 0.8 |
| *i*15:0(3-OH) | 416 | 1.2 |
| 16:0(3-OH) | 417 | 1.2 |
| *i*17:0(3-OH) | 709 | 2.0 |

[a]GLC-MS data, [b]Photometric assay, [c]HPLC (Pico-tag).

* Per-*O*-acetylated GlcN3N-ol can only be quantified by GLC analysis by approx. 50% compared to GlcNAc-ol, as determined by synthetic reference compound.

**Table II.** ESI-MS analysis of lipid A fractions obtained by reversed phase HPLC shown in Figure S1.

| Peak No. | Retention time, min | Yield in mg (%) | Mol. mass of the major peak |
|---|---|---|---|
| Wild-type | | | |
| 1a | 118.5 | 0.13 (5.8) | 1660.235 |
| 1b | 119.7 | 0.18 (8.3) | 1674.265 |
| **2** | **124.3** | **0.66 (25.5)** | **1716.300** |
| 3 | 127.3 | 0.06 (2.8) | 1716.301 |
| **5** | **131.9** | **0.55 (24.2)** | **1594.292** |
| 6 | ~ 133 | 0.19 (8.8) | 1608.306 |
| 6’ | 134.6 | 0.09 (4) | 1589.266 |
| | | | 1594.290 |
| Applied: | 2.1 mg | | |
| Total yield: | 1.86 mg (88.6%) | | |

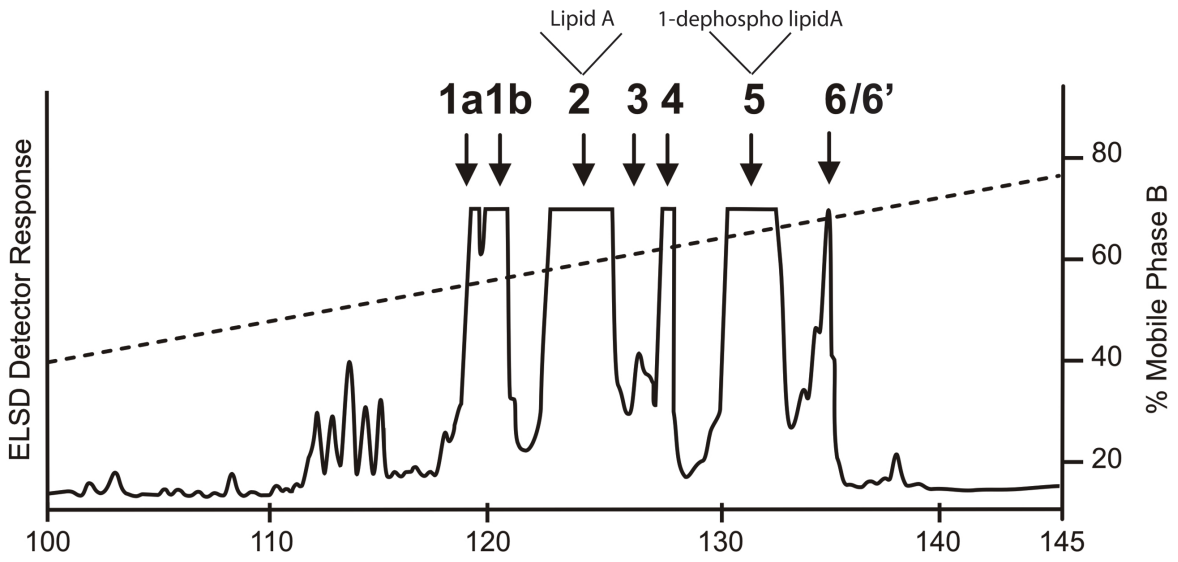
The major peaks shown in bold at *m/z* 1716.30 (peak 2) and *m/z* 1594.29 **(peak 5) represent the lipid A and the lipid A lacking the *P*-Etn group at C-1 of GlcN.**

## Supplemental materials

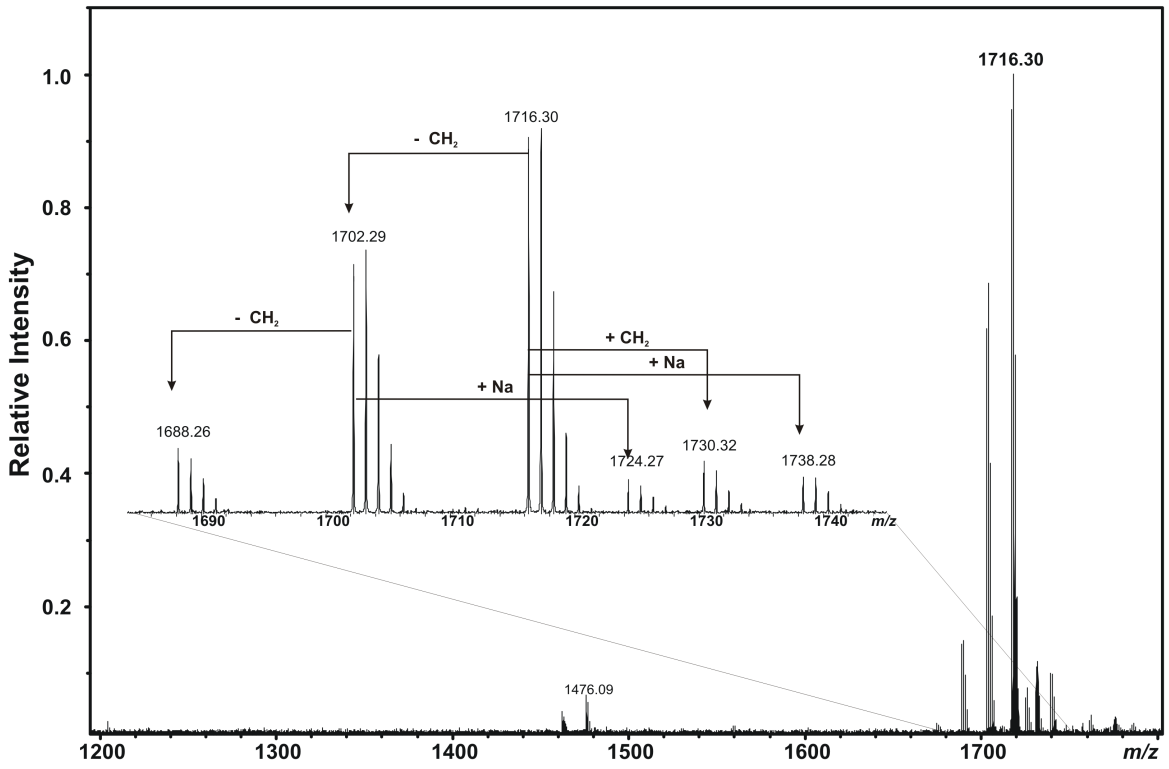
## Supplementary methods

**Human TLR2 activation assay.** HEK293 transfected with human TLR2 and CD14 were purchased from InvivoGen (HEKBlue$^{TM}$ hTLR2). Growth conditions and agonist assay were performed in accordance with recommendations of InvivoGen. Briefly, desired amount of the stimulus in a total volume of 20 µl (diluted in PBS) were added to a well of a flat-bottom 96-well plate (BD Falcon). 25000 HEKBlue$^{TM}$ hTLR2 cells in 180ul were added and the plate was incubated for 20-24h at 37°C and 5% $CO_2$. Detection followed the QUANTI-Blue$^{TM}$ protocol (InvivoGen). 20 µl of challenged cells were incubated with 180 µl detection reagent (QUANTI-Blue$^{TM}$, InvivoGen). Plates were incubated at 37°C and 5% $CO_2$ and developed color was measured using a spectrophotometer (BioRad) at 655nm. $Pam_3CSK_4$ (InvivoGen) was used as positive control.

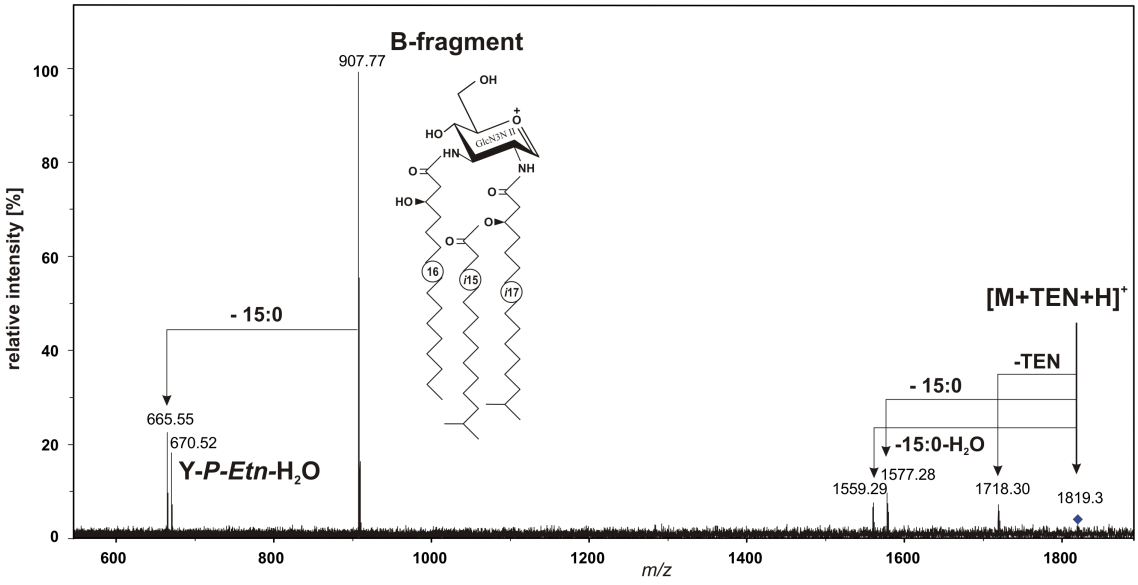
**Supplementary Figures**



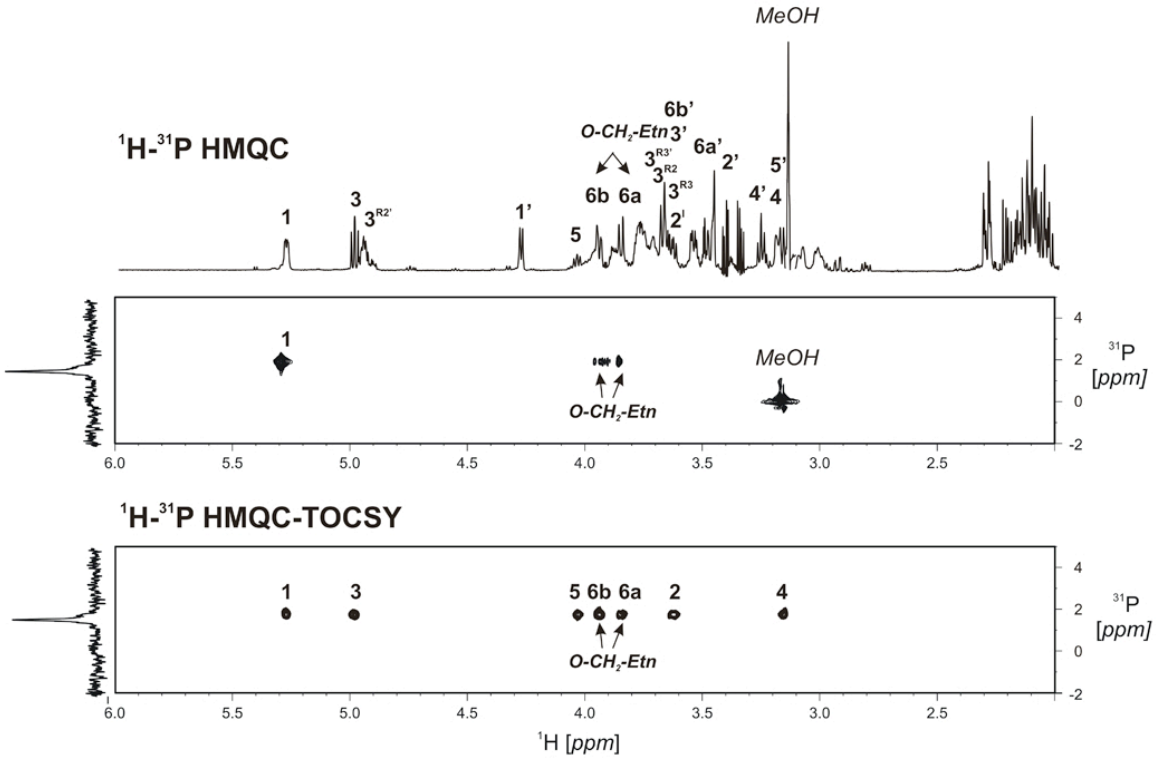

**Supplementary Figure 1.** HPLC elution profile of the lipid A from *C. canimorsus* 5. HPLC elution profiles of the semi-preparative fractionation of the lipid A from wild type *C. canimorsus* 5 (2.1 mg). Peak Nr. 2 (124.3 min) represents the intact lipid A and peak Nr. 5 (131.9 min) the 1-dephosphorylated lipid A (LA without *P*-Etn) as determined by ESI MS. The other peaks belong to lipid A with slightly modified fatty acids composition (Table II). For HPLC conditions see Materials and Methods.



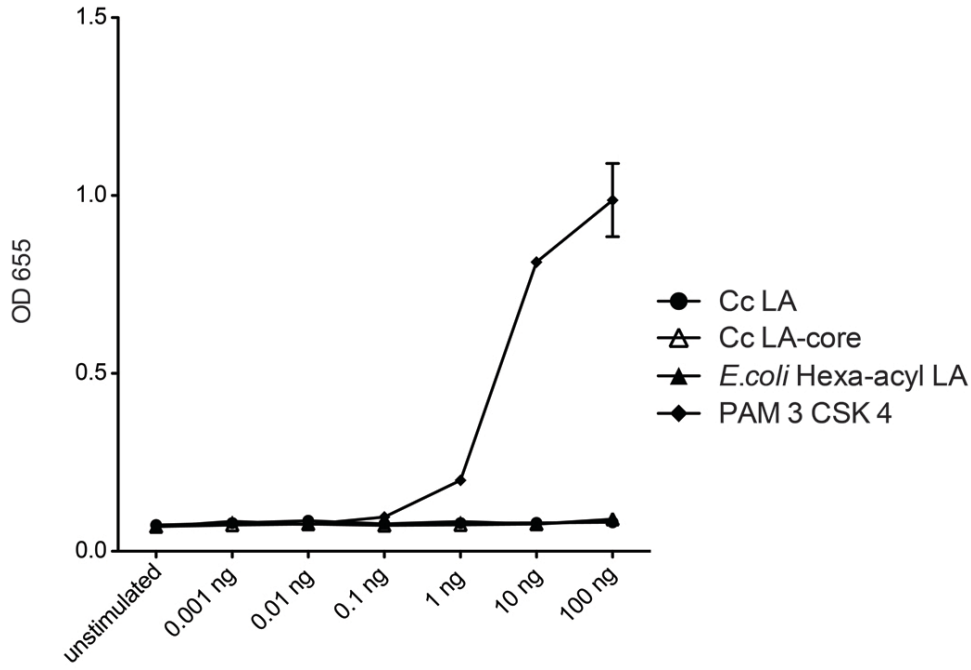

**Supplementary Figure 2**. Negative mode ESI mass spectrum of lipid A from *C. canimorsus* indicating heterogeneity in the fatty acids chain length (-$CH_2$-, Δ*m/z* = 14 u) as also shown in table II.




**Supplementary Figure 3.** CID-MS/MS (positive mode) of lipid A from *C. canimorsus* showing the B-fragment (non-reducing end) obtained from the parent ion $[M+TEN+H]^+$ [*m/z* 1819.3]. The abundant B-fragment ion is consistent with a GlcN3N carrying two primary fatty acids [16:0(3-OH) and *i*17:0(3-OH)] in amide linkage and one (*i*15:0) in ester linkage forming an acyloxyacyl residue [*i*17:0(3-*O*(*i*15:0)] and proves the hybrid backbone (GlcN3N'-GlcN) to be the major one (>95%) and the distribution of the fatty acids to be 3+2.




**Supplementary Figure 4.** $^1$H,$^{31}$P-HMQC (top) and $^1$H,$^{31}$P-HMQC-TOCSY (bottom) spectra (700 MHz) of lipid A in chloroform-methanol-water (20:10:1, v/v/v) at 27°C. The $^{31}$P NMR spectrum and the corresponding part of the $^1$H NMR spectrum are displayed along the F1 and F2 axes, respectively. Numerals refer to atoms in sugar and fatty acid residues denoted by letters as shown in Supplementary table I and Figure S2.




**Supplementary Figure 5.** Activation of human TLR2 with *C. canimorsus* (Cc) or *E. coli* lipid A (LA) or LA-core preparations. Indicated concentrations of purified lipid A or LA-core samples were assayed for TLR2 dependent NFκB activation with HEKBlue human TLR2 cells. The triacylated lipopeptide $Pam_3CSK_4$ was used a positive control. Data were combined from n=3 independent experiments, error bars indicated are standard error of the mean.

**Supplementary Table I.** $^{1}H$ (700 MHz) and $^{13}C$ (176.2 MHz) NMR data of the lipid A from *C. canimorsus* ($CDCl_3$/MeOD/$D_2O$, 40:10:1, v/v/v). Chemical shifts are referenced to internal $CDCl_3$ ($d_H$ 7.26, $d_C$ 77.0) at 27°C. For the assignment of the individual fatty acids (a-d, c') see Figure S4.

| $^{1}H$ | δ, ppm | J, Hz | $^{3}C$ | δ, ppm | $J_{C,P}$, Hz |
|---|---|---|---|---|---|
| **GlcN3N** | | | | | |
| H-1 | 4.28 | $J_{1,2}$ 8.0 | C-1 | 103.4 | |
| H-2 | 3.49 | * | C-2 | 52.9 | |
| H-3 | 3.68 | * | C-3 | 54.6 | |
| H-4 | 3.17 | * | C-4 | 68.3 | |
| H-5 | 3.20 | * | C-5 | 76.9 | |
| H-6a | 3.55 | * | C-6 | 60.0 | |
| H-6b | 3.68 | * | | | |
| **GlcN** | | | | | |
| H-1 | 5.29 | $J_{1,2}$ 2.9, $^{2}J_{1,P}$ 7.9 | C-1 | 92.8 | $^{2}J_{C-1,P}$ 4.7 |
| H-2 | 3.96 | $J_{2,3}$ 9.5 | C-2 | 51.4 | $^{3}J_{C-2,P}$ 7.2 |
| H-3 | 4.99 | $J_{3,4}$ 9.7 | C-3 | 72.7 | |
| H-4 | 3.27 | $J_{4,5}$ 9.5 | C-4 | 67.4 | |
| H-5 | 4.05 | * | C-5 | 72.6 | |
| H-6a | 3.64 | * | C-6 | 71.0 | |
| H-6b | 3.87 | * | | | |
| ***P*-Etn** | | | | | |
| H-1a,1b | 3.91, 3.98 | | C-1 | 61.7 | $^{2}J_{C-1,P}$ 4.3 |
| H-2a,2b | 3.04, 3.10 | | C-2 | 39.9 | $^{3}J_{C-2,P}$ 6.9 |
| **Fatty acids** | | | | | |
| **(a) R2, *i*17:0(3-OH)** | | | | | |
| | | | C-1 | 173.4[&] | |
| H-2a | 2.24 | | C-2 | 41.6 | |
| H-2b | 2.30 | | C-3 | 68.1[#] | |
| H-3 | 3.74 | | C-4 | 36.8 | |
| H-4a | 1.23 | | C-5 | 25.1 | |
| H-4b | 1.29 | | | | |
| H-5a,b | 1.02...1.13 | | C-6…C13 | 28.7...29.2 | |
| H-6 ... H-14 | 0.95 | | C-14 | 38.55 | |
| H-15 | 1.08 | | C-15 | 26.8 | |
| H-15Me | 0.68 | | C-15Me | 22.0 | |
| H-16 | 0.68 | | C-16 | 13.3 | |
| **(b) R3, *i*15:0(3-OH)** | | | | | |
| H-2a | 2.16 | | C-1 | 173.9[&] | |

| | | | |
|---|---|---|---|
| H-2b | 2.31 | C-2 | 40.8 |
| H-3 | 3.78 | C-3 | 67.9[#] |
| H-4a | 1.22 | C-4 | 36.6 |
| H-4b | 1.29 | C-5 | 25.1 |
| H-5a,b | 1.23 | | |
| H-6 ....H11 | 1.02...1.13 | C-6…C11 | 28.7...29.2 |
| H-12 | 0.95 | C-12 | 38.6 |
| H-13 | 1.08 | C-13 | 26.8 |
| H-13Me | 0.68 | C-13Me | 22.0 |
| H-14 | 0.68 | C-14 | 22.2 |

**(c) R2', *i*17:0(3-OH)**

| | | | |
|---|---|---|---|
| | | C-1 | 171.7[&] |
| H-2a | 2.13 | C-2 | 43.4 |
| H-2b | 2.06 | C-3 | 70.7 |
| H-3 | 4.95 | C-4 | 36.6 |
| H-4a | 1.22 | C-5 | 25.1 |
| H-4b | 1.29 | | |
| H-5a,b | 1.23 | | |
| H-6 ....H13 | 1.02...1.13 | C-6…C13 | 28.7...29.2 |
| H-14 | 0.95 | C-14 | 38.55 |
| H-15 | 1.08 | C-15 | 26.8 |
| H-15Me | 0.68 | C-15Me | 22.0 |
| H-16 | 0.68 | C-16 | 22.0 |

**(c') R2'', 15:0**

| | | | |
|---|---|---|---|
| | | C-1 | 174.0 |
| H-2a,b | 2.12 | C-2 | 34.0 |
| H-3a,b | 1.43 | C-3 | 31.4 |
| H-4[a],b | 1.06 | C-4 | 27.0 |
| H-5...11 | 1.012...1.131 | C-5...C-11 | 28.7...29.2 |
| H-12[a],b | 0.95 | C-12 | 38.6 |
| H-13[a],b | 1.08 | C-13 | 26.8 |
| H-13Me | 0.68 | C-13Me | 22.0 |
| H-14 | 0.68 | C-14 | 22.0 |

**(d) R3', 16:0(3-OH)**

| | | | |
|---|---|---|---|
| | | C-1 | 172.5[&] |
| H-2a | 2.13 | C-2 | 43.4 |
| H-2b | 2.06 | C-3 | 67.6 |
| H-3a,b | 3.80 | C-4 | 36.6 |
| H-4a,b | 1.22 | C-5 | 25.1 |
| H-5a,b | 1.23 | | |
| H-6 ....H13 | 1.02...1.13 | C-6…C13 | 28.7...29.2 |
| H-14a,b | 0.95 | C-14 | 38.6 |
| H-15a,b | 1.09 | C-15 | 22.1 |
| H-16 | 0.68 | C-16 | 13.3 |

[*]Non-resolved multiplet.
[&, #] Assignement is interchangeable.

**Supplementary Table II. Bacterial strains used in this study**

| **Bacterial strains** | **Description or genotype** | **Reference or source** |
|---|---|---|
| ***E. coli*** | | |
| F515 | Deep rough mutant | [49] |
| Top10 | F- *mcrA* Δ*(mrr-hsd*RMS-*mcr*BC*)* *φ80lac*ZΔM*15* Δ*lac*X74 *rec*A1 *ara*D139 Δ*(araleu)*7697 *gal*U *gal*K *rps*L, *end*A1 *nup*G. $Sm^r$ | Invitrogen |
| ***C. canimorsus*** | | |
| *Cc5* | Human fatal septicemia after dog bite 1995 | [9], [5] |

## 2.2 Manuscript in preparation: Detoxification of lipid A by *Capnocytophaga canimorsus*

**Author contributions:** SI, GC and UZ conceived and designed the experiments. SI performed the experiments. SI analyzed the data. SI and GC wrote the paper.

**Statement of my work:** My contribution was the data of figures 2, 3 and 4.

# Detoxification of lipid A by *Capnocytophaga canimorsus*

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Running head: *C. canimorsus* lipid A modification

## Introduction

Gram-negative bacteria have evolved different strategies to modify the lipid A structure in order to reduce recognition by the host and sensitivity to cationic antimicrobial peptides (CAMPs) (Coats et al., 2009; Dixon and Darveau, 2005; Hajjar et al., 2002; Mata-Haro et al., 2007; Price et al., 1995; Wang et al., 2004; Wang et al., 2006). One of these consists in the modification of the 1 or 4' phosphates of lipid A (Coats et al., 2009; Curtis et al., 2011; Herrera et al., 2010; Ingram et al., 2010b; Mata-Haro et al., 2007; Wang et al., 2006). 4' phosphatases (LpxF) have been described in *Rhizobium leguminosarum*, *Rhizobium etli*, *Porphyromonas gingivalis, Francisella* species and *Helicobacter pylori* (Coats et al., 2009; Cullen et al., 2011; Ingram et al., 2010b; Wang et al., 2007). Deletion of *lpxF* and the resulting presence of the 4' phosphate on lipid A leads to increased endotoxicity (Coats et al., 2009; Cullen et al., 2011), but decreased resistance to CAMPs (Cullen et al., 2011; Ingram et al., 2010b). In the case of *Francisella* and *H. pylori* virulence is reduced (Cullen et al., 2011; Kanistanon et al., 2011; Wang et al., 2007). 1 phosphatases (LpxE) have been identified in *H. pylori*, *P. gingivalis*, *R. etli* and others (Coats et al., 2009; Cullen et al., 2011; Ingram et al., 2010a; Ingram et al., 2010b; Tran et al., 2004; Tran et al., 2006; Wang et al., 2004). Deletion of *lpxE* and the resulting presence of the 1 phosphate on lipid A leads to a slightly increased endotoxicity (Coats et al., 2009) and CAMP sensitivity (Ingram et al., 2010b). After dephosphorylation, the 1 position can be further modified. *H. pylori* is known to add a phosphoethanolamine (*P*-Etn) to the 1 position of lipid A (Cox et al., 2003; Kim et al., 2006; Tran et al., 2004). This happens via a two-step mechanism, which first involves 1 dephosphorylation by LpxE and subsequent *P*-Etn transfer by a phosphoethanolamine transferase (EptA) (Tran et al., 2004; Tran et al., 2006).

We have previously identified the lipid A structure of *C. canimorsus* (Ittig et al., submitted) and found a *P-Etn* group attached to the 1 end of lipid A and the lack of a 4' phosphate (Fig. 1 A). *C. canimorsus* lipid A consists of a β-(1'→6)-linked GlcN3N'-GlcN disaccharide, to which 3-hydroxy-15-methylhexadecanoic acid, 3-hydroxy-13-methyltetradecanoic acid, 3-O-(13-methyltetradecanoyl)-15-methylhexadecanoic acid, and 3-hydroxyhexadecanoic acid are attached at positions 2, 3, 2', and 3', respectively. This is in contrast to the potent TLR4 agonist, the *E. coli* lipid A consisting of a β-(1'→6)-linked GlcN disaccharide that is phosphorylated at positions 1 and 4' and carries four (R)-3-hydroxymyristate chains (at positions 2', 3', 2 and 3). The 2' and 3' 3-hydroxylated acyl groups in GlcN' are further esterified with laurate and myristate, respectively (Raetz, 1990).

In agreement with *H. pylori* we have identified *lpxE* and *eptA* in the genome of *C. canimorsus* 5 and found the overlapping genes to be organized in one operon. We show here that the deletion of *lpxE* or *eptA* leads to increased endotoxicity and decreased resistance to CAMPs, 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 found similar to 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.

## Results

**Identification of enzymes leading to the 1 *P*-Etn on lipid A**. We have investigated the enzymes leading to the addition of an ethanolamine (Etn) at the 1 Phosphate (*P*) of lipid A. In *H. pylori*, the addition of a *P*-Etn at the 1 position has been proposed to result from a two-step mechanism (Tran et al., 2006). In a first step the 1 phosphate is removed by a phosphatase (LpxE), and subsequently a *P*-Etn-transferase (EptA or PmrC, YjdB) adds a *P*-Etn to the 1 position of lipid A (Tran et al., 2006). In *H. pylori lpxE* and *eptA* are encoded by one operon (Hp0021-Hp0022). In the genome of *C. canimorsus*, we identified *Ccan*16950 as *eptA*. Search for a lipid A phosphatase were based on *lpxE* and/or *lpxF* sequences from *P. gingivalis (Coats et al., 2009)*, *F. novicida* (Wang et al., 2007), *Rhizobium etli* (Ingram et al., 2010b) *H. pylori* (Cullen et al., 2011; Tran et al., 2006) and on all available *Bacteroidetes*-group *pgpB* sequences. Three *lpxE/F* candidates have been found in the *C. canimorsus 5* genome (*Ccan*16960, *Ccan*14540 and *Ccan*6070). All candidates have been deleted and only deletion of *Ccan* 16960 affected endotoxicity (data not shown). Interestingly, *Ccan16960* forms an operon with *eptA* and the two genes overlap by 20 base pairs (bp). Following the operon organisation of *H. pylori*, *Ccan*16960 has been annotated as *lpxE*. This annotation has been validated by mutagenesis and impact on endotoxicity and CAMP resistance. The *C. canimorsus* lpxE-eptA operon formed by *Ccan*16950 and *Ccan*16960 is depicted in Figure 2.

**LpxE and EptA impact endotoxicity.** To study the impact on endotoxicity of the removal of the 1 *P* or the addition of an *P*-Etn to the free 1 position of lipid A, we engineered Δ*eptA* and Δ*lpxE* mutations and we monitored endotoxicity using a HEK293

cell line overexpressing human TLR4/MD-2/CD14 and a secreted reporter protein (HEKBlue human TLR4 cell line). Activation of this cell line greatly 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 ΔlpxE-eptA* exhibited identical endotoxicity as *C. canimorsus* Δ*lpxE* (Fig. 3 A), again suggesting a two-step enzymatic mechanism in which EptA adds the *P*-Etn only after the removal of the lipid A 1 phosphate by LpxE (Tran et al., 2004; Tran et al., 2006).

**Δ*eptA* and Δ*lpxE* mutations are non-polar**

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). Δ*lpxE* could be complemented with *lpxE* or *lpxE-eptA* in trans, but not with *eptA* alone (Fig. 3 B). Δ*eptA* was complemented with *eptA* or *lpxE-eptA* in trans (Fig. 3 C), and *ΔlpxF-eptA* was complemented with *lpxE-eptA* in trans (Fig. 3 D).

**LpxE and EptA affect resistance to Polymyxin B.**

Lipid A modifications have been shown not only to affect endotoxicity, but also to alter resistance to CAMPs such as Polymyxin B (Ingram et al., 2010b; Lee et al., 2004; Raetz, 1990; Raetz et al., 2007). Hence, we monitored the minimum inhibitory concentration of Polymyxin B for *C. canimorsus* 5, Δ*lpxE*, Δ*eptA* and the double-knockout Δ*lpxE-eptA*. *C. canimorsus* 5 wt was highly resistant to Polymyxin B, as it was still able to grow in the presence of 512 mg/L Polymyxin B (MIC of 1024 mg/L) (Fig. 4). The MIC decreased to 512 mg/L for Δ*eptA* mutant bacteria and to 128 mg/L for *C.*

*canimorsus* Δ*lpxE* bacteria, showing an increased sensitivity to Polymyxin B (Fig. 4). The difference between the two mutants can be explained by the fact that EptA simultaneously adds a negative and a positive charge, whereas LpxE only removes a negative charge. Therefore the Δ*lpxE* mutation leads to a higher increase in negative charge interacting with Polymyxin B. The *C. canimorsus* Δ*lpxE-eptA* double-KO had the same MIC as the single Δ*lpxE* mutant (Fig. 4). This is in agreement with the proposed two-step enzymatic mechanism of LpxE and EptA, in which EptA can add the P-Etn only after the removal of the lipid A 1 phosphate (Tran et al., 2004; Tran et al., 2006).

**Discussion**

The *P*-Etn modification at position 1 was shown here to be important for low endotoxicity and Polymyxin B resistance of *C. canimorsus*, as has been shown in *H. pylori* (Cullen et al., 2011). In *C. canimorsus*, as in *H. pylori*, the enzymes responsible for the *P*-Etn modification, LpxE and EptA, are encoded by one operon. The identical phenotype in endotoxicity and Polymyxin B sensitivity of Δ*lpxE* and Δ*lpxF-eptA* suggests that the *P*-Etn containing lipid A is synthesized by a similar two-step enzymatic process as in *H. pylori* (Tran et al., 2004; Tran et al., 2006). In *H. pylori*, lipid A also carries a *P*-Etn group at position 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, where dephosphorylation is necessary for substitution of 1 phosphate with *P*-Etn (Cullen et al., 2011; Tran et al., 2004; Tran et al., 2006).

We found in both assays, endotoxicity and Polymyxin B sensitivity, that Δ*lpxE* had a more severe effect than Δ*eptA*. 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 proposed process, the Δ*lpxE* mutation leads to an increase of a negative charge (the phosphate) compared to the wt, while Δ*eptA* would result in a free hydroxy-group at the 1 end of lipid A as compared to the *P*-Etn in the wt. As net negative charges are important for interaction with CAMPs as well as TLR4/MD-2, 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 and that the annotation of *lpxE* and *eptA* in *C. canimorsus* 5 is correct.

It is noteworthy that one could expect the mutation of *eptA* not to affect charge dependent mechanisms, as no net charge change is expected. Still Δ*eptA* showed a small effect on Polymyxin B sensitivity and a more pronounced effect on endotoxicity. This suggests that the positive charge on the Etn might not only shield the negative charge of the phosphate, but that there may exist some repulsion between the positive charges on CAMPs and the positively charged residues on TLR4/MD-2 exists.

The genetic association of *lpxE* and *eptA* genes suggests that this modification of lipid A is essential for survival in the dog's mouth environment, the habitat of *C. canimorsus*. This lipid A modification might as well favour human infections.

## 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 (Shin et al., 2007) 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 resuspended in PBS. Selective agents were added at the following concentrations: erythromycin, 10 mg/ml; cefoxitin, 10 mg/ml; gentamicin, 20 mg/ml; ampicillin, 100 mg/ml.

**Genetic manipulations of *C. canimorsus*.** Genetic manipulations of *Cc5* wt has been described in ref (Mally and Cornelis, 2008). Briefly, replacement cassettes with flanking regions spanning approximately 500 bp homologous to direct *lpxE or eptA* framing regions were constructed with a three-fragment overlapping-PCR strategy. As the ATG of *eptA* is within the coding region of *lpxE*, around 100bp upstream of the *eptA* ATG were not deleted in *lpxE* single KO. 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* insertion cassette. The *ermF* resistance cassette was amplified from plasmid pMM13 DNA 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 consisted in *lpxE::ermF* or *eptA::ermF* insertion cassettes respectively and were then digested with *Pst*I and *Spe*I for cloning into the appropriate sites of the *C. canimorsus* suicide vector pMM25 (Mally and Cornelis, 2008). Resulting plasmids were transferred by RP4-mediated conjugative DNA transfer from *E. coli* S17-1 to *C. canimorsus 5* to allow integration of the insertion cassette. Transconjugants were then selected for presence of the *ermF* cassette 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 (Mally and Cornelis, 2008). Full length *lpxF, eptA* or *lpxF-eptA* 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, MD-2, CD14 and a secreted NFκB dependent reporter were purchased from InvivoGen (HEKBlue$^{TM}$ hTLR4). Growth conditions and endotoxicity assay were as recommended by InvivoGen. Briefly, desired amounts of LPS or lipid A 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$^{TM}$ hTLR4 cells in 180 µl were then added and the plate was incubated for 20-24h at 37°C and 5% $CO_2$. If the antagonistic activity of a compound on the action of *E. coli* O111 LPS was assayed, the compound was added in a total volume of 10 µl (diluted in PBS), 25000 HEKBlue$^{TM}$ hTLR4 cells in 180 µl were added and the plate was incubated for 3h at 37°C and 5% $CO_2$. Then the cells were stimulated with 5 ng/ml *E.*

*coli* O111 LPS and the plate was incubated as above. Detection 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 colour developed was measured at 655nm using a spectrophotometer (BioRad).

**Polymyxin B sensitivity assay.** Polymyxin B sulphate was obtained from Sigma-Aldrich. The agar dilution method was performed based on the CLSI/NCCLS recommendations (Ferraro and NCCLS/CLSI, 2003). 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 condition). Plates were incubated and examined for growth of visible colonies after 48h and 72h.

**Genome annotation.** *Blast*-p search tool (Altschul et al., 1997) against the *C. canimorsus* 5 genome (Manfredi et al., 2011) was used. Search sequences were obtained from the National Center for Biotechnology Information. All available *Bacteroidetes*-group sequences were used as search if available, but 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* sequences from *P. gingivalis (Coats et al., 2009)*, *F. novicida* (Wang et al., 2006), *R. etli* (Ingram et al., 2010b), *H. pylori* (Cullen et al., 2011; Tran et al., 2006) and on all available *Bacteroidetes*-group *pgpB* sequences.

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## Tables

### Table 1. Bacterial strains used in this study

| | | |
|---|---|---|
| *Cc5* | Human fatal septicemia after dog bite 1995 | (Shin et al., 2007) |
| *Cc5 ΔlpxE* | Replacement of *Ccan_16960* by *ermF;* $Em^r$ (primer 6493-6498) | This study |
| *Cc5 ΔeptA* | Replacement of *Ccan_16950* by *ermF*; $Em^r$ (primer 6499-6504) | This study |
| *Cc5 ΔlpxE-eptA* | Replacement of *Ccan_16960-16950* by *ermF;* $Em^r$ (primer 6493-6495 and 6502-6504) | This study |

### Table 2. Oligonucleotides used in this study

| Ref. | Name | Sequence 5'-3' | Restriction | 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 | CCCTGCAGTGTTCCTCGCCCTGT | *Pst*I | *eptA* | A |

| | | | | | |
|---|---|---|---|---|---|
| | | TAC | | | |
| 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* | |

## Figures

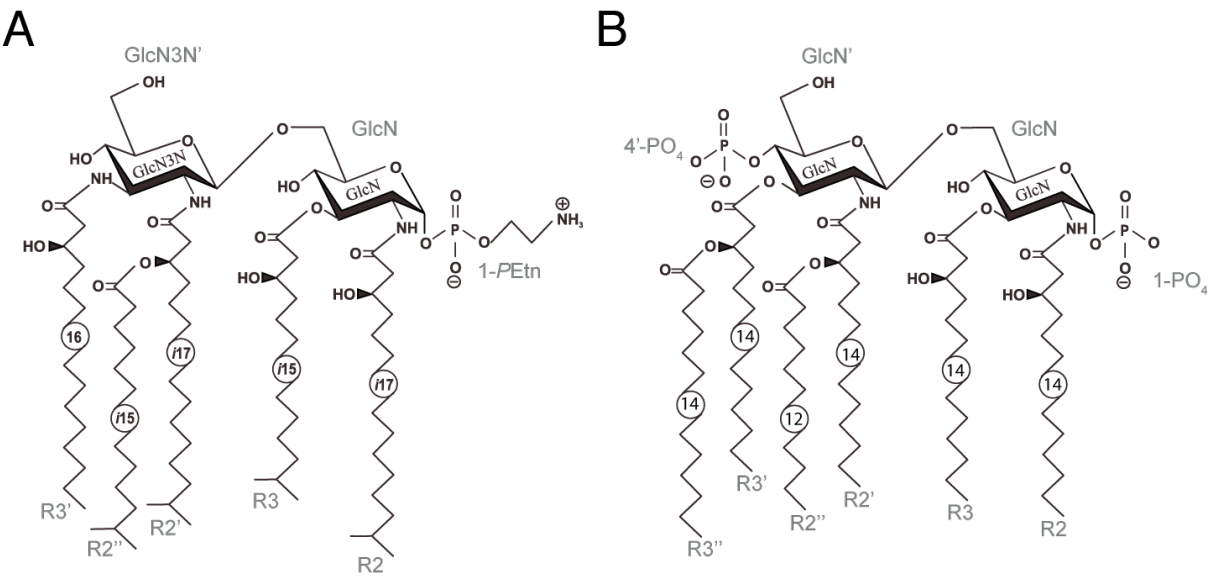


**Figure 1.** Structures of *C. canimorsus* lipid A (A) and *E. coli* lipid A (B). (A) *C. canimorsus* lipid A consists of a β-(1'→6)-linked GlcN3N'-GlcN disaccharide, to which 3-hydroxy-15-methylhexadecanoic acid, 3-hydroxy-13-methyltetradecanoic acid, 3-O-(13-methyltetradecanoyl)-15-methylhexadecanoic acid, and 3-hydroxyhexadecanoic acid are attached at positions 2, 3, 2', and 3', respectively. The disaccharide carries a positively charged ethanolamine at the 1 phosphate and lacks a 4' phosphate. (B) Structure of *E. coli* hexa-acylated lipid A. *E. coli* lipid A consists of a β-(1'→6)-linked GlcN disaccharide that is phosphorylated at positions 1 and 4' and carries four (R)-3-hydroxymyristate chains (at positions 2', 3', 2 and 3). The 2' and 3' 3-hydroxylated acyl groups in GlcN' are further esterified with laurate and myristate, respectively (Raetz, 1990).

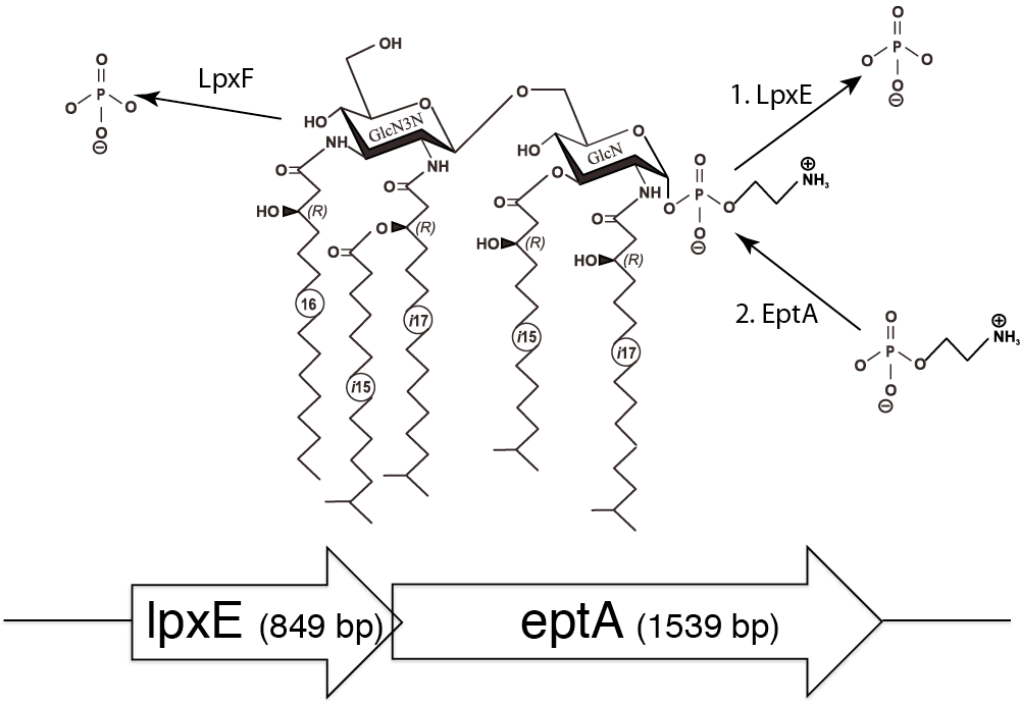


**Figure 2.** Proposed enzymatic activity of LpxE, EptA and LpxF (top) and schematic representation of the *lpxE-eptA* operon (bottom)(drawn to scale).

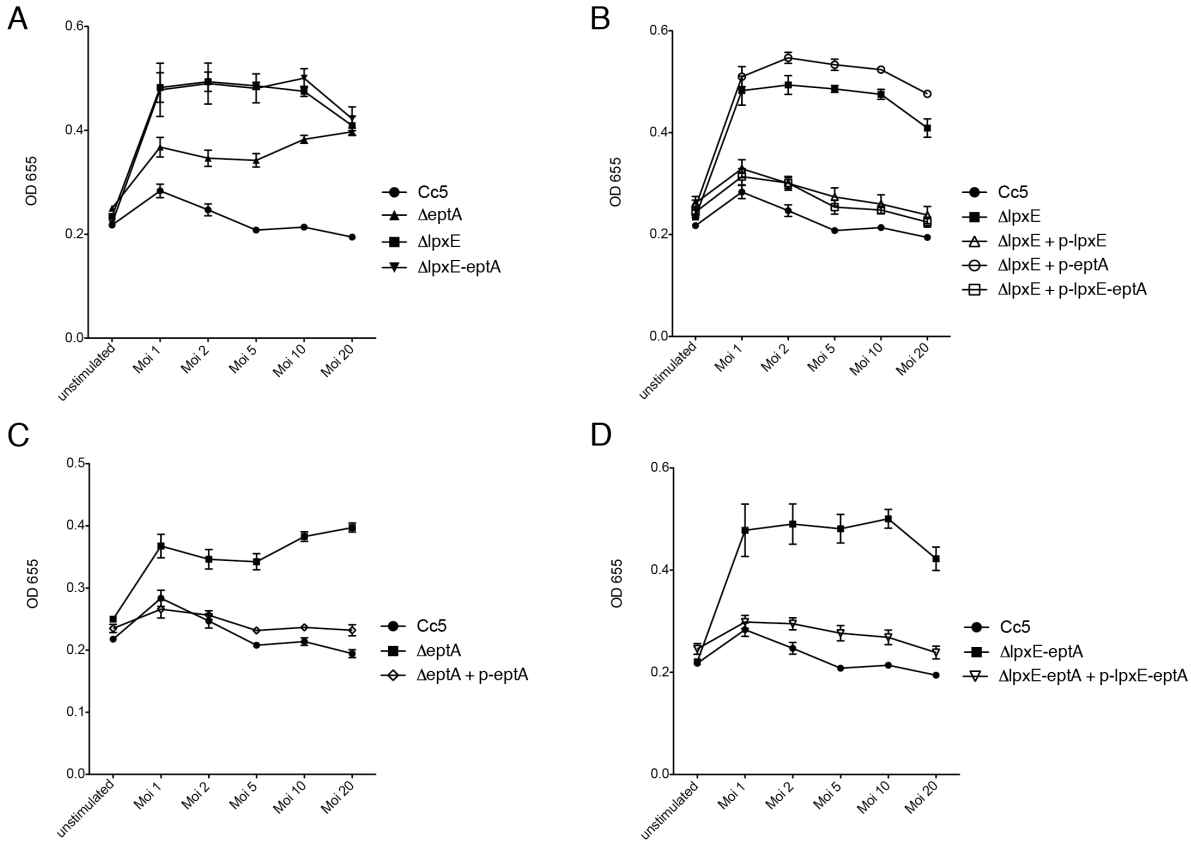


**Figure 3.** Effect of deletion of *lpxE* or *eptA* on endotoxicity (A) Endotoxic activity of heat killed *C. canimorsus* (Cc) wildt-ype (Cc5), Δ*lpxE*, Δ*eptA* or Δ*lpxE-eptA* bacteria. Indicated multiplicity of infection (MOI) of heat killed bacteria were assayed for TLR4 dependent NFκB activation with HekBlue human TLR4 cells. Data were combined from n=3 independent experiments, error bars indicated are standard error of the mean. (B-D) Endotoxic activity of heat killed *C. canimorsus* 5 wild-type (Cc5), Δ*lpxE*, Δ*eptA* or Δ*lpxE-eptA* bacteria and effect of the presence of indicated plasmid (p-) in trans. (A-D) Indicated multiplicity of infection (MOI) of heat killed bacteria were assayed for TLR4 dependent NFκB activation with HekBlue human TLR4 cells. All mutations showed to be non-polar. Data were combined from n=3 independent experiments, error bars indicated are standard error of the mean.

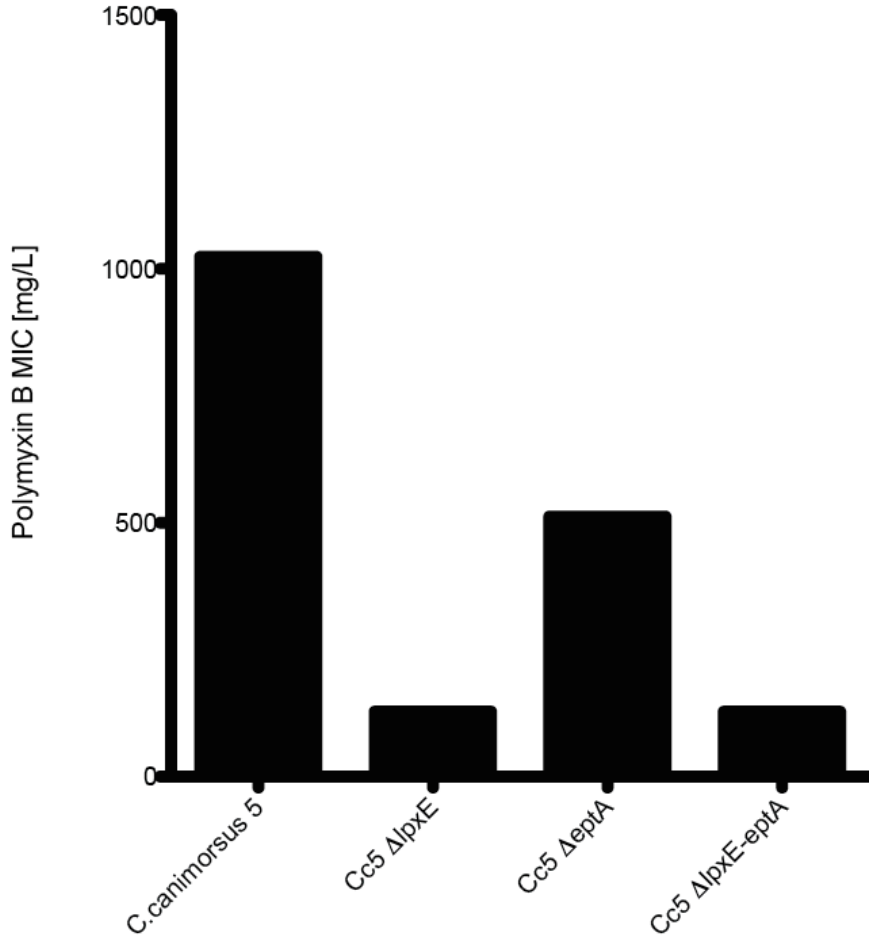


**Figure 4.** Effect of deletion of *lpxE* or *eptA* on resistance to Polymyxin B. Minimum inhibitory concentration (MIC) of Polymyxin B for *C. canimorsus* (Cc) wild-type (Cc5), Δ*lpxE*, Δ*eptA* or Δ*lpxE-eptA.* Polymyxin B MIC was determined using the Agar dilution method. Data were combined from n=4 independent experiments, where MIC measured were identical.

# 3 O-antigens of *Capnocytophaga canimorsus*

## 3.1 Manuscript in preparation: Structure, biosynthesis and function of *Capnocytophaga canimorsus* 5 O-antigens

**Author contributions:** SI, CF, HS, GC and UZ conceived and designed the experiments. SI, CF, HS and UZ performed the experiments. SI, CF, HS and UZ analyzed the data. SI and GC wrote the paper.

**Statement of my work:** My contribution was the data of figures 2, 3, 5, 6, 7, 8 and S3. The Transposon-screen and serum sensitivity assays were performed by CF and HS. UZ performed all chemical analysis.

# Structure, biosynthesis and function of *Capnocytophaga canimorsus* 5 O-antigens

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Running head: Structure and function of *C. canimorsus* O-antigens

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**Abstract**

*C. canimorsus* is a usual member of dog's oral flora that was discovered in patients that underwent dramatic infections after having been bitten, scratched or licked by a dog. The structure of the LPS from *Capnocytophaga canimorsus* 5 (Cc5) was determined by chemical analysis, GLC-MS, ESI FT-ICR MS and NMR spectroscopy. Two different O-antigens (LPS I and LPS II) were found to be co-expressed. LPS I consists of repeating units of N-Acetylfucosamine (FucNAc), glucuronic acid (GlcA), N-Acetylquinovosamine (QuiNAc) and N-galacturonoyl-2-aminoglycerol (GalANgro) while LPS II O-antigen consists of five repeating units of N-Acetylglucosamine (GlcNAc) and L-Rhamnose (L-Rha). Several transposon mutants sensitive to complement killing isolated by a large screen turned out to be also sensitive to killing by Polymyxin B. All the mutations mapped in a 28-kb locus consisting of 29 genes involved in the biosynthesis and assembly of the sugars identified in LPS I and LPS II. All serum- and polymyxin-sensitive mutants lacked LPS I but also a high molecular weight polysaccharide reacting with a specific anti LPS I antiserum. We inferred that this polysaccharide was a type 1 or 4 capsule consisting of the LPS I repeating units. The K-antigen, formed by LPS I and the related capsule, but not LPS II, were found to be assembled by a *wzx/wzy* dependent process. Deletion of *wzz* lead to deregulation of the length of LPS I, to the loss of the LPS I dependent capsule and to an altered surface as detected by TEM. Summarizing, we show that the *C. canimorsus* 5 K-antigen is responsible for the complement and Polymyxin B resistance.

## Introduction

*Capnocytophaga canimorsus* (formerly Centers for Disease Control group DF-2) are capnophilic Gram-negative bacteria that belong to the family of *Flavobacteriaceae* in the phylum *Bacteroidetes. C. canimorsus* is found in the normal oral flora of dogs and cats. It is rarely but regularly isolated from dog or cat bite infections since its discovery in 1976 (Bobo and Newton, 1976; Brenner et al., 1989). These infections occur, worldwide, with an approximate frequency of one per million inhabitants per year. They generally begin with flu symptoms and evolve in a few days into fulminant septicaemia and peripheral gangrene with mortality as high as 40 % (Bailie et al., 1978; Bobo and Newton, 1976; Le Moal et al., 2003; Pers et al., 1996; Westwell et al., 1989). Splenectomy, alcohol abuse and immunosuppression have been associated with a number of cases, but more than 40% of the patients have no obvious risk factor (Lion et al., 1996). Recent observations help understanding the infectiveness of *C. canimorsus* for humans. *C. canimorsus* are able to escape complement killing and phagocytosis by human polymorphonuclear leukocytes (PMN's) (Meyer et al., 2008; Shin et al., 2009). They also escape detection and phagocytosis by macrophages, which results in a lack of release of pro-inflammatory cytokines (Shin et al., 2007). In addition to the passive evasion from innate immunity, some strains are able to block the killing of *Escherichia coli* phagocytosed by macrophages (Mally et al., 2009; Meyer et al., 2008) and to block the onset of pro-inflammatory signalling induced by an *E. coli* lipopolysaccharide (LPS) stimulus (Shin et al., 2007). *C. canimorsus* also has the unusual property to deglycosylate mammalian proteins, including IgG and surface glycoproteins from phagocytes (Mally 2008; Manfredi 2011; Renzi et al., 2011).

Gram-negative bacteria have a complex set of surface polysaccharides, which

contribute to pathogenicity as well as commensalism (Bravo et al., 2008; Cardoso et al., 2006; Grossman et al., 1987; Lerouge and Vanderleyden, 2002; Murray et al., 2003; Nesper et al., 2002; Pluschke et al., 1983a; Porat et al., 1992; Raynaud et al., 2007; Ugalde et al., 2000). These include the lipopolysaccharide (LPS) as well as capsules or other exopolysaccharides (Whitfield, 2006). The LPS of gram-negative bacteria, a major component of the outer membrane, consists of three regions: the lipid A, the core oligosaccharide, and the O-antigen. The O antigen is synthesized independently of the lipid A-core (Raetz, 1990) and generally consists of several repeats of an oligosaccharide called the O-unit. Three pathways have been described for LPS biosynthesis and translocation and they essentially differ by their export mechanism. They are called Wzy-dependent, ABC-transporter dependent and synthase dependent (Raetz and Whitfield, 2002). The O-antigen greatly varies in between and within species, providing the main basis for serotyping. It can be a virulence factor contributing to serum resistance (Murray et al., 2003; Murray et al., 2005; Murray et al., 2006; Nesper et al., 2001; Nesper et al., 2002; Pluschke et al., 1983a; Pluschke et al., 1983b; Raynaud et al., 2007; Slaney et al., 2006; Ugalde et al., 2000; West et al., 2005). O-antigen deficient strains of different bacteria have generally reduced virulence (Raynaud et al., 2007; Ugalde et al., 2000).

In a previous study we reported that *C. canimorsus* resist killing by human complement by virtue of a polysaccharide structure, likely the LPS. A mutant hypersensitive to killing by complement via the antibody-dependent classical pathway, called Y1C12, was indeed found to be affected in a glycosyltransferase gene (Shin et al., 2009). This mutant was missing a polysaccharide structure, the LPS or a capsule, but was still endowed with other polysaccharide structures. Here we characterize the different polysaccharide structures present at the surface of *C. canimorsus* 5 and we

characterize Y1C12 and other complement-sensitive mutants. We show that *C. canimorsus* 5 expresses two LPS O-chains (I and II) and a capsule made of the O chain from LPS I. Synthesis of both chains and their assembly is encoded in one large locus. Mutants that are sensitive to complement are also sensitive to Polymyxin B and they are all affected in the biosynthesis of LPS I.

## Results

### Structure of two different O-antigens from *C. canimorsus* 5

The composition of the O-antigens expressed by *C. canimorsus 5* was determined using electrospray-ionisation mass spectrometry (ESI-MS) and gas liquid chromatography mass spectrometry (GLC-MS) or using an NMR based technology (described in Zähringer et al, 2011, submitted). Two different LPS O-antigens (LPS I and LPS II) were identified. LPS I O-antigen consists of repeating units of N-Acetylfucosamine (FucNAc), glucuronic acid (GlcA), N-Acetylquinovosamine (QuiNAc) and N-galacturonoyl-2-aminoglycerol (GalA and NGro: GalANgro) (Fig. 1 A). The aminoglycerol attached to the galacturonic acids mask the negative charges of the carboxygroup at position 2 of the uronic acid (GalA). LPS II O-antigen consists of only five alternating units of N-Acetylglucosamine (GlcNAc) and L-Rhamnose (L-Rha) (Fig. 1 B). Mutant Y1C12 affected in a glycosyltransferase synthesized only LPS II, hinting that Y1C12 is affected in a gene involved in synthesis of LPS I only.

**LPS I is expressed on each single *C. canimorsus* 5 bacterium**

In order to see, if each individual *C. canimorsus* 5 bacterium co-expresses LPS I and LPS II, we generated an antiserum specific to LPS I by absorbing an antiserum raised against total bacteria with a large excess of intact Y1C12 bacteria. We stained *C. canimorsus* 5 wt (Fig. 2 A) and Y1C12 (Fig. 2 B) bacteria by immunofluorescence using this LPS I antibody. The surface of every *C. canimorsus* 5 cell was stained all around while none of the Y1C12 bacteria were stained (Fig. 2). Thus all *C. canimorsus* 5 bacteria express LPS I or a closely related antigen. At least some if not all of *C. canimorsus* 5 bacteria must co-express LPS II since both LPS were purified from the same culture. Hence we infer that LPS I and II can be co-expressed by *C. canimorsus* 5.

***C. canimorsus* 5 also has a capsular antigen related to LPS I**

Since several polysaccharides were observed in our previous work, we used the anti-LPS I serum to identify the band corresponding to LPS I. We purified bacterial polysaccharide structures by digesting whole bacteria with proteinase K followed by immunoblotting using anti-*C. canimorsus* 5 and anti-LPS I antibody (Fig. 3). As a control purified LPS samples from *C. canimorsus* 5 wt and Y1C12 bacteria were used. Bands at molecular weight (MW) lower than LPS II were assigned as lipid A plus core (LA-core). Bands at MW higher than LPS I were assigned as B (3-5 five clustered bands). Bands labeled A and B were present in the wt and all Tn mutants. These were non-LPS bands, as they were not present in purified LPS samples. They are also not structural elements related to LPS I as they were not stained with the anti-LPS I antibody (Fig. 3 B). In contrast, the band labeled HMW was missing Y1C12 (Fig. 3 A and B) and it was stained with the anti-LPS I antibody (Fig 3. B) although it was not

present in *C. canimorsus* 5 LPS purifications. This indicated that *C. canimorsus* 5 encodes a high molecular weight structural element made at least partially of subunits identical to LPS I. This could be a capsule of type 1 or 4 that is made out of LPS repeating units (Whitfield, 2006). LPS I and the related capsule thus represents the *C. canimorsus* K-antigen.

**Identification of the genes encoding the O-antigens of *C. canimorsus* 5**

In order to identify the genes encoding these O antigens, we sought to identify more complement-sensitive mutants. By screening a transposon mutant library (*Tn*4351) of *C. canimorsus* 5, we isolated four clones (hereafter designated Y1D1, Y3A2, U5D4, X1B12) whose survival rate in normal human serum (NHS) was severely decreased compared to that of the *C. canimorsus* 5 wt. As shown in figure 4, 10% NHS reduced the viable counts of all these mutants by almost 5 log10 units after 3 h of incubation while heat inactivated (HI) NHS had no significant effect indicating that the serum sensitivity involved complement-dependent killing (Fig. 4).

Mutant bacteria Y1D1, Y3A2, X1B12 and U5D4 all lacked the LPS I band (Fig. 5 A), thus resembling Y1C12. Although *Tn*4351 often leads to cointegration, all the five mutants considered were bona-fide transposition mutants (Supplementary Fig. 1). The integration site of Y1C12, Y1D1, Y3A2, U5D4 and X1B12 were mapped on the genome sequence (Manfredi, Pagni, Cornelis, 2011) and found to be located in different genes clustered in a 28-kb locus, including the glycosyltransferase (Y1C12) identified earlier (Shin et al., 2009) (Fig. 6 A). This locus encodes 29 genes, whereof 23 are predicted to be involved in sugar synthesis, transfer or export. The gene cluster is directly flanked by transposase genes *Ccan*23450 and *Ccan* 22980, indicating the possibility of horizontal acquisition. This *C. canimorsus* 5 O-antigen gene cluster encodes the biosynthesis of

sugars for LPS II (L-Rha: RmlA-D) as well as genes required for biosynthesis of sugars incorporated in LPS I (FucNAc: FnlA-C, QuiNAc: WbtA-C, GlcA: Ugd and GalA: Ugd and Uge). The O-antigen repeat-units of LPS I and II and the putative role of genes from the *C. canimorsus* 5 "O-antigen cluster" are summarized in figure 6 B. Interestingly, all serum sensitive transposon mutants mapped in genes required for biosynthesis of LPS I (Fig. 6 A). The annotation of the gene mutated in Y1C12 was now refined as *wbuB*, a predicted FucNAc transferase (Shin et al., 2009). In Y1D1 the transposon inserted in *wbtA* (*wbtA* in *F. tularensis* or *wbpM* in *P. aeruginosa*), a gene required for QuiNAc biosynthesis (Belanger et al., 1999; Liu et al., 2008; Samuel and Reeves, 2003). For mutant Y3A2, the Tn was found in *uge*, which encodes the enzyme for the conversion of GlcA into GalA. The transposon generating U5D4 and X1B12 was located in *fnlC*, encoding the enzyme required for the last step in FucNAc biosynthesis. Y1C12, X1B12 and U5D4 synthesized a LPS, which migrated slightly below the LPS I, band (labeled LPS* in Fig. 5 A). Interestingly, the LPS* band was only detected for the mutants affecting FucNAc synthesis or transfer. The band labeled LPS* was not detected with the anti-LPS I antibody (Fig. 5 B), indicating that the LPS* is not identical to LPS I. It might be that LPS* is a version of LPS I without FucNAc and that the FucNAc residues are essential for recognition by the anti-LPS I antiserum and for serum resistance. This would match the reported role of FucNAc in pathogenesis of several bacteria (Kneidinger et al., 2003).

The mapping of the new Tn mutants not only allowed to annotate accurately the LPS biosynthesis genes but it confirmed that LPS I but not LPS II is involved in the mechanism of serum resistance of *C. canimorsus* 5. By purification and analysis of Y1C12 LPS we could clearly show, that Y1C12 (therefore *wbuB*) affects biosynthesis of LPS I but not II. For Y1C12 polar effects have been excluded by complementation

studies (Shin et al., 2009).

**The length of the O-chains from LPS I is controlled by Wzz**

The polysaccharides from a fifth complement-sensitive mutant (M1C12) presented an unusual migration in gel electrophoresis. Instead of a single band, it appeared as a ladder of products with a higher mass than wt LPS I. No additional bands below the LPS I band appeared and the size difference in between each single step of the laddering fits more with 5-times the tetrasaccharide repeat than a single tetrasaccharide unit (Fig. 5). In this mutant the Tn inserted in a gene homologous to *E. coli wza* (Fig. 7). *E. coli wza* is thought to play a role in capsular export by forming a multimeric putative translocation channel (Whitfield, 2006). The gene just downstream of *wza* is an homologue of *E. coli wzz*. *wzz* (formerly *cld* or *ro* 1) is considered as the regulator of the O-antigen polymerase, Wzy, since *E. coli wzz* mutants have a random O-chain length distribution (Franco et al., 1996; Franco et al., 1998; Raetz and Whitfield, 2002). The abnormal chain length distribution observed in M1C12 LPS I (Fig. 5, Fig. S3) suggests that, in this mutant, the insertion had a polar effect and that the silencing of *wzz* was responsible for the phenotype. This interpretation was confirmed by engineering and analyzing single-gene deletions of *wza, wzz* and *wza-wzz*. Single deletion of *wzz*, which is the last gene in the operon, leads to the same laddering as in M1C12 and to the loss of the HMW-band (Fig. S3). The observed laddering of LPS I O-antigen but not of LPS II O -antigen upon deletion of *wzz* indicated that LPS I assembles by the *wzx/wzy* pathway.

**Deletion of *wzz* leads to an altered bacterial surface**

Bacterial samples were Immunogold labeled using the anti-LPS I antibody and

examined by transmission electron microscopy (TEM). The gold particles localized to the surface of *C. canimorsus* 5 (Fig. 8 A and B, left side), whereas only background labeling was detected on the surface of Y1C12 (Fig. 8 A right side). Besides the Immunogold labeling, no difference in the surface structure of *C. canimorsus* 5 wt and Y1C12 mutant was detectable (Fig. 8 A). The M1C12 mutant bacteria, which show deregulated O-antigen polymerization, had an abnormal surface compared to wt. It was ruptured by little spikes unlike the rather smooth surface of wt bacteria (Fig. 8 B). The surface protruding spikes were labeled by anti-LPS I antibodies (see arrowhead Fig. 8 B, right side), indicating that they probably represented the ultra-long LPS I made by M1C12 (Fig. 5, Fig. S3). As both, Y1C12 and M1C12, lack the HMW structural element, these spikes cannot account for the observed surface alteration.

**LPS I is essential for Polymyxin B resistance**

We have seen here before that LPS I protects *C. canimorsus* from the bactericidal action of complement. As the LPS O-chain has been reported to protect some bacteria from cationic antimicrobial peptides (CAMP) (Cardoso et al., 2006), we monitored the sensitivity to Polymyxin B of wt and mutant Cc5 bacteria. *C. canimorsus* 5 wt was found to be highly resistant (MIC = 1024 mg/L) while all LPS I/capsule mutants showed a decreased resistance (Fig. 9). Therefore the K-antigen is not only important for serum resistance but as well for resistance to CAMPs.

## Discussion

The O-antigen is known to play an important role in serum resistance and virulence of pathogenic bacteria (Bravo et al., 2008; Grossman et al., 1987; Murray et al., 2003; Murray et al., 2005; Murray et al., 2006; Raynaud et al., 2007; Ugalde et al., 2000). In particular this was shown to apply to *P. gingivalis,* which is phylogenetically related and occupies a similar niche. *P. gingivalis* were reported to rely on a surface polysaccharide for serum resistance (Slaney et al., 2006). We have investigated the structure of archetypal *C. canimorsus* 5 LPS and found two different O-antigens, called LPS I and LPS II. The expression of LPS I and LPS II is shown not to be mutually exclusive and a single bacterium can have both LPS present at its surface.

*C. canimorsus* 5 LPS I O-antigen consists of repeating units of L-FucNAc, GlcA, QuiNAc and GalANgro while LPS II O-antigen consists of five repeating units of GlcNAc and L-Rha. . The aminoglycerol attached to the uronic acid masks the negative charge of the uronic acid. This O-unit resembles the one of *V. cholerae* H11 (non O1), which besides neuraminic acid contains GlcANgro, QuiNAc and GalANgro (Vinogradov et al., 1992; Vinogradov et al., 1993). L-FucNAc residues in capsular structures or LPS have been associated with pathogenicity in *P. aeruginosa* (O4, O11, O12) and *S. aureus* (serotype 5 and 8) (Kneidinger et al., 2003). Overall, the presence of a L-Rha containing LPS glycoform and a more complex LPS glycoform containing three to five distinct sugars in a single cell reminds of *P. aeruginosa* (Lam et al.).

In addition to the lipid A linked form, *C. canimorsus* seems to build a high molecular weight (HMW) structure out of sugars of LPS I, likely a capsule. Capsules made of sugars of the LPS O-unit have been described and are called capsules type 1 and 4 (Whitfield, 2006). Type 1 capsules generally contain uronic acids (Whitfield, 2006) as

were detected in *C. canimorsus* LPS I. The *C. canimorsus* LPS I (in accordance with the *E. coli* nomenclature LPS I would be the $K_{LPS}$) and its corresponding HMW-form thus represent one K-antigen, as seen in some *E. coli* strains, consisting of the LPS and a related capsule of type 1 or 4 (Whitfield, 2006).

In previous study we have reported that Y1C12 was deficient in a putative glycosyltransferase. We could show that a polysaccharide structure dependent on this glycosyltransferase, likely the LPS, protected *C. canimorsus* from deposition of membrane-attack-complex (MAC) and therefore killing by serum (Shin et al., 2009). We have now analyzed the LPS structure of this mutant strain and found that it lacked the K-antigen. We infer that the serum resistance of *C. canimorsus* is based on the K-antigen. Further all serum sensitive mutants mapped into one single gene cluster, that we call the O-antigen cluster. It encodes not only the biosynthesis of sugars for LPS I (FucNAc: FnlA-C, QuiNAc: WbtA-C, GlcA: Ugd and GalA: Ugd and Uge) but also for LPS II (L-Rha: RmlA-D). All serum sensitive Tn mutants mapped in genes required for biosynthesis of LPS I. This suggests again that LPS I but not LPS II is involved in the mechanism of serum resistance of *C. canimorsus* 5. As is the case in *V. cholerae* (Nesper et al., 2001; Nesper et al., 2002), the serum sensitive strains equally showed increased sensitivity to CAMPs. Concluding, LPS I and its related HMW-form protect *C. canimorsus* from CAMPs and human complement.

LPS I and the related capsule but not LPS II is translocated via the Wzy-dependent pathway. To affect LPS II but not LPS I we have tried to mutate *wzt*, as LPS II might be exported via the *wzm/wzt* pathway (Raetz and Whitfield, 2002). Deletion mutation in the predicted C. canimorsus 5 *wzt* gene did not affect the polysaccharide structures visualized by Western-blotting (data not shown). Further, we tried to delete *rmlA*, *rmlC* or *rmlD* to interfere with LPS II. All these mutations showed to be lethal, which probably

reflects the fact, that a Rhamnose is present in the core. Perturbation of LPS I chain length by deletion of *wzz* led to sensitivity to serum and CAMPs. Even if generally a longer O-antigen is considered to protect better from complement (Porat et al., 1992), in the case of *C. canimorsus* unregulated length of the O-chain of LPS I led to serum sensitivity. Deletion of *wzz* did not lead to a uniform prolonged LPS I, which might promote improved serum resistance, but to a dispersed chain length. The regular surface structure was thus changed and spike-like protrusions were seen. Further Δ*wzz* bacteria did not have the HMW-form of LPS I, the proposed capsule. This capsule might be lost in Δ*wzz* bacteria because the sugars are used up for the ultra-long LPS I or because it is dependent on *wzz*. If the resulting serum sensitivity of Δ*wzz* bacteria is based on the loss of the capsule dependent on LPS I or on the irregular surface structure based on the various LPS I species present could not be evaluated. But the LPS I laddering observed for Δ*wzz* bacteria starts only at higher molecular weight than the wt LPS I. Hence the O-chain of LPS I of Δ*wzz* bacteria is never shorter as the wt LPS I. Studies in *E. coli* and *Salmonella* reported correlation of O-antigen length and resistance to complement (Bravo et al., 2008; Murray et al., 2003; Murray et al., 2005; Murray et al., 2006; Porat et al., 1992). Hence the LPS I of Δ*wzz* bacteria should be at least as protective as the one of *C. canimorsus* 5 wt. Based on this assumption the LPS I dependent capsule seems to be the complement and CAMP resistance providing structure. Attempts to prove this hypothesis by mutation of genes involved in capsular export (*wzb*, *wzc*) failed. Neither mutation affected the band pattern seen by Western-blotting, either because of bad annotation even if all possible candidates have been tested or because the *C. canimorsus* capsule is exported independently of the genes with homology to *wzb* and *wzc*.

## Materials and methods

**Bacterial strains and growth conditions.** The following bacterial strains were used: *C. canimorsus* strains *C. canimorsus* 5, isolated from human infection as described previously (31); *E. coli* Top10 (Invitrogen). *C. canimorsus* bacteria were routinely grown on heart infusion agar (Difco) supplemented with 5% sheep blood (Oxoid) for 2 days at 37°C in the presence of 5% CO2. Selective agents were added at the following concentrations: erythromycin at 10  g/ml, gentamicin at 20 g/ml.

**Antisera.** Anti-rabbit IgG-FITC was obtained from Southern Biotech. Polyclonal serum against *C. canimorsus* 5 was generated from rabbits by immunization with HK *C. canimorsus* 5 (Laboratoire d'Hormonologie, Marloie, Belgium). The Y1C12-absorbed serum was prepared by incubating twice an excess amount of Y1C12 mutant bacteria (harvested from blood plates and washed in phosphate-buffered saline [PBS]) with anti-*C. canimorsus* 5 serum at 4°C for 12 h. Bacteria were removed by repeated centrifugation**.**

**LPS isolation.** *C. canimorsus* were harvested from 600 blood plates in phosphate buffered saline (PBS) and washed with ddH2O, ethanol (300ml) and acetone (300ml), followed each time by centrifugation at 18000 x g for 30 min.

**Transposon mutant screen for serum bactericidal assay.** Random Transposon4351 mutants were generated as described previously (Mally et al., 2008). Transposon (*Tn*) mutant bacteria were grown in 100 μl heart infusion broth (Difco) supplemented with 10% (vol/vol) fetal bovine serum (Invitrogen) in 96-well plates for 48 h without shaking

in a 37°C incubator with 5% CO2. Subsequently they were diluted in a new plate to an OD590 of 0.025. 50 μl of bacterial suspension (6.25 x 105 CFU) was incubated with either 10% NHS or HI NHS as a control in a 100 μl total volume. After 3 h of incubation, a microliter-range aliquot was spotted onto blood agar using a metal stamper and incubated for 48 h. The insertion site of the *Transposon* was mapped by arbitrary PCR as described previously (Mally et al., 2008).

**Serum sensitivity.** Bacteria were harvested by gently scraping colonies off the agar surface. They were washed and resuspended in PBS to an OD600 of 0.4. NHS from healthy volunteers was pooled, aliquoted, and stored at 80°C. Serum was HI at 55°C for 30 min. A total of 1 x 107 bacteria were incubated in 10% NHS in PBS at 37°C with 5% CO2. Serial dilutions were plated onto blood plates, and viable colonies were counted after 48 h of incubation.

**Gene annotation.** Blast-p search tool (Altschul et al., 1997) against the *C. canimorsus* 5 genome (Manfredi et al.) was used. Search sequences were obtained from the National Center for Biotechnology Information. All available search sequences for a corresponding gene of interest were used. The highest scoring subjects over all the searches have been annotated as corresponding enzymes.

**Immunoblotting of proteinase K-resistant structures.** Bacteria were harvested from blood-agar plates, washed once in 1ml of PBS and adjusted to an OD600 of 1.5 in PBS. 500µl bacterial suspension was pelleted and dissolved in 125µl ddH2O loading buffer (1% sodium dodecyl sulfate [SDS], 10% glycerol, 50 mM dithiothreitol, 0.02% bromophenol blue, 45 mM Tris (pH 6.8)). Samples were boiled at 99°C for 10 minutes. Proteinase K (50µg/ml final concentration) was added and samples were incubated at 37°C overnight. After incubation samples were boiled again for 10 minutes at 99°C and a second volume of proteinase K (equal to the first) was added. Samples were incubated at 55°C for 3 hours, boiled again for 5 minutes at 99°C and loaded on a 15% SDS-PAGE. Samples were analyzed by western blotting.

**Polymyxin B sensitivity assay.** Polymyxin B sulphate was obtained from Sigma-Aldrich. The agar dilution method was performed based on the CLSI/NCCLS recommendations (Ferraro, 2003). Briefly, 104 bacteria diluted in PBS were spotted in 2µl on HIA 5% sheep blood plates containing Polymyxin B ranging from 0.5 mg/L to 1024 mg/L (2-fold increase per condition). Plates were examined for growth of visible colonies after 48h and 72h.

**Immunofluorescence.** Falcon culture slides (Becton Dickinson) were coated with poly-D-lysine. Bacteria from blood agar plates were adjusted to an OD600 of 1 in PBS. Slides were washed four times with PBS. 100µ of bacterial suspension, corresponding to 5 x 107 CFU, was added to the slides and incubated for 1 h at 37°C. Slides were washed four times with PBS before being fixed with 3% paraformaldehyde for 15 min at RT. Bacteria were labeled with anti-*C. canimorsus* 5 or Y1C12-absorbed anti-*C. canimorsus* 5 antiserum diluted 1:100 in PBS-BSA for 1h at RT. Slides were washed

four times in PBS. FITC-conjugated secondary antibody was added (dilution 1:100 in PBS-BSA), and slides were incubated at RT for 45 min. Slides were washed four times with PBS, mounted with anti-fade reagent (Vector Laboratories), and analyzed by use of a Leica Dmire2 microscope. Pictures were taken using a digital camera (Hamamatsu Photonics) and OpenLab software (version 3.1.2).

**Electron microscopy.** Bacteria were harvested from blood agar plates and washed twice in PBS (1x) and adjusted to an OD600 of 1.0 in PBS. Bacteria were fixed with 2% glutaraldehyde and prepared for EM analysis.

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## Figures

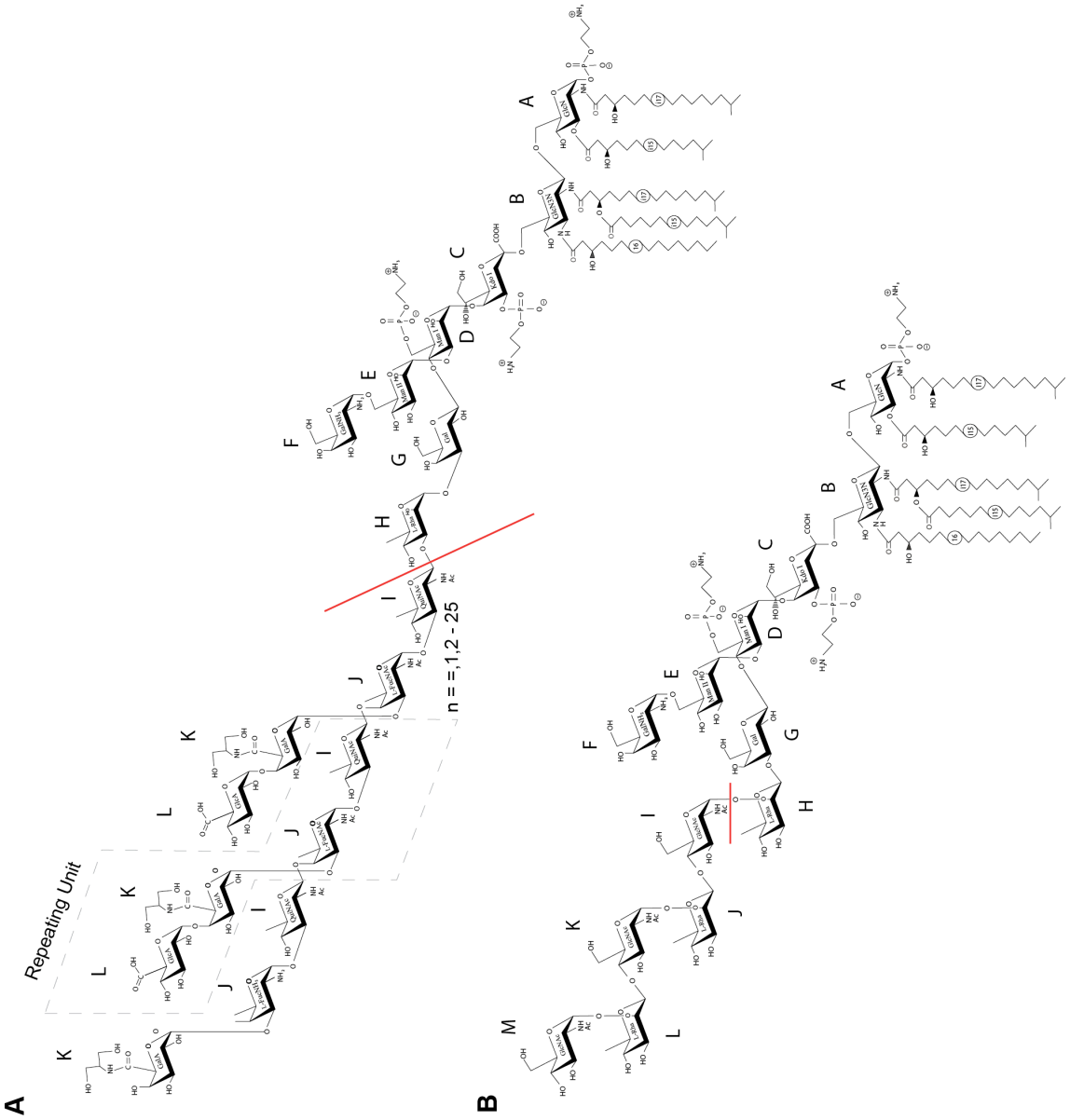



**Figure 1. Structure of the complete LPS of *C. canimorsus* 5.** (A) *C. canimorsus* 5 LPS I O-antigen shows repeating units of N-Acetylfucosamine (FucNAc), glucuronic acid (GlcA), N-Acetylquinovosamine (QuiNAc) and N-galacturonoyl-2-aminoglycerol (GalA and NGro: GalANgro). The red line indicates the end of the core-oligosaccharide. (B) *C. canimorsus* 5 LPS II O-antigen shows repeating units of N-Acetylglucosamine

(GlcNAc) and L-Rhamnose (L-Rha). The O-polysaccharide starts with the GlcNAc labeled „I“. The red line indicates the end of the core-oligosaccharide.

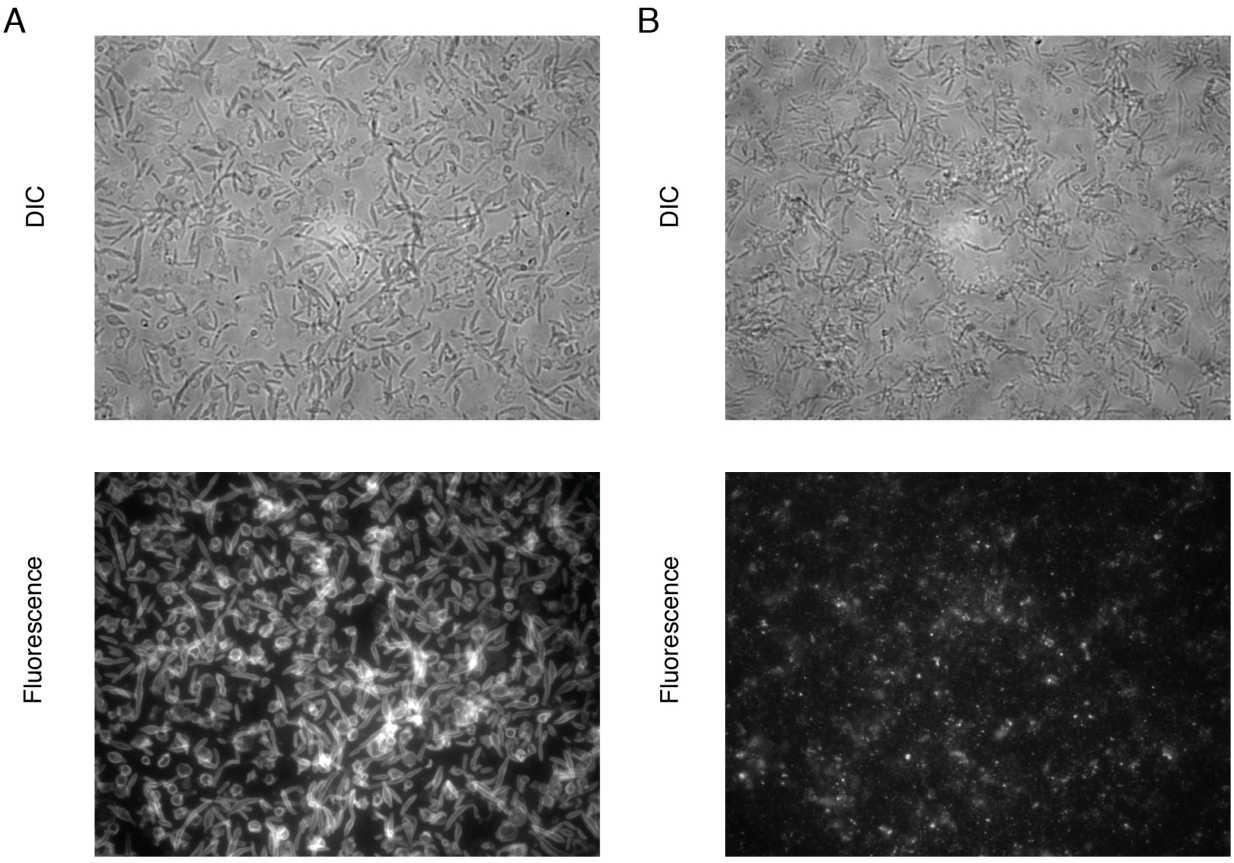



**Figure 2. LPS I is expressed by each single *C. canimorsus* 5 bacterium.** Presence of LPS I was tested by immunofluorescence on paraformaldehyde fixed but not permeabilized bacteria using Y1C12-absorbed anti-*C. canimorsus* 5 antiserum followed by anti- rabbit IgG conjugated to FITC. (A) DIC and immunofluorescence picture of *C. canimorsus* 5. (B) DIC and immunofluorescence picture of Y1C12 Transposon-mutant.

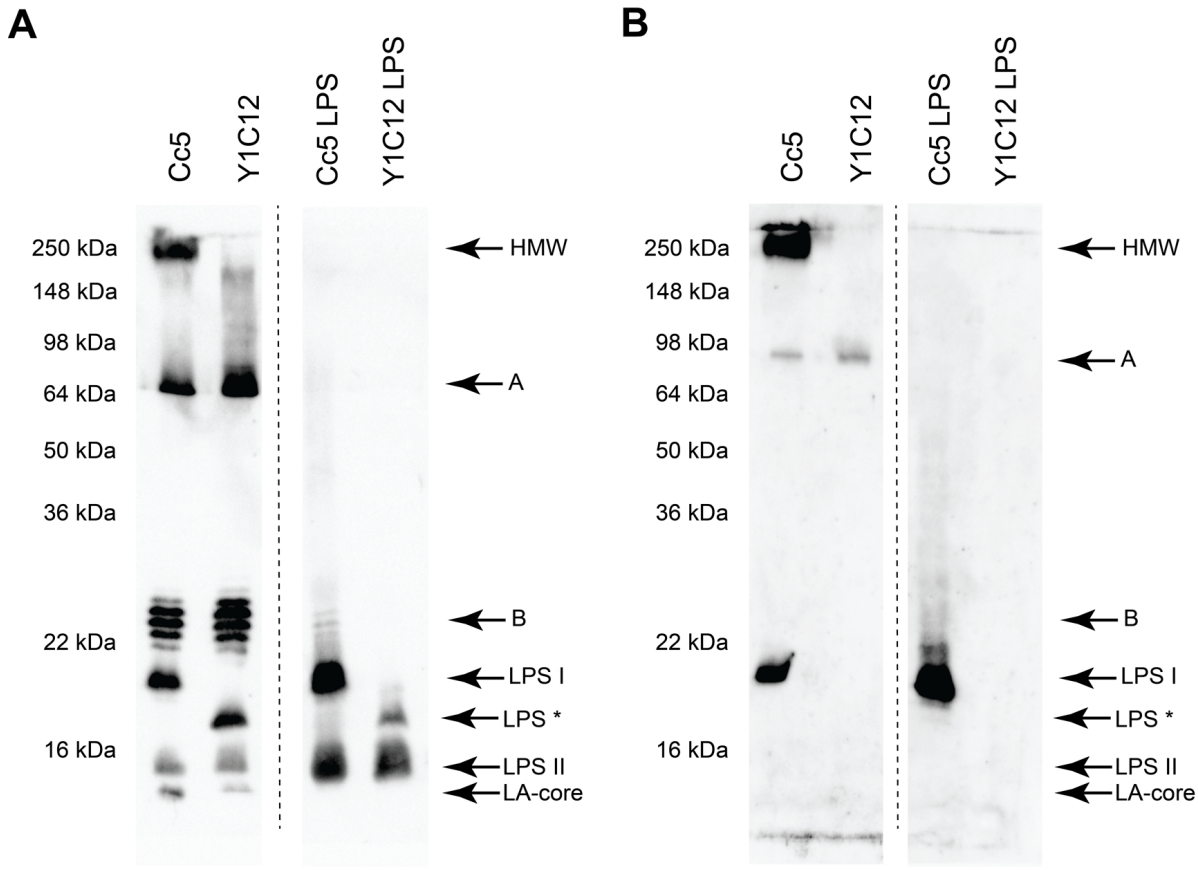



**Figure 3. Y1C12 mutant has alterations in LPS I.** (A) Immunoblotting analysis of proteinase K-treated *C. canimorsus* 5 and Y1C12 mutated *C. canimorsus* 5 and of LPS isolated from *C. canimorsus* 5 and Y1C12 using anti-*C. canimorsus* 5 antibody. (B) Immunoblotting analysis as described above (A) using anti-LPS I antibody.

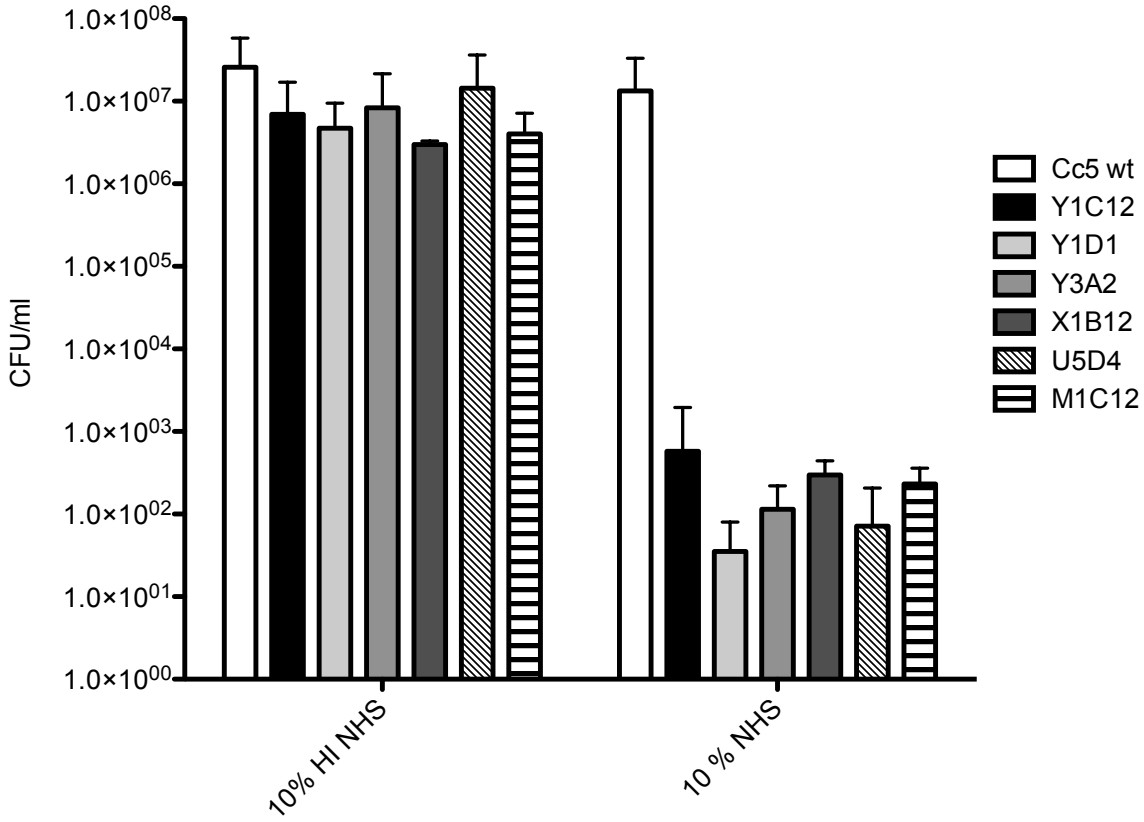



**Figure 4. The Transposon mutants are serum sensitive.** Total CFU present after incubation of wt or *Transposon*4351 mutated *C. canimorsus* in 10% heat inactivated normal human serum (HI NHS) or 10% normal human serum (NHS) for 180 min at 37°C. Mean value of thee independent experiments.

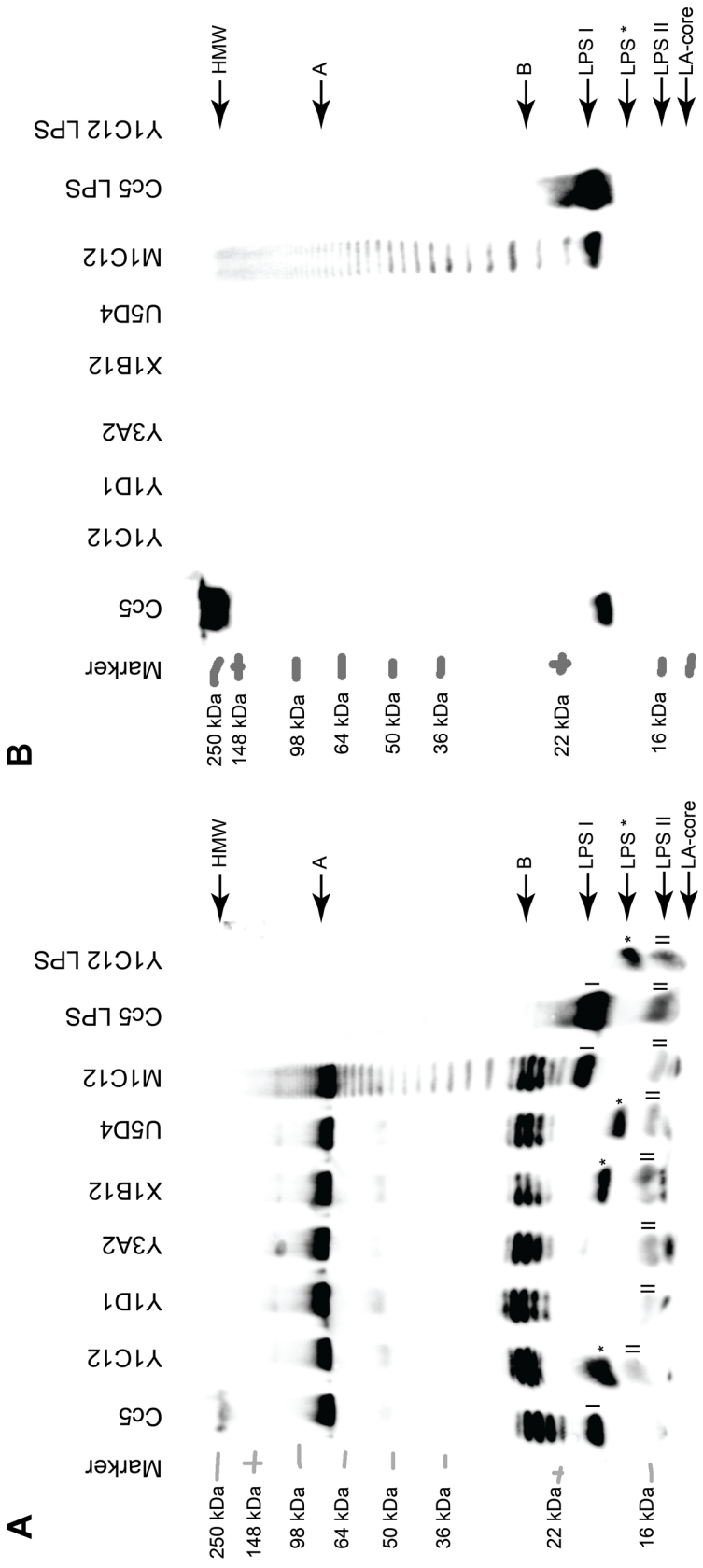



**Figure 5. Transposon mutants have an alteration in LPS I.** (A) Immunoblotting analysis of proteinase K-treated *C. canimorsus* 5 and Tn mutated *C. canimorsus* 5 and of LPS isolated from *C. canimorsus* 5 and Y1C12 using anti-*C. canimorsus* 5 antibody. (B) Immunoblotting analysis as described above (for A) using anti-LPS I antibody.

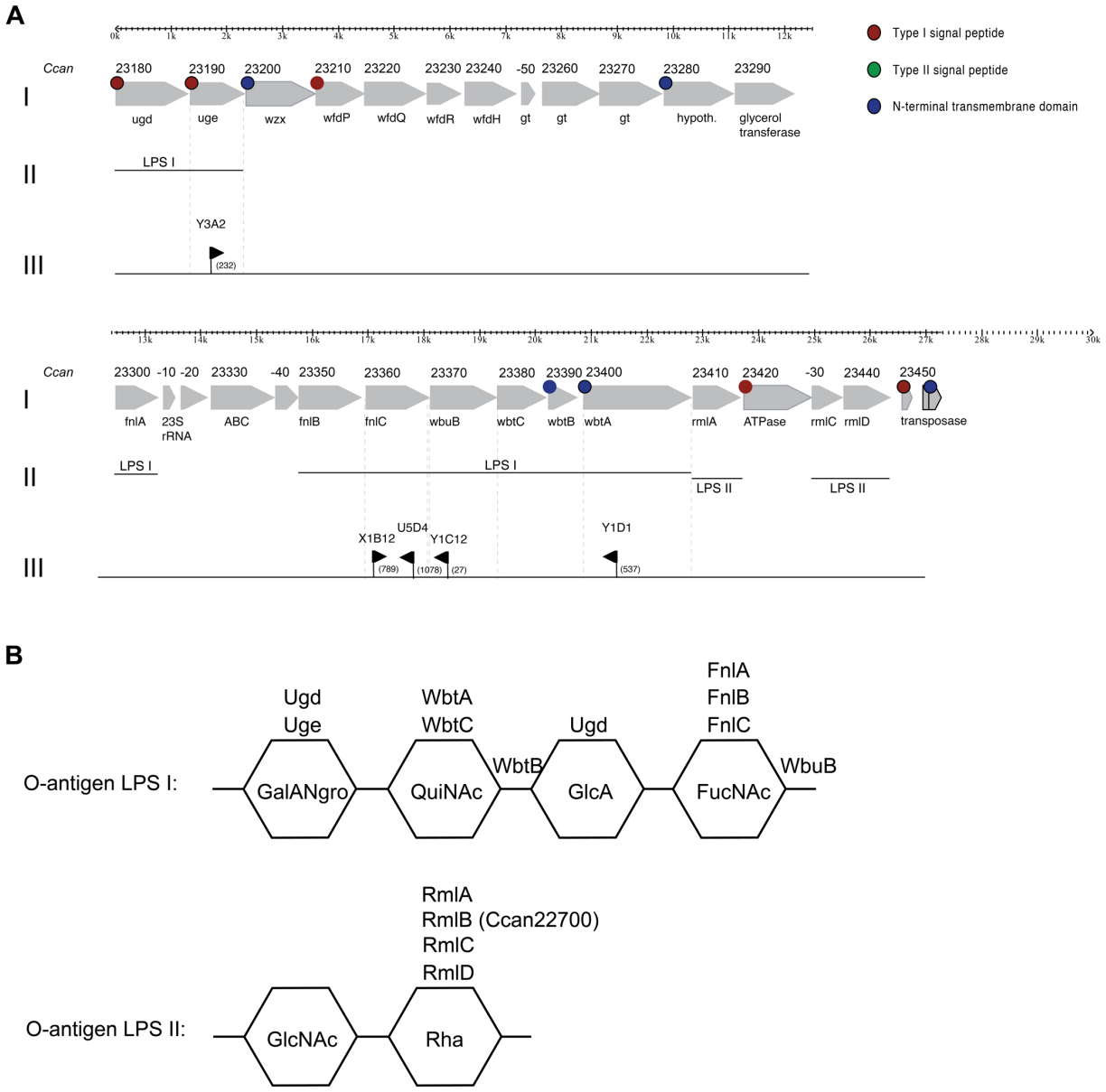



**Figure 6. The *C. canimorsus* 5 O-antigen cluster.** (A) Genetic organization of the O-antigen cluster of *C. canimorsus* 5. (I) Extents, orientation and names of the genes forming the *C. canimorsus* 5 O-antigen cluster. Type I and II signal peptides as well as N-terminal transmembrane domains are indicated with circles. (II) The *C. canimorsus* 5 O-antigen cluster can be divided into two regions. One for the synthesis, assembly and transport of LPS I. The other region encodes genes for the synthesis, assembly and transport of LPS II. (III) Position of transposon (*Tn*4531) insertions. Flags indicate

direction of *Tn*4531 insertion, numbers in brackets transposon integration site. (B) Schematic structure of the O-antigen subunits of *C. canimorsus* 5 and assignment of putative functions to the genes of the O-antigen cluster**.** Single O-repeat units are shown, with sugar residues and glycosidic linkages indicated. LPS I: N-Acetylfucosamine (FucNAc), glucuronic acid (GlcA), N-Acetylquinovosamine (QuiNAc) and N-galacturonoyl-2-aminoglycerol (GalANGro). LPS II: N-Acetylglucosamine (GlcNAc) and L-Rhamnose (L-Rha).

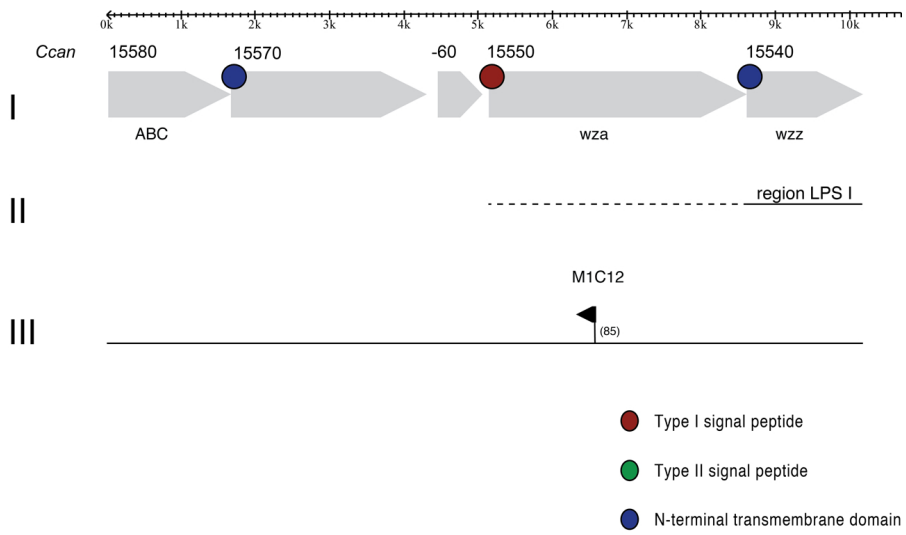



**Figure 7. Genetic organization of the wzz operon of *C. canimorsus*.** (A) Extents, orientation and names of the genes forming the *C. canimorsus* 5 wzz operon. Type I and II signal peptides as well as N-terminal transmembrane domains are indicated with circles. (B) Assignment of corresponding region according to the division of the *C. canimorsus* 5 O-antigen cluster into two regions. Region 1 encodes for the synthesis, assembly and transport of LPS I. Region 2 encodes genes for the synthesis, assembly and transport of LPS II. (C) Position of *Transposon*4531 insertions. Flags indicate direction of Transposon4531 insertion, numbers in brackets transposon integration site.

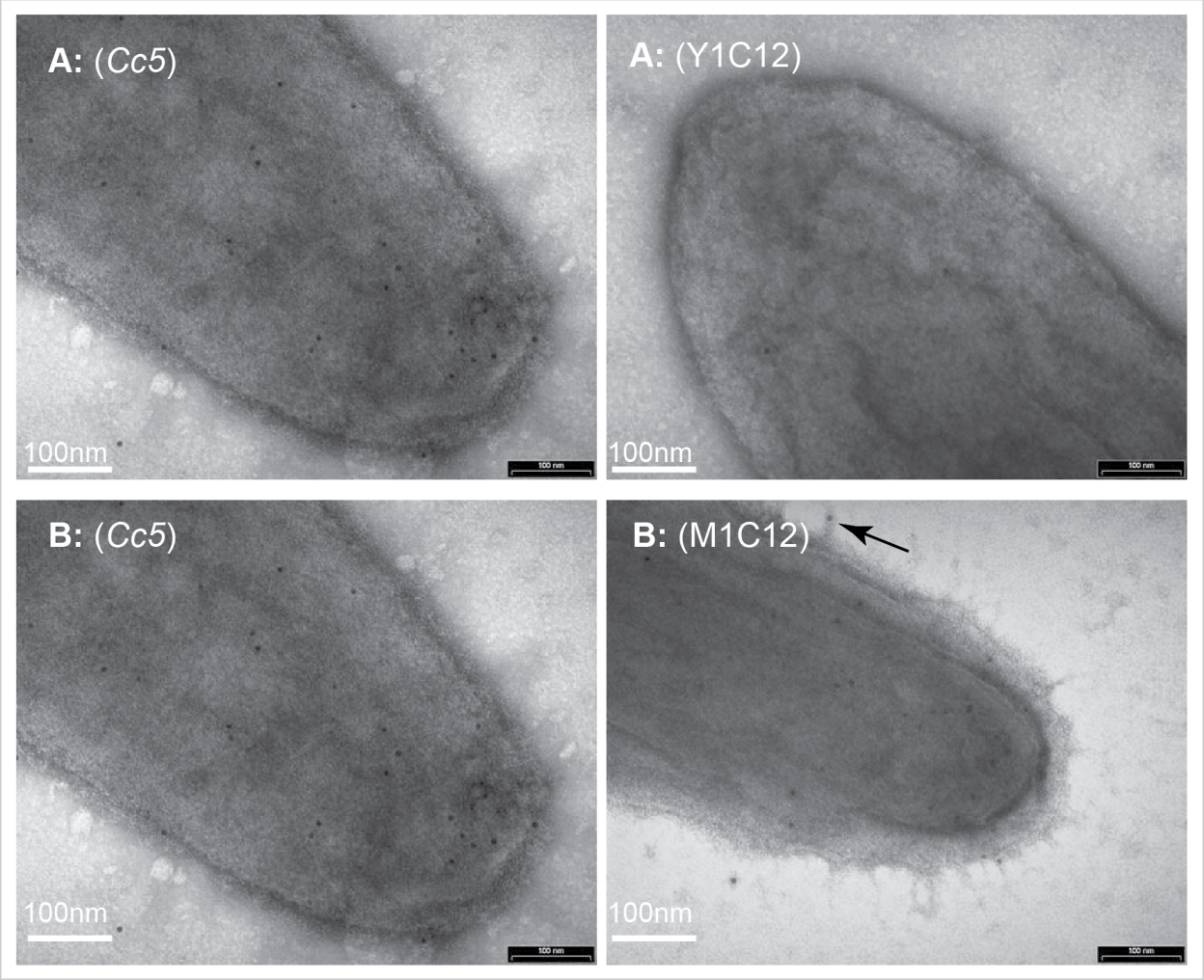



**Figure 8. M1C12 shows altered surface structure.** Bacteria were ImmunoGold labelled using Y1C12-absorbed anti-*C. canimorsus* 5 antiserum (anti-LPS I antibody) and were analyzed by transmission electron microscopy (TEM). (A) ImmunoGold *C. canimorsus* 5 (left) and Y1C12 (right). (B): ImmunoGold *C. canimorsus* 5 (left) and M1C12 (right).

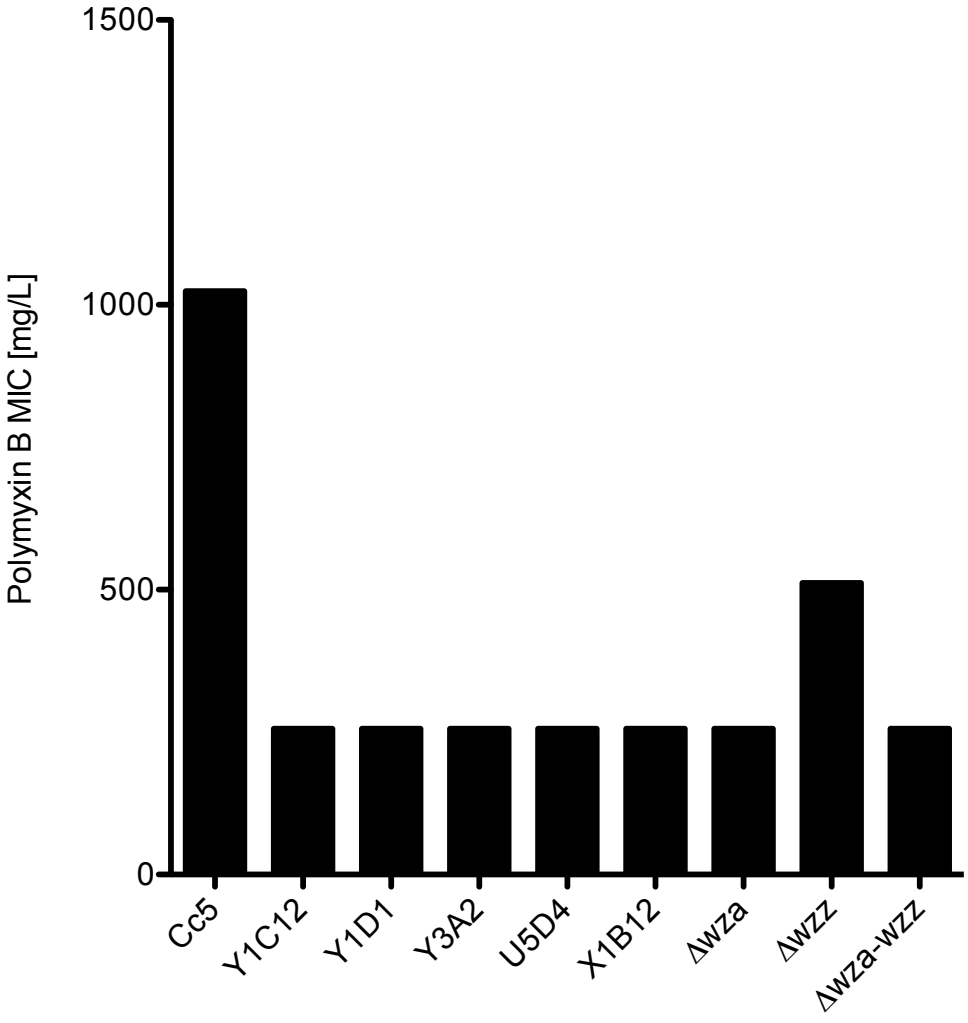
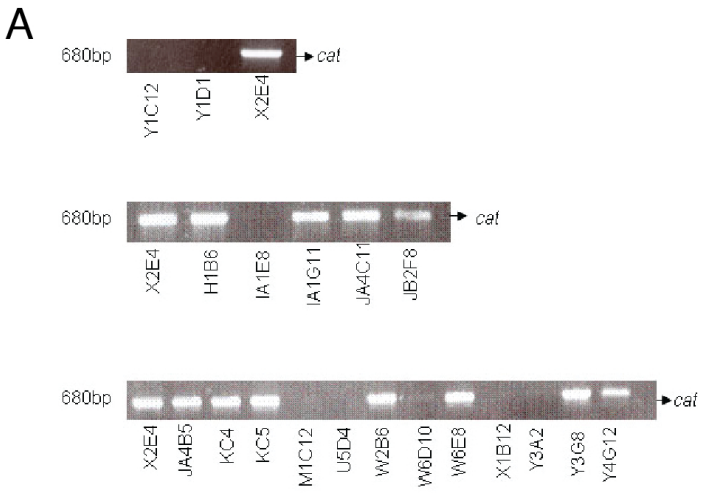
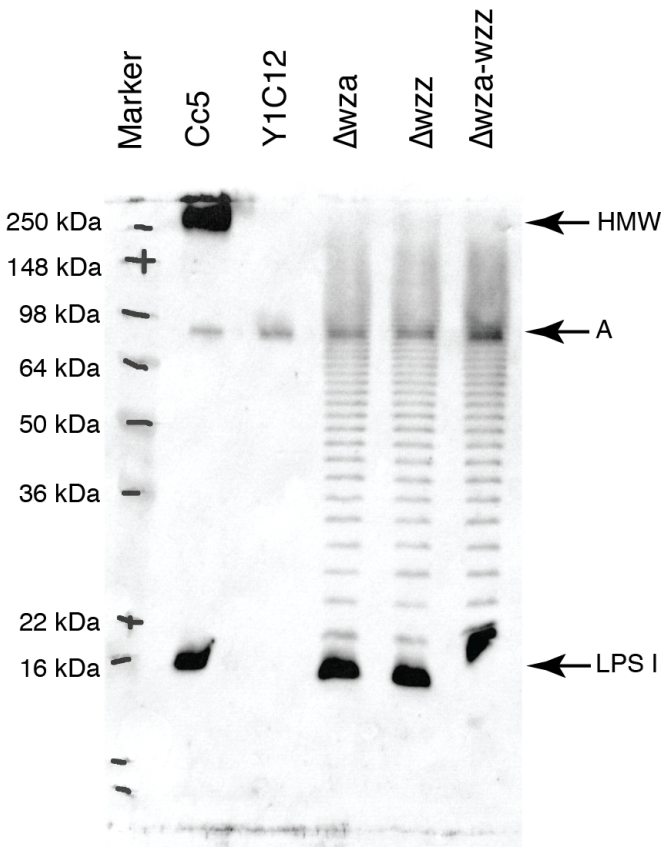



**Figure 9. Transposon mutants are sensitive to Polymyxin B.** Minimum inhibitory concentration (MIC) of Polymyxin B for *C. canimorsus* (Cc) wild-type (*C. canimorsus* 5), the transposon mutants and Δ*wza*, Δ*wzz* or Δ*wza-wzz.* Polymyxin B MIC was determined using the Agar dilution method.




**Supplementary figure 1.** To test for vector cointegration, the chloramphenicol acetyltransferase gene (cat), which is present on the Transposon4351 delivery vector pEP4351, was amplified as a 633-bp PCR product from genomic DNA using primers 3576 and 3577. All mutants described in this study did show not to have the vector cointegrated.


**Supplementary figure 2.** Biosynthesis pathways for the sugars in *C. canimorsus* 5 O-antigens. Adapted from (Liu et al., 2008).




**Supplementary figure 3. Single gene deletion of *wzz* leads to the laddering effect observed in M1C12.** Immunoblotting analysis of proteinase K-treated *C. canimorsus* 5, Y1C12 and of *C. canimorsus* 5 Δ*wza*, Δ*wzz* or Δ*wza-wzz* using anti-LPS I antibody.

# 4 Immune evasion by *Capnocytophaga canimorsus*

**Statement of my work:** My contribution was the data of figures 1, 2, 3, 5, 6, 7, 8. The Transposon-screen on NO-release and mapping of the mutants was performed by H. Shin, M. Mally and C Pfaff-Paroz. The metabolite analysis was done in collaboration with the group op S Gresziek by M. Gentner.

## 4.1 Introduction

In previous studies *C. canimorsus* was found to inhibit LPS stimulated murine macrophages from releasing nitric oxide (NO) and TNFα (Shin et al., 2007). Manuela Mally, Hwain Shin and Cécile Pfaff have performed a genome wide Transposon (Tn)-screen and have identified mutants unable to interfere with the NO release of macrophages. The mutants have been mapped and found to affect genes of different functional categories. Following up this work, I tried to unravel the mechanism behind this inhibition. My work led to the conclusion that the common link between all these mutations is that they all slightly reduce growth, hinting that the agent responsible for the blockade of NO release could be a metabolite. In good agreement with this hypothesis, it turned out that the agent is a small thermostable molecule but the identification attempts were unsuccessful.

I have further analyzed at which step *C. canimorsus* interferes with LPS sensing and signaling by macrophages (chapter 4.3).

## 4.2 Growth dependent effects of *Capnocytophaga canimorsus* on the innate immune system involve a small heat stable molecule

### 4.2.1 The genes hit by the Tn-mutation belong to different functional categories.

The insertion site of the Tn was mapped by arbitrary PCR. These Tn mutants have been termed X2E4, Y2F12, Y4B5, X7B9, W2E9 and Y4G6. The predicted function of the Tn mutants deficient in growth and blocking the LPS induced NO release are listed in **table 1**. The genes hit by the Tn in W2E9 and Y4G6 have previously been described as important in eukaryotic glycoprotein deglycosylation and thus nutrient acquisition (Mally et al., 2008; Renzi et al., 2011). The gene hit in the case of W2E9 is a sialidase, which has been shown to cleave terminal sialic acid from eukaryotic glycoproteins (Mally et al., 2008). The Tn in the Y4G6 mutant maps into locus PUL5 (Manfredi et al., 2011a; Manfredi et al., 2011b; Renzi et al., 2011). This locus has been reported to encode the N-glycan glycoprotein deglycosylation complex of Cc5 (Renzi et al., 2011). The genes hit by the Tn in X2E4 and X7B9 encode nucleotide or amino acid biosynthesis, respectively. The difference in growth of these mutants between RAW and J774 macrophages **(Fig. 1 A and C)** might reflect the different growth media used for these two cell lines (DMEM and RPMI1640, respectively). Y2F12 mutation hits a gene in locus

PUL11 [see (Manfredi et al., 2011b)]. PUL11 consists of two genes with sequence homology to *Bacteroides thetaiotaomicron* starch utilization system (sus) genes *susC* and *susD (Anderson and Salyers, 1989; Reeves et al., 1997; Shipman et al., 2000),* termed *camN* and *camO*, as well as of two hypothetical proteins termed *camA* and *camB*. Like Y4G6 impacting PUL5, Y2F12 impacting PUL11 might be important in nutrient acquisition. Summarizing, the common feature of the genes affecting the inhibition of NO release play a role in growth on eukaryotic cells.

**Table1.** Name of the Tn mutant, orf (*Ccan*) number of the gene in which the Tn insertion mapped and predicted function of the targeted gene are indicated.

| **name** | **Tn-Insertion in *Ccan* Nr.** | **Predicted function as** | **Reference** |
|---|---|---|---|
| X2E4 | *Ccan*_3130 | Dihydroorotase | this study |
| Y2F12 | *Ccan*_20110 | hypothetical | this study |
| Y4B5 | *Ccan*_05080 | hypothetical | this study |
| X7B9 | *Ccan*_06510 | Glutamine synthetase | this study |
| W2E9 | *Ccan*_04790 | Sialidase | (Mally et al., 2008) |
| Y4G6 | *Ccan*_08710 | SusD of PUL5 | (Renzi et al., 2011) |

### 4.2.2 The inhibitory effect of *C. canimorsus* on NO release by macrophages is growth dependent.

Tested for growth on J774.1 macrophages, all Tn mutants showed decreased growth capacity as compared to the Cc5 wt **(Fig. 1 D)**. Only X2E4 showed almost wt growth on J774.1 macrophages, but also exhibited an intermediary phenotype in the NO release assay. The same Tn mutants were as well assayed for growth and preventing the LPS induced NO release using RAW murine macrophages. Surprisingly, differences using J774.1 or RAW murine macrophages were found **(Fig. 1 A/B and C/D)**. Mainly X7B9 and X2E4 exhibited differences in growth on RAW macrophages as compared to J774.1 cells. Overall, a positive correlation between a wt growth and the ability to block the LPS induced NO release was found for both murine macrophage cell lines.

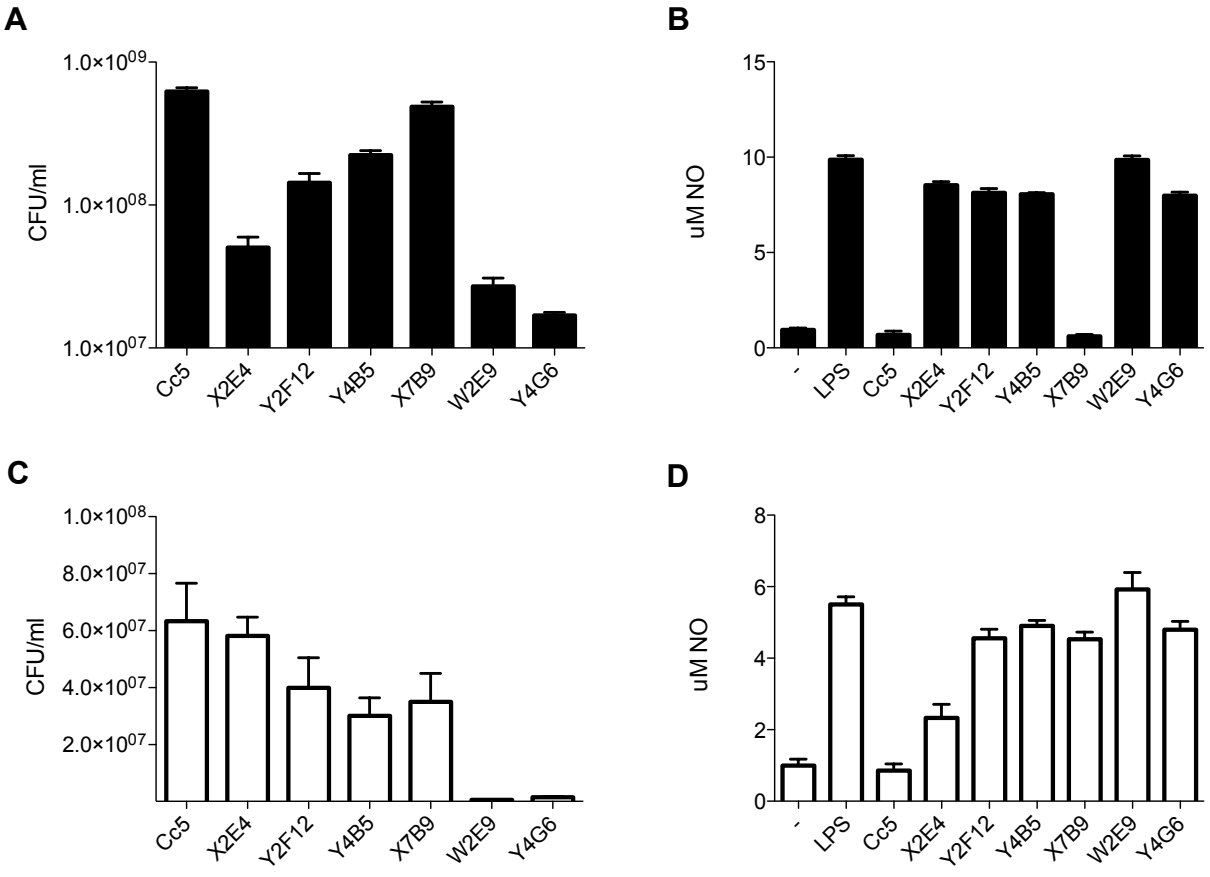



**Figure 1. *C. canimorsus* 5 Tn-mutants are deficient in growth on macrophages and in blocking the LPS induced NO release by murine macrophages.** (A) Growth on RAW264.7 macrophages 24 hours post-infection with MOI 50 of *C. canimorsus 5* wild-type (Cc5) and the Tn mutants and (B) NO release of RAW264.7 macrophages stimulated with LPS and infected with the indicated strain at MOI 50 for 24h. (C) Growth on J774.1 macrophages 24 hours post-infection with MOI 50 of Cc5 and the Tn mutants and (D) NO release of J774.1 macrophages stimulated with LPS and infected with the indicated strain at MOI 50 for 24h.

### 4.2.3 *C. canimorsus* growth on eukaryotic cells and ability to block the NO release by LPS stimulated macrophages are positively correlated.

Multiple patient-derived *C. canimorsus* strains (Cc2-Cc12) as well as dog mouth derived strains (D7-D101) were tested for growth on RAW macrophages and blocking the LPS induced NO release **(Fig. 2)**. Only few strains were able to block the LPS induced NO release **(Fig. 2 B)**, and exactly these strains were found to grow best **(Fig. 2 A)**. Only D101, which seems to grow above the average, was found not to block the NO release by LPS stimulated macrophages.

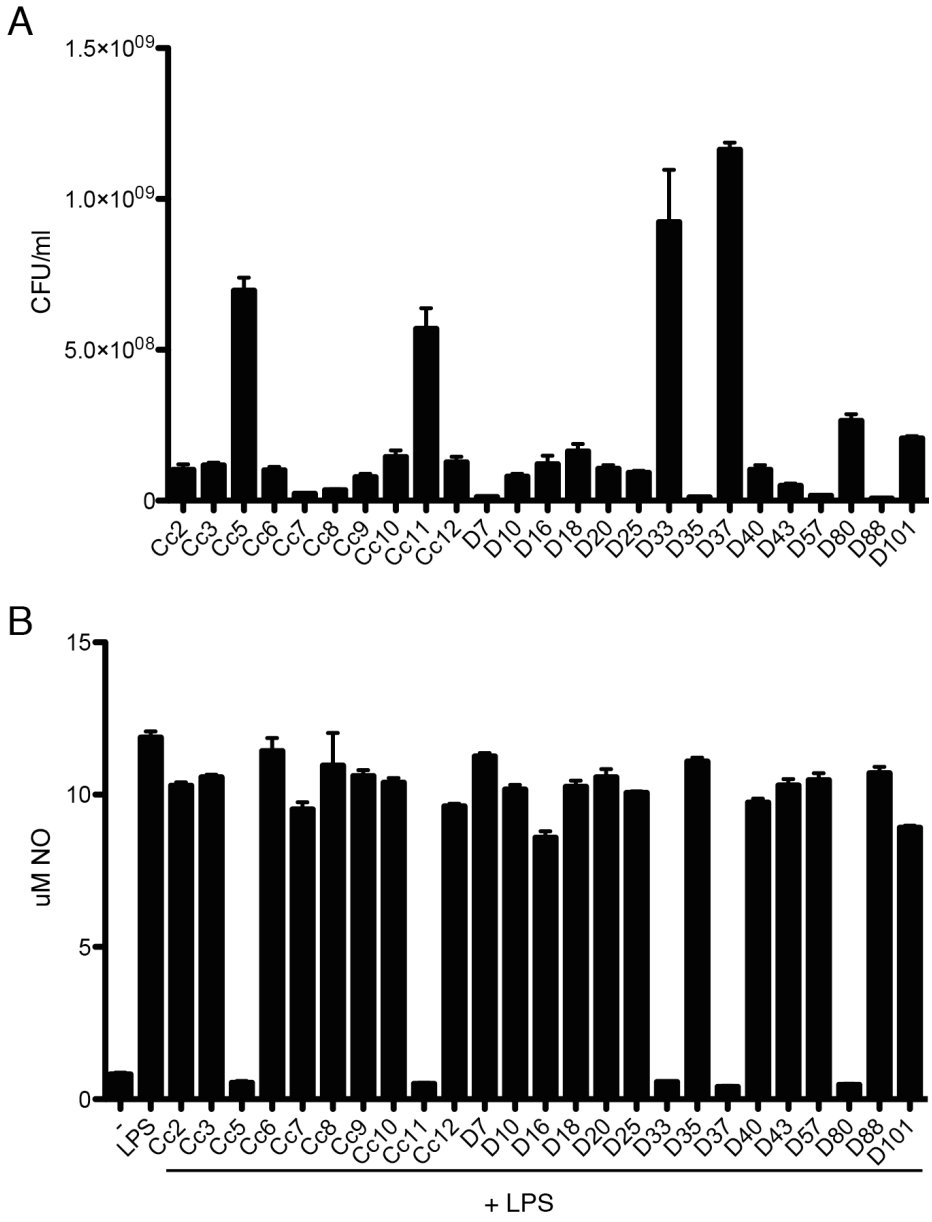



**Figure 2. Only a restricted set of *C. canimorsus* strains has the ability to grow well on macrophages and to block the NO release by LPS stimulated macrophages.** (A) Growth on RAW264.7 macrophages 24 hours post-infection with *C. canimorsus* (MOI 50) patient derived strains (labeled Cc2-12) and strains isolated from dog's mouth (labeled d7-d101). (B) NO release of RAW264.7 macrophages stimulated with LPS and infected with the indicated strain at MOI 50 for 24h.

### 4.2.4 The NO release blocking factor is secreted, small and heat stable.

To address the question, if *C. canimorsus* interfere with macrophages by releasing a factor, we performed assays with conditioned medium (CM). To prepare Cc5 CM, RAW murine macrophages were infected with Cc5 for 24h, the supernatant was collected and sterile filtered (0.22 μm) and occasionally further processed. The Cc5 CM was found to mediate the immunomodulatory effect, even if filtered through a 3kDa-cutoff filter unit **(Fig. 3 A)**. The effect of Cc5 CM on RAW macrophages was found to be concentration dependent, as dilution with fresh growth medium led to an increase in NO release **(Fig. 3 B)**. Increasing MOI and time of infection also affected the blocking of the NO release **(Fig. 3 C)**, again pinpointing the concentration dependency of the observed effect. The NO release blocking molecule was further found to be heat stable **(Fig. 3 D)**. Interestingly, growth medium that has been left on macrophages for 24 h (termed old medium) affected the NO release as well, showing the dependency of RAW macrophages on proper cell culture conditions for effective response to bacterial stimuli as LPS. Nevertheless, the effect of *C. canimorsus* conditioned medium was found to be significantly stronger than the effect of "old medium". The effect on NO-release by *C. canimorsus* conditioned medium was not restored upon addition of fresh FCS **(Fig. 3 E)**. Therefore the effect seems not to be due to the bacterial consumption of a medium component but rather due to the release of a small thermostable molecule affecting murine macrophage function.

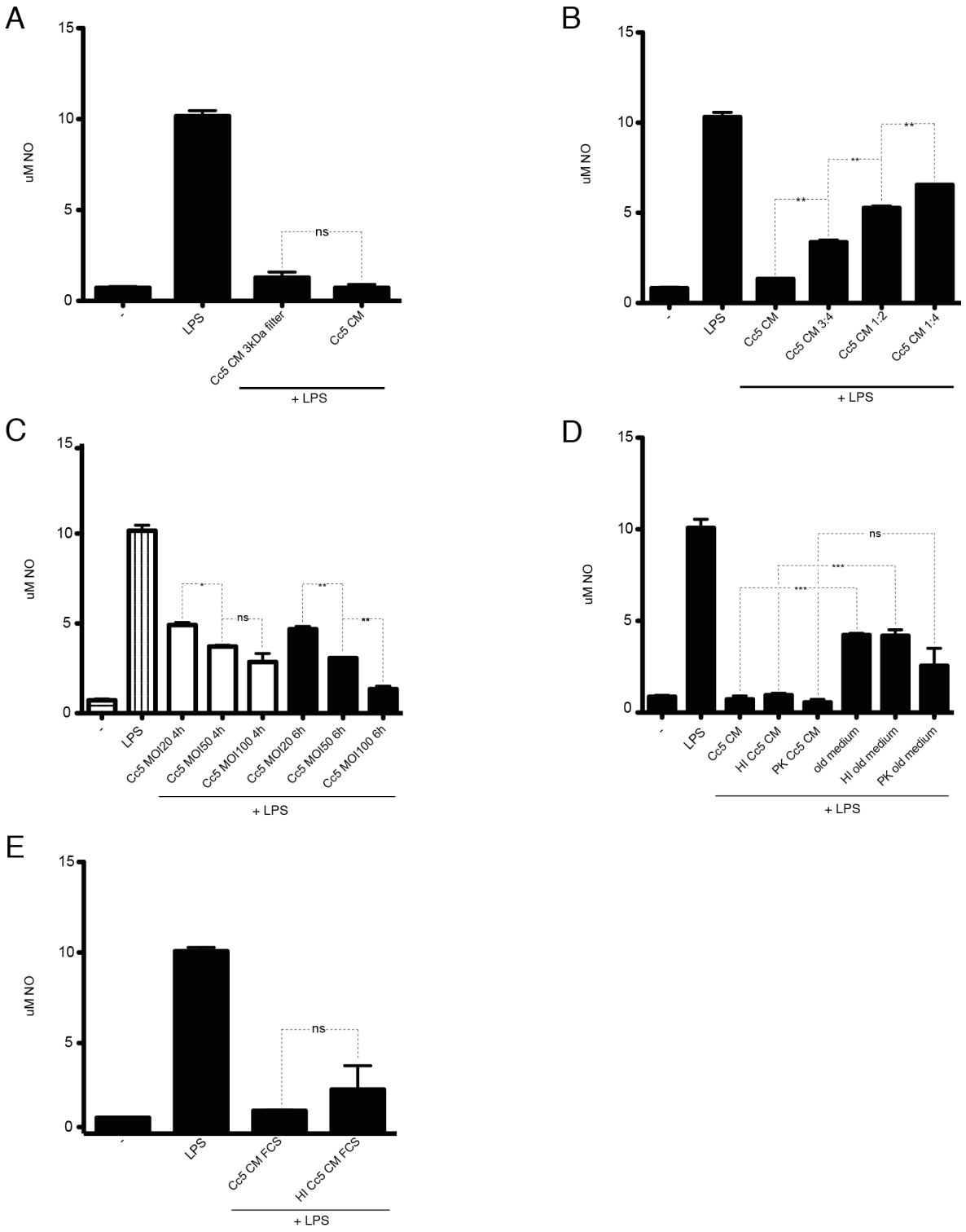



**Figure 3. The NO release blocking factor is secreted, small and heat stable. The effect is concentration dependent and can't be compensated by addition of extra FCS.** (A) RAW murine macrophages were infected at MOI 50 with *C. canimorsus* 5. 24h

post infection the supernatant was collected and sterile filtered (0.22 um), called conditioned medium. In one case the conditioned medium filtered through a 3kDa filter unit. This conditioned medium was put on pre-seeded RAW cells and they were stimulated with LPS for further 24h before NO release was measured. (B) *C. canimorsus* 5 conditioned medium was prepared as in (A) and diluted with fresh medium to the extent indicated and was put on pre-seeded RAW cells. They were then stimulated with LPS for further 24h before NO release was measured. (C) RAW macrophages were stimulated with LPS and co-infected with the indicated MOI of *C. canimorsus* 5 for 4h or 6h before NO release was measured. (D) *C. canimorsus* 5 conditioned medium was heat inactivated (HI) or treated with proteinase K (PK). As control the same treatment was performed with medium that has been on top of RAW macrophages for 24h (labeled “old medium”). The treated medium was put on pre-seeded RAW cells and they were stimulated with LPS for further 24h before NO release was measured. (E) *C. canimorsus* 5 conditioned medium was supplemented with extra FCS (10% v/v) and put on pre-seeded RAW cells and they were stimulated with LPS for further 24h before NO release was measured.

### 4.2.5 Succinate and Acetate are the main metabolic end product in *C. canimorsus* 5 culture supernatant.

In order to identify the Cc5 released molecule that affects macrophages, NMR analysis of Cc5 CM was performed. NMR samples were prepared from 400 µl of Cc5 CM by adding 5% $D_2O$ as described in the materials section. An overview spectrum of the supernatant from infected cultures is depicted in **Fig. 4 A**. Resonances close to water (4.78 ppm) are obscured due to solvent suppression. Selected regions from the spectra from the infected (+) and not-infected (-) cultures, as well as of 3 mM Succinate (suc) and 3 mM Acetate (ac) dissolved in (-) medium are shown **(Fig. 4 B)**. In the infected sample (+), two resonances (2.39 ppm and 1.91 ppm) are more intense than in the non-infected control (-). Data from *C. ochracea* (Kapke et al., 1980) indicate that Succinate and/or Acetate are the metabolites most likely to have higher concentrations. This assumption was confirmed by the observation of the respective resonances (2.39 ppm, suc) and (1.91 ppm, ac) in the control samples prepared from Succinate (suc) and Acetate (ac) dissolved in (-) medium. Using the NMR peak intensities of the supernatant and control spectra concentrations of these metabolites were determined. Succinate was found at 1.82 mM in the infected sample (and at 0.14 mM in the uninfected reference), while Acetate was found at 1.75 mM in the infected cell culture supernatant (compared to 0.17 mM for the uninfected reference) **(Fig. 4 C)**. Cc5 CM as prepared for the NMR analysis (without $NaN_3$) was confirmed to have full activity on RAW macrophages **(Fig. 5 A)**, thus measured concentrations must be sufficient to fulfill the immune suppression.

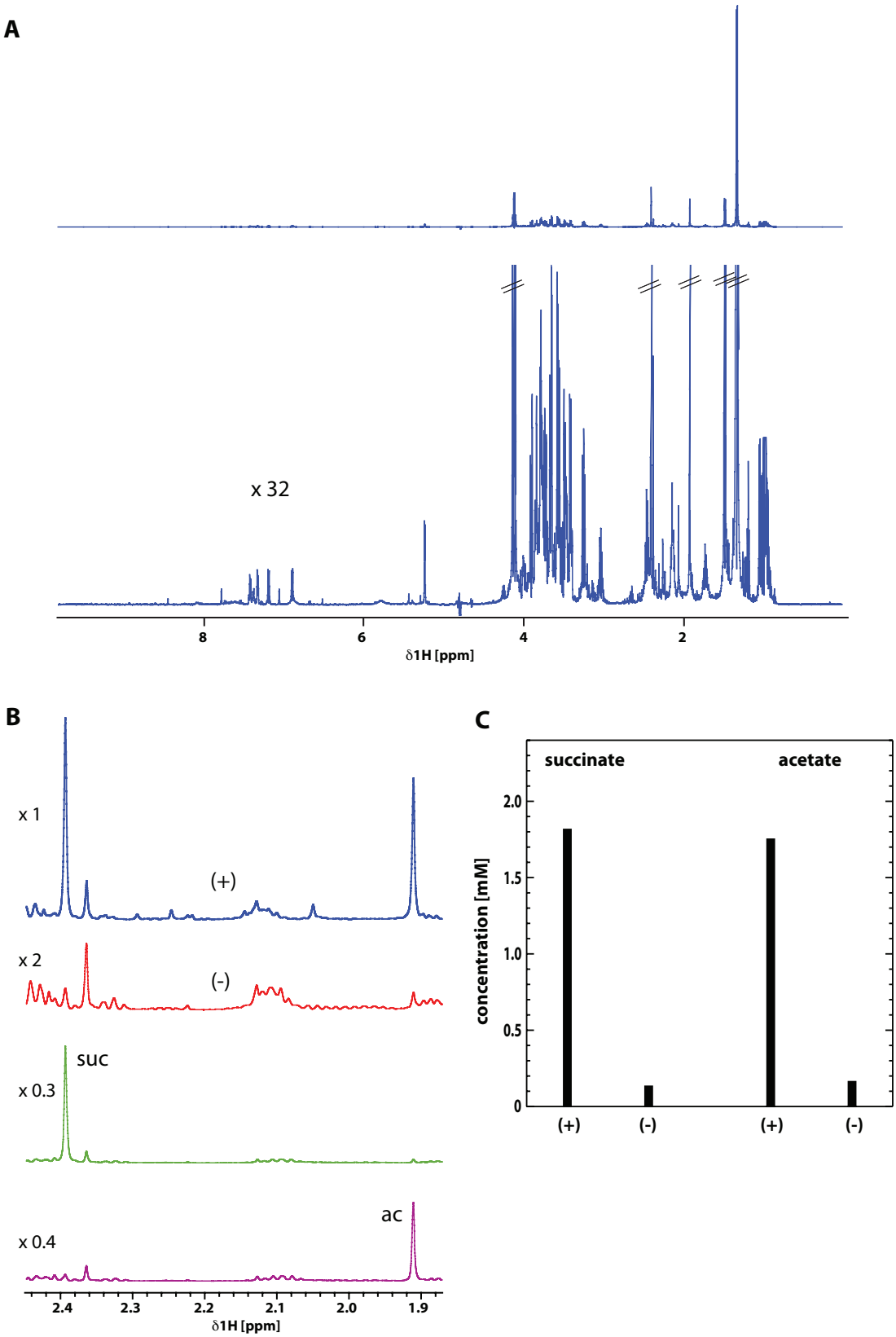



**Figure 4. NMR analysis of the supernatant of Raw 264.7 macrophages cultures infected (+) or not-infected (-) with Cc5 bacteria.** (A) Overview spectrum of *C. canimorsus* 5 conditioned medium as described in the material and methods.

Resonances close to water (4.78 ppm) are obscured due to solvent suppression. (B) Selected regions from the spectra from the infected (+) and not-infected (-) cultures, as well as of 3 mM Succinate (suc) and 3 mM Acetate (ac) dissolved in (-) medium. In the infected sample (+), two resonances (2.39 ppm and 1.91 ppm) are more intense than in the non-infected control (-). Data from *C. ochracea* (Kapke et al., 1980) indicate that Succinate and/or Acetate are the metabolites most likely to have higher concentrations. This assumption was confirmed by the observation of the respective resonances (2.39 ppm, suc) and (1.91 ppm, ac) in the control samples prepared from Succinate (suc) and Acetate (ac) dissolved in (-) medium. (C) Using the NMR peak intensities of the supernatant and control spectra, the following concentrations of these metabolites are determined: 1.82 mM (suc,+), 0.14 mM (suc,-), 1.75 mM (ac,+), and 0.17 mM (ac,-).

### 4.2.6 Neither Succinate nor Acetate modulate macrophage immune response to LPS at relevant concentration.

To see if Succinate or Acetate is the Cc5 factor affecting macrophages, we have performed further assays. Medium that has been left for 24h on RAW macrophages was supplemented with Succinate, Acetate or Succinate and Acetate. The medium of pre-seeded RAW macrophages was replaced with this Succinate/Acetate containing media and the cells were stimulated with LPS followed by an NO release assay. Up to 10mM Succinate, Acetate and Succinate/Acetate did not affect LPS induced NO release by RAW macrophages **(Fig. 5 B)**. 10mM Succinate or Acetate is at least 5 times more than was measured in Cc5 CM. Hence, neither Succinate nor Acetate nor a

combination of both account for the inhibition of the LPS induced NO release by macrophages.

### 4.2.7 Ammonia at relevant concentration is not responsible for the block of LPS induced NO release.

Another small molecule produced by bacteria with possible effects on macrophages is ammonia. To determine the effect of ammonia on murine macrophages, different concentrations of ammonia were assayed for interference with LPS induced NO release **(Fig. 5 C)**. 50 and 100 mM ammonia drastically influenced NO release **(Fig. 5 C)**. The concentration of ammonia in *C. canimorsus* CM (prepared as before) was measured. All tested strains exhibited similar amount of ammonia **(Fig. 5 D)**, while only Cc5 affects NO release by RAW macrophages. The concentration of ammonia measured in Cc5 conditioned medium is at least five fold too low to influence LPS induced NO release **(Fig. 5 C and D)**.

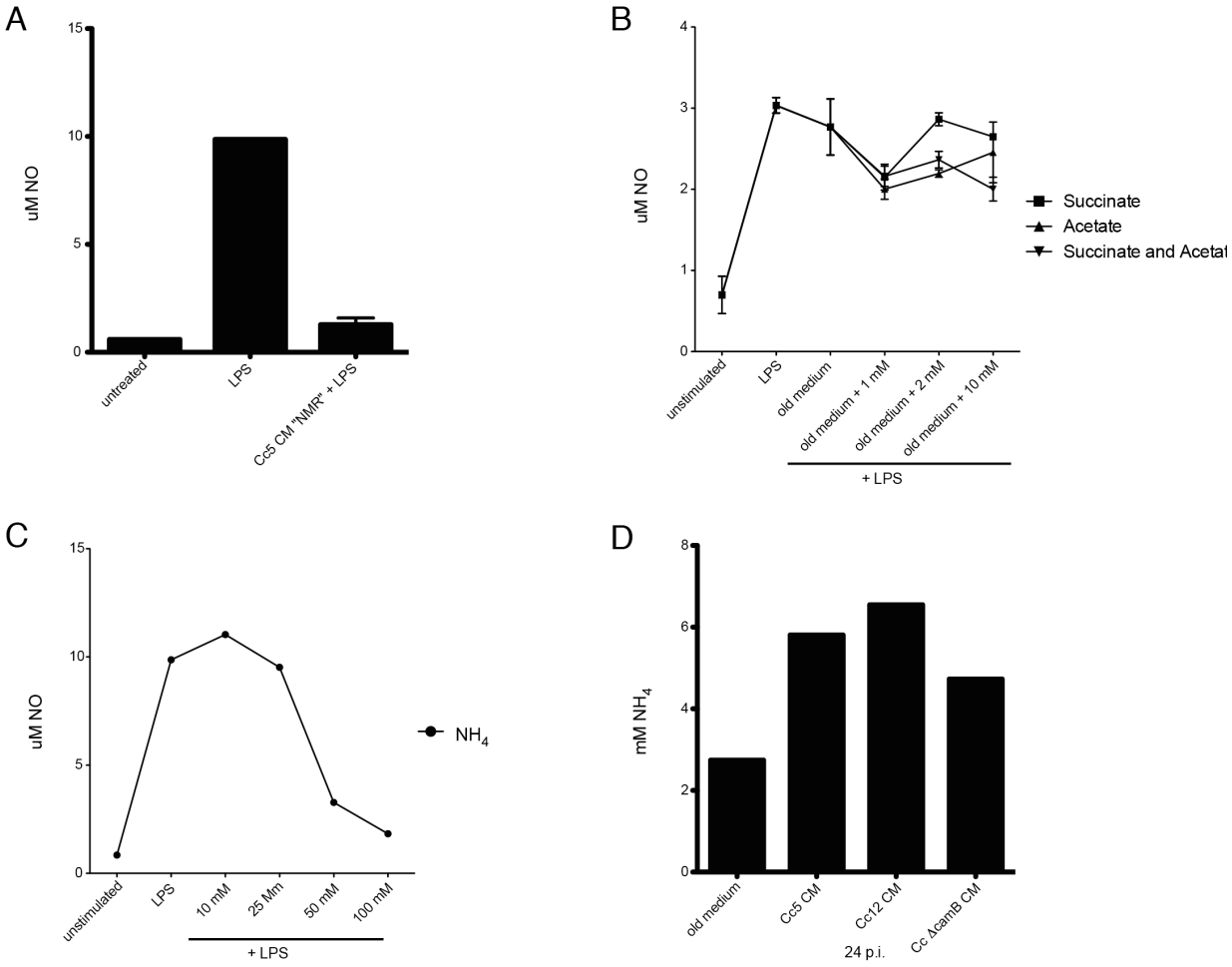



**Figure 5. Succinate, Acetate and ammonia are not responsible for the *C. canimorsus* 5 dependent blocking of the NO release by LPS stimulated macrophages.** (A) RAW murine macrophages were infected at MOI 50 with *C. canimorsus* 5. 24h post infection the supernatant was collected and sterile filtered (0.22 um), called conditioned medium. Then the conditioned medium was processed as for NMR analysis (see methods section). This conditioned medium was put on pre-seeded RAW cells and they were stimulated with LPS for further 24h before NO release was measured. (B) Medium that has been on top of RAW macrophages for 24h (labeled "old medium") was complemented with the indicated amount of Succinate, Acetate or Succinate and Acetate. This medium was put on pre-seeded RAW cells and they were stimulated with LPS for further 24h before NO release was measured. (C) as in (B) but

with indicated concentration of $NaNH_4$. (D) Amount of $NH_4$ present in the medium of RAW cells infected with the indicated *C. canimorsus* strain at MOI 50 for 24h.

### 4.2.8 pH change upon infection is not responsible for the block of LPS induced NO release.

To address the question, if a drop in pH upon release of metabolic byproducts could account for the inhibition if LPS induced NO release, we assessed the pH change in the culture supernatant upon infection of RAW macrophages. As might be expected from the difference in growth capacity **(Fig. 1 B and D),** Cc5 led to a stronger acidification of the growth medium 24 hours after infection than the Y2F12 related Cc5 Δ*camB* **(Fig. 6 A)**. To estimate the importance of the pH change, the pH of Cc5 CM was adjusted to 7.9 or 7.65. The capacity of the Cc5 CM to block the LPS induced NO release by RAW macrophages was found not depend on the reduced pH **(Fig. 6 B).**

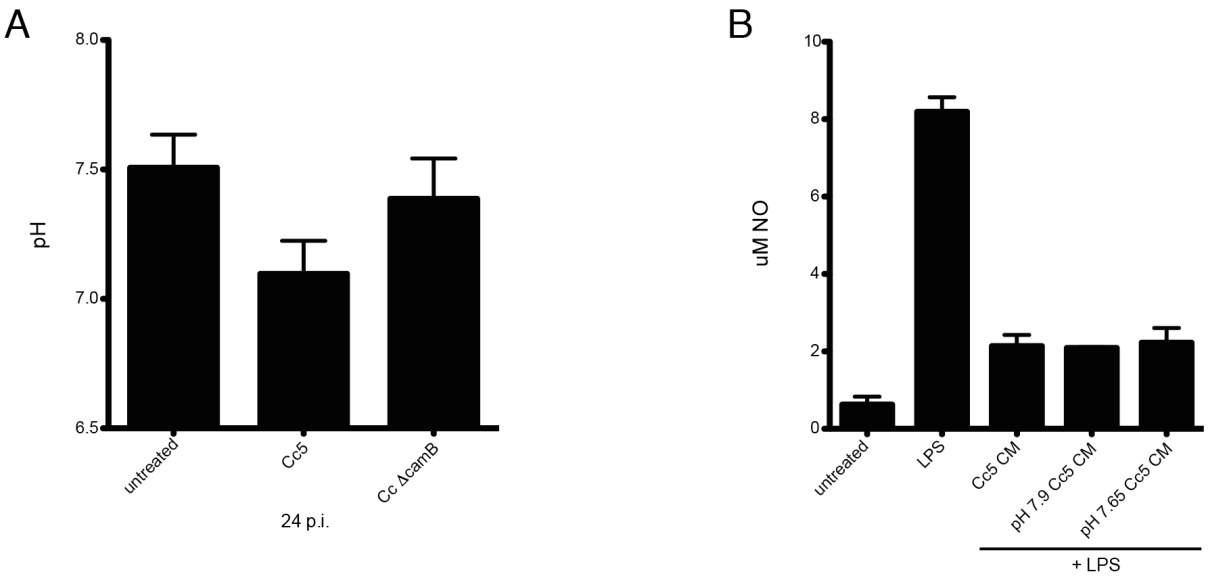



**Figure 6. The effect of *C. canimorsus* on LPS stimulated macrophages is not mediated by changes in pH.** (A) RAW murine macrophages were infected at MOI 50 with the indicated *C. canimorsus* strain. 24h post infection the supernatant was collected and sterile filtered (0.22 um) to yield conditioned medium. Then the pH was measured using a pH-meter. (B) *C. canimorsus* 5 conditioned medium prepared as in (A) was titrated to pH 7.65 or 7.9. This medium was put on pre-seeded RAW cells and they were stimulated with LPS for further 24h before NO release was measured.

### 4.2.9 Discussion

In a genome wide Tn-screen we have identified several mutants deficient in blocking the NO release of LPS stimulated murine macrophages, an effect of *C. canimorsus* 5 on macrophages described previously (Shin et al., 2007). We have compared the growth capacity on macrophages and the above-mentioned immunosuppressive effect of the Tn mutants and a *C. canimorsus* strain collection. Interestingly, a positive correlation was found for growth on macrophages and the ability to modulate the NO release of LPS stimulated macrophages. The relation of growth on eukaryotic cells and blocking the NO release as well matches the genes hit by the Tn. In mutants affected in blocking the LPS induced NO release the Tn located in genes for general metabolism and in genes known to be important for nutrient acquisition (Mally et al., 2008; Manfredi et al., 2011b; Renzi et al., 2011). The striking growth dependency hints at a metabolite rather than an enzyme dependent effect of *C. canimorsus* on macrophages.

We further show that *C. canimorsus* affects LPS stimulated macrophages by releasing a small (<3kDa), soluble and heat stable factor. Dilution of the factor-containing media led to a decrease in the immunosuppressive action, again pinpointing the concentration-dependency. The size, heat-stability and the striking effect of even a 1:2 dilution suggest a non-enzymatic effect of *C. canimorsus* on macrophages. This is in contrast to a non-identified immunosuppressive factor of *C. ochracea*, which is heat-labile and protease sensitive (Ochiai et al., 1998). Extra addition of FCS was shown here not to diminish the immunosuppressive effect, confirming that a factor is released rather than simple consumption of essential media components. *C. canimorsus* thus releases a small, heat-stable factor that interferes with macrophage stimulation by LPS.

To identify the small molecule involved in blocking the LPS induced NO release by macrophages, we have analyzed the culture supernatant of *C. canimorsus* infected macrophages. By NMR analysis Succinate (1.5 mM) and Acetate (2.2 mM) have been identified as the main metabolites released by *C. canimorsus*. However, Succinate and Acetate were shown here not be not responsible for the blocking of the LPS induced NO release by macrophages. Butyrate, a small molecule and known bacterial metabolic byproduct with immunosuppressing function, was identified only in trace amounts (data not shown). Other candidates like ammonia or a general effect of the pH drop upon infection have as well been ruled out. Therefore, the *C. canimorsus* released, small, heat-stable molecule affecting macrophages remains to be identified.

Testing old growth medium we could show that macrophages rely on optimal media conditions for proper response to LPS. The small molecule released by *C. canimorsus* might reduce the fitness of the cells under cell culture conditions used. Such cells would respond far less to LPS. It might thus be that the observed effect of conditioned medium is related to the cell culture condition without media flux and does not reflect the in vivo situation in the blood adequately.

### 4.2.10 Materials and Methods

**Chemicals.** Succinate (disodium Succinate), Acetate and Ammonium chloride were purchased from Sigma.

**Bacterial strains and growth conditions.** The strains used in this study are listed in **Table 2**. *E. coli* strains were routinely grown in LB broth at 37°C. *C. canimorsus* 5 (Shin et al., 2007) 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% $CO_2$. Selective agents were added at the following concentrations: erythromycin, 10 mg/ml; cefoxitin, 10 mg/ml; gentamicin, 20 mg/ml; ampicillin, 100 mg/ml. Heat inactivated *Y. enterocolitica* E40 were prepared by taking 1 ml of a o/n culture, centrifugation at 14000 rpm for 1 min, aspiration of the supernatant and resuspension in 1 ml PBS. The bacterial suspension was boiled at 99°C under agitation for 2 hours.

**Genetic manipulations of *C. canimorsus*.** Genetic manipulations of *Cc5* wt has been described (Mally and Cornelis, 2008). Briefly, replacement cassettes with flanking regions spanning approximately 500 bp homologous to direct *cam*- framing regions were constructed with a three-fragment overlapping-PCR strategy. Final PCR product consisted in *camB::ermF* insertion cassette and was then digested with PstI and SpeI for cloning into the appropriate sites of the *C. canimorsus* suicide vector pMM25. Resulting plasmids were transferred by RP4-mediated conjugative DNA transfer from *E. coli* S17-1 to *C. canimorsus 5* to allow integration of the insertion cassette.

Transconjugants were then selected for presence of the *ermF* cassette and checked for sensitivity to cefoxitin. Deletion of the appropriate regions was verified by PCR.

**Genome wide Tn4351 screen for blocking LPS induced NO release by murine J774.1 macrophages.** Random Tn4351 mutants were generated as described previously (Mally and Cornelis, 2008). J774.1 macrophages were seeded at $3x10^4$ cells/well in 100µl in 96-well plates and incubated o/n. Tn mutant bacteria were grown in 96-well plates and subsequently diluted in a new plate to an $OD_{590}$ of 0.12. 10 µl bacterial solution was added to the corresponding well to be infected (MOI of 20). The macrophages were stimulated with an MOI = 20 equivalent of heat inactivated *Y. enterocolitica* E40 (prepared as described above). The cells were incubated for 24 h at 37°C and 5% $CO_2$. Triplicates of 50 µl of each sample was mixed with 50 µl of modified Griess regent in 96-well plates. The plates were incubated in the dark for 10 min and absorption was read at 575nm using a using a spectrophotometer (BioRad). The insertion site of the Tn was mapped by arbitrary PCR as described previously (Mally et al., 2008). The Tn insertion in Y2F12 is such, that it only affects *camB*, as was shown by complementation studies (Mally, 2008). Hence, in certain experiments the Tn mutant is replaced by a deletion mutation of *camB*.

**Cell culture and growth of *C. canimorsus* on murine macrophages.** J774.1 murine monocyte-macrophages (ATCC TIB-67) were cultured in RPMI-1640 (Invitrogen) supplemented with 2 mM L-Glutamine (Invitrogen), 1 mM sodium pyruvate and 10% (v/v) fetal calf serum (Invitrogen). RAW264.7 murine macrophages (ATCC TIB-71) were cultured in DMEM (Invitrogen) supplemented with 1 mM sodium pyruvate and 10% (v/v) fetal calf serum (Invitrogen). Cells were grown in medium without antibiotics in a

humidified atmosphere enriched with 5% CO2 at 37°C. Cells were seeded in 24-well plates at $0.5\times10^5$ cell/ml and incubated o/n. Bacteria were harvested by gently scraping colonies off the agar surface and resuspending them in PBS. The cells were infected with a total of $5\times10^6$ bacteria per well (MOI = 50) in a final volume of 1.033 ml medium for 24 h. Samples were diluted in PBS and plated on HIA 5% sheep blood plates, incubated for 2 days at % CO2 at 37°C before colonies were counted.

**Nitric-oxide (NO) release assay.** J774.1 or RAW264.7 murine macrophages were seeded in 24-well plates at $0.5\times10^5$ cell/ml and incubated o/n. Bacteria were harvested by gently scraping colonies off the agar surface and resuspending them in PBS. The cells were infected with a total of $5\times10^6$ bacteria per well (MOI = 50) and stimulated with an MOI = 50 equivalent of heat inactivated *Y. enterocolitica* E40 as LPS stimulus (prepared as described above) in a final volume of 1.066 ml medium and incubated for 24 h. Triplicates of 50 µl of each sample was mixed with 50 µl of modified Griess regent in 96-well plates. The plates were incubated in the dark for 10 min and absorption was read at 575nm using a spectrophotometer (BioRad).

**NO release assay with conditioned medium and modified conditioned media.** RAW264.7 murine macrophages were seeded in 24-well plates at $0.5\times10^5$ cell/ml and incubated o/n. Bacteria were harvested by gently scraping colonies off the agar surface and resuspending them in PBS. The cells were infected with a total of $5\times10^6$ bacteria per well (MOI = 50) and incubated for 24 h. The supernatant was then taken and sterile filtered (0.22 um filter, Millipore) to give conditioned medium. This conditioned medium was replacing the growth medium of RAW macrophages seeded in 24-well plates the

day before (at $0.5\times10^5$ cell/ml). These cells were stimulated with an MOI = 50 equivalent of heat inactivated *Y. enterocolitica* E40 as LPS stimulus (prepared as described above) in a final volume of 1.033 ml medium and incubated for 24 h. Triplicates of 50 µl of each sample was mixed with 50 µl of modified Griess regent in 96-well plates. The plates were incubated in the dark for 10 min and absorption was read at 575nm using a using a spectrophotometer (BioRad). The conditioned medium was modified by further filtration trough a 3kDa filter unit (Vivaspin, Sartorius), it was diluted with fresh growth medium, heat treated (99°C, 20 min), the pH was adjusted using 1M phosphoric acid or supplemented with different concentration of Succinate, Acetate or Ammonium.

**Identification of the main metabolic end product in Cc5 culture supernatants.** *Cc5* were grown for 24 hours in the presence of murine macrophages (Raw 264.7) in DMEM supplemented with 1 mM Na-Pyruvate and 10% v/v fetal calf serum. The medium was collected and the bacteria were pelleted by centrifugation (5 minutes, 15000 rcf, 4 °C). 0.1% $NaN_3$ was added to the supernatant and the pH was adjusted to 7.5 with phosphate-buffer (500 mM, pH 8). The medium was finally passed through a 0.22 um filter and a 3 kDa cut-off filter (Vivaspin, Sartorius). NMR samples were prepared from 400 µl of this medium by adding 5% $D_2O$ and placed into 5 mm standard NMR tubes. NMR measurements were carried out at 24 ˚C on a Bruker Avance DRX 600 spectrometer equipped with a triple resonance pulse field gradient probe. 1D proton NMR spectra were recorded with the excitation sculpting scheme (pulseprogram zgesgp in the standard Bruker library) as described previously (Hwang and Shaka, 1998) achieving water suppression by gradient dephasing of the water resonance. The proton carrier was set to the water frequency for solvent suppression. Spectra were

recorded with 57344 complex points and acquisition times of 1.99 seconds. The total experimental time was 3 minutes and 26 seconds for the accumulation of 64 transients. Spectra were processed and evaluated using the software Topspin 2.1.6 (Bruker).

**Ammonium concentration determination.** Ammonium concentration in *C. canimorsus* conditioned medium was determined using the ammonia assay kit (Sigma) according to the manufacturers instructions.

**pH measurement.** pH was determined using a pH-meter (Mettler Toledo).

**Statistical analysis.** Unpaired two-tailed students t-test was performed using Prism software (graphpad). Where * means $p < 0.05$, ** that $p < 0.01$ and *** that $p < 0.005$.

**Table 2. Bacterial strains used in this study**

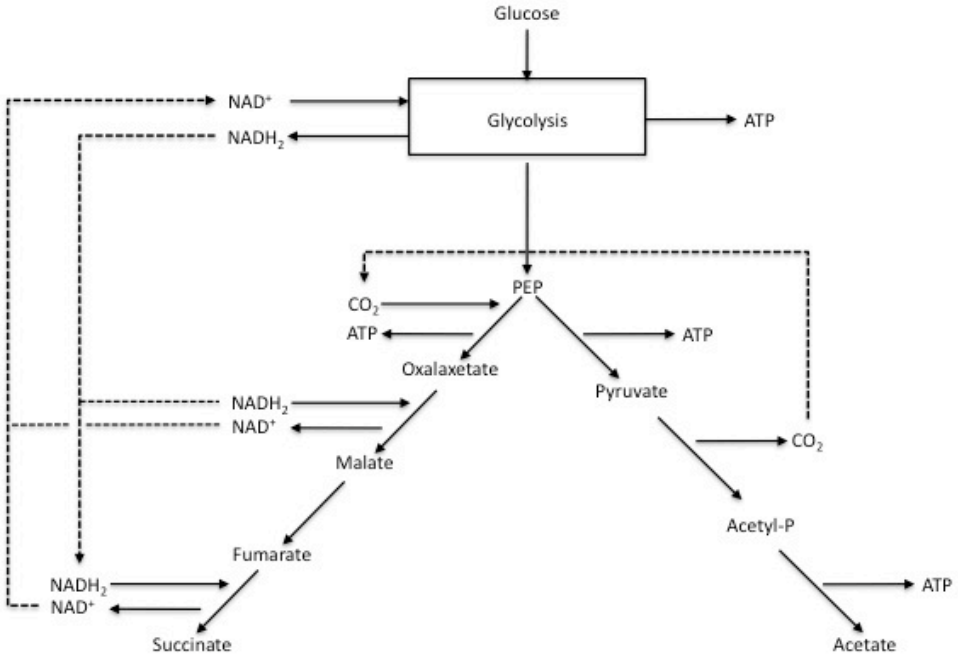
| Bacterial strains | Description or genotype | Reference or source |
|---|---|---|
| ***E. coli*** | | |
| S17-1 | *hsdR17 recA1* RP4-2-*tet::*Mu1*kan::*Tn7; $Sm^r$ | (Simon et al., 1983) |
| Top10 | F- *mcrA* Δ*(mrr-hsd*RMS-*mcr*BC*)* *φ80lacZ*ΔM*15* Δ*lac*X74 *rec*A1 *ara*D139 Δ*(araleu)*7697 *gal*U *gal*K *rps*L*, end*A1 *nup*G*.* $Sm^r$ | Invitrogen |
| ***Y. enterocolitica*** | | |
| E40 | - | (Sory et al., 1995) |
| ***C. canimorsus*** | | |
| *Cc5* | Human fatal septicemia after dog bite 1995 | (Manfredi et al., 2011a; Shin et al., 2007) |
| *Cc2* | Human fatal septicemia after dog bite | (Shin et al., 2007) |
| *Cc3* | Human septicemia | (Shin et al., 2007) |
| *Cc6* | Human infection, from Katholic University Leuven, Belgium, 1996 | This study |
| *Cc7* | Human septicemia | (Shin et al., 2007) |
| *Cc8* | Human septicemia, from Prof. M. Delmée, Louvain, Belgium, 2004 | This study |
| *Cc9* | Human septicemia, BCCM/LMG 11510 | (Shin et al., |

| | | |
|---|---|---|
| | | 2007) |
| *Cc10* | Human fatal septicemia after dog bite, ATCC 35978 | (Shin et al., 2007) |
| *Cc11* | Human septicemia, BCCM/LMG 11511 | (Shin et al., 2007) |
| *Cc12* | Human fatal septicemia after dog bite, ATCC 35979 | (Shin et al., 2007) |
| *X2E4* | Δ*Ccan*_3130, Dihydroorotase | This study |
| *Y2F12* | Δ*Ccan*_20110, hypothetical | This study |
| *Y4B5* | Δ*Ccan*_05080, hypothetical | This study |
| *X7B9* | Δ*Ccan*_06510, Glutamine synthetase | This study |
| *W2E9* | Δ*Ccan*_04790, Sialidase | This study |
| *Y4G6* | Δ*Ccan*_08710, SusD of PUL5 | This study |
| Δ*camB* | Mutation of the last gene in the Y2F12 operon | (Mally, 2008) |
| *CcD7* | Isolated from dogs mouth | (Mally et al., 2009) |
| *CcD10* | Isolated from dogs mouth | (Mally et al., 2009) |
| *CcD16* | Isolated from dogs mouth | (Mally et al., 2009) |
| *CcD18* | Isolated from dogs mouth | (Mally et al., 2009) |
| *CcD20* | Isolated from dogs mouth | (Mally et al., 2009) |
| *CcD25* | Isolated from dogs mouth | (Mally et al., 2009) |
| *CcD33* | Isolated from dogs mouth | (Mally et al., 2009) |
| *CcD35* | Isolated from dogs mouth | (Mally et al., 2009) |
| *CcD37* | Isolated from dogs mouth | (Mally et al., 2009) |
| *CcD40* | Isolated from dogs mouth | (Mally et al., |

| | | |
|---|---|---|
| | | 2009) |
| *CcD43* | Isolated from dogs mouth | (Mally et al., 2009) |
| *CcD57* | Isolated from dogs mouth | (Mally et al., 2009) |
| *CcD80* | Isolated from dogs mouth | (Mally et al., 2009) |
| *CcD88* | Isolated from dogs mouth | (Mally et al., 2009) |
| *CcD101* | Isolated from dogs mouth | (Mally et al., 2009) |

### 4.2.11 Supplementaries: Metabolism of *C. canimorsus* 5

Short chain fatty acids (SCFA) are major end products of bacterial metabolism. Acetate and Succinate (a dicarboxylic acid) were reported to be the main fermentation output of *Bacteroides fragilis*, *Bacteroides ovatus* (Macfarlane and Macfarlane, 2003; Macy et al., 1978), *Capnocytophaga ochracea* (Calmes et al., 1980) and *Capnocytophaga haemolytica* (Yamamoto et al., 1994). Succinate might be decarboxylated to propionate in C-limiting conditions to regenerate $CO_2$ (Macy et al., 1978). As in *C. ochracea*, Succinate is probably built from Phosphoenolpyruvate (PEP) via intermediate production of OxalAcetate, Malate and Fumarate (Kapke et al., 1980) (see Fig. S1 for a metabolic map). During this process bacteria dispose of reducing equivalents accumulated during glycolysis and might generate ATP. Converting PEP to OxalAcetate by PEP carboxykinase (PEPCK; *Ccan*15480) or PEP carboxylase (PEPC; *Ccan*10960) is furthermore a way for bacteria to fix $CO_2$. This is in contrast to *C. ochracea* that has shown to fix $CO_2$ almost exclusively by PEPCK (Kapke et al., 1980). $CO_2$ dependent glucose fermentation leading to Succinate production reflects the capnophilia of *Capnocytophaga canimorsus*. Moreover released Succinate can be metabolised by cross-feeding bacteria (Kolenbrander et al., 2002; Macfarlane and Macfarlane, 2003). In accordance with *C. ochracea*, Acetate might be formed from PEP, the intermediates being Pyruvate and Acetyl-phosphate. Acetate formation increases ATP yield compared to Succinate formation, but does not recycle $NADH_2$ (see Fig. S1 for a metabolic map).




**Supplementary Figure 1.** Proposes metabolic pathway of Succinate and Acetate generation by *C. canimorsus*. Succinate is probably built from Phosphoenolpyruvate (PEP) via intermediate production of OxalAcetate, Malate and Fumarate. During this process bacteria dispose of reducing equivalents accumulated during glycolysis and might generate ATP. Converting PEP to OxalAcetate by PEP carboxykinase (PEPCK; *Ccan*15480) or PEP carboxylase (PEPC; *Ccan*10960) is furthermore a way for bacteria to fix $CO_2$. Acetate might be formed from PEP, the intermediates being Pyruvate and Acetyl-phosphate. Acetate formation increases ATP yield compared to Succinate formation, but does not recycle $NADH_2$.

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## 4.3 Effects of *Capnocytophaga canimorsus* on LPS sensing and signaling by macrophages

### 4.3.1 *C. canimorsus* 5 affects murine TLR4, murine CD14, phosphorylation of p38 and transcription of iNOS.

To find out at which step *C. canimorsus* interferes, we have analyzed key proteins involved in LPS sensing and signaling. Macrophages sense the LPS stimulus via the TLR4/MD-2/CD14 receptor complex {Medzhitov, 1997 #20;Shimazu, 1999 #21;Ulevitch, 1999 #22}. The signaling cascade following Toll-like receptor 4 (TLR4) dimerization includes mitogen activated protein kinases (MAPK) like p38 and JNK, NFκB nuclear translocation {Cario, 2000 #23} followed by inducible NO synthase (iNOS) dependent production and release of NO, release of TNFα and other proinflammatory cytokines {Beutler, 2000 #24}. Cell-lysates of *C. canimorsus* infected and LPS stimulated RAW macrophages were analyzed by Western-blotting for p38, p-p38 (phospho p38), TLR4 and iNOS. Cc5 infection was found to inhibit LPS induced phosphorylation of p38, while total p38 level was similar **(Fig. 7 A)**. *C. canimorsus* strains not able to block the NO release did not affect LPS dependent phosphorylation of p38 **(Fig. 7 A)**, as shown for Cc12 and the Y2F12 related mutant Cc5 Δ*camB*. In uninfected cells, LPS stimulation led as expected to transcription of iNOS. Again Cc5 but not Cc12 or Cc5 Δ*camB* appeared to inhibit expression of iNOS upon LPS stimulation **(Fig. 7 B)**. By RT-PCR we have further observed that the transcription of iNOS mRNA upon LPS stimulation is blocked by Cc5. Again, Cc12 and Cc5 Δ*camB* did not alter iNOS mRNA levels upon LPS stimulation **(Fig. 7 D)**. In accordance with the lack of downstream signaling events as phosphorylation of p38 and iNOS transcription, TLR4 was shifted in size upon Cc5 infection, but not or only partially upon infection with Cc12 or Cc5 Δ*camB* **(Fig. 7 C)**. The shifted TLR4 band might represent proteolytically cleaved or deglycosylated TLR4. It might be that the smaller TLR4 cannot respond to

LPS and thus the cells lack all TLR4 dependent downstream signaling events. Even more CD14, one of the co-receptors of TLR4, was found slightly size shifted upon infection with Cc5, but not or far less upon infection with Cc5 Δ*camB* **(Fig. 7 E)**. In the case of CD14 the lysed cells have been treated with PNGaseF, cleaving all N-linked sugars, to see if the size shift is due to complete deglycosylation of N-linked sugars. The observed size shift of CD14 upon PNGaseF treatment is by far bigger than the observed smeary shift upon Cc5 infection. Therefore Cc5 infection leads either to proteolytic cleavage of CD14, only partial deglycosylation of N-linked glycans or deglycosylation of the O-linked sugars present on CD14. Summarizing, Cc5 infected and LPS stimulated macrophages lack TLR4 downstream signaling events and exhibit size-shifted versions of TLR4 and CD14.

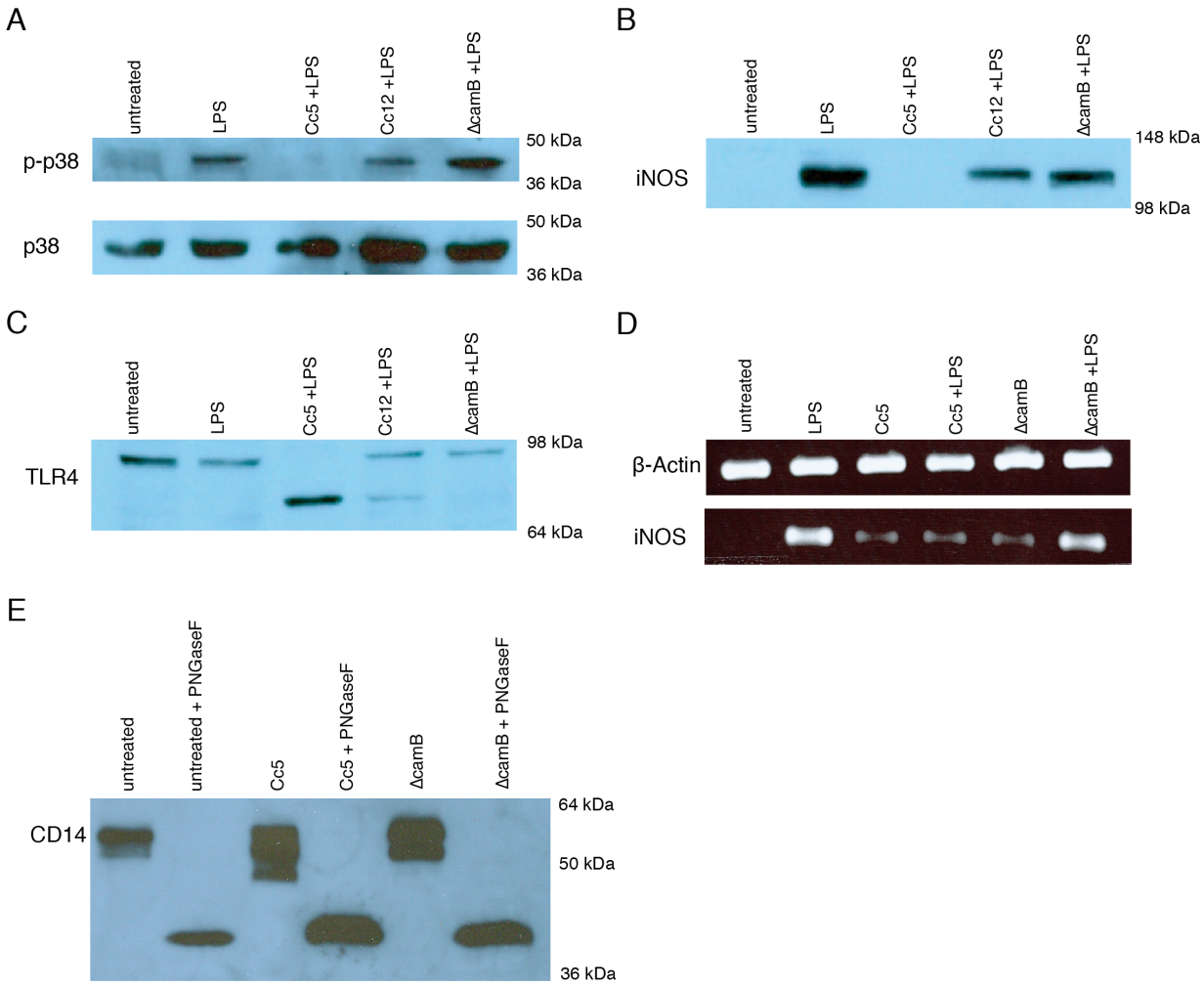


**Figure 7. *C. canimorsus* 5 prevents LPS dependent phosphorylation of p38, induction of iNOS and affects TLR4 and CD14.** (A-C) J774.1 macrophages were infected for 24h with the indicated strain at MOI 50, stimulated with LPS and further incubated for another 24h. Cells were washed, lysed and boiled before the samples were charged on SDS-page followed by western-blot analysis. (A) Western-blot anti-p38 and anti- phospho-p38. (B) Western-blot anti-iNOS. (C) Western-blot anti-TLR4. (D) iNOS RT-PCR on J774.1 macrophages infected and stimulated as in (A-C). β-actin was included as a control. (E) Western-blot anti-CD14.

### 4.3.2 Recombinant human TLR4 slightly shifts in size upon treatment with Cc5, but less upon treatment with Y2F12 related mutant Cc5 Δ*camB*.

In order to determine the nature of the size-shifting effect of Cc5 on TLR4, recombinant human (rh) TLR4 and MD-2 were incubated with a suspension of *C. canimorsus*. Western-blot revealed recombinant TLR4 (**Fig. 8 A**, up) and MD2 (**Fig. 8 A**, down). While rh MD-2 stayed unaffected by the Cc5 treatment, rh TLR4 slightly shifted in size upon Cc5 treatment. The small size shift can clearly not correspond to a complete deglycosylation of TLR4, which carries several big branches of high-mannose type N-linked sugars. To differentiate proteolytically cleaved or deglycosylated TLR4, we performed lectin staining with Galanthus nivalis agglutinin lectin (GNA) staining terminal mannose linked to mannose **(Fig. 8 B)**, with *Sambucus nigra* lectin (SNA) that recognizes terminal sialic acid (2-6) linked to Gal or to GalNAc **(Fig. 8 C)**, and with Datura stramonium agglutinin lectin (DSA) labeling Gal linked to GlcNAc **(Fig. 8 D)**. In agreement with the presence of a sialidase in Cc5 [see (Mally et al., 2008)], the SNA signal was decreased upon treatment with Cc5. While hybrid and complex N-linked glycoproteins have been reported to be almost completely cleaved by the Cc5 endo-b-N-acetylglucosaminidase GpdG (Renzi et al., 2011), the high-mannose N-linked sugars on TLR4 seem not to be cleaved to this extent. This is in agreement with other endo-b-N-acetylglucosaminidases that were reported not to be active on high-mannose type polysaccharide branches. Hence, it seems that only terminal sialic acids are cleaved off the glycan chains on TLR4 by Cc5. This might account for the minimal size shift observed with rh TLR4.

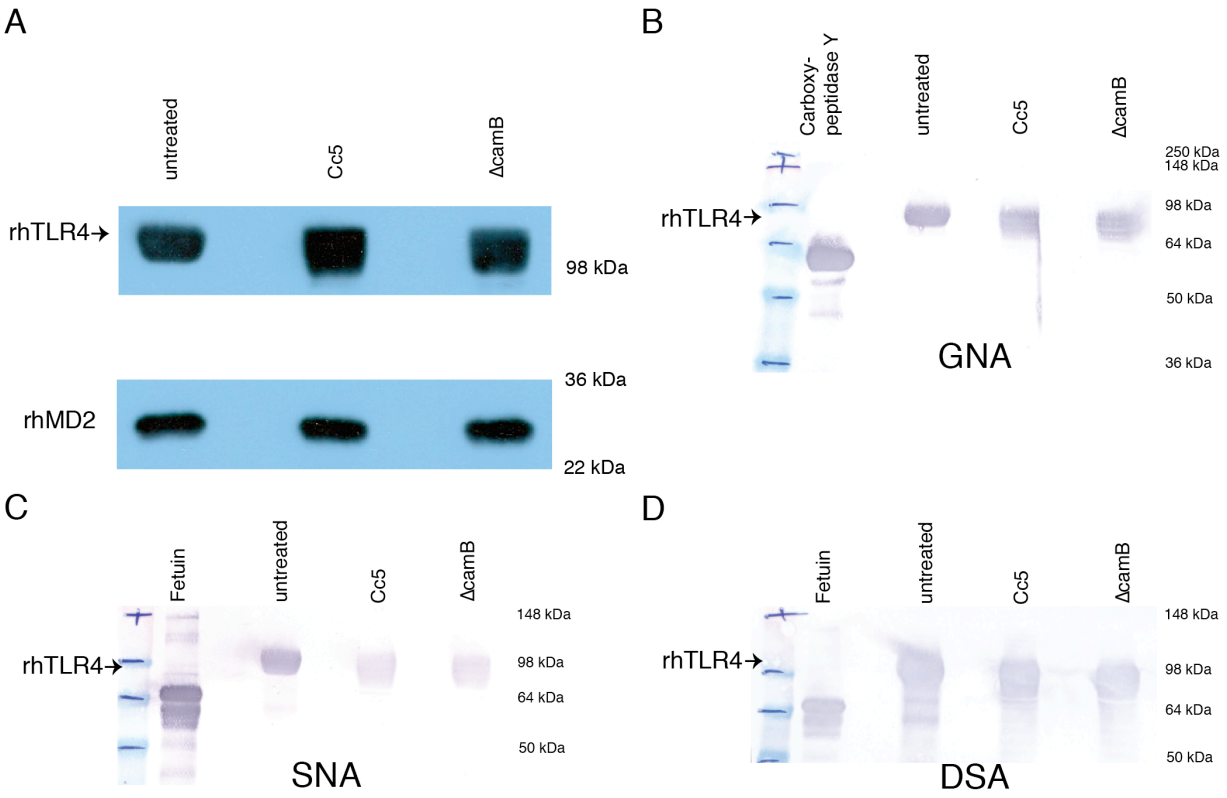


**Figure 8. Recombinant human TLR4 slightly shifts in size upon treatment with *C. canimorsus* 5.** (A-D) Recombinant human TLR4/MD-2 were incubated with either DPBS or with a suspension of *C. canimorsus* ($OD_{600}$ = 12) in DPBS. The samples were incubated for 90 min at 37°C, the tubes were centrifuged twice and supernatant was recovered, boiled and charged on SDS-Page followed by Western-blot analysis. (A) Western-blot anti-Flag revealing recombinant TLR4 (up) and MD2 (down). (B-D) TLR4 glycosylation state was determined by staining with the Galanthus nivalis agglutinin lectin (GNA) staining terminal mannose linked to mannose (B), the *Sambucus nigra* lectin (SNA) that recognizes terminal sialic acid (2-6) linked to Gal or to GalNAc (C), and Datura stramonium agglutinin lectin (DSA) labeling Gal linked to GlcNAc (D).

## Discussion

To elucidate the level at which *C. canimorsus* interferes with the macrophage signaling pathway we studied key events upon LPS stimulation. Following LPS stimulation, *C. canimorsus* infected macrophages lack iNOS transcription, as shown by RT-PCR and Westernblotting, which explains perfectly the absence of NO in the culture supernatant. More upstream in the TLR4 signaling pathway we found p38 not to be phosphorylated when the cells were stimulated with LPS and infected with *C. canimorsus* 5. This indicates that the immunosuppression occurs upstream or at the level of the MAPK. For an extracellular bacterium like *C. canimorsus*, the most obvious target for the interference with the TLR4 signaling pathway is the surface exposed TLR4 itself or one of the TLR4 related proteins like CD14, MD-2 or LBP. Infected cells clearly showed a size shifted TLR4 band in Western blot analysis, indicating that *C. canimorsus* interferes with the LPS signaling at the level of TLR4. To assess whether the size shift was due to proteolysis or deglycosylation, purified recombinant TLR4 was incubated with *C. canimorsus* and analyzed for size and glycosylation. The high-Mannose type N-linked glycosylation of TLR4 seems not to be affected by *C. canimorsus*, which is in contrast to the almost complete cleavage of hybrid and complex N-linked glycan chains (Renzi et al., 2011). Only terminal sialic acid were found to be cleaved off by *C. canimorsus*, which is in agreement with the presence of a sialidase (Mally et al., 2008). The observed small size shift of recombinant human TLR4 could be due to the partial loss of terminal sialic acids. However, the observed big size shift of TLR4 in the case of infected macrophages can't be explained by the minimal deglycosylation found and might be due to proteolysis, either by bacterial proteases or by host proteases. It has to be noted that the prolonged infection time (48h) with *C. canimorsus* led in some cases

to some cell death, whereat only the faster growing *C. canimorsus* strains affected cell viability (data not shown). Cell death and the related proteases, like Caspases, could therefore account for the observed limited size-shift of TLR4 in the case of infected macrophages. Caspase3 was shown to be slightly activated after 48h infection with Cc5 or Cc11 (data not shown). But it could as well be that the recombinant TLR4/MD-2 were not incubated long enough with *C. canimorsus* to obtain the full deglycosylation, as has been seen with IgM deglycosylation (F. Renzi, personal communication). Therefore, the observed size shift of TLR4 upon infection of murine macrophages could be due to deglycosylation, even if this could not be validated using recombinant soluble TLR4. As TLR4 has been shown to rely on glycosylation for its function (da Silva Correia and Ulevitch, 2002), affecting its glycan chains will reduce the reactivity towards LPS. Besides the effect on TLR4, CD14 was shown to be cleaved by *C. canimorsus* 5, either by proteolysis, incomplete N-linked or O-linked deglycosylation.

Thus, the observed effect on TLR4 and CD14, either by proteolysis or deglycosylation, could be the cause of the non-responsiveness of the macrophages to LPS. Unfortunately, the fitness-reducing effect of the released small molecule and the related Caspase/host protease dependent protein cleavage can’t be distinguished easily from direct bacterial effects on TLR4 and CD14.

### 4.3.3 Materials and Methods

**Bacterial strains and growth conditions.** The strains used in this study are listed in **Table 2**. *E. coli* strains were routinely grown in LB broth at 37°C. *C. canimorsus* 5 (Shin et al., 2007) 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% $CO_2$. Selective agents were added at the following concentrations: erythromycin, 10 mg/ml; cefoxitin, 10 mg/ml; gentamicin, 20 mg/ml; ampicillin, 100 mg/ml. Heat inactivated *Y. enterocolitica* E40 were prepared by taking 1 ml of a o/n culture, centrifugation at 14000 rpm for 1 min, aspiration of the supernatant and resuspension in 1 ml PBS. The bacterial suspension was boiled at 99°C under agitation for 2 hours.

**Immunoblotting for p38, p-p38, TLR4, CD14 and iNOS.** J774.1 macrophages were seeded at $3\times10^5$ cells/2ml in 6-well plates and incubated for 4h. Cells were infected with *C. canimorsus* (MOI 50) and incubated for 20h. Then cells were stimulated with an MOI = 50 equivalent of heat inactivated *Y. enterocolitica* E40 as LPS stimulus an incubated for further 24h. Cells were washed with PBS and collected in 1ml cold PBS by scraping them off the surface using cell scrapers (Corning). Cells were pelleted by centrifugation (6 min, 20 000 x g, 4°C) and resuspended in 100µl Phospho-Safe-reagent (Novagen) and then mixed by pipetting and vortexing followed by incubation for 10 min on ice. After centrifugation (6 min, 20 000 x g, 4°C) the supernatant was recovered, 5x SDS-PAGE sample buffer was added and the samples were boiled for 5 min at 95°C. Proteins were separated on 12% SDS-PAGE gels and transferred to Nitrocellulose membranes (Amersham). The membranes were probed with anti-p38, anti-phospho-

p38 (both Cell Signaling Technologies, dilution 1:1000), anti-TLR4 (Santa Cruz, sc-16240, diluted 1:500), anti-CD14 (Santa Cruz, sc-73794, diluted 1:500) and anti-iNOS (Santa Cruz, sc-650, dilution 1:500) antibodies. PNGaseF treatment of cell-lysates were performed according to the supplier (New England BioLabs, P0704).

**RT-PCR.** J774.1 macrophages were seeded at $5\times10^5$ cells/2ml in 6-well plates and incubated for 4h. Then they were infected with *C. canimorsus* at a MOI of 50 and stimulated with an MOI = 50 equivalent of heat inactivated *Y. enterocolitica* E40 as LPS stimulus (prepared as described above) and incubated for 24h. RNA was extracted using the RNeasy kit (Quiagen) according to the manufacturers instruction. An additional DNase I digest was introduced with 0.25 U/µg RNA for 15 min at 37°C and stopped by addition of final 2.5 mM EDTA and heat inactivation at 75°C for 10 min. Subsequent reverse transcription was performed with 200 U Superscript II/µg RNA in first strand buffer (Invitrogen), 10 mM DTT, 40 U RNAseOUT (Invitrogen) and 0.5 µg $dT_{12\text{-}18}$ for 60 min at 42°C and stopped at 70°C for 15 min. 10% of cDNA preparation or of a preparation made without addition of reverse transcriptase was subjected to PCR using primers TGCATGGACCAGTATAAGGCAAGC and AGCTTCTGGTCGATGTCATG-AGCAA (5' to 3') for iNOS and primers TAAAACGCAGCTCAGTAACAGTCCG and TGGAATCCTGTGGCATCCATGAAAC for β-actin (30 amplification cycles for β-actin and 35 for iNOS).

**TLR4 deglycosylation assay and lectin stain.** Bacteria were collected from blood agar plates and resuspended in DPBS at $OD_{600}$ = 12. 100 µl of bacterial suspensions were then incubated with 25 µl of a TLR4/MD-2 (R&D Systems, 3146-TM/CF) solution (1 mg/ml) for 90 minutes at 37°C. As negative control, TLR4/MD-2 solution was

incubated for 90 minutes at 37°C with 25 µl DPBS. Samples were then centrifuged twice for 6 min at 10000 × g at 4°C, supernatant was collected and loaded in a 12% SDS gel. Samples were analyzed by immunoblotting (anti-Flag, M2 anti-Flag antibody, Sigma) and lectin staining. Lectin staining with Galanthus nivalis agglutinin lectin (GNA), Sambucus nigra lectin (SNA) and Datura stramonium agglutinin lectin (DSA) were performed according to manufacturer recommendations (DIG Glycan Differentiation Kit, 11210238001, Roche).

### 4.3.4 References


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# *5* The *cam* locus is a bona-fide PUL.

## 5.1 Preface

Among all the Tn mutants identified, one (Y2F12) seemed to be of particular interest, as the operon hit by the Tn was found only present in the wt strains able to prevent LPS stimulated macrophages from releasing NO. The operon affected by this Tn mutant was termed Cam (*C. canimorsus* active mechanism) In addition it presented all the characteristics of a PUL.

## 5.2 Results

### 5.2.1 PUL11 is only present in few *C. canimorsus* strains

All dog isolates and the clinical isolates were tested for their capacity to block NO release by macrophages challenged with LPS. Only a small subset of the tested *C. canimorsus* strains were able to block the LPS induced NO release by macrophages (Mally et al., 2009). The capacity to block NO was correlated to the presence of PUL 11 (Mally, unpublished data). *Cc5* PUL11 is composed of two genes with sequence homology to *B. thetaiotaomicron susC* and *susD,* termed *camN* and *camO*, as well as of two hypothetical proteins termed *camA* and *camB*. PUL11 appeared to be important for proper growth on eukaryotic cells **(Fig. 1 B and D)**, as the Tn mutant Y2F12 affecting *camB* exhibited a small growth defect. In order to find a possible correlation, we assessed the presence of PUL11 in several *C. canimorsus* strains and the growth of these strains **(**for growth see **Fig. 2)**. We thus tested the presence of *camA* and *camB*

at gene and protein level, as susC- and susD-like proteins might be shuffled between strains (P. Manfredi, personal communication). Only few strains appeared to encode *camA* and *camB*, while the two genes were always found associated **(Fig. 9)**. Interestingly, PUL11 genes were found exclusively present in strains growing well on eukaryotic cells and being able to block the NO release of LPS stimulated murine macrophages **(Fig. 2)**. This hints at a role of PUL11 in growth on macrophages, as has been shown for PUL5 (Manfredi et al., 2011b; Renzi et al., 2011).

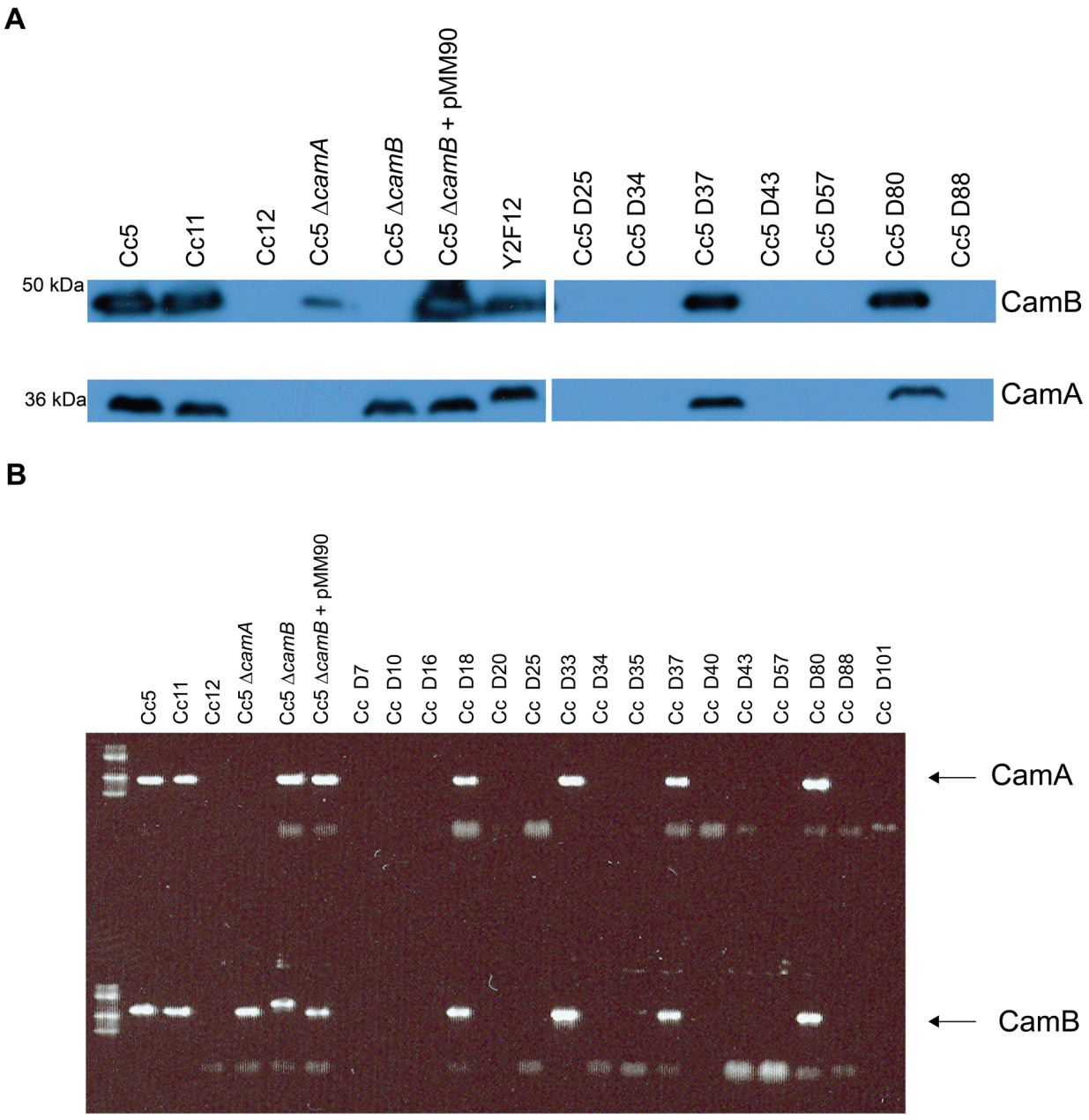


**Figure 9. Presence of *camA* and *camB* in different *C. canimorsus* strains.** (A) Western-blot anti-CamA and anti-CamB on total cell extract of indicated *C. canimorsus* strain. (B) PCR on *camA* and *camB*, revealed by agarose gel electrophoresis.

### 5.2.2 PUL11 encoded proteins CamO, A, B and sialidase are copurified: PUL11 is a bona-fide PUL

*C. canimorsus* 5 PUL5 has been shown to assemble in a multi-protein complex including genes of PUL5 and sialidase (Manfredi et al., 2011b). In a similar assay we have purified the PUL11 encoded CamB bearing a Strep- and His-tag. CamO and CamA were shown to co-purify with CamB **(Fig. 10 A)**. The copurification of CamN, the SusC-like protein, was not assayed due to the lack of appropriate antibody. As for PUL5, PUL11 proteins were found to copurify sialidase **(Fig. 10 A)**. This suggests that PUL11 forms a multi-protein complex including sialidase. Therefore PUL11 seems to be a bona-fide PUL. The copurification of sialidase hints at a role of PUL11 in glycan degradation, where CamA or CamB could be glycosyl hydrolases. The single proteins of PUL11 have previously been identified as surface-exposed (Mally, 2008; Manfredi et al., 2011b), fulfilling another requirement for a PUL.

Surprisingly, CamB, encoded by the last gene in PUL11, was found to be released into the conditioned medium **(Fig. 10 B)**. CamB, like CamA, which has been found only in trace amounts in the conditioned medium (data not shown), is a lipoprotein. Hence, one would expect it to be tightly anchored in the outer membrane of the bacteria. But in some way CamB seems to be released by *C. canimorsus* from the membrane. The released CamB did not show any size-shift **(Fig. 10 B)**, hinting that even the lipid part might still be present on the released protein.

PUL11 fulfills all requirements for a PUL encoded glycan degradation system. But the enzymatic action of the possible glycosyl hydrolases encoded by PUL11, CamA and CamB, is unknown. To assess the enzymatic activity of CamB, it was expressed in *C. canimorsus* 5 and purified over a Strep-tag (pSI09 and pSI10) **(Fig. 10 D)**. The resulting double-band in the elution fractions was analyzed by Western-blot using anti-Strep and

anti-CamB antiserum and by mass spectrometry. Both bands **(see Fig. 10 D)** represent CamB, the small size difference might be due to changes in lipidation, cleavage of the signal peptide or by difference in post-translational bacterial modification as glycosylation. The elution fractions were tested for activity towards several umbelliferyl-fused sugars **(Fig. 10 C)** (GlcNAc, α-Mannoside, β-Mannoside, Chitin and the sialidase substrate acetylneuraminic acid called MUAN; not shown and negative for all elution fractions: Galactose, Fucose and sulfate). The copurified sialidase likely accounts for the cleavage of MUAN. The cleavage of GlcNAc might be due to CamB. But it can't be completely excluded that one of the few contaminants accounts for cleavage of GlcNAc, especially as elution fraction 3 as well exhibits an activity on GlcNAc **(Fig. 10 C)**, while it lacks both CamB bands **(Fig. 10 D)**.

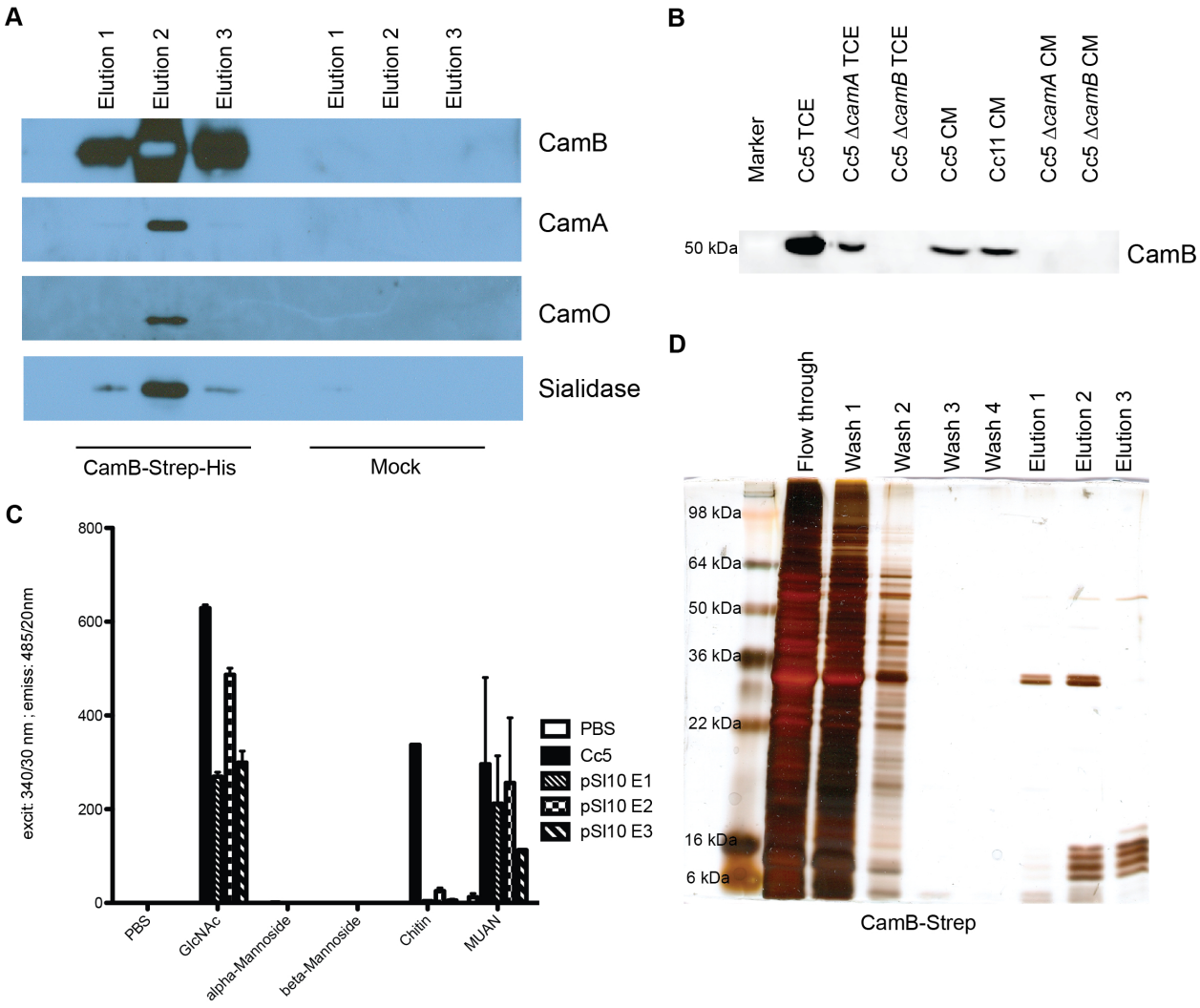


**Figure 10. Characterization of PUL11.** (A) Copurification of CamA, CamO and sialidase with CamB-Strep-His following a His- and Strep-tag purification visualized by Western-blotting. (B) Presence of CamB in *C. canimorsus* conditioned medium (CM) identified by Western-blotting. As a positive-control total cell extract (TCE) was included. (C) Activity of CamB-Strep purification elutions 1, 2 and 3 (E1-E3) assayed on methylumbelliferyl-conjugated sugars indicated, where MUAN is the conjugate with acetylneuraminic acid. (D) Steps in CamB-Strep (pSI09/10) purification revealed by silverstaining of a 12% SDS-PAGE.

## 5.3 Methods

**Immunoblotting.** *C. canimorsus* were scraped from plates and resuspended in PBS. $OD_{600}$ was set to 1 and 1ml of this suspension was centrifuged to pellet the bacteria. The pellet was then resuspended in 100ul of distilled water. 25ul SDS loading dye was added and the samples were boiled for 5 min, to give a total cell extract (TCE). 12.5ul of each sample were loaded on a 12% SDS PAGE, blotted and analyzed using antisera against CamA, CamB, CamO or sialidase (Mally, 2008).

Samples from conditioned medium (prepared as described above) were precipitated with trichloroacetic acid (TCA). To do so, 1.8 ml of conditioned medium were supplemented with 200ul cold TCA and incubated at 4°C o/n. Then the sample was centrifuged (20'000 x rcf, 4°C, 30min), the supernatant was discarded and the pellet was washed with 600ul ice-cold acetone, followed by another centrifugation step as above. The supernatant was discarded and the pellet was air dried, resuspended in 160ul distilled water plus 40ul SDS loading dye and boiled for 5 min. 25 ul of a sample were charged per slot on a 12 % SDS PAGE.

**PCR identifying presence of genes.** PCR was carried out on single colonies picked with a toothpick using Taq DNA polymerase (NEB). *camA* was amplified using primer 4200 and 4201, *camB* with 4332 and 4254 (primers are listed in the Appendix, table A2). PCRs were charged on 1% agarose gels and visualized using Ethidiumbromide.

**Purification of CamB over a Strep-tag.** *C. canimorsus* 5 bearing pSI09 or pSI10 (Appendix, table A1) were grown on HIA cfx plates for 2 days. Colonies from 10 plates

were collected by scraping and resuspension in PBS. $OD_{600}$ was set to 6 and 1ml of this suspension was added to a 1.5 ml Eppendorf tube and the bacteria were pelleted by centrifugation (10'000 x rcf, 3 min, 4°C). 500ul of a lysis buffer (0.2% Triton X-100, 1% NP-40, 1% sodium deoxycholate, 25mM Tris HCl, 150mM NaCl, pH 7.5) were added per tube. The pellets were resupended by vortexing and pipetting and incubated for 15 min on ice. The debris was pelleted by centrifugation (20'000 x rcf, 15 min, 4°C) and the supernatants were taken and pooled. 500 µl - 1 ml Streptactin resin (IBA) was loaded on a small column (BioRad) and equilibrated with 5 ml Buffer W (100 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 8.0). The resin was taken and mixed with the bacterial lysate diluted 1:3 in PBS and incubated o/n on a turning wheel at 4°C. The resin was then load again on the column and was wash 5 times with 5 ml Buffer W, before the elution with 3 times 500 µl - 1 ml (adapted to the resin volume) Buffer E (100 mM Tris, 150 mM NaCl, 1 mM EDTA, 2.5 mM Desthiobiotin, pH 8.0). Purification over a Strep- and His-tag was performed as previously described (Manfredi et al., 2011b).

**Hydrolysis of methylumbelliferyl-conjugated sugars/compounds.** 50ul of 0.2 mM methylumbelliferyl-conjugated sugar (in 0.25M sodium acetate, pH 7,5) were mixed in a black 96-well plate with 50 ul of CamB-Strep elution fraction 1-3 or 50ul of a $OD_{600}$ = 0.4 suspension of *C. canimorsus* in PBS. The plates were incubated o/n at 37°C. Free methylumbelliferyl was measured u sing a Synergy2 multiplate-reader (BioTek) at an excitation of 340/30nm, using an emission filter of 485/20nm. All methylumbelliferyl-conjugated sugars wee purchased from Sigma (GlcNAc: M2133, Galactose: M1633, Sulfate: M7133, Fucose: M8527, acetylneuraminic acid, MUAN: M8639, Chitin: now within CS1030, β-Mannoside, M0905, α-Mannoside: M3657)

**Plasmids.** Plasmids used are listed in the Appendix, table A2.

## 5.4 Discussion

The proteins encoded by PUL11 have been reported to be exposed to the bacterial surface (Mally, 2008; Manfredi et al., 2011b). Here we show that most of them can be copurified and that they even seem to associate with sialidase. These data show that PUL11 is indeed a bona-fide PUL. The copurification of sialidase hints at a role in glycan foraging, as has been shown for PUL5 (Mally et al., 2008; Manfredi et al., 2011b; Renzi et al., 2011). We show here that the proteins of PUL11 with possible enzymatic activity, termed CamA and CamB, are present in only few *C. canimorsus* strains. Exactly these strains were shown to grow best on macrophages and to block LPS stimulated macrophages from NO release. Hence, PUL11 might be required for nutrient acquisition in cell culture conditions leading to improved growth and, indirectly, to the described immunosuppressive effect. We have further tried to identify the role of PUL11 in glycan degradation. Purified CamB was found inactive towards several sugar substrates. However, it has been shown that the growth supporting effect of PUL11 can be replaced by a laminarinase/chitinase (a glucanase), an enzyme releasing glucose from polyglucans (Master thesis, Claudio Cadel). ΔPUL11 bacteria supplemented with laminarinase were further shown to block the LPS induced NO release by macrophages (Master thesis, Claudio Cadel). This indicates that the small molecule interfering with the immune response of macrophages is not directly related to PUL11. Whether and at which step PUL11 plays a role in deglycosylation of eukaryotic glycoproteins and if it has a laminarinase activity remains to be clarified.

## 5.5 References


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# 6 Appendix

## 6.1 Appendix 1: Lipid A of *Capnocytophaga canimorsus*: Additional results

**Statement of my work:** My contribution was the data of all figures (1-14) in this section.

### 6.1.1 *E. coli* penta-acyl lipid A is, in contrast to *C. canimorsus* lipid A, a TLR4 antagonist

In order to evaluate the TLR4 agonistic and antagonistic potential of *C. canimorsus* 5 (Cc5) lipid A, we compared it in endotoxicity assays not only to lipid IVa, but as well to *E. coli* F515 penta-acylated lipid A. *E. coli* F515 lipid A lacks one acyloxyacyl-residue [14:0-3-*O*(14:0)] and corresponds to a *lpxM* deletion. Endotoxic activity of *C. canimorsus* lipid A in comparison to *E. coli* F515 lipid A was assayed using two different methods: (i) Indicated concentrations of purified lipid A samples were assayed for TLR4 dependent NFκB activation with Hek293 cells overexpressing human TLR4/MD2/CD14 and a secreted reporter gene (HekBlue human TLR4 cell line), (ii) purified LPS samples were assayed for induction of TNFα release by human THP-1 macrophages. TNFα release was measured using ELISA. In both assays *C. canimorsus* lipid A and *E. coli* F515 penta-acyl lipid A showed to be not endotoxic **(Fig. 1 A, C and Fig. 1 B, D)**. But *E. coli* penta-acyl lipid A showed to be a potent antagonist on the activity of *E. coli* O111 LPS **(Fig. 1 B and D)**, being around 10-fold less inhibitory as lipid IVa. As has previously been shown, Cc5 lipid A is no antagonist of the interaction *E. coli* O111 LPS and human TLR4/MD2 **(Fig. 1 B and D or Chapter 2.1, Fig. 7)**.

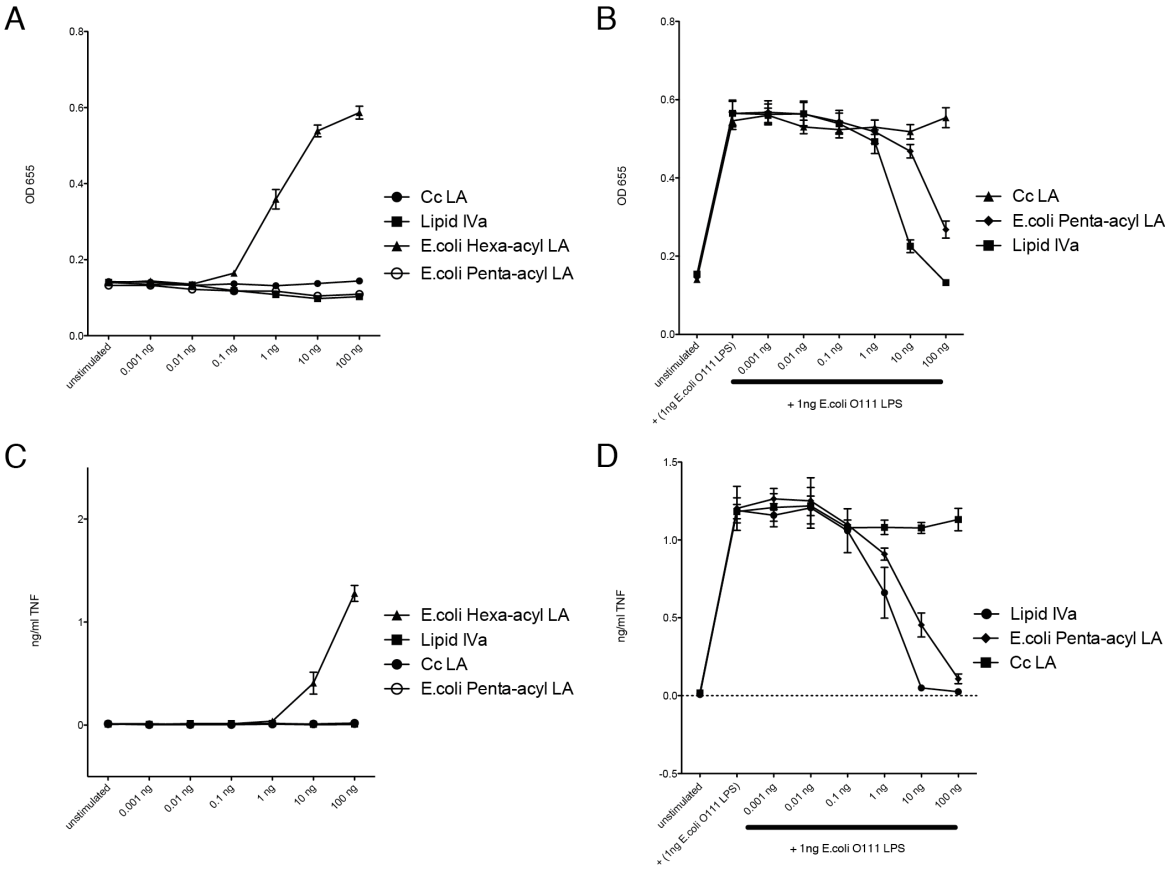


**Figure 1. Endotoxic activity of *C. canimorsus* (Cc) lipid A (LA) and antagonistic activity of Cc LA on the action of *E. coli* O111 LPS.** (A) Indicated concentrations of purified LA were assayed for TLR4 dependent NFκB activation with HekBlue human TLR4 cells. (C) Purified LA were assayed for induction of TNFα release by human THP-1 macrophages. (B) HekBlue human TLR4 cells were preincubated for 3h with purified LA at the concentration indicated. Then the cells were stimulated with 1ng *E. coli* O111 LPS for further 20-24h and TLR4 dependent NFκB activation was measured. (D) Human THP-1 macrophages were preincubated for 3h with purified LA samples at the concentration indicated. Then the cells were stimulated with 1ng *E. coli* O111 LPS for further 20h and TNFα release was measured.

### 6.1.2 *C. canimorsus* is highly resistant to human β-Defensin 2

Cc5 has been shown to be highly resistant to the CAMP Polymyxin B **(Chapter 2.2 Fig. 4)**, which is derived from *Bacillus polymyxa*. More physiological CAMPs, which Cc5 might encounter in dog's mouth and in human infections are Defensins (Lehrer et al., 1993; Mathews et al., 1999; Miyasaki et al., 1990; Pazgier et al., 2006; Pazgier et al., 2007). Human β-Defensin 2 (DEFB2) are expressed on the skin, oral and pulmonary epithelia, conjunctiva, cornea, astrocytes, gut epithelia and on epidermal and gingival keranocytes (Pazgier et al., 2006). To address the DEFB2 resistance, resuspended *C. canimorsus* 5 wt, Δ*lpxE*, Δ*eptA* or Δ*lpxE-eptA* were incubated for 30 min or 2h with various amounts of DEFB2. All *C. canimorsus* strains tested were found to be resistant to even the highest concentration of DEFB2 used **(Fig. 2)**. *C. canimorsus* seems therefore to be highly resistant to Defensins and CAMPs in general, even in comparison to other high resistant bacteria as *P. gingivalis* (Curtis et al.). This might be due to the lack of negative charges in the lipid A as well as in the O-antigens expressed (see chapters 2 and 3). Higher concentrations of DEFB2 might be needed to lay open the difference between Cc5 and the lipid A mutants.

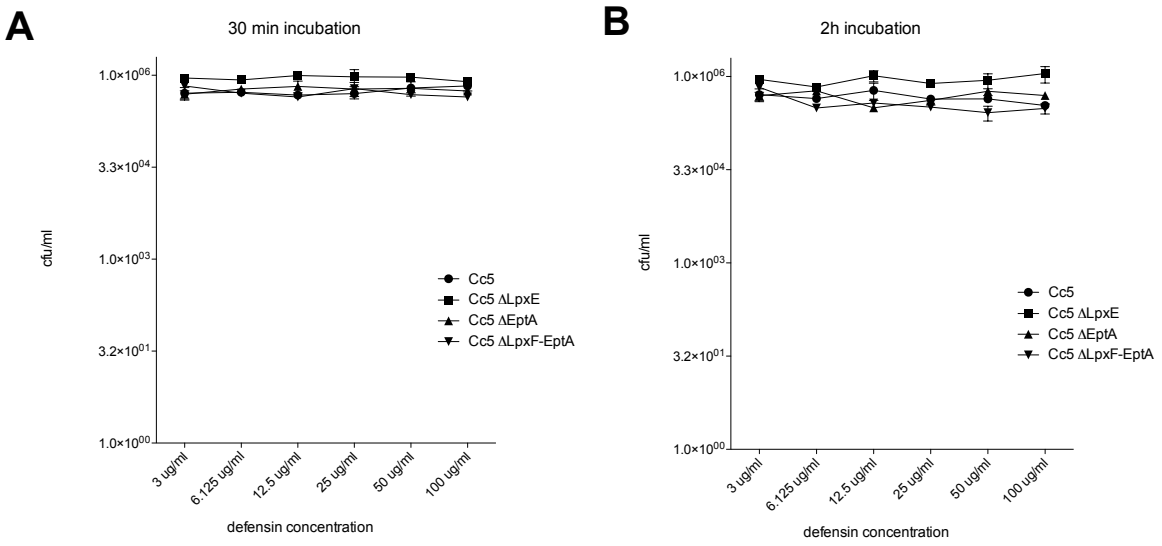


**Figure 2. Human β-Defensin 2 (DEFB2) resistance of *C. canimorsus* wt and lipid A mutants.** Resuspended *C. canimorsus* 5 wt (Cc5), Δ*lpxE*, Δ*eptA* or Δ*lpxE-eptA* were resuspended in PBS and incubated with the indicated amount of DEFB2 for 30 min (A) or 2 h (B). After incubation with DEFB2 the suspension was diluted and plated for cfu counting.

### 6.1.3 *Ccan*6070 and *Ccan*14540 are not lipid A phosphatases

Search for a lipid A phosphatase were based on *lpxE* and/or *lpxF* sequences from *P. gingivalis* (Coats et al., 2009a; Coats et al., 2009b), *F. novicida* (Wang et al., 2006), *R. etli* (Ingram et al.), *H. pylori* (Tran et al., 2004) and on all available CFB-group *pgpB* sequences. Three *lpxE/F* candidates have been found in the *C. canimorsus 5* genome (*Ccan*16960, *Ccan*14540 and *Ccan*6070). All candidates have been deleted, whereas only deletion of *Ccan*16960 affected endotoxicity **(chapter 2.2 Fig. 3)**. Deletion of

*Ccan*6070 **(Fig. 3 B)** or *Ccan*14540 **(Fig. 3 A)** did not lead to a strong increase in endotoxicity, which would be expected in case of a deletion of a lipid A phosphatase. Hence, we conclude that *Ccan*14540 and *Ccan*6070 are not lipid A phosphatases.

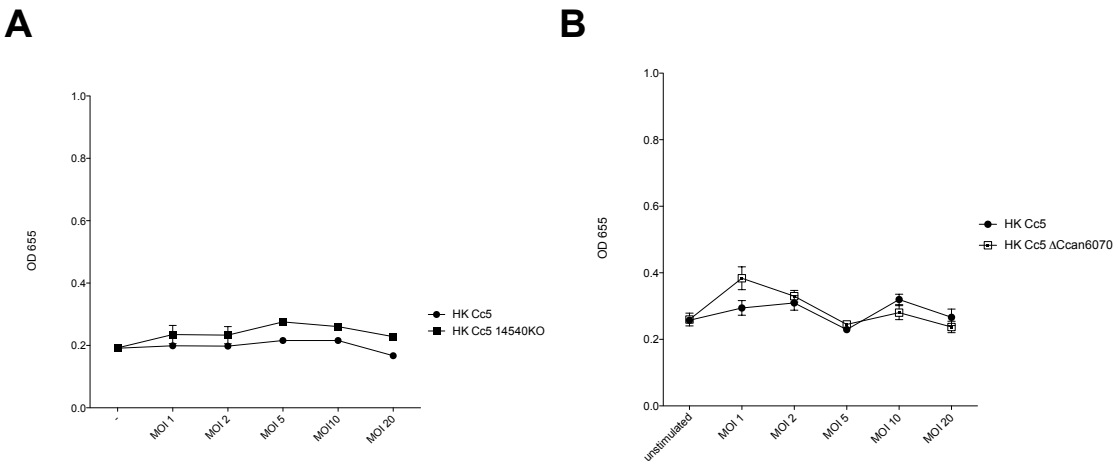


**Figure 3. Endotoxic activity of heat killed *C. canimorsus* (Cc) wild-type (Cc5), Δ*Ccan14540* or Δ*Ccan6070* bacteria.** (A-B) Indicated multiplicity of infection (MOI) of heat killed (HK) bacteria were assayed for TLR4 dependent NFκB activation with HekBlue human TLR4 cells.

### 6.1.4 *Ccan*16940 is not a lipid A acyltransferase

The *C. canimorsus lpxE-eptA* operon (*Ccan*16960-50) is followed by a gene (*Ccan*16940) annotated as putative acyltransferase. The control of the acylation of lipid A by acyltransferases and deacylases offers another way to modulate endotoxicity (Curtis et al.; Kawasaki et al., 2004). To address the activity of *Ccan*16940 on lipid A, we engineered a deletion mutant of *Ccan*16940 and analyzed it for endotoxicity. A triple KO *Ccan*16960-40 was further compared in endotoxicity to the double KO *Ccan*16960-50 (corresponding Δ*lpxE-eptA*). As Δ*Ccan*16940 showed similar endotoxicity than the Cc5 wt **(Fig. 4)** and Δ*Ccan*16960-40 was as endotoxic as Δ*Ccan*16960-50 **(Fig. 4)**, we concluded, that *Ccan*16940 does not acylate lipid A. This was further conformed by complementation of Δ*lpxE/eptA* **(chapter 2.2 Fig. 3)**. It thus seems as if *Ccan*16940 is not involved in lipid A modification.

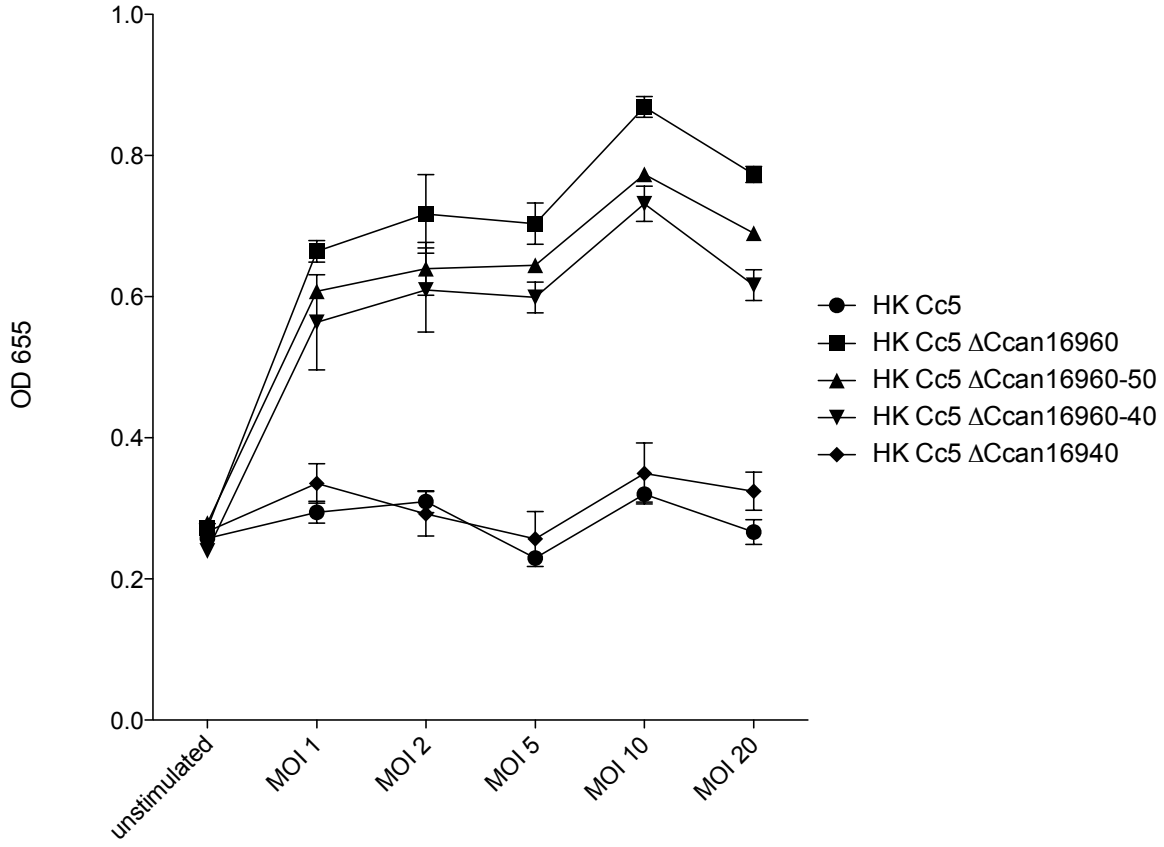


**Figure 4. Endotoxic activity of heat killed (HK) *C. canimorsus* (Cc) wild-type (Cc5), Δ*16940, Δ16960, Δ16960-50 and Δ16960-40* bacteria.** Indicated multiplicity of infection (MOI) of heat-killed bacteria were assayed for TLR4 dependent NFκB activation with HekBlue human TLR4 cells.

### *6.1.5 C. canimorsus* LpxE is non-functional and EptA is toxic in *E. coli*

The function of the corresponding *lpxE-eptA* operon in *H. pylori* (Hp0021-Hp0022) has been validated by expression of these genes in *E. coli* (Tran et al., 2004). Even though the function of *C. canimorsus lpxE* and *eptA* is currently confirmed by structural analysis of the lipid A of deletion mutants, we have tried to express *C. canimorsus* LpxE and EptA in *E. coli* K12. To render the *E. coli* K12 lipid A more similar to *C. canimorsus* lipid A, we have used a *E. coli* K12 Δ*lpxM* background strain featuring a penta-acylated lipid A. This should render the *E. coli* lipid A a better substrate for *C. canimorsus* lipid A modifying enzymes like LpxE and EptA. Interestingly, expression of *C. canimorsus* EptA had strong toxic effects in *E. coli* top 10, which was used for cloning of the plasmid. By sequencing we found that only *E. coli* top 10 colonies having a mobile genetic element inserted upstream of the EptA start codon were able to grow (Data not shown). We hypothesize that Cc EptA modifies even 1 phosphorylated *E. coli* lipid A and might lead to membrane instability. Further *C. canimorsus* EptA might put a *P*-Etn group at several other positions like sugars in the core oligosaccharide and might thus lead to problems with membrane integrity. Constant pUC19 driven expression of Cc5 LpxE (pSI65) in *E. coli* K12 Δ*lpxM* did not alter endotoxicity **(Fig. 5)**. pUC19 driven expression of Cc5 LpxE-EptA (pSI68) in *E. coli* K12 Δ*lpxM* increased the endotoxic activity **(Fig. 5)**. As the expression of LpxE alone had no effect, and Cc EptA seemed to have a striking toxic activity in *E. coli* K12 Δ*lpxM*, the effect of coexpression of LpxE-EptA might be attributed to EptA. Coexpression of Cc LpxE-EptA in *E. coli* K12 Δ*lpxM* might be less toxic due to a lower expression level of EptA, as the pUC19 ribosome-binding site (rbs) for *eptA* in pSI65 has been changed to the *C. canimorsus* rbs for *eptA* in pSi68. The observed effect on endotoxicity resulting upon presence of pSI68 might

be explained by differences from 1 phosphate lipid A to 1 *P*-*P*Etn resulting from *C. canimorsus* EptA activity in *E. coli*.

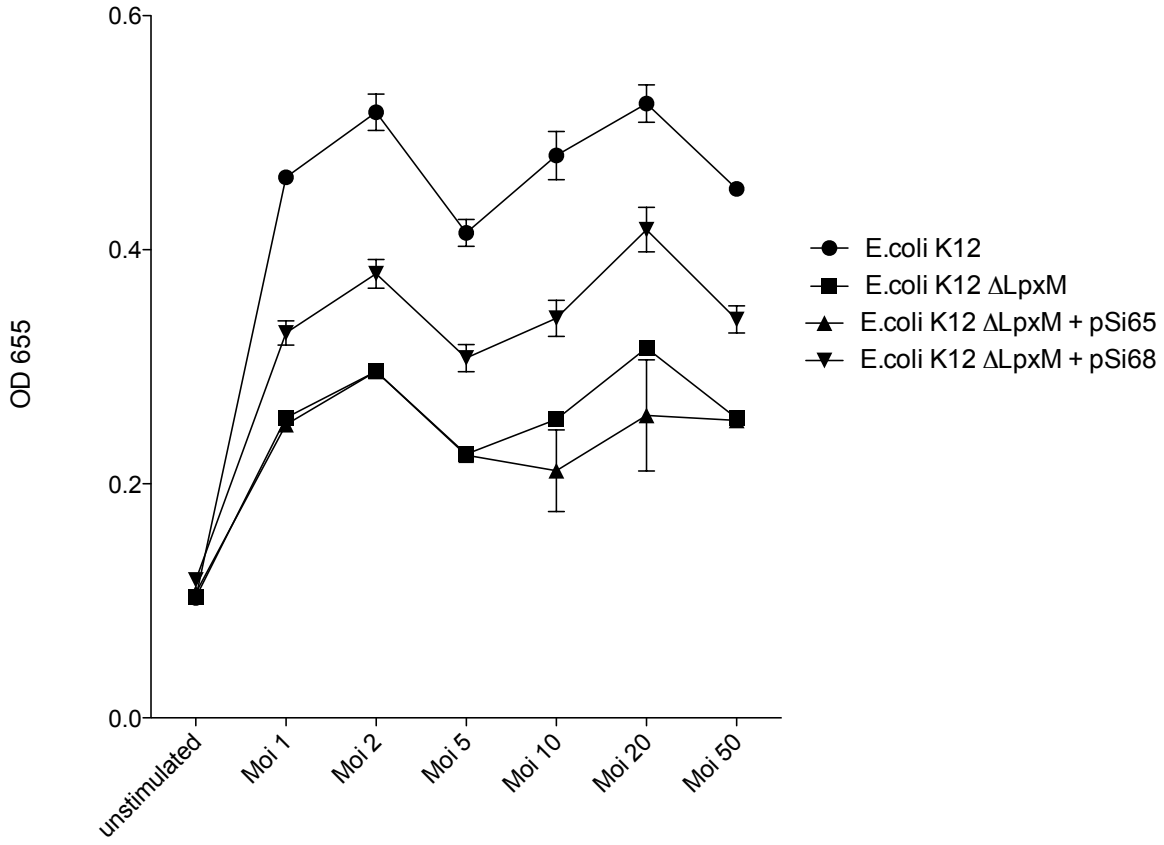


**Figure 5. Endotoxic activity of heat killed *E. coli* K12, *E. coli* K12 Δ*lpxM, and E. coli* K12 Δ*lpxM* with a plasmid encoding *C. canimorsus lpxE* (pSi65) or *lpxE-eptA* (pSi68).** Indicated multiplicity of infection (MOI) of heat-killed bacteria were assayed for TLR4 dependent NFκB activation with HekBlue human TLR4 cells.

### 6.1.6 *C. canimorsus* LPS is neither an agonist nor an antagonist of murine TLR4

Murine TLR4 is known to react different than the human receptor to some underacylated lipid A. Lipid IVa is an antagonist of the human TLR4 but an agonist of the murine TLR4 (Golenbock et al., 1991; Means et al., 2000). We therefore assayed the ability of Cc5 LPS to stimulate murine TLR4 by measuring nitric oxide (NO) release from murine macrophages (RAW 264.7). While Cc5 LPS showed a 100-fold reduced induction of human TLR4 than *E. coli* O111 LPS, Cc5 LPS did not stimulate murine macrophages at all to release NO **(Fig. 6)**. This difference might be due to changes in the TLR4 sequence and therefore variation in lipid A binding between the human and the murine TLR4, as has been suggested for lipid IVa (Meng et al., 2010).

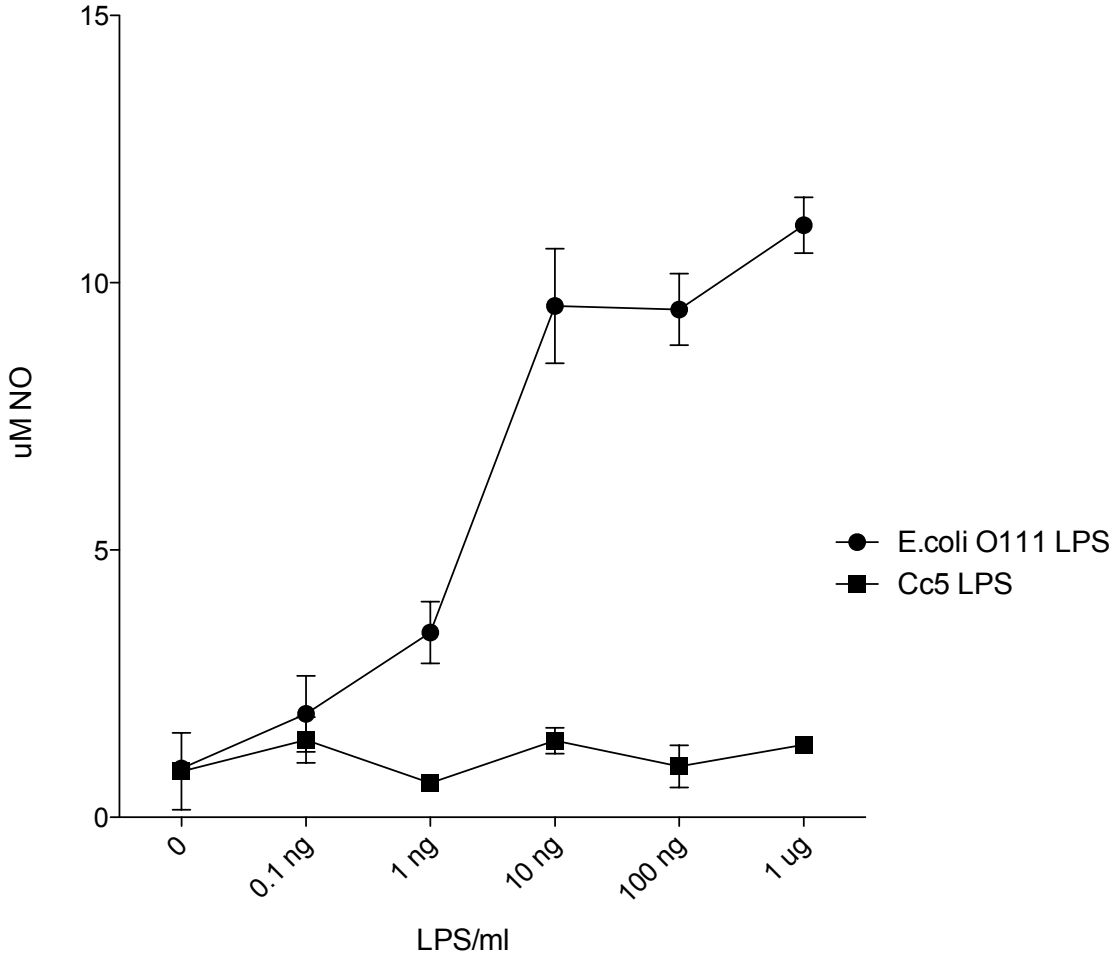


**Figure 6. Activity of *C. canimorsus* (Cc) LPS on murine TLR4.** RAW264.7 murine macrophages were stimulated with indicated amount of LPS for 24 h. Release of nitric oxide (NO) was measured using modified Griess reagent.

Antagonistic activity of *C. canimorsus* LPS on the action of *E. coli* O111 LPS on murine TLR4 was further examined. RAW264.7 murine macrophages were preincubated for 3h with purified Cc5 LPS samples at the concentration indicated. Then the cells were stimulated with 10 ng/ml, 100 ng/ml or 1 ug/ml *E. coli* O111 LPS for further 24h and NO

release was measured. *C. canimorsus* LPS showed to be no antagonist of *E. coli* O111 LPS binding to murine TLR4 **(Fig. 7)**. Summarizing, *C. canimorsus* LPS seems neither to be an agonist nor an antagonist for murine TLR4, while it was shown to be a partial agonist of the human TLR4. To our knowledge this is the first example of an LPS with greater reactivity towards the human than the murine TLR4/MD-2/CD14. Further work will be needed to elucidate which sequence variation between the human and murine TLR4/MD-2/CD14 is responsible for the observed difference in activity.

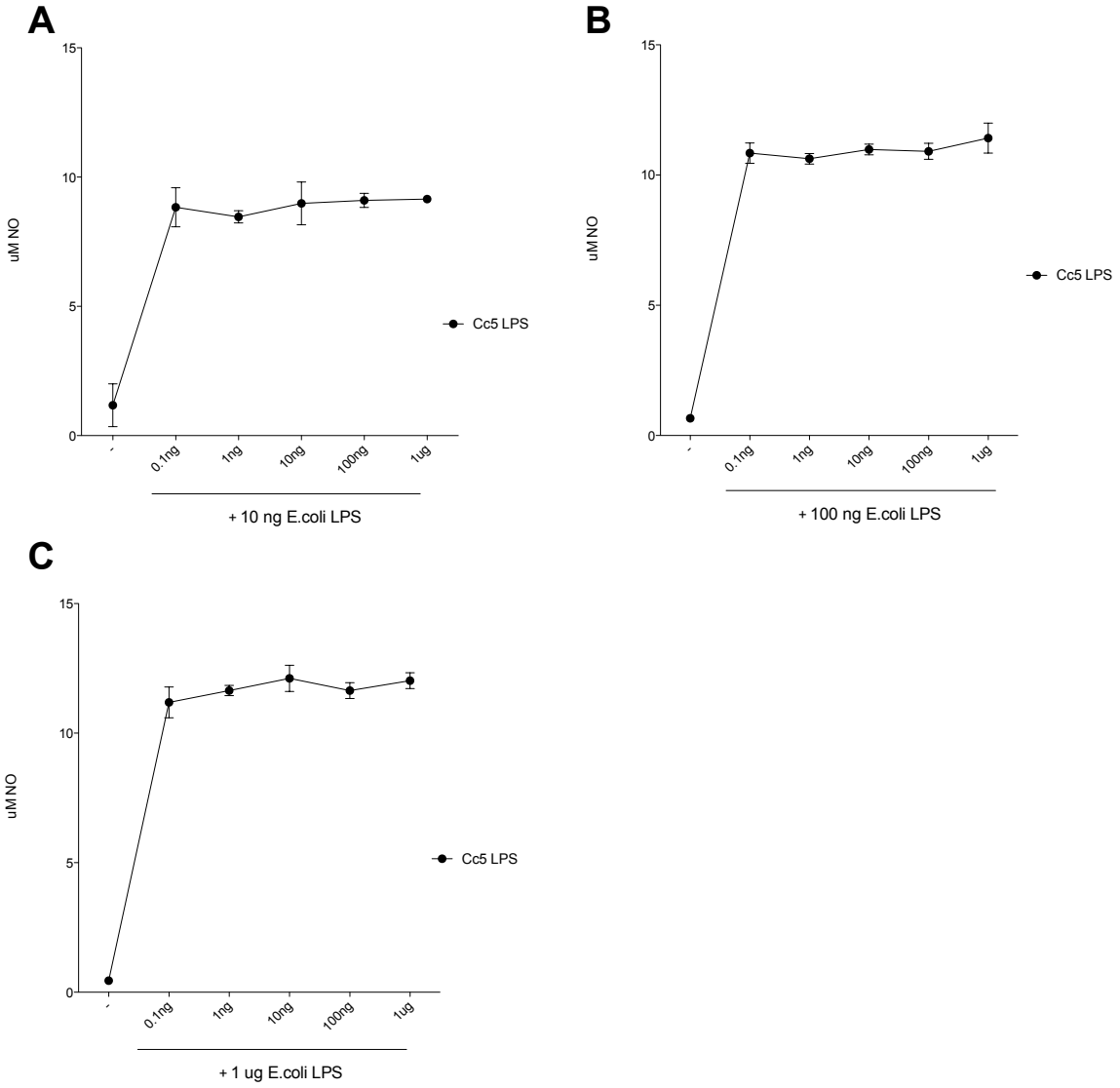


**Figure 7. Antagonistic activity of *C. canimorsus* LPS on the action of *E. coli* O111 LPS on murine TLR4.** RAW264.7 murine macrophages were preincubated for 3h with the indicated amount (amount indicated is per ml) of *C. canimorsus* (Cc) LPS, stimulated with 10 ng/ml (A), 100 ng/ml (B) or 1 ug/ml (C) *E. coli* O111 LPS and further incubated for 24h. Release of nitric oxide (NO) was measured using modified Griess reagent.

### 6.1.7 Comparison of endotoxicity of different *C. canimorsus* strains

In order to see if low endotoxicity is a common feature of all Cc strains, we tested all patient derived strains (except the slow growing strain Cc8) and several dog mouth derived strains for activity on murine macrophages. Murine RAW264.7 macrophages were incubated for 24h with heat-killed bacteria (amount corresponding to MOI 50) and NO release was measured. All strains exhibited similar capacity to activate murine macrophages **(Fig. 8)**. In this assay, stimuli for TLR4, TLR2 or other pattern-recognition receptors (PRR) can't be distinguished. As purified Cc5 LPS has been found not to be active on murine TLR4 **(Fig. 7)**, the *C. canimorsus* dependent activation of murine macrophages might rather reflect TLR2 stimulation. Nevertheless, none of the strains tested seems to express a LPS with a increased activity on murine TLR4.

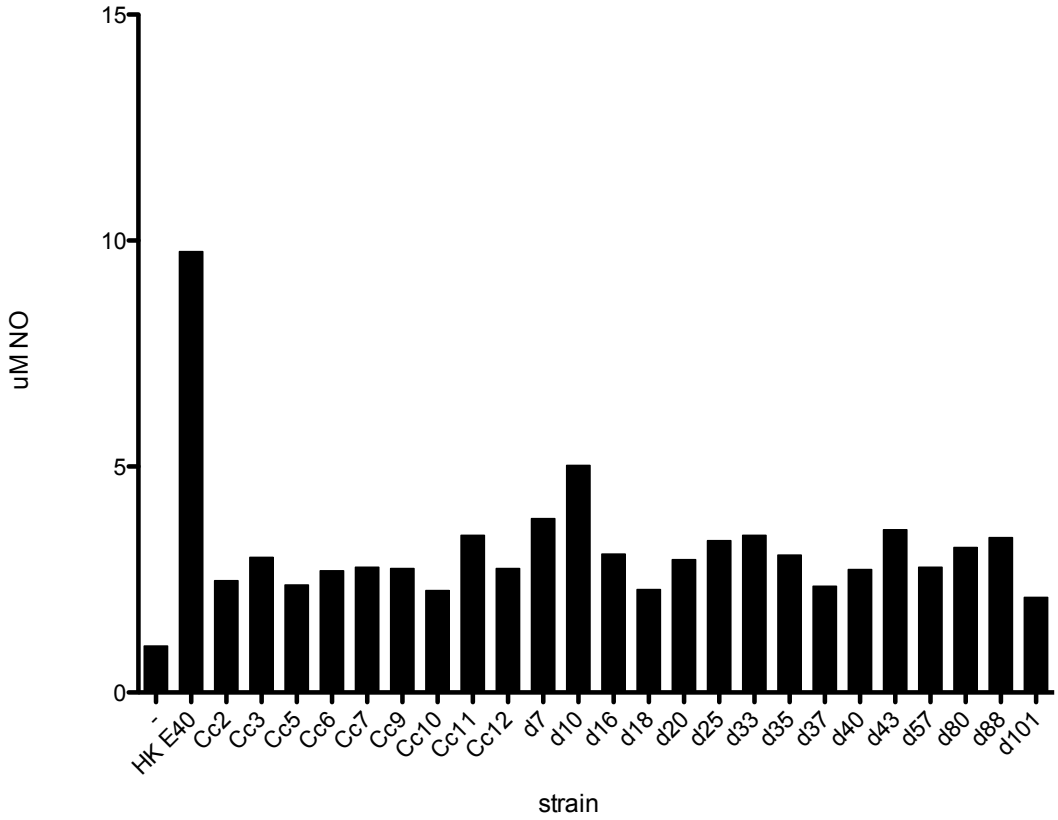


**Figure 8. NO release of RAW264.7 murine macrophages after stimulation for 24h with heat-killed (HK) bacteria.** Macrophages were stimulated with heat-killed (HK) bacteria at MOI 50 and incubated for 24h. Release of nitric oxide (NO) was measured using modified Griess reagent. Patient derived strains are marked with Cc (Cc2-Cc12), dog mouth derived strains are marked with d (d7-d101). HK *Yersinia enterocolitica* MRS40 (E40) was used as positive control.

In the same NO release assay other *Capnocytophaga* species and the slow growing *C. canimorsus* strain Cc8 were compared to Cc5. To make differences better visible,

several MOIs were tested. Interestingly, Cc8, a patient derived strain (Prof. Dr. Michel Delmée, Université Catholique de Louvain) showed increased macrophages stimulation **(Fig. 9)**, while Cc5, *C. cynodegmi, C. ochracea* and *C. gingivalis* activated murine macrophages to a similar extent **(Fig. 9)**. Stimuli for TLR4, TLR2 or other pattern-recognition receptors (PRR) could not be distinguished with this method. We were interested to see if the increased macrophage activation was due to changes in lipid A. Therefore the same samples were assayed for TLR4 dependent NFκB activation with Hek293 cells overexpressing human TLR4/MD-2/CD14 and a secreted reporter gene. Again Cc8 showed to lead to higher stimulation than Cc5 and than other *Capnocytophaga* species **(Fig. 10)**. It might thus well be that the lipid A of Cc8 is different from Cc5 and might either carry a 1 or 4' phosphate or have alterations in the acyl chains. To compare Cc8 to Cc5, we analyzed several genes involved in lipid A synthesis or modification. The sequence of *lpxK*, the 4' kinase, was identical in Cc5 and Cc8 (data not shown). Cc5 and Cc8 *lpxL*, a late acyltransferase, showed only few sequence variation **(Fig. 11)**. The presence of the *lpxE-eptA* operon in Cc8 was confirmed by PCR on *lpxE, eptA* and *lpxE-eptA* **(Fig. 12)**. Therefore the 1-position of Cc8 lipid A might be modified as in Cc5. The higher endotoxicity of Cc8 LPS could be explained by alterations in acylation (encoding of *lpxM*, incorporation of shorter acyl chains) or changes in 4' dephosphorylation. The gene encoding the lipid A 4' phosphatase, LpxF, is not known in *C. canimorsus* 5 **(Chapter 2.1, Fig. 3)**. It might be that Cc8 doesn't encode *lpxF*, and therefore still has a phosphate attached to the 4' position of lipid A, which would explain the increased endotoxicity. A genome wide Transposon-insertion screen on endotoxicity could lead to the identification of Cc5 *lpxF* and would allow comparison to Cc8. Unravelling the structure of Cc8 lipid A would solve the question of variations to Cc5 lipid A.

*C. ochracea*, *C. cynodegmi*, *C. gingivalis* and *C. canimorsus* 5 stimulated murine macrophages to a similar extent **(Fig. 9)**. This includes several stimuli for TLR4, TLR2 or other PRRs. Therefore we have tested heat-killed bacteria in an only TLR4 dependent cell activation assay. The endotoxic activity of the tested *Capnocytophaga* species varied **(Fig. 10)**. Cc5 and *C. ochracea* showed weakest endotoxicity (in agreement with (Yoshimura et al., 2002)). *C. gingivalis* exhibited a slightly increased endotoxic activity. *C. cynodegmi*, besides *C. canimorsus* the other member of dog mouth's flora, showed higher endotoxicity. Neither *C. ochracea*, *C. cynodegmi* nor *C. gingivalis* encode the *C. canimorsus lpxE-eptA* operon **(Fig. 12)**. Of course only minor nucleotide sequence variation would abolish PCR amplification, hence *C. ochracea*, *C. cynodegmi* or *C. gingivalis* might still encode *lpxE* and *eptA*.

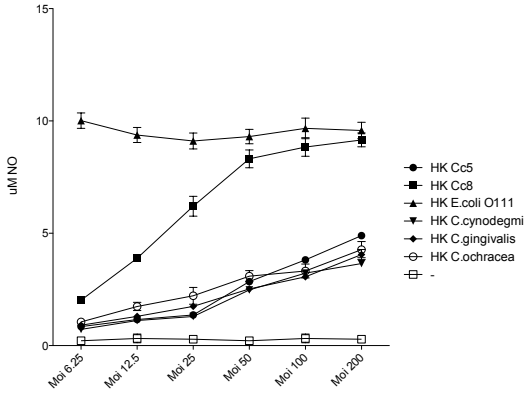


**Figure 9. NO release of RAW264.7 murine macrophages after stimulation for 24h with heat-killed (HK) bacteria.** Macrophages were stimulated with heat-killed (HK) bacteria at MOI indicated and incubated for 24h. Release of nitric oxide (NO) was measured using modified Griess reagent.

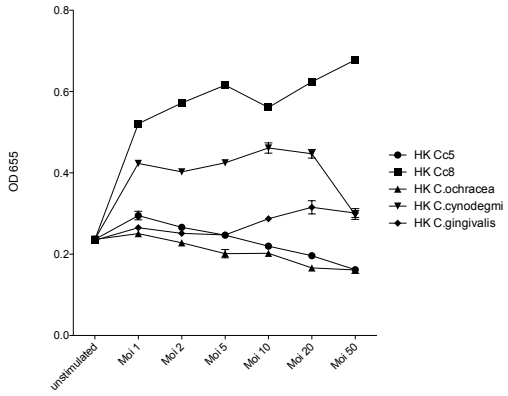


**Figure 10. Endotoxic activity of *C. canimorsus* (Cc) 5, Cc8 and other *Capnocytophaga* species.** Indicated MOI of heat killed (HK) bacteria were assayed for TLR4 dependent NFκB activation with HekBlue human TLR4 cells.

```
CLUSTAL 2.0.12 multiple sequence alignment


Cc5_LpxL_seq           MGNLLIYILAFPVIWLVSVLPFRWLYIFSDFVYLFVYKIFKYRVEVVRKNLSIAFPDKTK 60
Cc5_LpxL_database      -MNLLIYILAFPVIWLVSVLPFRWLYIFSDFVYLFVYKIFKYRVEVVRKNLSIAFPDKTK 59
Cc8_LpxL               MGNLLIYILAFPVIWLVSVLPFRWLYIFSDFVYLFVYKIFKYRVEVVRKNLSIAFPDKTE 60
                         ************************************************************:

Cc5_LpxL_seq           AEKRNIERKFYHHMCDMFLEMVKSYHMSEKEIKKRMVYTNIELIKSYENSRSIIFLCGHY 120
Cc5_LpxL_database      AEKRNIERKFYHHMCDMFLEMVKSYHMSEKEIKKRMVYTNIELIKSYENSRSIIFLCGHY 119
Cc8_LpxL               AEKRNIERKFYHHMCDMFLEMVKSYHMSEKEIKKRMVYTNIELIKPYENSRSIIFLCGHY 120
                       *******************************************.**************

Cc5_LpxL_seq           ASYEWLMSLGYFLKHKSYGLYTPITNPYFDRLVKKIRMKHRGFLISRYAAASEMKKHRDE 180
Cc5_LpxL_database      ASYEWLMSLGYFLKHKSYGLYTPITNPYFDRLVKKIRMKHRGFLISRYAAASEMKKHRDE 179
Cc8_LpxL               ASYEWLMSLGYFLKHKSYGLYTPITNPYFDRLVKKIRMKHQAFLISRYAAASEMKKHRDE 180
                       ******************************************:.******************

Cc5_LpxL_seq           NTIACYGFAADQSPSSSKSYRREFLGKIVPVFTGAERLGKQLNTVMVYAKIEKVKRGYYQ 240
Cc5_LpxL_database      NTIACYGFAADQSPSSSKSYRREFLGKIVPVFTGAERLGKQLNTVMVYAKIEKVKRGYYQ 239
Cc8_LpxL               NTIACYGFAADQSPSSSKSYRREFLGKIVPVFTGAERLGKQLNTVMVYAKIEKVKRGYYQ 240
                       ************************************************************

Cc5_LpxL_seq           CTFEILAENPNEMPNYQITDLFFERLNQQIYQKPEYYLWTHNRFKRM 287
Cc5_LpxL_database      CTFEILAENPNEMPNYQITDLFFERLNQQIYQKPEYYLWTHNRFKRM 286
Cc8_LpxL               CTFEILAENPNEMPNYQITDLFFERLNQQIYQKPEYYLWTHNRFKRM 287
                       *********************************************
```

**Figure 11. ClustalW multiple sequence alignment of *C. canimorsus* 5 (Cc5) and *C. canimorsus* 8 (Cc8) *lpxL*.** *lpxL* was cloned in pUC19 with primers 6317/6242 (for Cc5)

and 6338/6242 (for Cc8) and sequenced with standard sequencing primers for pUC19 (Microsynth, pUCM13-52, pUCM13r-57). Cc5 *lpxL* sequence was taken from genome database (marked as _database) and re-sequenced (marked as _seq). Primer 6317 couldn't be used for cloning of Cc8 *lpxL* cloning, as a polymorphism leads to an internal HindIII site.

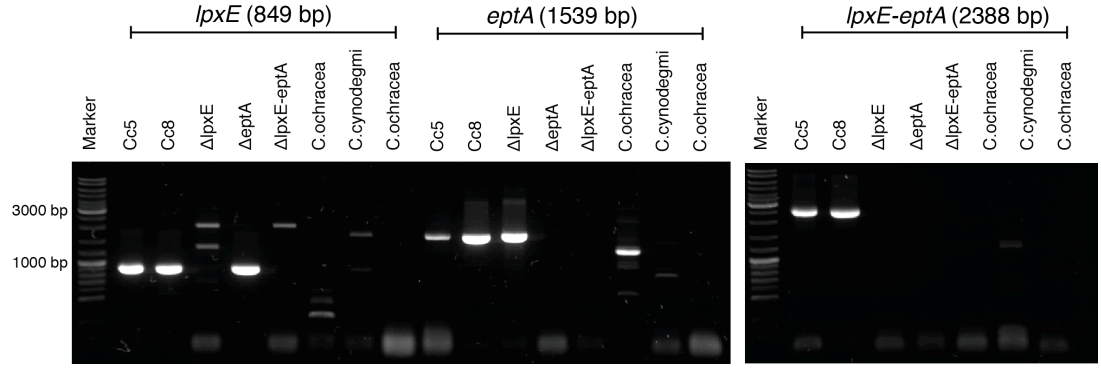


**Figure 12. PCR amplification of *lpxF*, *eptA* or *lpxF-eptA*** using primers 6646/6647, 6648/6649 or 6646/6649, respectively.

### 6.1.8 *C. canimorsus* 5 and *C. canimorsus* 8 LpxL partially restore *E. coli* Δ*lpxL* phenotype

In order to compare LpxL of Cc5 and Cc8 we have expressed the genes in *E. coli* K12 Δ*lpxL* (Bainbridge et al., 2006). Cc5 LpxL is a (R)-3-hydroxy-13-methyltetradecanoic acid transferase. This acyl chain is not synthesized by *E. coli*. Due to the known fuzziness of LpxL, it might most probably attach palmitate in *E. coli (*personal communication, U. Zähringer). Cc8 LpxL might have an altered acyl chain specificity than the one of Cc5 and could preferentially add palmitate or other by *E. coli* synthesized acyl chains. Therefore a difference in endotoxicity for *E. coli* K12 Δ*lpxL* with the plasmid encoding Cc5 *lpxL* (pSI49 and pSI51) or Cc8 *lpxL* (pSI50 and pSI52) might be measured. To address this issue, heat-killed bacteria were assayed for TLR4 dependent NFκB activation with the HekBlue human TLR4 cell line. Expression of Cc5 and Cc8 *lpxL* in *E. coli* K12 Δ*lpxL* leaded to a similar increase in endotoxicity as compared to *E. coli* K12 Δ*lpxL* **(Fig. 13 and 14)**. This suggests that Cc8 *lpxL* might as well be a (R)-3-hydroxy-13-methyltetradecanoic acid transferase (or a transferase of another acyl chain synthesized by *C. canimorsus* and not by *E. coli*).

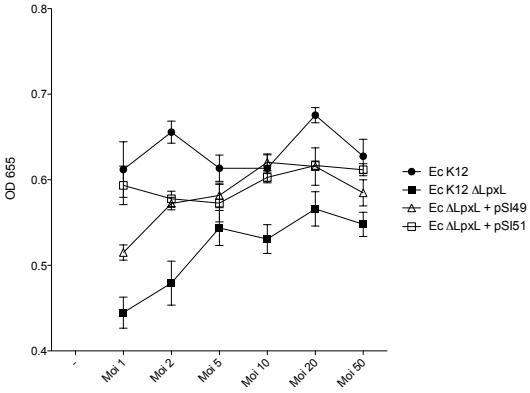


**Figure 13. Endotoxic activity of *E. coli* (Ec) K12, *E. coli* K12 Δ*lpxL, E. coli* K12 Δ*lpxL* with the plasmid encoding Cc5 *lpxL* (pSI49 and pSI51).** Indicated MOI of heat killed (HK) bacteria were assayed for TLR4 dependent NFκB activation with HekBlue human TLR4 cells.

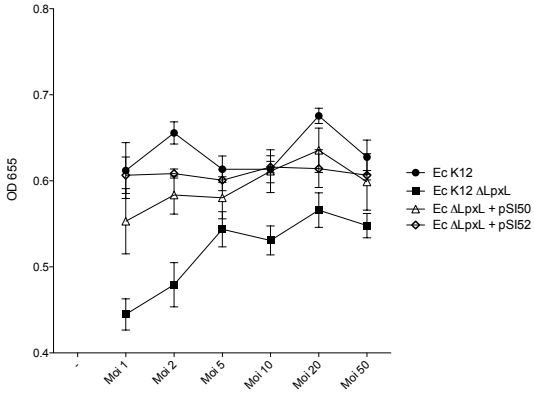


**Figure 14. Endotoxic activity of *E. coli* (Ec) K12, *E. coli* K12 Δ*lpxL, E. coli* K12 Δ*lpxL* with the plasmid encoding Cc8 *lpxL* (pSI50 and pSI52).** Indicated MOI of heat killed (HK) bacteria were assayed for TLR4 dependent NFκB activation with HekBlue human TLR4 cells.

### 6.1.9 Additional Methods

**Sensitivity of *C. canimorsus* to Human β-Defensin 2.** The resistance of *C. canimorsus* to DEFB2 was measured using the protocol previously described (Curtis et al.). Human β-defensin 2 (GIGNPVTCLKSGAICHPVFCPRRYKGIGYCGLPGTKCCLL, with disulfide bonds C-1–C-5, C-2–C-4, and C-3–C-6) were supplied by Peptides International. Briefly, $1x10^6$ cfu/ml *C. canimorsus* bacteria were incubated with 3-100 μg/ml DEFB2 in PBS for 30 min or 2h. cfu/ml was then determined using dilution and plating.

**NO release by RAW264.7 or J774 murine macrophages.** Murine monocyte-macrophage J774.1 (ATCC TIB-67) and murine monocyte-macrophage RAW264.7 (ATCC TIB-71) were cultured as recommended by the American Type Culture Collection. $1x10^5$ cells were seeded in 24-well plates (1 ml/well) and incubated for 24h at 37°C and 5% $CO_2$. Cells were stimulated as indicated and incubated for further 24h. Nitric oxide (NO) production was the estimated as the amount of nitrite released in the culture medium, by use of modified Griess reagent (Sigma).

**Plasmids.** All Plasmids are described in the Appendix, table A1.

### 6.1.10 Discussion

*C. canimorsus* lipid A and *E. coli* F515 penta-acyl lipid A both showed to be not endotoxic. *E. coli* penta-acyl lipid A showed to be a potent antagonist on the activity of *E. coli* O111 LPS, being around 10-fold less inhibitory as lipid IVa. This is in contrast to Cc5 lipid A, which was found not to be an antagonist of the interaction *E. coli* O111 LPS and human TLR4/MD-2. Lipid IVa binds deeper into the MD-2 pocket and is turned by 180° as compared to *E. coli* hexa-acyl and probably penta-acyl lipid A (Ohto et al., 2007; Park et al., 2009). The binding of lipid IVa to MD-2 could therefore be more stable as the penta-acylated *E. coli* lipid A. Lipid IVa therefore is a more potent antagonist as *E. coli* penta-acyl lipid A. The discrepancy in agonism and antagonism of *E. coli* penta-acyl lipid A and Cc5 lipid A can be explained by differences in binding to MD-2. The free lipid A of *C. canimorsus* was found not to bind to human MD-2 even if LBP and CD14 are present **(chapter 2.1 Fig. 5)**. Hence it can't act as an antagonist, as the antagonism mainly takes place at the level of MD-2 (Coats et al., 2007). This is in contrast to *E. coli* penta-acyl lipid A that seems to be transported from CD14 onto MD-2 and thus competes for its binding with the agonist.

CAMPs are important members of the innate immune system and have a broad range bactericidal activity. CAMP binding to bacteria relies on charge-charge interactions. Reducing the negative charge on the bacterial surface reduces the interaction with CAMPs and thus the fatal insertion of CAMPs in the bacterial membrane. Defensins are small cystein-rich CAMPs and several Defensins are expressed in the human oral cavity (Mathews et al., 1999), and likely in dog's mouth. *C. canimorsus* has therefore to cope with CAMPs in its natural habitat and accordingly exhibited a high CAMP

resistance. We could demonstrate that besides high resistance to Polymyxin B, *C. canimorsus* highly resists to the action of human Defensins, even in comparison to other highly resistant bacteria as *P. gingivalis* (Curtis et al.). Altering the net negative charge of *C. canimorsus* lipid A had a strong influence on Polymyxin B resistance, and very probably on CAMP resistance in general. This feature of *C. canimorsus* might as well be important for human infections, as human CAMPs (as Defensins) are present in peripheral blood (Pazgier et al., 2006) and are released by polymorphonucleated neutrophils (Lehrer et al., 1993). Besides the effect on endotoxic activity, lipid A modifications alter *C. canimorsus* resistance to CAMPs and thus favour persistence in dog's oral cavity as well as human infections.

### 6.1.11 References


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## 6.2 Appendix 2: Table A1: Plasmids constructed

| Name | Insert | template | in plasmid | cleavage sites | remarks | primer used |
|---|---|---|---|---|---|---|
| pSI01 | 3 fragment PCR: orf 568::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | orf568 is Ef-Tu; lethal deletion | 5106-5111 |
| pSI02 | 3 fragment PCR: orf 958::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | orf958 is pitrilysin | 5100-5105 |
| pSI03 | 3 fragment PCR: orf 1890::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | | 5094-5099 |
| pSI04 | orf 958 | Cc5 genomic DNA | pMM47 | NcoI/XbaI | orf958 is pitrilysin | 5213/5214 |
| pSI05 | orf 958-His (read-through to His on plasmid) | Cc5 genomic DNA | pMM47 | NcoI/XbaI | orf958 is pitrilysin | 5213/5319 |
| pSI06 | Mutation in CamA zinc binding motif: HEFSH to HEFSA | pMM82 | - | - | iPCR | 5183/5184 |
| pSI07 | Mutation in CamA zinc binding motif: HEFSH to HDFSH | pMM82 | - | - | iPCR | 5185/5186 |
| pSI08 | CamB-13Aa linker-His (orf981) | Cc5 genomic DNA | pMM47 | NcoI/XbaI | CamB is the 4. gene in Cc5 PUL11 | 4339/5238 |
| pSI09 | CamB-13Aa linker-Strep | Cc5 genomic DNA | pMM47 | NcoI/XbaI | CamB is the 4. gene in Cc5 PUL11 | 4339/5481 |
| pSI10 | CamB-13Aa linker-Strep | Cc5 genomic DNA | pMAPA OmpACc | NcoI/XbaI | CamB is the 4. gene in Cc5 PUL11 | 4339/5481 |
| pSI11 | CamB-13Aa linker-StrepHis | Cc5 genomic DNA | pMM47 | NcoI/XbaI | CamB is the 4. gene in Cc5 PUL11 | 4339/5482 |
| pSi12 | CamB-13Aa linker-StrepHis | Cc5 genomic DNA | pMAPA OmpACc | NcoI/XbaI | CamB is the 4. gene in Cc5 PUL11 | 4339/5482 |
| pSI13 | CamB-13Aa linker-His for E. coli | Cc5 genomic DNA | pET22b+ | NdeI/XhoI | CamB with Signalpeptide | 4332/5305 |
| pSI14 | CamB-13Aa linker-His for E. coli | Cc5 genomic DNA | pET22b+ | NdeI/XhoI | CamB without Signalpeptide | 4333/5305 |
| pSI15 | orf608 Cc5 (LuxS) | Cc5 genomic DNA | pMAPA OmpAFJ | NcoI/XbaI | orf608 is a homologue to LuxS | 6012/6013 |
| pSI16 | orf608 Cc5 (LuxS) | Cc5 genomic DNA | pMAPA OmpACc | NcoI/XbaI | orf608 is a homologue to LuxS | 6012/6013 |
| pSI17 | orf608 Cc5 (LuxS) | Cc5 genomic DNA | pMM47 | NcoI/XbaI | orf608 is a homologue to LuxS | 6012/6013 |
| pSI18 | LpxK E. coli O111 | E. coli O111 genomic DNA | pMAPA OmpAFJ | NcoI/XbaI | | 6163/6164 |
| pSI19 | LpxK E. coli O111 | E. coli O111 genomic DNA | pMM47 | NcoI/XbaI | | 6163/6164 |
| pSI20 | LpxK E. coli BL21 | E. coli BL21 genomic DNA | pMAPA OmpAFJ | NcoI/XbaI | | 6163/6164 |
| pSI21 | LpxK E. coli BL21 | E. coli BL21 genomic DNA | pMM47 | NcoI/XbaI | | 6163/6164 |
| pSI22 | LpxL E. coli O111 | E. coli O111 genomic DNA | pMAPA OmpAFJ | NcoI/XbaI | | 6165/6166 |

| | | | | | | |
|---|---|---|---|---|---|---|
| pSI23 | LpxL E. coli O111 | E. coli O111 genomic DNA | pMAPA OmpACc | NcoI/XbaI | | 6165/6166 |
| pSI24 | LpxL E. coli O111 | E. coli O111 genomic DNA | pMM47 | NcoI/XbaI | | 6165/6166 |
| pSI25 | LpxL E. coli BL21 | E. coli BL21 genomic DNA | pMAPA OmpAFJ | NcoI/XbaI | | 6165/6166 |
| pSI26 | LpxL E. coli BL21 | E. coli BL21 genomic DNA | pMAPA OmpACc | NcoI/XbaI | | 6165/6166 |
| pSI27 | LpxL E. coli BL21 | E. coli BL21 genomic DNA | pMM47 | NcoI/XbaI | | 6165/6166 |
| pSI28 | LpxK Campylobacter jejuni | C. jejuni genomic DNA | pMM47 | NcoI/XbaI | | 6263/6264 |
| pSI29 | LpxK Campylobacter jejuni | C. jejuni genomic DNA | pMAPA OmpACc | NcoI/XbaI | | 6263/6264 |
| pSI30 | LpxL Campylobacter jejuni | C. jejuni genomic DNA | pMM47 | NcoI/XbaI | | 6265/6266 |
| pSI31 | LpxL Campylobacter jejuni | C. jejuni genomic DNA | pMAPA OmpACc | NcoI/XbaI | | 6265/6266 |
| pSI32 | LpxK Cc5 | Cc5 genomic DNA | pMM47 | NcoI/XbaI | Ccan17380 is LpxK | 6208/6209 |
| pSI33 | LpxK Cc5 | Cc5 genomic DNA | pMAPA OmpACc | NcoI/XbaI | Ccan17380 is LpxK | 6208/6209 |
| pSI34 | LpxL Cc5 | Cc5 genomic DNA | pMM47 | NcoI/XbaI | Ccan6750 is LpxL | 6241/6242 |
| pSI35 | LpxL Cc5 | Cc5 genomic DNA | pMAPA OmpACc | NcoI/XbaI | Ccan6750 is LpxL | 6241/6242 |
| pSI36 | LpxK Cc8.1 | Cc8.1 genomic DNA | pMM47 | NcoI/XbaI | | 6208/6209 |
| pSI37 | LpxK Cc8.1 | Cc8.1 genomic DNA | pMAPA OmpACc | NcoI/XbaI | | 6208/6209 |
| pSI38 | LpxL Cc8.1 | Cc8.1 genomic DNA | pMM47 | NcoI/XbaI | | 6241/6242 |
| pSI39 | LpxL Cc8.1 | Cc8.1 genomic DNA | pMAPA OmpACc | NcoI/XbaI | | 6241/6242 |
| pSI40 | 3 fragment PCR: Ccan17380::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | Ccan17380 is LpxK | 6210-6215 |
| pSI41 | 3 fragment PCR: Ccan6750::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | Ccan6750 is LpxL | 6243-6248 |
| pSI42 | 3 fragment PCR: Ccan14540::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | Ccan14540 is a candidate for LpxF | 6362-6367 |
| pSI45 | Ccan6750 (LpxL) Cc5 | Cc5 genomic DNA | pUC19 | HindIII/XbaI | ATG pUC19 | 6317/6242 |
| pSI46 | LpxL Cc8.1 | Cc8.1 genomic DNA | pUC19 | SphI/XbaI | ATG pUC19 | 6317/6242 |
| pSI49 | Ccan6750 (LpxL) Cc5 | Cc5 genomic DNA | pUC19 | SphI/XbaI | own ATG in frame with ATG pUC19 | 6474/6242 |
| pSI50 | LpxL Cc8.1 | Cc8.1 genomic DNA | pUC19 | SphI/XbaI | own ATG in frame with ATG pUC19 | 6474/6242 |
| pSI51 | Ccan6750 (LpxL) Cc5 | Cc5 genomic DNA | pUC19 | SphI/XbaI | own ATG, out of frame with ATG pUC19 | 6475/6242 |

| | | | | | | |
|---|---|---|---|---|---|---|
| pSI52 | LpxL Cc8.1 | Cc8.1 genomic DNA | pUC19 | SphI/XbaI | own ATG, out of frame with ATG pUC19 | 6475/6242 |
| pSI53 | Ccan14540 Cc5 | Cc5 genomic DNA | pUC19 | SphI/XbaI | ATG pUC19 | 6471/6369 |
| pSI54 | Ccan14540-like from Cc8.1 | Cc8.1 genomic DNA | pUC19 | SphI/XbaI | ATG pUC19 | 6471/6369 |
| pSI55 | Ccan14540 Cc5 | Cc5 genomic DNA | pUC19 | SphI/XbaI | own ATG in frame with ATG pUC19 | 6472/6369 |
| pSI56 | Ccan14540-like from Cc8.1 | Cc8.1 genomic DNA | pUC19 | SphI/XbaI | own ATG in frame with ATG pUC19 | 6472/6369 |
| pSI57 | Ccan14540 Cc5 | Cc5 genomic DNA | pUC19 | SphI/XbaI | own ATG, out of frame with ATG pUC19 | 6473/6369 |
| pSI58 | Ccan14540-like from Cc8.1 | Cc8.1 genomic DNA | pUC19 | SphI/XbaI | own ATG, out of frame with ATG pUC19 | 6473/6369 |
| pSI59 | Ccan16960 | Cc5 genomic DNA | pMM47 | NcoI/XbaI | Ccan16960 is LpxE, ! Overlap with Ccan16950! | 6646/6647 |
| pSI60 | Ccan16950 | Cc5 genomic DNA | pMM47 | NcoI/XhoI | Ccan16950 is EptA, !overlap with Ccan16960! | 6648/6649 |
| pSI61 | Ccan16940 | Cc5 genomic DNA | pMM47 | NcoI/XhoI | | 6650/6651 |
| pSI62 | Ccan16960-50 | Cc5 genomic DNA | pMM47 | NcoI/XhoI | | 6646/6649 |
| pSI63 | Ccan16960-40 | Cc5 genomic DNA | pMM47 | NcoI/XhoI | | 6646/6651 |
| pSI64 | Ccan16950-40 | Cc5 genomic DNA | pMM47 | NcoI/XhoI | | 6648/6651 |
| pSI65 | Ccan16960 | Cc5 genomic DNA | pUC19 | SphI/XbaI | own ATG in frame with ATG pUC19 | 6652/6647 |
| pSI66 | Ccan16950 | Cc5 genomic DNA | pUC19 | SphI/SacI | own ATG in frame with ATG pUC19 | 6653/6654 |
| pSI67 | Ccan16940 | Cc5 genomic DNA | pUC19 | SphI/SacI | own ATG in frame with ATG pUC19 | 6655/6656 |
| pSI68 | Ccan16960-50 | Cc5 genomic DNA | pUC19 | SphI/SacI | own ATG in frame with ATG pUC19 | 6652/6654 |
| pSI69 | Ccan16960-40 | Cc5 genomic DNA | pUC19 | SphI/SacI | own ATG in frame with ATG pUC19 | 6652/6656 |
| pSI70 | Ccan16950-40 | Cc5 genomic DNA | pUC19 | SphI/SacI | own ATG in frame with ATG pUC19 | 6653/6656 |
| pSI71 | pMM47 with Promoter Ccan16960 | Cc5 genomic DNA | pMM47 | SalI/NcoI | | 6644/6645 |
| pSI72 | LpxM E. coli O111 | E. coli O111 genomic DNA | pMAPA OmpAFJ | NcoI/XbaI | | 6167/6168 |
| pSI73 | 3 fragment PCR: Ccan16960::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | Ccan16960 is LpxE, ! Overlap with Ccan16950! | 6493-6498 |
| pSI74 | 3 fragment PCR: Ccan16950::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | Ccan16950 is EptA, !overlap with Ccan16960! | 6499-6504 |

| | | | | | | |
|---|---|---|---|---|---|---|
| pSI75 | 3 fragment PCR: Ccan16940::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | | 6505-6510 |
| pSI76 | 3 fragment PCR: Ccan16960::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | | 6493-5/6502-04 |
| pSI77 | 3 fragment PCR: Ccan16960::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | | 6493-5/6508-10 |
| pSI78 | 3 fragment PCR: Ccan6070::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | Ccan6070 is a candidate for LpxF | 6511-6516 |
| pSI79 | 3 fragment PCR: Ccan12100::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | Ccan12100 is a homologue to wzt | 6600-6605 |
| pSI80 | 3 fragment PCR: Ccan8390::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | Ccan8390 is a homologue to wzb | 6553-6558 |
| pSI81 | 3 fragment PCR: Ccan17350::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | Ccan17350 is a homologue to wzc | 6559-6564 |
| pSI82 | 3 fragment PCR: Ccan15550::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | Ccan15550 is a homologue to wza | 6541-6546 |
| pSI83 | 3 fragment PCR: Ccan15540::ermF | Cc5 genomic DNA | pMM25 | SalI/SpeI | Ccan15540 is wzz | 6547-6552 |
| pSI84 | 3 fragment PCR: Ccan15550::ermF | Cc5 genomic DNA | pMM25 | SalI/SpeI | | 6565, 6542-6546/6547-6552 |
| pSI85 | 3 fragment PCR: Ccan23290::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | Ccan23290 belongs to the O-antigen cluster | 6517-6522 |
| pSI86 | 3 fragment PCR: Ccan23410::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | Ccan23410 is a homologue to rmlA | 6523-6528 |
| pSI87 | 3 fragment PCR: Ccan23430::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | Ccan23430 is a homologue to rmlC | 6529-6534 |
| pSI88 | 3 fragment PCR: Ccan23440::ermF | Cc5 genomic DNA | pMM25 | PstI/SpeI | Ccan23440 is a homologue to rmlD | 6535-6540 |

## 6.3 Appendix 3: Table A2: Oligonucleotide primers

| Number | Primer SI | Keyword | Sequence | sense or antisense | gene locus | organism | remarks |
|---|---|---|---|---|---|---|---|
| 5094 | 1 | KO orf 1890 | cactgcaggcttctgcggtaacagtagg | s | orf 1890 | Cc5 | |
| 5095 | 2 | KO orf 1890 | gagtagataaaagcactgttcaatgcttgcgtaacctac | as | orf 1890 | Cc5 | |
| 5096 | 3 | KO orf 1890 | cacaagcaggtgtaggttacgcaagcattgaacagtgcttttatctactccgatagcttc | s | orf 1890 | Cc5 | |
| 5097 | 4 | KO orf 1890 | catcattgtctctgtctctgtgaggacaaccctacgaaggatgaaatttttcagggacaac | as | orf 1890 | Cc5 | |
| 5098 | 5 | KO orf 1890 | aaaaatttcatccttcgtagggttgtcctcacagagacag | s | orf 1890 | Cc5 | |
| 5099 | 6 | KO orf 1890 | caactagtcaagttgatttcaacacgacgg | as | orf 1890 | Cc5 | |
| 5100 | 7 | KO orf 958 | cactgcaggaagggacatttattaactgc | s | orf 958 | Cc5 | |
| 5101 | 8 | KO orf 958 | gagtagataaaagcactgttgtacgtgtagggtcttcgtc | as | orf 958 | Cc5 | |
| 5102 | 9 | KO orf 958 | tggggcaaaagacgaagaccctacacgtacaacagtgcttttatctactccgatagcttc | s | orf 958 | Cc5 | |
| 5103 | 10 | KO orf 958 | gttttataggctcttcttgaatatccactcctacgaaggatgaaatttttcagggacaac | as | orf 958 | Cc5 | |
| 5104 | 11 | KO orf 958 | aaaaatttcatccttcgtaggagtggatattcaagaagag | s | orf 958 | Cc5 | |
| 5105 | 12 | KO orf 958 | caactagtctggcaagtaatccaaatcc | as | orf 958 | Cc5 | |
| 5106 | 13 | KO orf 568 | cactgcaggctgctatcactaaagtattg | s | orf 568 | Cc5 | |
| 5107 | 14 | KO orf 568 | gagtagataaaagcactgttcttggaatacctacctgacg | as | orf 568 | Cc5 | |
| 5108 | 15 | KO orf 568 | tcttttgggtcgtcaggtaggtattccaagaacagtgcttttatctactccgatagcttc | s | orf 568 | Cc5 | |
| 5109 | 16 | KO orf 568 | ctcttctttactcaagatatacacctcagcctacgaaggatgaaatttttcagggacaac | as | orf 568 | Cc5 | |
| 5110 | 17 | KO orf 568 | aaaaatttcatccttcgtaggctgaggtgtatatcttgag | s | orf 568 | Cc5 | |
| 5111 | 18 | KO orf 568 | caactagtgttacctgaccagcacctac | as | orf 568 | Cc5 | |
| 5148 | 19 | RevT-PCR gp47phox | tccccagccagcactatg | s | p47 phox! | J774 | wrong primer: are for p47phox |
| 5149 | 20 | RevT-PCR gp47phox | cagagatgaccgtggcaac | as | p47 phox! | J774 | wrong primer: are for p47phox |
| 5181 | 21 | RevT-PCR gp91phox | gcatccagaaatgcaagaca | s | gp91phox | J774 | |
| 5182 | 22 | RevT-PCR gp91phox | catcaaggcctctgctctct | as | gp91phox | J774 | |
| 5183 | 23 | quickchange HExxH orf 982 (camA) | cacgaattctcagccattatgcaccaaaccattgac | s | orf 982 | Cc5 | |
| 5184 | 24 | quickchange HExxH orf 982 (camA) | tggtgcataatggctgagaattcgtgaaaaatggttc | as | orf982 | Cc5 | |
| 5185 | 25 | quickchange HExxH orf 982 (camA) | cacgacttctcacacattatgcaccaaaccattgac | s | orf 982 | Cc5 | |
| 5186 | 26 | quickchange HExxH orf 982 (camA) | tggtgcataatgtgtgagaagtcgtgaaaaatggttc | as | orf 982 | Cc5 | |
| 5213 | 27 | Complementation of KO orf958 | cgtaccatgggaaagggacatttattaactgc | s | orf 958 | Cc5 | |
| 5214 | 28 | Complementation of KO orf958 | gttctagatttttttagtgctttctggcaagtaatc | as | orf 958 | Cc5 | |
| 5215 | 29 | Complementation of KO orf958 | gttctagatttttttagtgctttctggcaagtaatc | as | orf 958 | Cc5 | |
| 5216 | 30 | Detection of orf 958 in div Cc strains | tgccgctaccttagacgaat | s | orf 958 | Cc5 | |
| 5217 | 31 | Detection of orf 958 in div Cc strains | attcgcgctcagagatgagt | as | orf 958 | Cc5 | |
| 5218 | 32 | RevT-PCR iNOS (NOS2) | ctgcatggaccagtataaggcaagc | s | iNOS | J774 | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 5219 | 33 | RevT-PCR iNOS (NOS2) | agcttctggtcgatgtcatgagcaa | as | iNOS | J774 | |
| 5220 | 34 | RevT-PCR p67phox | tctaagaagctggcgctctc | s | p67phox | J774 | |
| 5221 | 35 | RevT-PCR p67phox | gcgtctgagttttccctttg | as | p67phox | J774 | |
| 5238 | 36 | Complementation of KO CamB with C-term 13 Aa linker-His tagged CamB | actctagaccagctcctgccccagcgccacctgctttgttgagaataatccaaaaatc | as | orf 981 | Cc5 | |
| 5305 | 37 | CamB in E. coli with 13 Aa linker-His | ccgctcgagttctagaccagctcctgccccagcgccacctgctttgttgagaataatccaaaaatc | as | orf 981 | Cc5 | |
| 5319 | 38 | Complementation of KO orf958 | gttctagattatttttagtgctttctggcaagtaatc | as | orf 981 | Cc5 | |
| 5427 | 39 | camNOAB in pEt22b+ (for expression in E. coli) | gggaattgcatatgggactaaaaattaaaaagatg | s | orf 984-981 | Cc5 | |
| 5428 | 40 | camNOAB in pEt22b+ (for expression in E. coli) | ccgctcgagttatttgttgagaataatccaaaaatc | as | orf 984-981 | Cc5 | |
| 5429 | 41 | camNOAB in pEt22b+ (for expression in E. coli) | gggaattgcatatgaaacataaaattttaacatatag | s | orf 983-981 | Cc5 | |
| 5430 | 42 | CamB N-term His in pET15 (for expression in E. coli) | gctttagacatatggacaaagagtccgtttttg | s | orf 981 | Cc5 | |
| 5431 | 43 | CamB N-term His in pET15 (for expression in E. coli) | gtgtatggatccttatttgttgagaataatccaaaaatc | as | orf 981 | Cc5 | |
| 5432 | 44 | CamB N-term His in pET15 (for expression in E. coli) | gctttagacatatggcaggtggcgctggggcaggagctgacaaagagtccgtttttg | s | orf 981 | Cc5 | |
| 5433 | 45 | CamB N-term His in pET15 (for expression in E. coli) | cgtttagacatatggacgatgacgataaggacaaagagtccgtttttg | s | orf 981 | Cc5 | |
| 5442 | 46 | human TLR4 (glu24-lys631) with 10xHi-tag and Cd33 signal peptide, for pcDNA3 | gttaaagcttcagacatgccgctgctgctactgctgcccctgctgtgggcaggggccctg gct gaaagctgggagccctg | s | TLR4 | human | |
| 5443 | 47 | human TLR4 (glu24-lys631) with 10xHi-tag and Cd33 signal peptide, for pcDNA3 | gcttctcgagtcattactactagtgatgatggtgatggtgatggtgatgatgcttattcatctg acaggtgatattc | as | TLR4 | human | |
| 5452 | 48 | CamB expression in E. coli, 2step PCR! | cataccatggga aaaaaaataaaacaactaataggcgtattggtggcattacttaccta gggggatgtcaacaagacaaag | s | orf 981 | Cc5 | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 5453 | 49 | CamB expression in E. coli, 2step PCR! | gggggatgtcaacaagacaaagag gacgatgacgataag catcatcaccatcaccac tccgtttttgagaaacctgc | s | orf 981 | Cc5 | |
| 5454 | 50 | CamB expression in E. coli, 2step PCR! | gggggatgtcaacaagacaaagag gacgatgacgataag tggagccacccgcagttcgaaaaa tccgtttttgagaaacctgc | s | orf 981 | Cc5 | |
| 5455 | 51 | CamB expression in E. coli, 2step PCR! | gggggatgtcaacaagacaaagag ctggaagttctgttccaggggccc tccgtttttgagaaacctgc | s | orf 981 | Cc5 | |
| 5456 | 52 | CamB expression in E. coli, 2step PCR! | gggggatgtcaacaagacaaagag ctggaagttctgttccaggggccc catcatcaccatcaccac tccgtttttgagaaacctgc | s | orf 981 | Cc5 | |
| 5457 | 53 | CamB expression in E. coli, 2step PCR! | gggggatgtcaacaagacaaagag atcgaaggtcgt catcatcaccatcaccac tccgtttttgagaaacctgc | s | orf 981 | Cc5 | |
| 5458 | 54 | CamB expression in E. coli, 2step PCR! | gggggatgtcaacaagacaaagag atcgaaggtcgt tggagccacccgcagttcgaaaaa tccgtttttgagaaacctgc | s | orf 981 | Cc5 | |
| 5481 | 55 | CamB expression in Cc, 2step PCR! | actctagattatttttcgaactgcgggtggctccatgcgccagctcctgcagctcctgcccc agcgccacctgctttgttgagaataatccaaaaatc | as | orf 981 | Cc5 | |
| 5482 | 56 | CamB expression in Cc, 2step PCR! | actctagacctttttcgaactgcgggtggctccatgcgccagctcctgcagctcctgcccc agcgccacctgctttgttgagaataatccaaaaatc | as | orf 981 | Cc5 | |
| 5483 | 57 | sequencing pcDNA, SP6 | atttaggtgacactatag | s | sequencing | - | |
| 5484 | 58 | sequencing pcDNA, T7 | taatacgactcactataggg | as | sequencing | - | |
| 5615 | 59 | CamB expression in Cc, C-term 13 Aa linker and OneStrep-tag | actctagattatttctcgaactgcgggtggctccacgctgaacctcccgatccaccgccag aacctccacctttttcgaactgcgggtg | s | orf 981 | Cc5 | |
| 5616 | 60 | CamB expression in Cc, C-term 13 Aa linker and OneStrep-tag | ccttttcgaactgcgggtggctccaagcgctagctcctgcagctcctgccccagcgccac ctgctttgttgagaataatccaaaaatctgc | s | orf 981 | Cc5 | |
| 5617 | 61 | KO of camN (orf 984) | cactgcagatgggactaaaaattaaaaagatgatg | s | orf 984 | Cc5 | |
| 5618 | 62 | KO of camN (orf 984) | gagtagataaaagcactgttccatagatggaagttgctccac | as | orf 984 | Cc5 | |
| 5619 | 63 | KO of camN (orf 984) | ccatagatggaagttgctccacgcacacgaacagtgcttttatctactccgatagcttc | s | orf 984 | Cc5 | |
| 5620 | 64 | KO of camN (orf 984) | caatttttcacgctcctgaaaatatacctcctacgaaggatgaaatttttcagggacaac | as | orf 984 | Cc5 | |
| 5621 | 65 | KO of camN (orf 984) | aaaaatttcatccttcgtaggaggtatattttcaggagcgtg | s | orf 984 | Cc5 | |
| 5622 | 66 | KO of camN (orf 984) | caactagtttataatcctactcttaatgttaatgtcatttg | as | orf 984 | Cc5 | |
| 5651 | 67 | CamA-Strep-Stop-CamB in pET22b+ | cgggtggctccatgcactagctcctgcagctcctgccccagcgccacctgcgttaatgtta tctaaatctacattgtcaagatttgc | as | orf 982 | Cc5 | |
| 5652 | 68 | CamA-Strep-Stop-CamB in pET22b+ | gcaggagctgcaggagctagtgcatggagccacccgcagttcgaaaaataaaatacc tatgaaaaaaataaaacaactaataggcg | s | orf 982 | Cc5 | |
| 5653 | 69 | CamA-OneStrep-Stop-CamB in pET22b+ | cgatccaccgccagaacctccacctttttcgaactgcgggtggctccaagcgcttgcact agctcctgcgttaatgttatctaaatctacattgtcaag | as | orf 982 | Cc5 | |
| 5654 | 70 | CamA-OneStrep-Stop-CamB in pET22b+ | aaaaggtggaggttctggcggtggatcgggaggttcagcgtggagccacccgcagttc gagaaataaaatacctatgaaaaaaataaaacaactaatag | s | orf 982 | Cc5 | |
| 5696 | 71 | recombinant human TLR4 in pFlag-CMV -> N-term Flag-tag | gttaaagcttgaaagctgggagccctg | s | TLR4 | human | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 5697 | 72 | recombinant human TLR4 in pFlag-CMV -> N-term Flag-tag | gcttgtcgactcattactactacttattcatctgacaggtgatattc | as | TLR4 | human | |
| 5698 | 73 | orf 958 Cc5 mutation in zinc binding site | gctcatttttttgatcatcttctctttgaaggaac | s | orf 982 | Cc5 | |
| 5699 | 74 | orf 958 Cc5 mutation in zinc binding site | caaagagaagatgatcaaaaaaatgagcgaatcctg | as | orf 982 | Cc5 | |
| 5700 | 75 | orf 958 Cc5 mutation in zinc binding site | caggattcgctgctttttttgaacatcttctctttg | s | orf 982 | Cc5 | |
| 5701 | 76 | orf 958 Cc5 mutation in zinc binding site | gatgttcaaaaaaagcagcgaatcctgtacgtgtag | as | orf 982 | Cc5 | |
| 5702 | 77 | orf 958 Cc5 mutation in zinc binding site | gctcatttttttgaagctcttctctttgaaggaactaaaaatattg | s | orf 982 | Cc5 | |
| 5703 | 78 | orf 958 Cc5 mutation in zinc binding site | cttcaaagagaagagcttcaaaaaaatgagcgaatcctg | as | orf 982 | Cc5 | |
| 5704 | 79 | human MD2 in pCMVtag3b; templat: AA0099571, Hwains plasmid with hMD2 | ccgcggatccctgttttcttccatatttactgaagctcag | s | MD-2 | human | |
| 5705 | 80 | human MD2 in pCMVtag3b; templat: AA0099571, Hwains plasmid with hMD2 | cccgctcgagctaatttgaattaggttggtgtaggatg | as | MD-2 | human | |
| 5712 | 81 | human MD2 in pCMVtag3b; templat: AA0099571, Hwains plasmid with hMD2 | ccgcctgcagctgttttcttccatatttactgaagctcag | s | MD-2 | human | |
| 5673 | 63_new | KO of camN (orf 984), new primer "C" | cgtgtgggtggagcaacttccatctatggaacagtgcttttatctactccgatagcttc | s | orf 984 | Cc5 | |
| 6012 | 82 | Cc5 luxS (=orf 608) in pMM47 (for expression in Cc) | cgtaccatggagaaaatagccagtttttgtattg | s | orf 608 | Cc5 | |
| 6013 | 83 | Cc5 luxS (=orf 608) in pMM47 (for expression in Cc) | cagttctagactatttaggataaaccaaattttcttc | as | orf 608 | Cc5 | |
| 6014 | 84 | human TLR4 with a C-term Flag tag | gcttctcgagtcattactactacttgtcgtcatcgtctttgtagtccttattcatctgacaggtgatattc | as | TLR4 | human | |
| 6096 | 85 | Cc5 luxS (=orf 608) with a C-term His-tag in pMM47 (for expression in Cc) | cagttctagagctttaggataaaccaaattttcttctttc | as | orf 608 | Cc5 | |

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|---|---|---|---|---|---|---|---|
| 6097 | 86 | Cc5 luxS (=orf 608) with a C-term His-tag in pET vectors (for expression in E. coli) | cgtacatatggagaaaatagccagtttttgtattg | s | orf 608 | Cc5 | |
| 6098 | 87 | Cc5 luxS (=orf 608) with a C-term His-tag in pET vectors (for expression in E. coli) | cagtctcgagtgcgccagctcctgctttaggataaaccaaattttcttctttc | as | orf 608 | Cc5 | |
| 6099 | 88 | Cc5 pfs (together with luxS for AI2 Synthesis) in pMM47 (for expression in Cc) | cgtaccatggcaagaatagggattataggagctatgg | s | orf 20040 | Cc5 | |
| 6100 | 89 | Cc5 pfs (together with luxS for AI2 Synthesis) in pMM47 (for expression in Cc) | cagttctagactaaacctctaaggcatagtgtttaat | as | orf 20040 | Cc5 | |
| 6101 | 90 | Cc5 pfs (together with luxS for AI2 Synthesis) with C-term His-tag in pMM47 (for expression in Cc) | cagttctagagcaacctctaaggcatagtgtttaataag | as | orf 20040 | Cc5 | |
| 6102 | 91 | Cc5 pfs (together with luxS for AI2 Synthesis) with C-term His-tag in pET (for expression in E. coli) | cgtacatatgagaatagggattataggagctatgg | s | orf 20040 | Cc5 | |
| 6103 | 92 | Cc5 pfs (together with luxS for AI2 Synthesis) with C-term His-tag in pET (for expression in E. coli) | cagtctcgagtgcgccagctcctgcaacctctaaggcatagtgtttaataag | as | orf 20040 | Cc5 | |
| 6163 | 93 | E. coli lpxK in pMM47 (expression in Cc) | cgtaccatgggaatcgaaaaaatctggtctgg | s | lpxK | E. coli | |
| 6164 | 94 | E. coli lpxK in pMM47 (expression in Cc) | cgtatctagacagtctgcagctagttgccagaagccag | as | lpxK | E. coli | |
| 6165 | 95 | E. coli lpxL in pMM47 (expression in Cc) | cgtaccatgggaacgaatctacccaagttctcc | s | lpxL | E. coli | |
| 6166 | 96 | E. coli lpxL in pMM47 (expression in Cc) | cgtatctagattaatagcgtgaaggaacgc | as | lpxL | E. coli | |
| 6167 | 97 | E. coli lpxM in pMM47 (expression in Cc) | cgtaccatggaaacgaaaaaaaataatagcgaatac | s | lpxM | E. coli | |
| 6168 | 98 | E. coli lpxM in pMM47 (expression in Cc) | cgtatctagattatttgatgggataaagatctttg | as | lpxM | E. coli | |

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|---|---|---|---|---|---|---|---|
| 6208 | 99 | Cc lpxK (Ccan17380) in pMM47 (expression in Cc) | cgtaccatggtaagaattattcgatacttgctactgc | s | Ccan 17380 | Cc5/Cc8 | |
| 6209 | 100 | Cc lpxK (Ccan17380) in pMM47 (expression in Cc) | cgtatctagattacaaaaaaggttcagaaaatttaatc | as | Ccan 17380 | Cc5/Cc8 | |
| 6210 | 101 | KO Ccan17380 | ccctgcagccttccttagctacagctgc | s | Ccan 17380 | Cc5 | |
| 6211 | 102 | KO Ccan17380 | gagtagataaaagcactgttttttagttgttgaattgttgc | as | Ccan 17380 | Cc5 | |
| 6212 | 103 | KO Ccan17380 | cctctacctttgcaacaattcaacaactaaaaaaaacagtgcttttatctactccgatagcttc | s | Ccan 17380 | Cc5 | |
| 6213 | 104 | KO Ccan17380 | gatttttaatcaaagcaaaaatataagaaaattacagaccctacgaaggatgaaatttttc agggacaac | as | Ccan 17380 | Cc5 | |
| 6214 | 105 | KO Ccan17380 | aaaaatttcatccttcgtagggtctgtaattttcttatatttttgc | s | Ccan 17380 | Cc5 | |
| 6215 | 106 | KO Ccan17380 | caactagtcgttaaaatccttatgagttgc | as | Ccan 17380 | Cc5 | |
| 6216 | 107 | KO Cc luxS (orf 608) | ccctgcagcgaaagtaaatattttgaattaactcaac | s | orf 608 | Cc5 | |
| 6217 | 108 | KO Cc luxS (orf 608) | gagtagataaaagcactgttacaaatatgattttcaaaagattatttattc | as | orf 608 | Cc5 | |
| 6218 | 109 | KO Cc luxS (orf 608) | gaataaaacaagaataaataatctttgaaaatcatatttgtaacagtgcttttatctactcc gatagcttc | s | orf 608 | Cc5 | |
| 6219 | 110 | KO Cc luxS (orf 608) | gaaaatttttcaggatttcttgatatcattaggtaaacactacgaaggatgaaatttttcagg gacaac | as | orf 608 | Cc5 | |
| 6220 | 111 | KO Cc luxS (orf 608) | aaaaatttcatccttcgtagtgtttacctaatgatatcaagaaatcc | s | orf 608 | Cc5 | |
| 6221 | 112 | KO Cc luxS (orf 608) | caactagtggaatattgtcggcagtatc | as | orf 608 | Cc5 | |
| 6241 | 113 | Cc lpxL (ccan 6750) in pMM47 (expression in Cc) | cgtaccatgggaaacttattaatctacatacttgcttttcc | s | Ccan 6750 | Cc5/Cc8 | |
| 6242 | 114 | Cc lpxL (ccan 6750) in pMM47 (expression in Cc) | cgtatctagactacatccttttgaaacgattg | as | Ccan 6750 | Cc5/Cc8 | |
| 6243 | 115 | KO Cc lpxL (Ccan 6750) | ccctgcagcgaataaaacaaatacccaatagc | s | Ccan 6750 | Cc5 | |
| 6244 | 116 | KO Cc lpxL (Ccan 6750) | gagtagataaaagcactgttcaagggttaaaaatttagcaaatatag | as | Ccan 6750 | Cc5 | |
| 6245 | 117 | KO Cc lpxL (Ccan 6750) | ccgccaaatataactatatttgctaaatttttaaccccttgaacagtgcttttatctactccgata gcttc | s | Ccan 6750 | Cc5 | |
| 6246 | 118 | KO Cc lpxL (Ccan 6750) | ctaatcactaatctctacccccctcaccaactacgaaggatgaaatttttcagggacaac | as | Ccan 6750 | Cc5 | |
| 6247 | 119 | KO Cc lpxL (Ccan 6750) | aaaaatttcatccttcgtagttggtgaggggggtagag | s | Ccan 6750 | Cc5 | |
| 6248 | 120 | KO Cc lpxL (Ccan 6750) | caactagtgaggctatggctaatgcc | as | Ccan 6750 | Cc5 | |
| 6263 | 121 | C. jejuni lpxK in pMM47 (expression in Cc) | cgtaccatgggaagtgaagaaaaaaattatgaactttg | s | lpxK | C. jejuni | |

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|---|---|---|---|---|---|---|---|
| 6264 | 122 | C. jejuni lpxK in pMM47 (expression in Cc) | cgtatctagatcactgttcaaactccttgatataag | as | lpxK | C. jejuni | |
| 6265 | 123 | C. jejuni lpxL in pMM47 (expression in Cc) | cgtaccatgggaaaaaatagtgatagaatatatcttagtctttattatattttg | s | lpxL | C. jejuni | |
| 6266 | 124 | C. jejuni lpxL in pMM47 (expression in Cc) | cgtatctagatcattttgcacccttgtaaa | as | lpxL | C. jejuni | |
| 6317 | 125 | Cc lpxL in pUC19 (expression iin E. coli) | catgaagcttgaacttattaatctacatacttgcttttcc | s | Ccan 6750 | Cc5 | problem with Cc8.1: lpxL Cc8.1 has HindIII site! |
| 6318 | 126 | Cc lpxL in pUC19 (expression iin E. coli) | catgaagcttggtaagaattattcgatacttgctactgc | s | Ccan 17380 | Cc5 | |
| 6319 | 127 | KO lpxK in E. coli K12 (methode lambda red) | ggcgtttacgcgcaacttcacaaaatgcagtttggccaatgatcgaaaaatgtgtaggct ggagctgcttcg | s | lpxK | E.coli K12 | |
| 6320 | 128 | KO lpxK in E. coli K12 (methode lambda red) | attcatgactccatcaatcgaacgctgccgcggcgtaactagttgccagacatatgaatat cctccttag | as | lpxK | E.coli K12 | |
| 6338 | 129 | Cc lpxL in pUC19 (expression in E. coli) | catggcatgccaacttattaatctacatacttgcttttcc | s | Ccan 6750 / lpxL | Cc5/Cc8 | new SI125 |
| 6342 | 130 | RevT-PCR lpxK Cc5 | agcggttggttggtttctaa | s | Ccan 17380 | Cc5 | |
| 6343 | 131 | RevT-PCR lpxK Cc5 | ggcaacggttgtcttttcat | as | Ccan 17380 | Cc5 | |
| 6344 | 132 | RevT-PCR lpxK Cc5 | gcaaacgagatggtcaacct | s | Ccan 17380 | Cc5 | |
| 6345 | 133 | RevT-PCR lpxK Cc5 | tgatcgggaaatgcaatatg | as | Ccan 17380 | Cc5 | |
| 6346 | 134 | RevT-PCR lpxL Cc5 | tgcggacattatgccagtta | s | Ccan 6750 | Cc5 | |
| 6347 | 135 | RevT-PCR lpxL Cc5 | gttttcatcgcggtgttttt | as | Ccan 6750 | Cc5 | |
| 6348 | 136 | RevT-PCR lpxL Cc5 | acttgcttttccagtaatttgg | s | Ccan 6750 | Cc5 | |
| 6349 | 137 | RevT-PCR lpxL Cc5 | tgtttcgtttttctgctttcg | as | Ccan 6750 | Cc5 | |
| 6362 | 138 | KO Ccan14540 | ccctgcagtgcaatactcaacggattg | s | Ccan 14540 | Cc5 | |
| 6363 | 139 | KO Ccan14540 | gagtagataaaagcactgttcttatccttggtatcggtctatc | as | Ccan 14540 | Cc5 | |
| 6364 | 140 | KO Ccan14540 | cgaaagagagatagaccgataccaaggataagaacagtgcttttatctactccgatagc ttc | s | Ccan 14540 | Cc5 | |
| 6365 | 141 | KO Ccan14540 | caataccaaaaacaaaaaactatatttcataaaccttatttgtttctacgaaggatgaaattt ttcagggacaac | as | Ccan 14540 | Cc5 | |
| 6366 | 142 | KO Ccan14540 | aaaaatttcatccttcgtagaaacaaataaggtttatgaaatatagtttttg | s | Ccan 14540 | Cc5 | |
| 6367 | 143 | KO Ccan14540 | caactagtccgaaggcgaatacactacg | as | Ccan 14540 | Cc5 | |
| 6368 | 144 | Ccan14540 in pUC19 (expression in E. coli) | catgaagcttgcttgagtttatcattgagaaagac | s | Ccan 14540 | Cc5 | |
| 6369 | 145 | Ccan14540 in pUC19 (expression in E. coli) | cgtatctagatcaaatttggacatttacaatc | as | Ccan 14540 | Cc5 | |
| 6471 | 146 | Ccan14540 in pUC19 (expression in E. coli) | catggcatgcccttgagtttatcattgagaaagac | s | Ccan14540 | Cc5 | |
| 6472 | 147 | Ccan14540 in pUC19 (expression in E. coli) | catggcatgccatgcttgagtttatcattgagaaagac | s | Ccan 14540 | Cc5 | |

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|---|---|---|---|---|---|---|---|
| 6473 | 148 | Ccan14540 in pUC19 (expression in E. coli) | catggcatgcatgcttgagtttatcattgagaaagac | s | Ccan 14540 | Cc5 | |
| 6474 | 149 | Cc lpxL (Ccan6750) in pUC19 (expression in E. coli) | catggcatgccatgaacttattaatctacatacttgcttttcc | s | Ccan 6750 | Cc5 | |
| 6475 | 149_II | Cc lpxL (Ccan6750) in pUC19 (expression in E. coli) | catggcatgcatgaacttattaatctacatacttgcttttcc | s | Ccan 6750 | Cc5 | |
| 6493 | 150 | KO Ccan16960 (lpxE) | ccctgcagggcacgttcgtaccagtta | s | Ccan 16960 | Cc5 | |
| 6494 | 151 | KO Ccan16960 (lpxE) | gagtagataaaagcactgttatttgcttatttttgaatatttcgg | as | Ccan 16960 | Cc5 | |
| 6495 | 152 | KO Ccan16960 (lpxE) | cttatatttgccgccgaaatattcaaaataagcaaataacagtgcttttatctactccgatagcttc | s | Ccan 16960 | Cc5 | |
| 6496 | 153 | KO Ccan16960 (lpxE) | cttgcattatcttaacactcataaaaacaacactcccctacgaaggatgaaatttttcagggacaac | as | Ccan 16960 | Cc5 | |
| 6497 | 154 | KO Ccan16960 (lpxE) | aaaaatttcatccttcgtaggggagtgttgtttttatgagtgtt | s | Ccan 16960 | Cc5 | |
| 6498 | 155 | KO Ccan16960 (lpxE) | caactagtaaaccgtttcagtttgggt | as | Ccan 16960 | Cc5 | |
| 6499 | 156 | KO Ccan 16950 (eptA) | ccctgcagtgttcctcgccctgttac | s | Ccan 16950 | Cc5 | |
| 6500 | 157 | KO Ccan 16950 (eptA) | gagtagataaaagcactgttttattgatttttttaacataaaattttatc | as | Ccan 16950 | Cc5 | |
| 6501 | 158 | KO Ccan 16950 (eptA) | gttgtacttaatgataaaattttatgttaaaaaaaatcaataaaacagtgcttttatctactccgatagcttc | s | Ccan 16950 | Cc5 | |
| 6502 | 159 | KO Ccan 16950 (eptA) | atcttgtaaattacggattggtcattcaataattctacgaaggatgaaatttttcagggacaac | as | Ccan 16950 | Cc5 | |
| 6503 | 160 | KO Ccan 16950 (eptA) | aaaaatttcatccttcgtagaattattgaatgaccaatccg | s | Ccan 16950 | Cc5 | |
| 6504 | 161 | KO Ccan 16950 (eptA) | caactagttccacctcattgagattcac | as | Ccan 16950 | Cc5 | |
| 6505 | 162 | KO Ccan16940 (predicted acyltransferase, in lpxF/eptA operon) | ccctgcagtatccgtcacgttttgagc | s | Ccan 16940 | Cc5 | |
| 6506 | 163 | KO Ccan16940 (predicted acyltransferase, in lpxF/eptA operon) | gagtagataaaagcactgttcaaaaataaatcttgtaaattacggatt | as | Ccan 16940 | Cc5 | |
| 6507 | 164 | KO Ccan16940 (predicted acyltransferase, in lpxF/eptA operon) | tattgaatgaccaatccgtaatttacaagatttatttttgaacagtgctttatctactccgatagcttc | s | Ccan 16940 | Cc5 | |
| 6508 | 165 | KO Ccan16940 (predicted acyltransferase, in lpxF/eptA operon) | aatcaaacaacgtttttttatactcttttcaaaggaaactacgaaggatgaaatttttcagggacaac | as | Ccan 16940 | Cc5 | |
| 6509 | 166 | KO Ccan16940 (predicted acyltransferase, in lpxF/eptA operon) | aaaaatttcatccttcgtagtttcctttgaaaagagtataaaaaaac | s | Ccan 16940 | Cc5 | |

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|---|---|---|---|---|---|---|---|
| 6510 | 167 | KO Ccan16940 (predicted acyltransferase, in lpxF/eptA operon) | caactagtcgagccaatgacgagatt | as | Ccan 16940 | Cc5 | |
| 6511 | 168 | KO Ccan6070 | ccctgcagccaagcacaaccaattctc | s | Ccan 6070 | Cc5 | |
| 6512 | 169 | KO Ccan6070 | gagtagataaaagcactgtttatctttgtcgtttgaccctac | as | Ccan 6070 | Cc5 | |
| 6513 | 170 | KO Ccan6070 | gaagatgcctctggtagggtcaaacgacaaagataaacagtgcttttatctactccgatagcttc | s | Ccan 6070 | Cc5 | |
| 6514 | 171 | KO Ccan6070 | cagcaaaaaagcttttgtttttatattcatatgattaaaactacgaaggatgaaatttttcagggacaac | as | Ccan 6070 | Cc5 | |
| 6515 | 172 | KO Ccan6070 | aaaaatttcatccttcgtagttttaatcatatgaatataaaaacaaaagc | s | Ccan 6070 | Cc5 | |
| 6516 | 173 | KO Ccan6070 | caactagtttatcgggatgaaggtcg | as | Ccan 6070 | Cc5 | |
| 6517 | 174 | KO Ccan23290 | ccctgcagtggtacgaaatttttctgattc | s | Ccan 23290 | Cc5 | |
| 6518 | 175 | KO Ccan23290 | gagtagataaaagcactgttatttttttatttttttaacaaaaatttaatcc | as | Ccan 23290 | Cc5 | |
| 6519 | 176 | KO Ccan23290 | gtatatttagggattaaatttttgttaaaaaataaaaaataacagtgcttttatctactccgatagcttc | s | Ccan 23290 | Cc5 | |
| 6520 | 177 | KO Ccan23290 | caaacgaacccgtacctcctgtaatgagaagtgttctacgaaggatgaaatttttcagggacaac | as | Ccan 23290 | Cc5 | |
| 6521 | 178 | KO Ccan23290 | aaaaatttcatccttcgtagaacacttctcattacaggaggtac | s | Ccan 23290 | Cc5 | |
| 6522 | 179 | KO Ccan23290 | caactagtgaacctcgagaagccatt | as | Ccan 23290 | Cc5 | |
| 6523 | 180 | KO Ccan23410 (rmlA?) | ccctgcagattgaaaaaggaggccct | s | Ccan 23410 | Cc5 | |
| 6524 | 181 | KO Ccan23410 (rmlA?) | gagtagataaaagcactgttagtatttaatattttagtctatcttatttttatctaacac | as | Ccan 23410 | Cc5 | |
| 6525 | 182 | KO Ccan23410 (rmlA?) | ccgtttttgaagtgttagataaaaataagatagactaaaatattaaatactaacagtgctttatctactccgatagcttc | s | Ccan 23410 | Cc5 | |
| 6526 | 183 | KO Ccan23410 (rmlA?) | gttgctaaaaggtaagttgctaaaagatgaagaaaaactacgaaggatgaaatttttcagggacaac | as | Ccan 23410 | Cc5 | |
| 6527 | 184 | KO Ccan23410 (rmlA?) | aaaaatttcatccttcgtagtttttcttcatctttagcaacttac | s | Ccan 23410 | Cc5 | |
| 6528 | 185 | KO Ccan23410 (rmlA?) | caactagtccacgatatgccaaattg | as | Ccan 23410 | Cc5 | |
| 6529 | 186 | KO Ccan23430 (rmlC?) | ccctgcaggcaacgttttgaagaaacc | s | Ccan 23430 | Cc5 | |
| 6530 | 187 | KO Ccan23430 (rmlC?) | gagtagataaaagcactgttatgttgattataatcagcaatttaattta | as | Ccan 23430 | Cc5 | |
| 6531 | 188 | KO Ccan23430 (rmlC?) | gatattattttaaattaaattgctgattataatcaacataacagtgcttttatctactccgatagcttc | s | Ccan 23430 | Cc5 | |
| 6532 | 189 | KO Ccan23430 (rmlC?) | ctaatatgtttttcattgtttcaaatcgcatctacgaaggatgaaatttttcagggacaac | as | Ccan 23430 | Cc5 | |
| 6533 | 190 | KO Ccan23430 (rmlC?) | aaaaatttcatccttcgtagatgcgatttgaaacaatga | s | Ccan 23430 | Cc5 | |
| 6534 | 191 | KO Ccan23430 (rmlC?) | caactagtattcccaaaggaaagcg | as | Ccan 23430 | Cc5 | |

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|---|---|---|---|---|---|---|---|
| 6535 | 192 | KO Ccan23440 (rmlD?) | ccctgcagtgatgaacgcggatactt | s | Ccan 23440 | Cc5 | |
| 6536 | 193 | KO Ccan23440 (rmlD?) | gagtagataaaagcactgttttattcgtataggtttacatcaaaatc | as | Ccan 23440 | Cc5 | |
| 6537 | 194 | KO Ccan23440 (rmlD?) | cttttcacttcgattttgatgtaaacctatacgaataaaacagtgcttttatctactccgatagcttc | s | Ccan 23440 | Cc5 | |
| 6538 | 195 | KO Ccan23440 (rmlD?) | gattttgaaggtttatacttttttactttatccttatacttttttattctacgaaggatgaaatttttcaggacaac | as | Ccan 23440 | Cc5 | |
| 6539 | 196 | KO Ccan23440 (rmlD?) | aaaaatttcatccttcgtagaataaaaagtataaaggataaagtaaaaagtataaac | s | Ccan 23440 | Cc5 | |
| 6540 | 197 | KO Ccan23440 (rmlD?) | caactagtgcttccttatctaccccatc | as | Ccan 23440 | Cc5 | |
| 6541 | 198 | KO Ccan15550 (wza?) | ccctgcaggcctatatgcgtatggcac | s | Ccan 15550 | Cc5 | |
| 6542 | 199 | KO Ccan15550 (wza?) | gagtagataaaagcactgttcgttttttatgtatttgggca | as | Ccan 15550 | Cc5 | |
| 6543 | 200 | KO Ccan15550 (wza?) | gcactacatttgcccaaatacataaaaaacgaacagtgcttttatctactccgatagcttc | s | Ccan 15550 | Cc5 | |
| 6544 | 201 | KO Ccan15550 (wza?) | caatctcatctgtattactaactttattttcgcacataaactacgaaggatgaaatttttcagggacaac | as | Ccan 15550 | Cc5 | |
| 6545 | 202 | KO Ccan15550 (wza?) | aaaaatttcatccttcgtagtttatgtgcgaaaataaagttagtaatac | s | Ccan 15550 | Cc5 | |
| 6546 | 203 | KO Ccan15550 (wza?) | caactagtaatgcaggaagccctatg | as | Ccan 15550 | Cc5 | |
| 6547 | 204 | KO Ccan15540 (wzz) | cctggtcgacgcagctgatgacacagaat | s | Ccan 15540 | Cc5 | |
| 6548 | 205 | KO Ccan15540 (wzz) | gagtagataaaagcactgttaaattacttaaatgtattgaataacaaaact | as | Ccan 15540 | Cc5 | |
| 6549 | 206 | KO Ccan15540 (wzz) | caacaatgggagttttgttattcaatacatttaagtaatttaacagtgcttttatctactccgatagcttc | s | Ccan 15540 | Cc5 | |
| 6550 | 207 | KO Ccan15540 (wzz) | actcaacaacttttaacttttttgtatattcaaatgtcttaattctacgaaggatgaaatttttcaggacaac | as | Ccan 15540 | Cc5 | |
| 6551 | 208 | KO Ccan15540 (wzz) | aaaaatttcatccttcgtagaattaagacatttgaatatacaaaaagttaaa | s | Ccan 15540 | Cc5 | |
| 6552 | 209 | KO Ccan15540 (wzz) | caactagtcagcgcttatgccctta | as | Ccan 15540 | Cc5 | |
| 6553 | 210 | KO Ccan08390 (wzb) | cctggtcgaccaaaatgatggggcagta | s | Ccan 8390 | Cc5 | |
| 6554 | 211 | KO Ccan08390 (wzb) | gagtagataaaagcactgttatttttagtgtagttaattggagatttttatatt | as | Ccan 8390 | Cc5 | |
| 6555 | 212 | KO Ccan08390 (wzb) | ggatgaattgaatataaaaatctccaattaactacactaaaataacagtgcttttatctactccgatagcttc | s | Ccan 8390 | Cc5 | |
| 6556 | 213 | KO Ccan08390 (wzb) | taaagtttaccaaattccataaactttacccaaaactacgaaggatgaaatttttcaggagacaac | as | Ccan 8390 | Cc5 | |
| 6557 | 214 | KO Ccan08390 (wzb) | aaaaatttcatccttcgtagttttgggtaaagtttatggaattt | s | Ccan 8390 | Cc5 | |
| 6558 | 215 | KO Ccan08390 (wzb) | caactagtcttcacgtgccttacgtt | as | Ccan 8390 | Cc5 | |
| 6559 | 216 | KO Ccan 17350 (wzc) | ccctgcaggttcagcctatgtcactgc | s | Ccan 17350 | Cc5 | |
| 6560 | 217 | KO Ccan 17350 (wzc) | gagtagataaaagcactgttagtttgtgtgtatttacaaaagtgtaaa | as | Ccan 17350 | Cc5 | |
| 6561 | 218 | KO Ccan 17350 (wzc) | taattactacttttacacttttgtaaatacacacaaactaacagtgcttttatctactccgatagcttc | s | Ccan 17350 | Cc5 | |
| 6562 | 219 | KO Ccan 17350 (wzc) | gtctttttattgcttttgccatttaccttgtctacgaaggatgaaatttttcagggacaac | as | Ccan 17350 | Cc5 | |
| 6563 | 220 | KO Ccan 17350 (wzc) | aaaaatttcatccttcgtagacaaggtaaatggcaaaagc | s | Ccan 17350 | Cc5 | |
| 6564 | 221 | KO Ccan 17350 (wzc) | caactagtttcccaacacgttcactc | as | Ccan 17350 | Cc5 | |

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|---|---|---|---|---|---|---|---|
| 6565 | 222 | KO Ccan15550 A, SalI instead of PstI (if to join with KO Ccan 15540) | cctggtcgacgcctatatgcgtatggcac | s | Ccan 15550 | Cc5 | |
| 6644 | 223 | new pMM47 with promoter Ccan16960 | cgatgtcgaccagatgtcgtttttagtgtgatt | s | Ccan 16960 | Cc5 | |
| 6645 | 224 | new pMM47 with promoter Ccan16960 | cgatccatggttgcttattttgaatatttcgg | as | Ccan 16960 | Cc5 | |
| 6646 | 225 | Ccan16960 in pMM47 (expression in Cc) | cgtaccatggtttttaaagaatcagcaaataacc | s | Ccan 16960 | Cc5 | |
| 6647 | 226 | Ccan16960 in pMM47 (expression in Cc) | cagttctagattattgatttttttttaacataaaattttatc | as | Ccan 16960 | Cc5 | |
| 6648 | 227 | Ccan16950 in pMM47 (expression in Cc) | cgtaccatgggattaaaaaaaatcaataaatggactaaca | s | Ccan 16950 | Cc5 | |
| 6649 | 228 | Ccan16950 in pMM47 (expression in Cc) | gcttctcgagttagtcaaaaatgctcatttgc | as | Ccan 16950 | Cc5 | |
| 6650 | 229 | Ccan16940 in pMM47 (expression in Cc) | cgtaccatgggaaattattgaatgaccaatccg | s | Ccan 16940 | Cc5 | |
| 6651 | 230 | Ccan16940 in pMM47 (expression in Cc) | gcttctcgagttattcccgatcgccg | as | Ccan 16940 | Cc5 | |
| 6652 | 231 | Ccan16960 in pUC19 (expression in E. coli) | catggcatgccatggtttttaaagaatcagcaaata | s | Ccan 16960 | Cc5 | |
| 6653 | 232 | Ccan16950 in pUC19 (expression in E. coli) | catggcatgccatgttaaaaaaaatcaataaatggacta | s | Ccan 16950 | Cc5 | |
| 6654 | 233 | Ccan16950 in pUC19 (expression in E. coli) | catggagctcttagtcaaaaatgctcatttgc | as | Ccan 16950 | Cc5 | |
| 6655 | 234 | Ccan16940 in pUC19 (expression in E. coli) | catggcatgccatgatgggaatatttagaaagaatc | s | Ccan 16940 | Cc5 | |
| 6656 | 235 | Ccan16940 in pUC19 (expression in E. coli) | catggagctcttattcccgatcgccg | as | Ccan 16940 | Cc5 | |
| 6660 | 236 | KO Ccan 12100 (wzt) | ccctgcagaacggcgactatttcagtg | s | Ccan 12100 | Cc5 | |
| 6661 | 237 | KO Ccan 12100 (wzt) | gagtagataaaagcactgttaataaatgatttatatccattactattgacg | as | Ccan 12100 | Cc5 | |
| 6662 | 238 | KO Ccan 12100 (wzt) | atttagtaatcgtcaatagtaatggatataaatcatttattaacagtgcttttatctactccgatagcttc | s | Ccan 12100 | Cc5 | |
| 6663 | 239 | KO Ccan 12100 (wzt) | tccagagttttatgagttctatttgtaataaacgtaacataatctacgaaggatgaaatttttcagggacaac | as | Ccan 12100 | Cc5 | |
| 6664 | 240 | KO Ccan 12100 (wzt) | aaaaatttcatccttcgtagattatgttacgtttattacaaatagaactcata | s | Ccan 12100 | Cc5 | |
| 6665 | 241 | KO Ccan 12100 (wzt) | caactagttcgagtcaaaacagcaaaga | as | Ccan 12100 | Cc5 | |
| 6824 | 242 | Ccan15550 in pMM47 (expression in Cc) | cgtaccatgggtcctaaaattaaatttttatttctatttattttatgt | s | Ccan 15550 | Cc5 | |
| 6825 | 243 | Ccan15550 in pMM47 (expression in Cc) | cagttctagattacttaaatgtattgaataacaaaactcc | as | Ccan 15550 | Cc5 | |
| 6826 | 244 | Ccan15540 in pMM47 (expression in Cc) | cgtaccatgggttgcgaaaataaagttagtaatacagat | s | Ccan 15540 | Cc5 | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 6827 | 245 | Ccan15540 in pMM47 (expression in Cc) | cagttctagattaaacttttttcagcttttaactgtc | as | Ccan 15540 | Cc5 | |
| 6848 | 246 | human TLR4 in pcDNA3.1 | gttaaagcttatgccaggatgatgtctg | s | TLR4 | human | |
| 6849 | 247 | human TLR4 in pcDNA3.1 | gcttctcgagaagaaatgcctcaggaggt | as | TLR4 | human | |
| 6850 | 248 | mutation in human TLR4 | gtttggttctgggagaatttgcaaatgaaggaaacttggaaaagt | s | TLR4 | human | |
| 6851 | 249 | mutation in human TLR4 | acttttccaagtttccttcatttgcaaattctcccagaaccaaac | as | TLR4 | human | |
| 6852 | 250 | mutation in human TLR4 | aaggcttactttcacttccaacgcaggtgggaatgctttttca | s | TLR4 | human | |
| 6853 | 251 | mutation in human TLR4 | tgaaaaagcattcccacctgcgttggaagtgaaagtaagcctt | as | TLR4 | human | |
| 6888 | 252 | mutation in human TLR4 | atcgtttggttctgggagcatttagaaatgcaggaaacttggaaaagtttgac | s | TLR4 | human | |
| 6889 | 253 | mutation in human TLR4 | gtcaaacttttccaagtttcctgcatttctaaatgctcccagaaccaaacgat | as | TLR4 | human | |
| 6890 | 254 | mutation in human TLR4 | aatttagaaatgaaggaaacttggcaaagtttgccaaatctgctctagagggc | s | TLR4 | human | |
| 6891 | 255 | mutation in human TLR4 | gccctctagagcagatttggcaaactttgccaagtttccttcatttctaaatt | as | TLR4 | human | |
| 6892 | 256 | mutation in human TLR4 | attgaagaattccgattagcagccttagcctactacctcgatgatattattgacttatt | s | TLR4 | human | |
| 6893 | 257 | mutation in human TLR4 | aataagtcaataatatcatcgaggtagtaggctaaggctgctaatcggaattcttcaat | as | TLR4 | human | |
| 6894 | 258 | mutation in human TLR4 | aattaggcttcataagctgactttagcaaataattttgatagtttaaatgtaatgaaaa | s | TLR4 | human | |
| 6895 | 259 | mutation in human TLR4 | ttttcattacatttaaactatcaaaattatttgctaaagtcagcttatgaagcctaatt | as | TLR4 | human | |
| 6896 | 260 | mutation in human TLR4 | gccttgagtttctagatctcagtgcaaatggcttgagtttcaaagg | s | TLR4 | human | |
| 6897 | 261 | mutation in human TLR4 | cctttgaaactcaagccatttgcactgagatctagaaactcaaggc | as | TLR4 | human | |
| 6898 | 262 | mutation in human TLR4 | gccttgagtttctagatctcagtgcaaatggcttgagtttcgcaggttgctgttctcaaagtg | s | TLR4 | human | |
| 6899 | 263 | mutation in human TLR4 | cactttgagaacagcaacctgcgaaactcaagccatttgcactgagatctagaaactcaaggc | as | TLR4 | human | |
| 6901 | 264 | mutation in human TLR4 | ggcaacatttagaattagttaactgtgcatttggacagtttcccaca | s | TLR4 | human | |
| 6902 | 265 | mutation in human TLR4 | tgtgggaaactgtccaaatgcacagttaactaattctaaatgttgcc | as | TLR4 | human | |
| 6903 | 266 | mutation in human MD-2 | gagagactgtgaatacaacaatagcattctccttcaagggaataa | s | MD-2 | human | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 6904 | 267 | mutation in human MD-2 | ttattcccttgaaggagaatgctattgttgtattcacagtctctc | as | MD-2 | human | |
| 6905 | 268 | human MD-2 in pEFBOS | cgtatctagagatccctcgacctcga | s | MD-2 | human | |
| 6906 | 269 | human MD-2 in pEFBOS | cgtatctagagtcgcggccgc | as | MD-2 | human | |
| 6907 | 270 | sequncing of pEFBOS vector (fw, upstream of MCS) | ggcacctcgattagttctcg | s | sequencing | - | |

# 7 Acknowledgements

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**Marlise Amstutz**, the fairy godmother of the lab. “Feel Swiss, be clean” – as it is written in the lab. I admit to be very Swiss in this context and I thank you a lot for keeping the lab as organized as it is. Thanks for all your help with whatever problem and for being picky.

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**Present and past lab members**: Cécile Pfaff, Dr. Manuela Mally, Dr. Hwain Shin, Claudio Cadel, Loïc Sauteur, Giacomo Golfieri, Frederik Lauber, Dr. Ulrich Wiesand, Dr. Stefan Nicolet, Dr. Anna Rozhkova, Dr. Kristina Kolygo, Dr. Caroline Montagner, Dr. Fabian Commichau, Lisa Metzger, Christian Arquint, Cédric Cattin, Dr. Stefanie Wagner, Klaus Handloser, Oliver Märki, Dr. Catherine Müller and Dr. Andreas Diepold. You have all contributed to a cheerful atmosphere. Thanks for that.

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**Prof. Dr. Paul Jenö, Suzette Moes**, I thank you for your open door and your help whenever MS was needed. Thanks a lot for your support (especially in MS of glycan chains).

**Prof. Dr. Cécile Arrieumerlou, Prof. Dr. Christoph Dehio**, my committee members. Thanks a lot for your interest in the project and for your ideas related to it. You have provided additional guidance and have enriched the discussions about the direction to take. For this I am very thankful.

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**Sarah, Julian and David Ittig** – my family. Talk is cheap – I try to live my love for you three.

# 8 Curriculum vitae

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Phone: 0041 61 261 00 33
Simon.ittig@unibas.ch

# Curriculum Vitae

## Personal Data

| | |
|---|---|
| Date of birth | March 14th 1982 |
| Place of birth | Lucerne, Switzerland |
| Nationality | Swiss |
| Children | Julian (December 27th 2006), David (March 9th 2011) |

## Education and research experience

1.3.2008 – present:
PhD studies at the Biozentrum, University of Basel, in the group of Prof. Dr. Guy R. Cornelis: „Surface structures of *Capnocytophaga canimorsus* and their role in pathogenesis."

1.1.2008 – 28.2.2008:
PhD rotation at the Biozentrum, University of Basel, in the group of Prof. Dr.Cécile Arrieumerlou: „Role of Ca2+ signalling in *Shigella flexneri* infected and transactivated cells."

15.01.2007 - 01.10.2007:
Diploma thesis at University of Natural Resources and Applied Life Sciences, Vienna in the group of Prof. Dr. R. Grabherr: „Generation of a IgG producing CHO cell line exhibiting improved microcarrier attachment".

4.7.2005 – 29.7.2005:
EAWAG (Swiss Federal Institute for Environmental Science and Technology, ETH Zürich) in the group of Prof. Dr. Thomas Egli: "Physiological state of *E. coli* in anaerobic C-limited batch and chemostat culture".

2004 – 2007 :
ESBS école supérieure de Biotechnologie, Strasbourg, trinational Studies in Biotechnology (Université Louis Pasteur Strasbourg, TH Karlsruhe, Albert Ludwigs University Freiburg and University of Basel)

2002 – 2004:
University of Bern, Faculty for Chemistry and Biochemistry, Studies in Biochemistry

1995 – 2002:
Gymnasium: Kantonsschule Reussbühl (near Luzern)

## Diplomas

| | |
|---|---|
| 2007 | Master of Science in Biotechnology / Diplôme d'Ingénieur en Biotechnologie, ESBS, Strasbourg |
| 2006 | TOEIC English test |
| 2004 | 2nd pre-degree (2.Vordiplom) in Biochemistry at University of Bern |
| 2002 | Matura (typus A, Latin and classical Greek) |

## Teaching experience

Supervision of two Master students during the PhD (Giacomo Golfieri, 2008-2009, and Claudio Cadel, 2010-2011).

Teaching assistance in practical course at the Biozentrum of the University Basel (2008-2012, 6 fold one week, with Prof. Dr. Guy R. Cornelis and Prof. Dr. Cécile Arrieumerlou).

Mentoring of several Matura projects ("Maturaarbeiten") at AKAD, Zürich.

Oral and written examination at the internal Matura Pre-examination at AKAD Zürich (Biology and Chemistry).

## Publications

Ittig S, Lindner B, Stenta M, Manfredi P, Zdorovenko E, et al. (2012) The Lipopolysaccharide from *Capnocytophaga canimorsus* Reveals an Unexpected Role of the Core-Oligosaccharide in MD-2 Binding. PLoS Pathog 8(5): e1002667. doi:10.1371/journal.ppat.1002667

## Posters

Salome Meyer*, **Simon Ittig***, and Guy R. Cornelis; *Capnocytophaga canimorsus* interferes with the oxidative burst in macrophages; 22nd EMDS annual meeting, Brescia, Italy, 2008

**Simon Ittig***, Salome Casutt-Meyer*, Giacomo Golfieri Pablo Manferdi and Guy R. Cornelis; *Capnocytophaga canimorsus* interferes with the oxidative burst in macrophages; Biozentrum Symposium 2009

Francesco Renzi, Pablo Manfredi, **Simon Ittig**, Manuela Mally, Loïc Sauteur, Paul Jenö and Guy R. Cornelis, The genome sequence of C. canimorsus demonstrates the key

role of surface-exposed protein deglycosylation systems in growth and survival in host tissues; Biozentrum Symposium 2011

**Simon Ittig**, Chantal Fiechter, Ulrich Zähringer and Guy R. Cornelis; Low endotoxicity and complement resistance of Capnocytophaga canimorsus are based on structural features of the LPS; FEMS Microbiology congress 2011, Geneva, Switzerland

**Simon Ittig**, Pablo Manfredi, Buko Lindner, Ulrich Zähringer and Guy R. Cornelis; Endotoxic to the core: Structure and Function Relationship of Lipid A and Lipid A-core from *Capnocytophaga canimorsus* reveal an extended TLR4 binding mechanism; 10th anniversary of infection biology at Biozentrum symposium, 2011

## Grants

Fellow of the Werner Siemens Foundation at the international PhD program of the Biozentrum of the University of Basel (2008-2011)

## Languages

| | |
|---|---|
| German | Native language |
| French | Very good (oral and written) |
| English | Good |

## Informatics skills

Bioinformatics (SRS, Blast, Clustal…)
Charmm (basic knowledge)
Automated Image analysis (Cell Profiler, MetaXpress)

| | |
|---|---|
| Languages: | HTML (basic knowledge) |
| | Perl (basic knowledge) |

## References

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Es git e Bueb mit name Fritz
es git e Bueb mit name Fritz
und dä cha renne wi dr Blitz
und dä cha renne wi dr Blitz

Är rennt, dä unerhört Athlet
är rennt, dä unerhört Athlet
so schnäll, das me ne gar nid gseht
so schnäll, das me ne gar nid gseht

und wil er geng isch grennt bis jitz
und wil er geng isch grennt bis jitz
het ne no niemer gseh, dr Fritz
het ne no niemer gseh, dr Fritz

und ig sogar, dr Värslischmid
und ig sogar, dr Värslischmid
mues zuegäh: vilich gits ne nid
mues zuegäh: vilich gits ne nid

(Mani Matter, 1966)