Lysophospholipids - underestimated molecules of the unique phospholipidome of *Campylobacter jejuni*

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Lysofosfolipiden - onderschatte moleculen van het unieke fosfolipidoom van *Campylobacter jejuni*

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General Introduction

Campylobacteriosis

Campylobacteriosis is the most frequent zoonotic and foodborne gastrointestinal disease in humans in the European Union (EU) [1]. Most cases (80%-90%) are caused by the bacterium *Campylobacter jejuni* [1, 2]. A low intake of *C. jejuni* bacteria is already sufficient to cause disease [3]. Following exposure, *C. jejuni* travels through the gastrointestinal tract guided by chemotaxis and flagellar motility. During this process *C. jejuni* colonizes the lower intestinal tract. Symptoms may arise 2-5 days after *C. jejuni* ingestion. Early symptoms are fever, vomiting, and nausea which are followed by 3-7 days of watery or bloody diarrhea with abdominal cramps and pains [4]. Additionally, there is a risk of notable secondary sequelae, including peripheral neuropathies such as Guillain-Barré and Miller Fisher syndrome, and reactive arthritis [4].

Campylobacteriosis normally has a characteristic seasonality with a sharp increase of cases in the summer and early autumn [5]. The epidemiology of *Campylobacter* infection is notably different between developed countries and the developing world. In the former, the infections are mostly sporadic and the prevalence of asymptomatic infection is low. In developing countries, *Campylobacter* is often endemic, more frequently asymptomatic but a major cause of diarrhea in childhood [6, 7]. According to the World Health Organization (WHO), *Campylobacter* infections in developing countries in children under the age of 2 years are frequent and sometimes fatal. Since 2015, more than 246,000 cases of *Campylobacter* infection per year have been reported with a financial burden of approximately €2.4 billion annually [5].

Campylobacter jejuni

C. jejuni is a Gram-negative bacterium belonging to the class of *Campylobacterota* in the order *Campylobacteriales*. There are 31 different species and 10 sub-species within the genus *Campylobacter* [8]. The *Campylobacter* genus includes several clinically relevant species, including *C. jejuni*, *C. coli*, *C. fetus*, *C. lari*, and *C. upsaliensis* [9]. *C. jejuni* colonizes the gastrointestinal tract of a wide variety of animals, including poultry, cattle, sheep, and swine [4]. In birds, *C. jejuni* infection is mainly asymptomatic and colonization is stable over a long time period [4].

C. jejuni is a motile, slim spiral shaped bacterium with up to four windings but can transform into a coccoid form [10]. The width and length of the bacterium varies from 0.2-0.9 µm and 0.5-5 µm, respectively [11]. *C. jejuni* requires O_2 for growth, but it cannot grow under aerobic conditions. Its optimum oxygen tension is between 2-10%, but even at 0.3% O_2, the bacteria survive and grow slowly [12]. *C. jejuni* is a thermophilic species which is able to grow between 32°C and 44°C, with an optimum temperature of 41.5°C. *C. jejuni* does not grow below 30°C because

it lacks cold shock proteins which are essential for low-temperature adaptation [13]. Unlike most other bacteria, *C. jejuni* cannot utilize many common carbohydrates as carbon source. It lacks transporters to take up sugars such as glucose or galactose and the glycolytic enzymes glucokinase and 6-phosphofructokinase [14-17]. Instead *C. jejuni* prefers the amino acids serine, aspartate, proline, and glutamate and C4-dicarboxylates (fumarate, succinate, and malate) as primary energy source [18, 19].

C. jejuni pathogenesis

The consumption of contaminated chicken meat products is considered the primary source of human *C. jejuni* infection [5]. Other ways to get infected by *C. jejuni* are by contact with sheep, wild birds, contaminated water, and pet animals [20]. During the infection, *C. jejuni* is strongly associated with and inside intestinal epithelial cells [21]. Once *C. jejuni* has reached the epithelial cell barrier, immune cells are recruited in response to infection with the pathogen. Important receptors involved in this process are members of the Toll-like receptor (TLR) and Nod-like receptor (NLR) family. The stimulation of these receptors can lead to the activation of the transcription factor NF-kβ and the production of pro-inflammatory cytokines and chemokines, promoting a strong immune response to pathogens [22]. However, *C. jejuni* manages to evade some of these innate immune receptors to maximize their probability to colonize the host. For example, *C. jejuni* is not recognized by TLR5 due to absence of the TLR5 binding site in its flagellin, and TLR2 and TLR4 are also not activated by live *C. jejuni* [23].

Although *C. jejuni* lacks traditional virulence factors like a type III secretion system (T3SS) present in most enteropathogens, it possesses a complex array of virulence factors which aid to overcome the host defense. Identified factors include proteases, adhesins, invasion promoting proteins, a type VI secretion system, cytolethal distending toxin, and flagella-mediated motility [13, 24]. The identified (serine) proteases can damage the integrity of the epithelial monolayer [25, 26]. The best characterized *C. jejuni* adhesins are two fibronectin-binding proteins termed fibronectin (CadF) [27] and fibronectin-like protein A (FlpA) [28]. CadF and FlpA are highly conserved among *C. jejuni* strains and they are crucial for *C. jejuni* adherence to human intestinal epithelial cells and colonization of chickens [29]. After successful colonization, *C. jejuni* is capable to invade upper epithelial cells and to enter gut tissue cells *in vivo* and *in vitro* [30]. The *Campylobacter* invasion antigen B (CiaB) is secreted by *C. jejuni* to promote invasion of cultured epithelial cells [31]. Isogenic CiaB mutants yield reduced chicken colonization levels [32], suggesting that *C. jejuni* invasion may be an underestimated factor in chick colonization. Several other major bacterial pathogens have a secretory apparatus for direct injection of

effector molecules into host cells [33]. *C. jejuni* does not encode a syringe-like T3SS and direct evidence for CiaB injection is missing, but CiaB and other secreted Cia proteins (CiaA-H) require a functional flagellar export apparatus for their secretion and delivery into the cytosol of host cells [34-37]. *C. jejuni* possess a single flagellum situated at one or both poles of the bacterial body. Flagellar motility and chemotaxis are crucial for *C. jejuni* to efficiently colonize the host intestine [54]. The presence of the flagellar export system is also required for maximal cell invasion [36]. Type VI secretion systems (T6SS) are able to transport macromolecules from bacteria to neighboring cells. Approximately 10% of *C. jejuni* isolates carry a complete T6SS gene cluster, which has been shown to be cytotoxic toward red blood cells after down-regulation of the polysaccharide capsule [38].

Cytolethal distending toxin (CDT) is a widely distributed toxin in Gram-negative bacteria and has been identified as an important virulence factor produced by *C. jejuni* [39]. CDT holotoxin was found to induce eukaryotic cells to arrest in the G2/M phase of the cell cycle, preventing them from entering mitosis and eventually leading to cell death [39-41], but this factor is not required to establish disease. The exact role of CDT in *C. jejuni* pathogenesis is still cryptic [42].

C. jejuni cell envelope

The *C. jejuni* cell envelope comprises the inner and outer membrane separated by a peptidoglycan layer and is surrounded by a polysaccharide capsule [43]. The cytosolic (inner) membrane of *C. jejuni* is composed of a lipid bilayer with embedded proteins. The primary lipid components are phospholipids [44]. The bacterial outer membrane consists of an inner leaflet of phospholipids and outer leaflet of the glycolipid lipopolysaccharide (LPS) [45]. *C. jejuni* LPS lacks the O-antigen typically present in most enteropathogens and hence is named lipooligosaccharide (LOS). The LOS consists of core oligosaccharides forming the inner and outer core regions and a hydrophobic lipid A moiety [45, 46]. The outer membrane of *C. jejuni* also contains porin proteins which form hydrophilic ion permeable channels across the outer membrane and bacterial adhesins [47]. Other *C. jejuni* cell-surface carbohydrate containing structures include the lipid-anchored capsular polysaccharides (CPSs), *O*-linked glycosylated flagella, and *N*-linked glycoproteins [48]. CPSs have been shown to play roles in *C. jejuni* colonization and host immune response modulation [49].

C. jejuni membrane phospholipids

The bacterial membrane is an extremely important structure whose primary functions are to protect the inner components of the cell, to maintain the selective permeability of the cells through which nutrients can enter and wastes can leave

the bacteria, and to provide the structural foundation for energy production [44, 50]. Phospholipids are the key components of all cell membranes. They form a classical lipid bilayer structure which form a barrier for ions, proteins and other molecules. Most phospholipids consist of a phosphate containing hydrophilic head group and two hydrophobic fatty acids tails, which can vary in length from 12 to 24 carbon atoms, joined by an alcohol residue (usually a glycerol molecule) [51, 52]. The phospholipids of a bacterial membrane aid the function of membrane proteins, which perform functions essential for both cell physiology and disease progression. Depending on the lipid species and composition phospholipids can largely determine the local structure, dynamics, and even the activity of membrane proteins [53].

To survive changes in the environment, bacteria must adapt and change not only their protein repertoire but also their lipid composition. Therefor the bacterial membrane phospholipid composition is dynamic and continuously changing in response to environmental changes [54]. In this process, existing phospholipids are modified or replaced by newly formed lipids [55]. In the classical *de novo* biosynthetic pathway, a membrane phospholipid is derived from the acylation of *sn*-glycerol-3-phosphate (G3P) (Fig.1). Bacteria produce G3P from exogenous glycerol and/or from endogenous phosphorylated glycerol or dihydroxyacetone phosphate via glycerokinase (GyK) [56-58]. Through the ubiquitous bacterial phospholipid formation system (PlsX-PlsY) or the second PlsB-mediated route, G3P is acylated to form lysophosphatidic acid (lysoPA) [59, 60]. Following the acylation, lysoPA is further acylated by PlsC, which is expressed by almost all bacteria, to form the key intermediate phosphatidic acid (PA) [61]. PA is the basic lipid structure in bacteria and many different types of polar head groups can be attached to PA. Cytosine diphosphate (CDP)-diacylglycerol (CDP-DAG) is the key intermediate in phospholipid synthesis in bacteria, which is catalyzed by phosphatidate cytidylyltransferase (CdsA). Two major metabolic pathways start from CDP-DAG: phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) formation [51, 55, 62]. PE is the dominant phospholipid in most bacteria, which is produced by the decarboxylation of PS by PS decarboxylase (Psd) [51]. To form PG from CDP-DAG, the CMP is first exchanged to G3P by PG-phosphate synthase (PgsA). The resulting PG-phosphate is then rapidly dephosphorylated by phosphatidylglycerol phosphate phosphatase (PgpP) to form PG. Cardiolipin (CL) can be further synthesized from PG with the assistance of cardiolipin synthase (Cls). Lysophospholipids (LPLs) are intermediates or end products in the biosynthesis of cell membrane phospholipids, generated by the chemical and enzymatic processes of regiospecific phospholipases. Usually LPLs contain only one acyl chain instead of two [63, 64] and make up only a small fraction (<1%) of the bacterial membrane phospholipids [65]. Glycerophospholipids, including PE, PG, phosphatidylserine (PS),

phosphatidic acid (PA), phosphatidylinositol (PI) and cardiolipin (CL) can become cleaved by phospholipase A (PldA), leading to the accumulation of lysophosphatidylethanolamine (lysoPE), lysophosphatidylglycerol (lysoPG), lysophosphatidylserine (lysoPS), lysophos-phatidic acid (lysoPA), lysophosphatidylinositol (lysoPI) and lysocardiolipin (lysoCL). Key steps in the formation of phospholipids are illustrated in Fig. 1.

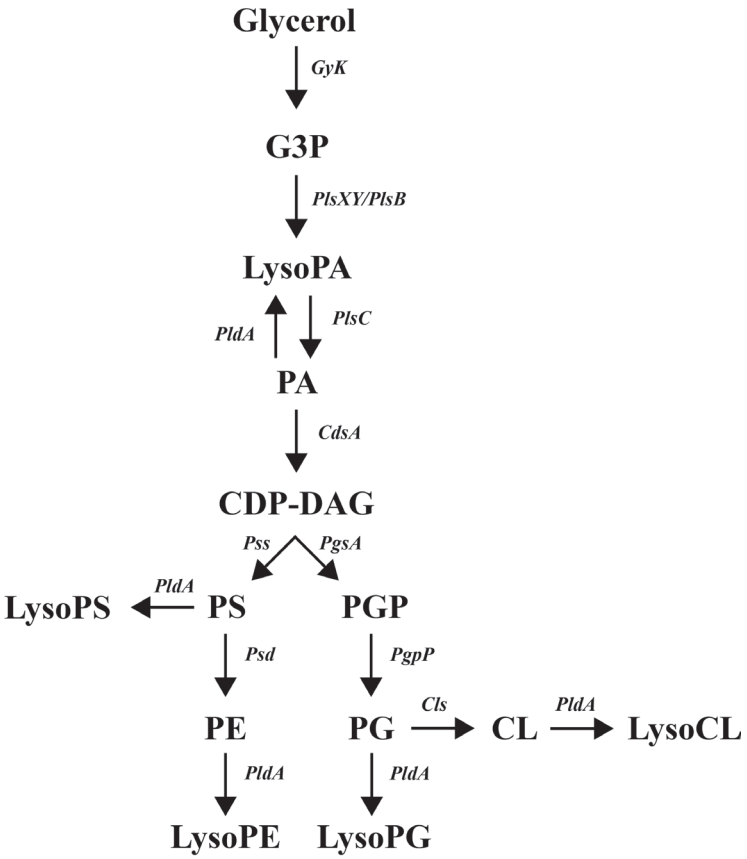

Figure 1. The *de novo* biosynthetic pathway of bacterial membrane lipids. The figure gives an overview of the metabolic pathways involved in bacterial membrane lipid formation. Enzymes that catalyze the reactions are indicated in italics. Abbreviations: G3P- glycerol-3-phosphate; LysoPA-lysophosphatidic acid; PA-phosphatidic acid; CDP-DAG-cytidine diphosphate-diacylglycerol; PS-phosphatidylserine; LysoPS-lysophosphatidylserine; PE-phosphatidylethanolamine; LysoPE-lysophosphatidylethanolamine; PGP-phosphatidylglycerol-phosphate; PG-phosphatidylglycerol; LysoPG-lysophosphatidyl-glycerol; CL-cardiolipin; LysoCL-lysocardiolipin; GyK-glycerokinase; Pls-acyltransferase; PldA-phospholipase A; CdsA-phosphatidate cytidylyltransferase; Pss-phosphatidylserine synthase; Psd-phosphatidylserine decarboxylase; PgsA-phosphatidyl-glycerolphosphate synthase; PgpP-phosphatidylglycerol phosphate phosphatase; Cls-cardiolipin synthase.

So far, the knowledge of the composition and adaptation of the phospholipidome of *C. jejuni* is very limited. Variation of PE and PG has been detected in continuous cultures of *C. jejuni* in response to changes in growth rate [66]. In another study when *C. jejuni* is changing from a spiral to coccoid form, the length of fatty acid acyl chain of the phospholipids has been shown to increase [67]. The induced changes in the fatty acid composition in these cocci result in leaky membranes, resulting in a decrease of cytoplasmic ATP. More recent, it has been reported that under the pressure of bile, the distribution of *C. jejuni* phospholipids in the cell membrane can vary through the regulation of the lipid asymmetry pathway (MLA) [68]. However, the dynamics of *C. jejuni* phospholipid composition under different growth conditions is still largely unknown.

C. jejuni phospholipase A

Bacterial membrane homeostasis and the ability of bacteria to modify the biophysical properties of membrane phospholipids are two essential factors for bacterial survival [55, 62]. As the important regulator of lipid homeostasis of cell membranes, phospholipase is important for maintaining the structural organization of cell membranes, which is indispensable for membrane proteins to be assembled correctly and to function [69]. Phospholipases remove one or more fatty acid tails from phospholipids and thus drives the formation of LPLs. Modification of the bacterial membrane phospholipid composition especially the accumulation of LPLs has been shown to play a crucial role in bacterial stress adaptation as needed after exposure to, for example, bile salts, heat and hydrogen chloride [70-74]. This suggests that phospholipases are crucial for bacterial life in a stressful environment.

***In vivo* challenges of *C. jejuni* – Bile salt exposure**

During colonization of the human intestine, *C. jejuni* is subjected to several stress conditions in the host. The presence of bile salt in the intestine is one of the factors that the host uses to induce changes in the gut microbiome [79]. Bile salts can act as antimicrobial agents by disrupting bacterial membranes [80]. When *C. jejuni* enters the intestine, it immediately encounters bile. Bile salts can pass the *C. jejuni* outer membrane directly or pass through porins [81, 82]. To survive and colonize the host gastrointestinal tract, *C. jejuni* must deal with the potentially stressful bile salts. However, in certain cases, bile salts can also be utilized by intestinal bacteria as nutrients or electron acceptors [80]. At high concentrations, bile salts will dissolve bacterial membrane lipids, causing leakage and cell death, but at low concentrations, bile salts might cause subtle effects on fluidity and permeability of the bacterial membrane [83-86]. In response to bile salts, bacteria are able to induce a change in membrane LPS/LOS architecture, membrane electric charge, hydrophobicity, lipid fluidity, and fatty acid composition to increase the bile resistance levels [87-90].

As an enteric pathogen, *C. jejuni* evolved multiple mechanisms to adapt bile salts stress. The multidrug efflux pump CmeABC, which has been shown to be essential for *C. jejuni* to colonize chickens, is the major mechanism of *C. jejuni* to resist the damaging effects of bile salt and is essential for *C. jejuni* to grow in bile salt-containing environments [91, 92]. CmeR and CbrR are two vital regulators of the *C. jejuni* CmeABC system [82, 93]. However, continuous growth of *C. jejuni* in bile salts induces the production of ROS which could result in DNA breaks. *C. jejuni* responds to the bile salt-induced ROS by altering global gene transcription in a manner consistent with a strategy to oxidative stress [94]. The *C. jejuni* AddAB DNA repair system is important to prevent the DNA damage caused by ROS [95]. *C. jejuni* uses the AddAB system instead of the RecBCD complex found in other bacteria such as *Escherichia coli* and *Bacillus subtilis* to carry out the nuclease, helicase, and Chi recognition activity required for homologous recombination [95, 96].

Bile salts have also been shown to enhance the pathogenic behavior of *C. jejuni* by inducing the expression of virulence genes, e.g. *ciaB*, *dccR*, *tlyA* and secretion of virulence proteins, e.g. invasion antigen B [97]. In addition, *C. jejuni* outer membrane vesicle (OMV) production has been reported to be stimulated by the presence of physiological concentrations of the bile salt through the changes in the expression of the maintenance of lipid asymmetry (MLA) pathway [68]. With the release of OMVs, the adhesion and invasion of *C. jejuni* to intestinal epithelial cells was increased as well [98]. Thus, the interaction between *C. jejuni* and bile salts in gastrointestinal tract may be important to facilitate *C. jejuni* colonization and virulence in the host gut.

In vivo challenges of *C. jejuni* – Iron acquisition

Iron is known as an essential compound for almost all organisms and can act as cofactor of enzymes to catalyze number of basic reactions like electron transport, energy metabolism and DNA synthesis [99]. Iron is essential for *C. jejuni* growth [100] but the availability of iron in the intestine is extremely low. Therefore, *C. jejuni* carries several uptake systems to acquire ferric iron from enterobactin and ferrichrome [101-103]. These are two important iron sources produced by the intestinal microbial flora and soil fungi [104, 105]. Because of the insolubility and toxicity of free Fe^{3+}, intracellular ferric iron in the host is normally bound to proteins in the form of hemin [106]. *C. jejuni* is capable to acquire iron from heme [101]. Erythrocyte hemolysis is a strategy employed by some bacteria to liberate heme for subsequent use as iron source. *C. jejuni* has been observed to be hemolytic with the assistance of the periplasmic binding protein CeuE, which is a component of the ferric uptake system, and the outer membrane phospholipase A which degrades membrane phospholipids [77, 107]. Ferrous iron is taken up by the *C. jejuni* high-affinity Feo ferrous transport system which plays an important role in *C. jejuni* colonization of the gastrointestinal tract [100, 108, 109].

Oxidative stress is closely linked with iron metabolism. In the intracellular environment, iron can be used as electron donor, but together with oxygen reactive oxygen species (ROS) can be formed during the Haber-Weiss reactions ($Fe^{3+} + O_2 \rightarrow Fe^{2+} + O_2$ and $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO$) [110, 111]. ROS are highly reactive and toxic for lipids, proteins, and DNA. To neutralize the ROS species, *C. jejuni* must detoxify these compounds by using different enzymes such as catalase, peroxidases, peroxiredoxin alkyl hydroperoxide reductase, superoxide dismutase, and other peroxiredoxins [110]. Catalase (KatA) which neutralizes H_2O_2 is activated when the H_2O_2 level in cytoplasm is high [112, 113], but also prevents the formation of ROS by limiting the acquisition and production of reactive iron in the cytoplasm.

***In vivo* challenges of *C. jejuni* – Low oxygen availability**

Oxygen levels in the intestine vary between 0% in the lumen to almost 10% near the epithelial cell surface [114, 115]. The microaerophilic nature of *C. jejuni* may drive the bacteria towards the optimal niche in the intestine and the low oxygen environment close to the epithelial surface. *C. jejuni* prefers the viscous mucosal matrix, where the oxygen concentration is low. *C. jejuni* thus needs to adjust its metabolism and growth rate in response to oxygen availability [116]. Thus, understanding the mechanisms of adaptation to low oxygen availability is key to elucidating how *C. jejuni* causes disease.

It is known that *C. jejuni* reduces the expression of genes which are involved in the catabolic and anabolic pathways, while it upregulates the fumarate respiration metabolism pathway in response to low oxygen availability [117]. Nevertheless, the sensor(s)/transcription factor(s) mediating oxygen regulation in *C. jejuni*, have not be identified. In other bacterial species, the expression of genes encoding electron transport proteins is under the control of the oxygen – responsive transcriptional regulator FNR [118]. *C. jejuni* lacks FNR homologues. The RacRS two-component system, Cj1491/Cj1492 two-component system and the LysR family regulator Cj1000 have been demonstrated to effect the expression of electron transport genes in *C. jejuni* [119-121]. A previous study in our lab showed that the RacRS system limited fumarate reduction in the presence of the energetically preferred electron acceptor nitrate under low oxygen conditions [121]. However, the way in which oxygen regulates gene expression and protein synthesis at a global level in *C. jejuni* is still poorly understood.

Aims and outline of the thesis

The molecular basis of *C. jejuni* infection is still largely unknown but it is clear that bacterial adaptation is crucial to successful colonization and pathogenesis [122, 123]. Bacterial virulence and metabolism often depend on the potential of a pathogen to adapt to the different environments encountered *in vivo*. The bacterial membrane plays

key role in this process as it serves as a chemical barrier and influences the activity of membrane proteins including nutrient transporters and potential virulence factors [124]. The membrane phospholipid composition is not a stable bacterial characteristic but can change in response to altered environmental conditions. This changes the organization and characteristics of the membrane lipids and proteins [125]. Better understanding of the fundamentals of bacterial membrane structure dynamics and its complementary regulatory structure is needed to fully appreciate its role in *C. jejuni* colonization and pathogenesis [126, 127]. So far, the phospholipidome of *C. jejuni* and its adaptation potential to new environments are poorly investigated. Published studies tend to hypothesize that *C. jejuni* has a relatively simple phospholipid composition of cell membranes compared to other pathogenic model bacteria [51]. The main aims of this thesis are to unravel the phospholipidome of *C. jejuni*, how it adapts to different *in vivo* challenges such as bile salt exposure and oxygen availability, and whether possible changes in membrane lipid composition influence the pathogenesis of this organism.

Chapter 2 provides an overview of the current knowledge of bacterial LPLs which are important eukaryotic cellular mediators but still only limited investigated in prokaryotes. In **Chapter 3** we determined the composition and dynamics of the phospholipidome of the *C. jejuni* and its effects on *C. jejuni* motility at low oxygen availability. In **Chapter 4** the role of the *C. jejuni* phospholipase PldA and its LPLs products during exposure to bile salt under low oxygen conditions has been investigated to learn more about the adaptive role of the membrane lipids in response to conditions encountered *in vivo*. In **Chapter 5** the biological effect of the *C. jejuni* LPLs on host cells was studied to assess their potential role as virulence factors. The novel findings are discussed and put in a broader perspective in **Chapter 6**.

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2

Biological functions of bacterial lysophospholipids

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Abstract

Lysophospholipids (LPLs) are lipid-derived metabolic intermediates in the cell membrane. The biological functions of LPLs are distinct from their corresponding phospholipids. In eukaryotic cells LPLs are important bioactive signaling molecules that regulate many important biological processes, but in bacteria the function of LPLs is still not fully defined. Bacterial LPLs are usually present in cells in very small amounts but can strongly increase under certain environmental conditions. In addition to their basic function as precursors in membrane lipid metabolism, the formation of distinct LPLs contributes to the proliferation of bacteria under harsh circumstances or may act as signaling molecules in bacterial pathogenesis. This review provides an overview of the current knowledge of the biological functions of bacterial LPLs including lysophosphatidylethanolamine (lysoPE), lysophosphatidic acid (lysoPA), lysophosphatidylcholine (lysoPC), lysophosphatidylglycerol (lysoPG), lysophosphatidylserine (lysoPS) and lysophosphatidylinositol (lysoPI) in bacterial adaptation, survival, and host-microbe interactions.

General characteristics of lysophospholipids

The primary function of phospholipids is to form the lipid bilayer that is characteristic of all prokaryotic and eukaryotic membranes. A small fraction of the phospholipids in the membrane are LPLs. In contrast to the canonical di-acylated phospholipids ubiquitously present in biological membranes, LPLs have only one fatty acid moiety conjugated to a central glycerol backbone [1]. In eukaryotes LPLs are regarded as bioactive signaling lipids which mediate a variety of cellular physiological responses [2-4]. Common LPLs are lysoPA, lysoPC, lysoPE, lysoPG, lysoPS and lysoPI [5, 6]. LPLs are generated as metabolic intermediates in the biosynthesis of cell membrane phospholipids or by cleavage of membrane phospholipids. Three different enzymes can cleave membrane phospholipids into LPLs: (1) regiospecific phospholipases can remove fatty acid tails from phospholipids [7], (2) during the formation of hepta-acylated LPS or triacylated phospholipid, the lipid A palmitoyltransferase PagP which is an enzyme present in the outer membrane of Gram-negative bacteria, can transfer a palmitate chain from phospholipids to hexa-acylated lipid A or another phospholipid, resulting in the formation of LPL [8-10], or (3) in the process of lipoprotein maturation in Gram-negative bacteria, apolipoprotein *N*-acyltransferase (Lnt) can transfer a fatty acid moiety from phospholipids to the lipoprotein precursor, resulting in the accumulation of LPL [11, 12] (Fig.1). Phospholipases differ in the specific bond they target in the phospholipid. Depending on the site of action they are classified into different subclasses A, B, C, and D. Phospholipase class A can be further divided based on the acyl chain that is cleaved. Phospholipase A_1 ($PldA_1$) hydrolyses the fatty acyl ester bond at the *sn*-1 position of the phospholipid resulting in the accumulation of (*Sn*)-1 LPL, while phospholipase A_2 ($PldA_2$) removes the acyl ester bond at the (*Sn*)-2 position of this molecule resulting in the formation of *sn*-2 LPL [13, 14].

In eukaryotic cells, the biological role of LPLs has been well-studied. Except as intermediate precursors in the biosynthesis of other cellular lipids, they are also potent messengers in G-protein-coupled signaling pathways that modulate particular immune responses by recruitment and activation of T cells, B cells and macrophages, and as multifunctional cellular growth factor in animals [15-17]. Although the concentration of LPLs in cells is very low, they are abundant in interstitial fluids and plasma. The precise content of LPLs in the body fluids is largely unknown, but lysoPC has been identified as the most abundant LPL in human plasma, with concentrations of 200-300 μM [18-20]. Other LPLs detected in human plasma are: lysoPE (10-50 μM) [19, 21-23], lysoPI (1-15 μM) [19, 24, 25], lysoPA (0.6-1 μM) [19, 26, 27], lysoPG (0.4 μM) [19, 22] and lysoPS (0.1 μM) [19, 22].

In bacteria, the role of the LPLs has barely been studied. Accumulation of LPLs in bacterial membranes is generally prevented either by their acylation to the corresponding di-acylated phospholipids or by phospholipase B, (also known as lysophospholipase which can cleave fatty acid tails of both phospholipids as well as

from LPLs) [28, 29]. Incorporation of lysolipids in lipid membranes creates instabilities in the lipid bilayer and thereby reduces its permeability barrier [6]. Exogenous $PldA_2$ of the host is therefore a potent antibacterial mechanism as it degrades the bacterial phospholipids to lysolipids [30]. Exogenous LPLs produced by the host have been demonstrated to accumulate in immune cells [31, 32] and help to reduce the bacterial infection [4]. LPLs derived from the host are also capable to trigger the release of the proinflammatory monomeric flagellin by the pathogenic *Salmonella* bacteria thereby enhancing the innate and inflammatory responses towards this bacterium [33]. LPLs produced by the hydrolysis of phospholipids by either endogenous PldA, exogenous $PldA_2$ or PagP and Lnt dependent enzymatic reactions should be rapidly cleared from the bacterial envelope to prevent their potential membrane-destabilizing effects [34, 35]. The LPL transporter LplT present in a number of Gram-negative bacterial species transports lysoPE, LysoPG and lysoCL to the cytoplasmic side of the inner membrane [35]. After transport by LplT, the LPLs are reacylated by acyl-ACP synthetase/LPL acyltransferase (Aas), giving LplT and Aas an essential role in defending Gram-negative bacteria from phospholipase attack [28]. In Gram-positive bacteria, LPLs are hardly be detected as they are toxic for these bacteria [36, 37].

Under normal conditions LPLs make up less than 1% of the total bacterial phospholipids content, however environmental stress can increase the amount of LPLs in the bacterial membranes [35, 38-40]. Several bacterial pathogens show increased release of LPLs after exposure to of bile salts, heat, and acid stress [40-44]. We recently have shown that under low oxygen conditions, high amounts of LPLs in *Campylobacter jejuni* are needed to allow the bacteria to be motile [38]. We and others have also shown that micromolar concentrations of distinct LPLs can be cytotoxic for eukaryotic cells [38, 45]. Therefore, LPLs may be an underestimated factor in bacterial pathogenesis and inflammation. The origin and function of the major bacterial LPL species is described below.

Biology of the major lysophospholipid species

Lysophosphatidic acid

Characteristics of lysoPA. The water-soluble lysoPA is the simplest form of lysoglycerophospholipids. In eukaryotic cells lysoPA is an important inflammatory marker which directly evokes various immunomodulatory responses, including apoptosis prevention, chemotaxis, cytokine and chemokine secretion, platelet aggregation, and wound healing enhancement [1, 17, 46, 47]. In bacteria, lysoPA is present at very low levels and is only known as an intermediate in biosynthesis of PA [48].

Bacterial lysoPA is considered the precursor for the synthesis of all membrane glycerolipids. LysoPA is synthesized by an acyl-ACP dependent glycerol-3-phosphate acyltransferase (Fig. 2) [49, 50]. Its biosynthesis occurs in the cytosol. Due

to lacking a binding site, lysoPA cannot be carried by the LplT transport system [51]. However, lysoPA can be produced from PA by phospholipase A (PldA) or can be made from lysoPC by autotoxin (ATX), an secreted lysophospholipase D present in some bacteria and eukaryotes (Fig 2) [1, 35, 52-54]. In addition to lysoPC, lysoPA is also easily converted *in vivo* from other LPLs including lysoPE, lysoPS and lysoPI via autotoxin in eukaryotic cells [55].

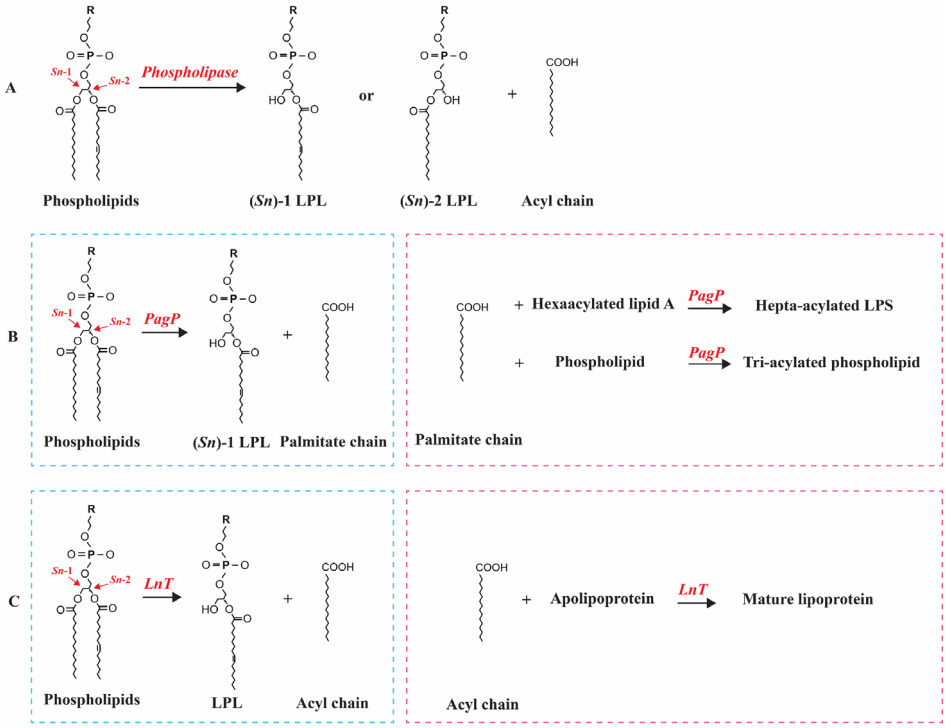

Figure 1. The bacterial LPL biosynthesis pathways. (A) LPL generated by phospholipase**. (B)** LPL produced by PagP as by-product in the formation of hepta-acylated LPS and tri-acylated PG. **(C)** LPL produced by Lnt as by-product in the lipoprotein *de novo* biosynthetic pathway. The blue dotted line represents the first step reaction, the pink dotted line represents the second step reaction. The involved enzymes and their cleavage sites are indicated in red.

Functions of lysoPA. The biophysiological roles of lysoPA in bacteria are largely unknown. Recent studies showed that reduced absorption of lysoPA might lead to a defect in flagella formation in *Helicobacter pylori* [56]. Flagella confer the motility of bacteria and are important to colonize and invade their host. Therefore, lysoPA could be an underestimated factor in bacterial pathogenesis.

Even though the role of lysoPA in bacteria is cryptic, lysoPA derived from bacteria may be important for animal health. For example, due to the changes in

gut microbiota, an increase of lysoPA and lysoPC has been observed in stools of mice suffering of colorectal tumorigenesis [57]. The dietary supplementation of *Lactobacillus plantarum* to weaned piglets would increase the relative abundance of the genus *Pantoea*, promoting the production of 1-palmitoyl lysoPA which can significantly improve the fat digestion and absorption pathway [58]. Therefore, the balance of gut health and gut microbiota-derived lysoPA seems important for intestinal homeostasis.

Effects of host-derived lysoPA on the bacteria-host interaction. In addition to lysoPA produced by bacteria, host-derived lysoPA can influence the course of bacterial infection. Host lysoPA plays an important role in alleviating bacteria-induced acute host diarrhea by increasing intestinal salt absorption and decreasing anion secretion [59, 60]. During the bacterial infection process, host lysoPA stabilizes the function and activation of mitogen-activated protein kinase / extracellular signal-regulated kinases (MAPK/ERK) pathway, the RhoA and Rho-associated kinase, proline-rich tyrosine kinase [61], Na^+/H^+ exchanger 3 [59], cystic fibrosis transmembrane conductance regulator [62] and Cl^-/OH^- exchanger [63], to maintain the intestinal ion homeostasis, and thus alleviate diarrhea. Besides, by inhibiting the bacterial endotoxin induced pro-inflammatory response through the activation of ERK 1/2, serine/threonine phosphatases, and P13 kinase signaling pathways [64], lysoPA is deemed as anti-inflammatory agent in bacteria-induced intestinal inflammation as well [54].

Role of lysoPA in host cells. The physiological role(s) of lysoPA in the eukaryotic host cell are primarily due to stimulation of the lysoPA receptor. By activating the lysoPA receptor, lysoPA can suppress the anti-tumor response in a cell intrinsic manner [65]. Mice with a defective lysoPA receptor showed increased intestinal barrier permeability and decreased H_2O_2-induced tight junction and adherens junction proteins expression in the colon [66]. In addition, bone marrow-derived dendritic cells from the mice had higher TNF-α levels and lower IL-10 secretion after lipopolysaccharide (LPS) stimulation [67]. LysoPA receptor activation by bacterial lysoPA has been associated with bacteria-induced hemolysis. Hemolysis caused by *Clostridium perfringens* is probably triggered through the activation of Ca^{2+} channels via the lysoPA receptor in erythrocytes which results in the lysis of the erythrocytes [68-70].

Lysophosphatidylcholine

Characteristics of lysoPC. LysoPC, also called lysolecithin, is a derivative of phosphatidylcholine (PC) that arises after cleavage of one fatty acid tail by a phospholipase. LysoPC can be further catabolized to lysoPA, and to choline via PC (Fig. 2) [35]. LysoPC is the most abundant LPL present in most animal tissues and is a central molecule in several physiological and pathological states [1, 71]. Only a few

bacteria are known to synthesize lysoPC, including *Agrobacterium tumefaciens*, *Pseudomonas aeruginosa*, *Chlamydia pneumonia*, *Flavobacterium johnsoniae,* and *Borrelia burgdorferi* [50, 72].

Like lysoPA, lysoPC is not a substrate of LplT. The bulky choline group likely prevents access of the enzyme to the LplT binding site [35]. Many bacterial species such as *Neisseria gonorrhoeae*, *Escherichia coli*, *Vibrio cholerae*, *Salmonella Typhimurium*, *H. pylori*, *C. jejuni*, *Bacteroidetes thetaiotamicron*, *Bacillus subtilis*, *Lactobacillus sp.*, and *C. perfringens* [50] cannot synthesize lysoPC, but might utilize lysoPC from the host. In the animal intestinal tract food-derived PC is degraded by pancreatic PldA to lysoPC [35, 73], suggesting that in general lysoPC is relatively non-toxic to the microbiota.

Function of lysoPC in bacteria. The biological role of lysoPC in eukaryotes has been well-documented. Besides by acting as a lipid precursor, lysoPC has for instance been associated with the severity of the inflammatory process [35]. Recent studies showed that decreased levels of lysoPC were associated with increased mortality risk [74]. In contrast, the physiological functions of bacterial lysoPC are still poorly understood. In one study endogenous lysoPC has been shown to regulate the function of bacterial membrane proteins [75]. In *Yersinia pseudotuberculosis* endogenous lysoPC regulates the activity of the outer membrane protein F, which functions as a size-selective filter for hydrophilic solutes [76]. The *E. coli* the large-conductance mechanosensitive channel (MscL), which is used by bacteria to respond to osmotic challenges in membrane tension, can be triggered to the fully open state by lysoPC [75]. The opened MscL is a wide water-filled pore that acts as an emergency safety valve for bacteria. *V. cholerae* has been reported to utilize lysoPC as nutrition or as resource to remodel its cell wall phospholipids [77].

Exogenous lysoPC has been shown to instantly kill the Gram-positive bacterium methicillin-resistant *Staphylococcus aureus* (MRSA) by causing increased membrane permeability [78]. It can also increase the sensitivity of *S. aureus* to antibiotics, like gentamicin [78]. LysoPC can have more indirect antimicrobial effects towards Gram-negatives as evident from an increased antibacterial activity of polymyxin B against *S. Typhimurium*, *Klebsiella pneumoniae* and *P. aeruginosa* [79-81]. This effect may be related lysoPC-induced leakiness of the bacterial membranes, facilitating the entry of antimicrobial agents [82].

Role of lysoPC in bacterial pathogenesis. In addition to altering the bacterial membrane architecture, exogenous lysoPC plays an essential role in cellular invasion and inflammation of bacterial pathogens [83]. The release of lysoPC from host cells after infection promotes the expression of *Salmonella* invasion proteins Sips and the *Salmonella* pathogenicity island 1 (SPI-1) encoded transcriptional regulator HilA to reinforce *Salmonella* invasion into the host [84]. In this process, host-derived lysoPC activates a cAMP-dependent signaling pathway leading to the production

and secretion of flagellin which in turn stimulates the host inflammatory and innate immune responses through activation of Toll-like receptor 5 [33]. Exogenous host-derived lysoPC reaches high levels within macrophages and phagosomes during *Mycobacterium tuberculosis* infection [31, 32]. The lysoPC is highly toxic and can disrupt and lyse the membranes of *Mycobacterium smegmatis* spheroplasts but due to the lysoplasmalogenase activity of the YhhN family membrane proteins they are not toxic for intact *M. smegmatis* [85]. Besides that, host-derived lysoPC induces multiple physiological reactions in bacteria. LysoPC released from the gut microbiota has been shown to impair intestinal barrier function and to play an important role in inflammatory bowel disease [86]. Four Gram-negative bacterial species *E. coli*, *Bilophila*, *Enterorhabdus* and *Gordonibacter* have been correlated with fecal lysoPC generation. The accumulation of lysoPC was shown to increase the inflammatory response and impair tight junctions *in vivo* and *vitro* [86]. The increased concentration of lysoPC originating from the gut microbiota might exacerbate colitis and damage the intestinal epithelial barrier.

Lysophosphatidylethanolamine

Characteristics of lysoPE. The major and perhaps most extensively studied LPL in bacteria is the zwitterionic lysoPE. LysoPE is mainly located in the outer membrane of Gram-negative bacteria and contributes to the positive curvature of the membrane [1, 87, 88]. Bacteria-derived lysoPE can be found in the environment as a release product of dead bacteria, but until now it has not been observed to be actively secreted by living bacteria into the environment [4]. In animals, lysoPE is only found in trace amounts in animal tissues, with the exception of plasma (there it is the second highest LPLs; 10 to 50 μM, or ~1% of total serum phospholipids) [1].

Bacterial lysoPE is formed by cleavage of PE by endogenous PldA or by the lipid A palmitoyltransferase PagP that transfers a palmitate chain from PE to hexa-acylated LPS or tri-acylated PG (Fig. 2) [8, 9, 35, 89]. Because lysoPEs induce a positive membrane curvature and have a detergent-like architecture, they are not considered suitable for being incorporated into the lipid bilayer structure and are rapidly converted into PE through lipid homeostasis processes [35, 90, 91]. However, the amount of lysoPE in the membranes of several bacterial pathogens increases during certain stress conditions [25]. Elevated oxygen concentrations increase the amount of lysoPE up to 34% of total phospholipid composition in the microaerophilic human pathogen *C. jejuni* [38]. The availability of glucose, increased temperature, or phenol biocide result in the accumulation of *Y. pseudotuberculosis* lysoPE from ~1% up to 16.3% [42, 92, 93]. Under phase stationary growth conditions, the maximum level of lysoPE in *Y. pseudotuberculosis* was reported up to 45% of the total phospholipids in cells grown at 8 °C [94]. In *V. cholerae* the presence of bile salts increases the amount of lysoPE from 2% to ~30% [40], while in *H. pylori* under pressure of acidified media,

the induced increase in lysoPE promotes the release of urease cytotoxin A [95, 96]. The increase of lysoPE during stress might be caused by the enhanced activity of the PldA as has been observed during phage lysis [97], colicin release [98], EDTA treatment [99] or heat shock [41] in *E. coli* [95, 96].

The length of the fatty acid tail of lysoPE in *E. coli* ranges from 14 to 18 carbon atoms with lysoPE 16:1 and 18:1 being most common [91, 100]. In *C. jejuni* the length of lysoPE varies from 12 to 20 carbon atoms and the fatty acid tails consist of saturated, unsaturated and cyclopropane-containing lipids. Here, lysoPE 14:0 and 19:0c are most dominant [38]. Three different lengths (16, 18, 20) of the fatty acid tail of lysoPE have been found in *V. cholerae*. In this pathogen, lysoPE 16:0 and 18:1 are the most dominant [101, 102]. In *H. pylori*, only lysoPE 18:1 has thus far been detected [103].

Role of lysoPE in bacteria. LysoPE is recognized as an essential growth factor and an important extracellular regulator in eukaryotic cells [104]. LysoPE seems also to have chaperone-like properties as it promotes the functional folding of citrate synthase and alpha-glucosidase after urea denaturation [41, 42]. LysoPEs are considered as molecular and chemical chaperones which directly affect the structure and function of membrane proteins in bacteria. Elevated membrane levels of lysoPE have been reported to allow enterobactin intake [91] and cause significant damage to the cell membrane, slow growth rates, and cause intercellular cytoplasm leakage and cell lysis [105]. In *E. coli* with high lysoPE levels the demarcation between the outer and inner membranes is missing and cytoplasmic granular degeneration and vacuolation appear [105]. The accumulation of lysoPE compromises the lipid asymmetry. The disruption of the outer membrane permeability barrier facilitates entry of molecules like enterobactin-ferric complex or vancomycin into the periplasm resulting in increased or suppressed bacterial growth [91].

LysoPE is also important for bacteria to tolerate environmental stresses. In *H. pylori* and *Y. pseudotuberculosis*, lysoPE generation is essential to adapt to an acid environment or to resist exposure to antibiotics, respectively [93, 96]. Bacterial acid resistance mechanisms may involve: (1) modification of membrane structure and outer membrane porins to block proton influx and periplasmic and cytoplasmic chaperones [106]; (2) activation the chloride-conducting (ClC) ion channel in cell membrane to allow protons enter the cell as uncharged HCl and then dissociated to H^+ and Cl^- to reduced intracellular pH [107, 108]; (3) remodeling of phospholipids in the internal membrane to decrease proton permeability [109]. Antibiotic resistance might be increased by lowering the permeability of bacterial membrane porin channel to inhibit the transport of antibiotic [93, 110]. Although, lysoPE can induce local changes in membrane curvature leading to alterations in function and conformation of membrane proteins, including porins [111]. How lysoPEs confer bacterial acid and antibiotic resistance remains to be elucidated.

Moreover, the redistribution of lysoPE to the inner membrane during stress

could change the deformation energy of the cell membrane bilayer, which determines the free energy in the protein conformation [112], and then act inversely on protein secretion, diffusion and insertion [113]. Therefore, the precise distribution of lysoPE in the bacterial membrane provides the structural basis for the proper assembly of certain membrane proteins which might be essential for bacteria [114, 115].

Antimicrobial properties of lysoPE. In addition to structural modification of cell membranes, lysoPEs have antimicrobial properties. LysoPE (16:1) of the housefly *Musca domestica* has been shown to have an antimicrobial activity [116]. Furthermore, lysoPEs isolated from Bacteroidetes *Chitinophaga* spp. have been claimed to have antimicrobial activity against the Gram-negative bacteria *Moraxella catarrhalis* and the Gram-positive *Micrococcus luteus* in a range of 4–16 μg/mL and 16–64 μg/mL, respectively [117]. The antimicrobial effect of lysoPE has been linked to selective inhibition of bacterial K^+-transport systems [116]. Bacteria need potassium for a number of cellular functions e.g. to maintain the activity of intracellular enzymes, pH homeostasis and membrane potential adjustment [118]. By blocking K^+-uptake transport in bacteria, lysoPE can become lethal for bacteria [116]. However, this hypothesis cannot entirely explain why lysoPE did not inhibit the growth of all Gram-positive bacteria since these bacteria possess only a single operative K+-uptake system, whereas Gram-negative bacteria use diverse transport systems for K^+-uptake and are thus not lethally affected by a selective system inhibition [119]. It is also possible that exogenous lysoPE could selectively integrate into the bacterial cell membrane and then permeabilize the cell. It has been reported that the increased level of lysoPE can lead to bacterial membrane collapse and trigger the autolysis mechanism that facilitates bacterial cell wall components and intracellular molecules such as the release of enzymes [95].

LysoPE and the bacteria-host interaction. Bacteria-derived LPLs can have an important effect on the host. LysoPEs, although ubiquitous in bacteria were long considered as not important in the regulation of a host-microbe interaction [120], this perception is changing. The larval metamorphosis of marine invertebrate hydrozoan *Hydractinia echinate* is dependent on bacterial LPL. Physical contact of larvae with bacterial LPLs (16:0/18:1 lysoPG, 18:0 lysoPE and 16:0 lysoPA) is needed to trigger the transformation of the larvae into the colonial adult stage [121]. LysoPEs released from commensal microbiota have been discovered to protect host intestinal epithelial barriers. They can rescue the disruption of the epithelial barrier in the colonic epithelium by sustaining the integrity of the intestinal epithelial barriers [4]. LysoPE secreted by bacteria can reverse the H_2O_2-induced inhibition of the expression of tight junction proteins and adherens junction proteins. These proteins are crucial for maintaining normal intestinal barrier function for the host [4, 122]. The lysoPEs produced by *Algoriphagus machipongonensis* were interpreted as activators and synergistic enhancers that promote single cells of the eukaryote *Salpingoeca rosetta* to develop

into multicellular rosettes, aid rosette structural integrity during its development, and facilitate rosette maturation. In this process, lysoPE recapitulates the initiation, stabilization, and maturation steps in rosette development by activating sulfonolipid rosette-inducing factors [120]. On the other hand, bacteria-derived lysoPE might be toxic towards host cells. For example, lysoPEs, especially the short chain containing lysoPEs of *C. jejuni* are able to permeabilize the host cell membrane via an oxidative stress-sensitive mechanism [45]. During bacterial infection with *Legionella pneumophila*, the accumulation of lysoPE as well as lysoPC in the mitochondrial membrane of host cells causes cell apoptosis by stimulating cytochrome C release from mitochondria [123]. The biological role of bacterial lysoPEs in host-microbe interaction can therefore be both beneficial and disruptive.

At this time, lysoPE-specific host cell receptors have not been identified. The lysoPA receptor has been reported to become activated by lysoPE directly or after transforming lysoPE into lysoPA by autotoxin in eukaryotic cells. The activation of lysoPA receptor by lysoPE results in protein kinase C (PKC) activation, which promotes tight junction formation and translocation of the adherens junction proteins, leading to an enhanced epithelial barrier integrity [124, 125].

Lysophosphatidylglycerol

Characteristics of lysoPG. LysoPG is another minor LPL that has been found in the bacterial membrane. It is produced by the hydrolysis of PG by $PldA_2$, or by PagP (Fig. 2) [35, 126]. Like lysoPE, lysoPG can be transported by LplT and then acylated by endogenous Aas in Gram-negative bacteria, but the remodeling rate for lysoPG is reported three times faster than lysoPE in *E. coli* [127]. The higher efficiency of lysoPG remodeling may be attributed to the substrate selectivity of Aas towards lysoPG because the substrate binding affinities and transport rate of LplT importation are similar between lysoPG and lysoPE [51]. LysoPG is barely detected in bacterial membranes probably because it can be easily reacylated to replenish phospholipids [36]. However, we recently showed that in *C. jejuni* which lacks the LplT transporter, the percentage of lysoPG can rise to 27% of the total phospholipids [38]. In *E. coli,* lysoPG may act as an anchor for capsular polysaccharide in the outer membrane [128].

In animal tissues, elevated lysoPG levels have been detected in acute coronary syndrome and may be related to the pathogenesis of cardiovascular diseases [22]. Parasite-derived lysoPG has been shown to contribute to the development of *Plasmodium falciparum*, a main pathogen of malaria cases, during its intraerythrocytic developmental cycle [129].

Lysophosphatidylserine

Characteristics of lysoPS. The distinguishing feature of lysoPS compared to

other LPLs is the head group, which is phospho-*L*-serine. This head group forms a phosphoester bond with the *sn*-3 hydroxide of the glycerol backbone, thus forming the glycerophospho-*L*-serine core [130]. Secretory PldA has been demonstrated to participate in production of lysoPS (Fig. 2) [1]. *In vivo*, lysoPS can be esterified with different fatty acids ranging from short chain C10 to long chain C24 [131].

Functions of lysoPS. It is known that eukaryotic lysoPSs are important bioactive lipids which play important roles in mammalian immunological processes, including macrophage activation, mast cell degranulation, leukemic cell stimulation, and regulatory T-cells maturation [132]. The tegumental surface membrane of some parasites like *Schistosoma mansoni* is enriched in lysoPS and lysoPE as well and these LPLs have been reported to affect the parasite-host interaction [133]. Like other LPLs, lysoPS is also detected in the bacterial membrane, but only at very low concentrations in a few species such as *Desulfovibrio* sp. [50]. The knowledge of physiological role of lysoPS in bacteria is therefore limited. The concentration of lysoPS in *E. coli* colonizing the intestine of gnotobiotic mice is elevated compared with germ-free mice [134]. It has been speculated that the lysoPS from *L. plantarum*-derived extracellular vesicles might be indispensable for lipid-mediated intercellular communication within or between living organisms [52]. Recently, lysoPS derived from gut microbiota was found to elicit a T helper 1 (Th1) cell immunopathological response in Crohn's disease, including promoting IFN-γ-producing $CD4^+$ T cell accumulation in the colon, enhancing Th1 cell effector functions, modulating Th1 cells bioenergetic metabolism and inducing Th1 cell epigenetic changes [135]. This suggests that bacterial lysoPS may be a relevant bioactive lipid factor in bacterial pathogenesis and intestinal inflammation progression.

Lysophosphatidylinositol

Characteristics of lysoPI. LysoPI is another subtype of endogenous lysoglycerophospholipids with an inositol as head group. It is generated by PldA through the hydrolysis of fatty acid tail of phosphatidylinositol (PI) (Fig. 2) [1]. LysoPI containing a 16:0, 18:0 or a 20:4 fatty acid tail are the most frequently found [1, 136]. The capacity to form PI and its derivative lysoPI is considered typical for eukaryotes [50], but *Actinomycetes*, *Myxococcus* some *δ-proteobacteria* are known to be rich in PI [50, 137].

Biological effects of lysoPI. In several eukaryotic cell types, lysoPI is capable to affect cell growth, differentiation and motility (sperm capacitation) [138, 139]. As lysoPI is barely detected in bacteria, the knowledge of its biology is mainly limited to a role in bacteria-host interaction [50]. The few reports are restricted to the effect of bacteria on lysoPI secretion by the host. *Lactobacillus apis* has been reported to promote lysoPI metabolism in bumblebees [140], whereas *H. pylori* has been detected to induce the generation of lysoPI in epithelial cells [141]. In another study *M. tuberculosis*-associated

lysoPI 18:0 has been found to increase in the plasma of patients with pulmonary tuberculosis (TB) [142], but it remains unclear whether this lysoPI is secreted from bacteria or from the host. In humans, where lysoPI has been well-studied, lysoPI is considered as an endocannabinoid neurotransmitter [143] which can induce endothelial cell activation associated with cellular proliferation, migration and tumorigenesis [144, 145]. The human GPR55 protein has been proposed as receptor for lysoPI [146]. Overall, it appears that lysoPI is mainly synthesized in eukaryotes where it plays a crucial role in a large number of biophysiological processes, but lysoPI is believed to be inextricably linked with gut microbiota.

Conclusions

The bacterial cell envelope is a multilayered outer barrier that protects the cell from harsh and capricious environments. As an important bioactive signaling molecule, LPLs can potentially affect the integrity of membrane lipid bilayer, leading to changes in the cellular structure and function. Recent studies confirm the indispensable role of LPLs played in bacterial invasion and the response to environmental stress. However, in this rapidly evolving field, the impact of bacterial LPLs on cell physiology is still relatively unknown. The present overview shows a role of LPLs in bacterial physiology and during the bacteria-host interaction. Environmental changes and the host immune response pose a huge threat to bacteria. Bacteria have evolved LPLs regulatory mechanisms, which could change the physical state of cell envelope and adapt to a variety of harsh conditions. Meanwhile the secretory LPLs are believed to have antimicrobial activity and show toxicity. Bacteria might take advantage of the synthesis of LPLs to compete with other microbes or to facilitate bacterial infection. On the other hand, exogenous host-derived LPLs can serve as carbon sources and could be utilized by bacteria to develop convenient reservoirs that help the pathogen to survive in the host during infection [77, 147, 148]. Clearly, the multiple roles that LPLs seem to play in bacteria, and how LPLs influence in host-bacteria interaction merits further investigation.

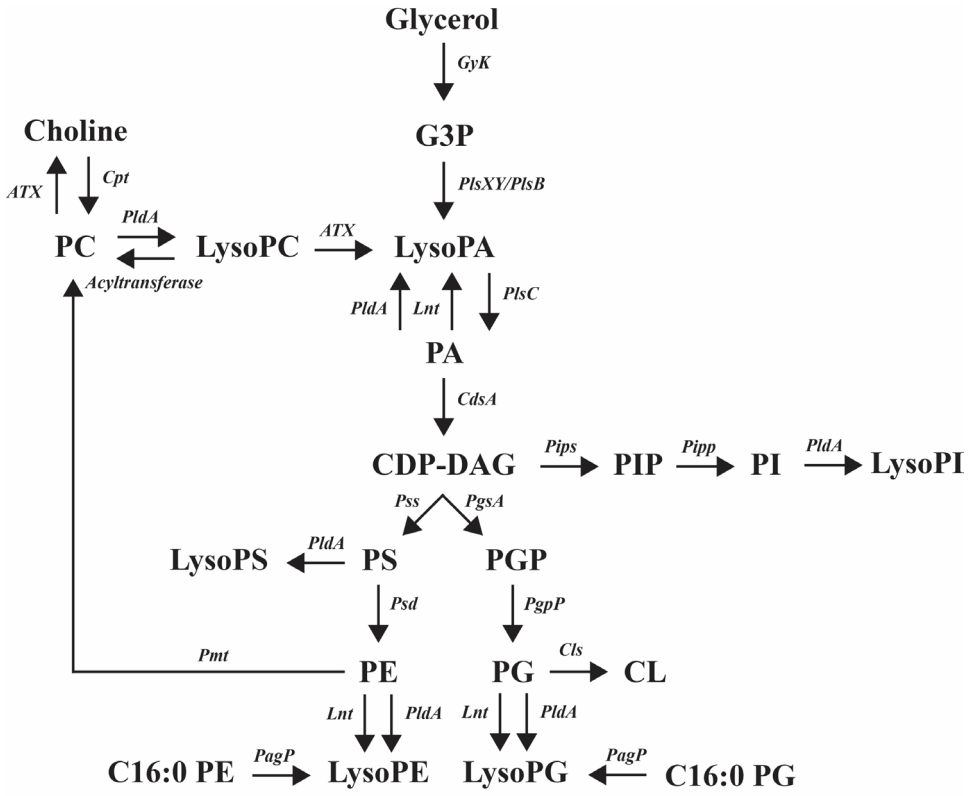

Figure 2. The *de novo* biosynthetic pathway of bacterial membrane phospholipids. The enzymes involved in the different catalyzed reactions are indicated in the text. Abbreviations: G3P-glycerol-3-phosphate; LysoPA-lysophosphatidic acid; PA-phosphatidic acid; CDP-DAG-cytidine diphosphate-diacylglycerol; PS-phosphatidylserine; LysoPS-lysophosphatidylserine; PE-phosphatidylethanolamine; LysoPE-lysophosphatidylethanolamine; PGP-phosphatidylglycerol-phosphate; PG-phosphatidyl-glycerol; LysoPG-lysophosphatidylglycerol; CL-cardiolipin; PIP-phosphatidylinositol phosphate; PI-phosphatidylinositol; LysoPI-lysophosphatidylinositol; PC-phosphatidylcholine; LysoPC-lysophosphatidylcholine; GyK-glycerokinase; Pls-acyltransferase; PldA-phospholipase A; Lnt-apolipoprotein *N*-acyltransferase; CdsA-phosphatidate cytidylyltransferase; Pips-phosphatidylinositolphosphate synthase; Pipp-phosphatidylinositolphosphate phosphatase; Pss-phosphatidylserine synthase; Psd-phosphatidylserine decarboxylase; PgsA-phosphatidylglycerolphosphate synthase; PgpP-phosphatidylglycerol phosphate phosphatase; Cls-cardiolipin synthase; PagP-lipid A palmitoyltransferase; Pmt-phospholipid N-methyltransferase; ATX-autotoxin.

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3

The unique phospholipidome of the enteric pathogen *Campylobacter jejuni*: lysophosholipids are required for motility at low oxygen availability

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Abstract

In response to changes in their environment bacteria need to change both their protein and phospholipid repertoire to match environmental requirements, but the dynamics of bacterial phospholipid composition under different growth conditions is still largely unknown. In the present study we investigated the phospholipidome of the bacterial pathogen *Campylobacter jejuni*. Transcription profiling on logarithmic and stationary phase grown cells of the microaerophilic human pathogen *Campylobacter jejuni* using RNA-seq revealed differential expression of putative phospholipid biosynthesis genes. By applying high performance liquid chromatography tandem-mass spectrometry (LC-MS/MS), we identified 203 phospholipid species representing the first determination of the phospholipidome of this pathogen. We identified nine different phospholipid classes carrying between 1 and 3 acyl chains. Phospholipidome analysis on bacteria of different ages (0-5 days) showed rapid changes in the ratio of phospholipids containing ethanolamine, or glycerol as phospholipid head group and in the number of cyclopropane bond containing fatty acids. Oxygen concentration influenced the percentage of lysophospholipids, and cyclo-propane bonds containing acyl chains. We show that large amounts of the phospholipids are lysophospholipids (30-45%), which mutant studies reveal are needed for normal *C. jejuni* motility at low oxygen conditions. *C. jejuni* possesses an unusual phospholipidome that is highly dynamic in response to environmental changes.

Introduction

Membrane phospholipids form a barrier for most molecules and influence the function of membrane proteins that play an essential role in a variety of cellular functions. Bacterial cytoplasmic membranes are composed of functionally diverse proteins embedded in a bilayer of phospholipids, while phospholipids form the inner leaflet of the asymmetric outer membrane of Gram-negative bacteria. Phospholipids in bacteria comprise about 10% of the dry weight of the cell and the synthesis of each mole of lipid requires about 32 moles of ATP, which represents a significant energy investment [1].

Most bacterial phospholipids consist of a variable head group connected to a phosphate group, a glycerol molecule and two fatty acids tails. The main phospholipids found in bacteria are phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL), lysyl-phosphatidylglycerol (LPG), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylcholine (PC) and phosphatidylserine (PS) [2]. PE and PG are the most abundant phospholipids in bacteria. In general, PE acts as a molecular chaperone that is essential for the proper folding of integral membrane proteins, while PG is required for protein translocation across the membrane [3, 4]. The hydrophobic fatty acid tails attached to the phospholipid head can vary in number and length (usually they contain between 12 and 24 carbon atoms), and in the number and position of unsaturated bonds [5]. Shorter lipid chains are less stiff and less viscous, making the membranes more flexible [6]. Membranes rich in unsaturated fatty acids are more fluid than those rich in saturated fatty acids [7]. Bacteria growing at increased temperature usually contain more saturated fatty acids in their membranes [8].

The membrane phospholipid composition is not a stable bacterial characteristic but can change in response to altered environmental conditions. During this adaptation, existing phospholipids can be either modified or degraded and replaced by newly synthesized lipids with characteristics that better match the environmental requirements [9]. Modification of existing phospholipids may involve the introduction of a cis-double bond into the fatty acids, causing the membrane to be more fluid, or a conversion of cis-unsaturated fatty acids to cyclopropane fatty acids by the enzyme cyclopropane fatty acid synthase (Cfa), resulting in a more stable membrane [10]. Furthermore, some bacteria possess a phospholipid *cis–trans* isomerase (Cti) that can replace the *cis* double bond with a *trans* double bond, leading to membranes that have a decreased permeability to solutes [9]. Phospholipids can also become cleaved by the enzyme phospholipase A (PldA), resulting in lysophospholipids that contain only one acyl chain instead of two [11]. Lysophospholipids usually make up only a small fraction (≤1%) of the bacterial membrane phospholipids, but they may accumulate in marine bacteria, bacterial pathogens and under certain conditions of environmental stress [12, 13]. Although specific phospholipid modifications are known to occur, a systematic analysis of the total set of bacterial phospholipids

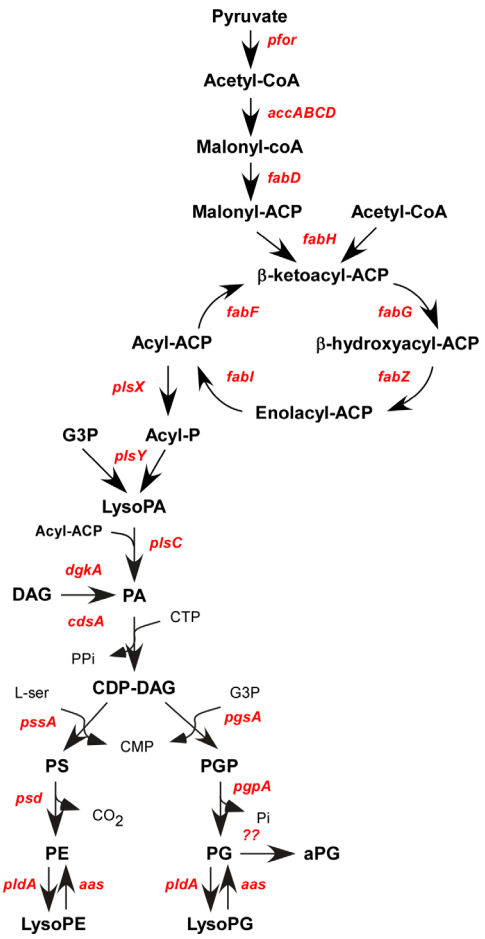
i.e. the bacterial phospholipidome and its dynamics in response to changes in the environment is still in its infancy, mainly due to the lack of high throughput analysis tools [2, 14].

The Gram-negative bacterium *Campylobacter jejuni* is one of the leading causes of bacterial foodborne illness causing an estimated 400 million cases of intestinal infection each year [15]. *C. jejuni* is microaerophilic and grows best at low O_2 and high CO_2 levels at a temperature of 42ºC. These conditions are present in the intestinal tract of poultry, which is often colonized by *C. jejuni* in very high numbers without eliciting overt clinical symptoms [16]. The bacterium has a typical spiral shape and is highly motile but can change into a coccoid nonmotile bacterium during the stationary phase in batch cultures. Thus far, the adaptation of *C. jejuni* to different growth environments has only been extensively studied at the metabolic and proteome level. Knowledge of the *C. jejuni* lipid repertoire is limited and mainly confined to the observation that *C. jejuni* contains the phospholipids PE and PG and produces the acyl chains C14:0, C16:0, C16:1, C18:1 and C19:0 cyclopropane [17, 18].

The genome sequence of *C. jejuni* strain 81116 indicates 22 genes likely involved in the biosynthesis or modification of phospholipids [9, 19]. For acyl phosphate production, the *C. jejuni accABCD* gene products likely convert acetyl-CoA to malonyl-CoA, which is used by the putative *fabB* gene product to link the malonyl group to an acyl carrier protein (ACP) (Fig. 1A). Malonyl-ACP is used by the *fabH* gene product to initiate the formation of a new acyl chain which is elongated by the putative FabGZI and FabF enzymes. Phospholipids may be formed by the PlsX–PlsY-mediated transfer of the produced acyl chain to the membrane, yielding lyso-phosphatidic acid (LysoPA) which can be acylated at the 2-position by PlsC, yielding phosphatidic acid (PA). This phosholipid may be further converted by the putative *cdsA, pssA, psd, pgsA* and *pgpA* gene products into phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). Modifications of the phospholipids may occur by the Cfa enzyme that converts *cis*-unsaturated fatty acids into cyclopropane fatty acids and by the PldA phospholipase that converts phospholipids into lysophospholipids. Despite putative lipid diversity, the *C. jejuni* genome appears to lack the genes encoding the stationary phase sigma factor RpoS and the transcription factor FabR that in many Gram-negative bacteria regulate phospholipid biosynthesis dependent on the growth phase [7, 20, 21].

In the present study, we applied a high throughput LC-MS approach to determine total lipid composition of *C. jejuni* under different environmental conditions. Our results indicate that the phospholipid composition of *C. jejuni* is highly dynamic and varies in response to the aging of the culture, and changes in oxygen availability. The phospholipidome of *C. jejuni* is characterized by a relatively high percentage of lysophospholipids, which we show influences *C. jejuni* motility under low oxygen conditions.

Fig. 1A)



B)

	Stat vs Log 5%
aas	-1.5
accA	-5.3
accB	1.2
accC-2	-1.4
accD	-1.1
cfa	7
cdsA	3.6
dgkA	nt
fabD	-3.6
fabF	-1.2
fabG	-6.5
fabH	-3
fabI	-6.2
fabZ	-4.7
pldA	-2.4
plsC	nt
plsX	-1
plsY	-1
pgpA	-2
pgsA	-3.6
psd	-1.2
pssA	-2.6

Fig. 1. Phospholipid biosynthesis pathway in *C. jejuni*. (A) Putative phospholipid synthesis pathway in *C. jejuni* based on the identified genes in the genome of strain 81116. Predicted genes are marked in red, substrates are marked in black. (B) RNA-seq analysis of the (putative) phospholipid genes. Fold changes were calculated for *C. jejuni* strain 81116 grown for 6 h (Log) vs 12 h (Stat) in HI at 42^{0}C under microaerophilic conditions (5% O_2, 10% $C0_2$, 10% H_2 and 75% N_2).

Results

The phospholipid biosynthesis genes of *C. jejuni* change in transcript abundance with growth phase

To investigate whether the transcription of the annotated phospholipid biosynthesis genes of *C. jejuni* strain 81116 (Fig.1A) is dependent on the growth phase of the culture, we performed RNA-seq analysis on the complete mRNA

content of logarithmic (6 h) and stationary phase (12 h) *C. jejuni* cultures. This revealed gene transcripts for all of the putative phospholipid biosynthesis genes. The majority of the genes, especially the fatty acid elongation genes *fabG, fabI* and *fabZ*, showed higher transcript levels in the logarithmic phase compared to the stationary phase (Fig. 1B). The *cfa* transcript on the other hand, was strongly upregulated in the stationary phase. The transcript differences indicate that *C. jejuni* phospholipid biosynthesis may change in different growth environments.

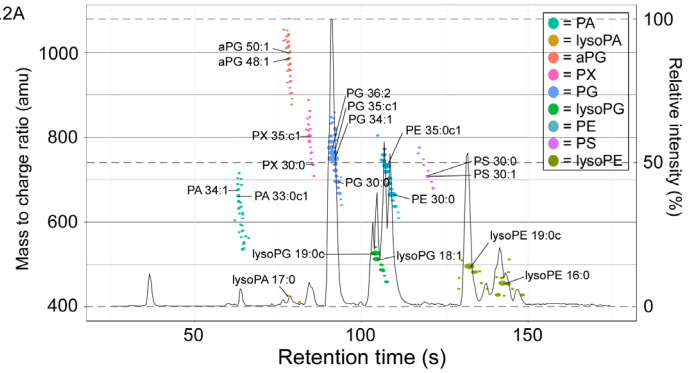
The unique phospholipidome of *C. jejuni*

The actual phospholipid composition of *C. jejuni* was first determined for strain 81116 grown in Heart Infusion (HI) medium (16 h, 42°C) under microaerophilic conditions (5% O_2, 10% CO_2, 10% H_2 and 75% N_2). Bacterial chloroform/methanol extracts were passaged over a HILIC column and analyzed by mass spectrometry. This resulted in the detection of 203 different phospholipids with the phospholipid head group ethanolamine, glycerol, hydrogen, serine, or nonidentifiable factor X. The lipids belonged to nine different lipid classes with PG lipids being most abundant (45%), followed by PE (28%), lysoPE (16%), PX (4%), lysoPG (3%), PA (2%), acyl-PG (1%), PS (<1%) and lysoPA (<1%) (Fig. 2A). Acyl-PG is a lipid class with three acyl chains of which one acyl chain is facing the opposite direction compared to the other two, the enzyme(s) responsible for the addition of the third acyl chain to PG is not known [2]. The novel lipid class designated as PX, possess a headgroup of a molecular mass of 133.075 (Fig. S1) which to our knowledge does not correspond to the masses of any of the known head groups. In agreement with the genome analysis *C. jejuni* lacked the phospholipids cardiolipin (CL), lysyl-phosphatidylglycerol (LPG) and phosphatidylinositol (PI). The fatty acid tails of the detected lipids varied in length between 12 and 21 carbon atoms. Each phospholipid class contained saturated, unsaturated and cyclopropane-containing lipids (Fig. 2B).

***C. jejuni* phospholipidome dynamics**

C. jejuni inhabits the oxygen-poor intestine of warm-blooded animals as a commensal, but it encounters higher oxygen levels when it causes bloody diarrhea in humans or when it lives outside the host in surface water [22,23]. As *C. jejuni* cannot grow anaerobically or under atmospheric oxygen levels, we studied the phospholipidomes of *C. jejuni* grown for a period of 0, 4, 8, 24, 36, 60 and 108 h (encompassing the logarithmic growth phase up to 8h, and both early and extended stationary phase) under low (0.3%) and high (10%) oxygen conditions. Reducing oxygen availability from 10% to 0.3% resulted in a lower bacterial growth rate (doubling time 5.57 h at 0.3% O_2 versus 1.8 h at 10% O_2), prolonged logarithmic (log) growth (60 h at 0.3% O_2 vs 8 h at 10% O_2) and higher number of viable bacteria (Fig. 3A).

Fig.2A



B)

PA	PG	PX	PE	PS	aPG	lysoPG	lysoPE	lysoPA
24:2	26:0	28:0	26:0	28:1	42:0	14:0	12:0	15:0c
24:1	27:0	30:1	27:0	28:0	43:0	15:0	14:1	16:1
24:0	28:1	30:0	28:1	29:0	44:1	16:1	14:0	17:0
25:1c1	28:0	31:0c1	28:0	30:1	44:0	16:0	14:0	
25:0c1	29:0c1	32:1	29:0	30:0	44:0	17:0c	15:0	
25:0	29:0	32:0	30:2	31:0c1	45:0c1	17:0	15:0	
26:2	30:2	33:0c1	30:1	32:1	46:2	18:1	16:1	
26:1	30:1	34:2	30:0	33:0c1	46:1	18:0	16:0	
26:0	30:0	34:1	31:0c1	34:1	46:0	19:0c	16:0	
27:1c1	31:1c1	34:0	31:0	35:1c1	47:0c1	19:0	17:0c	
27:0c1	31:0c1	35:1c1	32:2	35:0c1	48:2	20:1	17:0c	
27:0	31:0	35:0c1	32:1		48:1		17:0	
28:1	32:2	35:0	32:0		48:0		18:1	
28:0	32:1	36:2	33:1c1		49:1c1		18:1	
29:1c1	32:0	36:1	33:0c1		49:0c1		18:0	
29:0	33:0c1	36:0	34:2		49:0		19:0c	
30:2	33:0	37:1c1	34:1		50:3		19:0	
30:1	34:2	38:2	34:0		50:2		20:1	
30:0	34:1	38:0	35:1c1		50:1		20:0	
31:1c1	34:0	39:0	35:0c1		50:0			
31:0c1	35:1c1	41:0c1	35:0		51:1c1			
31:0	35:0c1		36:2		51:0c1			
32:2	35:0		36:1		51:0			
32:1	36:2		37:1c1		52:3			
32:0	36:1		38:2		52:2			
33:1c1	37:1c1		40:0		52:1			
33:0c1	37:0c1				52:0			
33:0	37:0				53:2c1			
34:1	38:2				53:1c1			
34:0	38:1				53:0c1			
35:1c1	38:0				53:0			
35:0c1	39:1c1				54:3			
36:2	39:0c1				54:2			
36:1	39:0				54:1			
37:1c1	40:1				54:0			
	40:0				55:2c1			
	41:1c1				55:1c1			
	41:0c1				57:2c1			
	42:0c2							

Fig. 2. The phospholipidome of *C. jejuni*. (A) A base peak chromatogram of a representative sample (straight gray line, right axis) with the identified phospholipids in *C. jejuni* with their mass to charge values (atomic mass units (amu), left axis) superimposed on top of it. A selection of the most intense phospholipids for each phospholipid class are labeled. The size of a dot is indicative of its intensity. PA= phosphatidic acid, LysoPA= lysophosphatidic acid, aPG= acyl-phosphatidylglycerol, PX= unknown, PG= phosphatidylglycerol, lysoPG= lysophosphatidylglycerol, PE= phosphatidylethanolamine, PS= phosphatidylserine and lysoPE=lysophosphatidylethanolamine. (B) All different lipid classes and -species identified in *C. jejuni* grown in HI at 42°C under microaerophilic conditions (5% O_2, 10% $C0_2$, 10% H_2 and 75% N_2).

Microscopic analyses of the cultures revealed that spiral shaped *C. jejuni* grown at 10% O_2 rapidly changed to coccoid bacteria shortly after entering stationary growth phase (Fig. 3B), which also have been observed but much slower under standard growth conditions [24]. At 0.3% O_2, most bacteria largely maintained their spiral-shape up to 108 h of growth (Fig. 3C).

Principal component analysis of the phospholipidomes of bacteria grown under the different oxygen conditions indicated major shifts in bacterial phospholipid composition with aging of the culture (Fig. 4). These changes occurred under both oxygen conditions tested but with clear differences. To better understand the dynamic nature of the *C. jejuni* phospholipidome, we specifically analyzed the changes in phospholipid composition of both different oxygen conditions.

Effect of oxygen and age on the *C. jejuni* lipid composition

Comparison of the total phospholipid composition of *C. jejuni* grown with either 10% or 0.3% oxygen for 4 and 8 h, respectively (log phase) identified PG, PE and lysoPE as most abundant phospholipid classes under both conditions (Fig. 5A & 5B). At 10% O_2, the percentage of lysoPE in the log phase was slightly lower than after growth with 0.3% O_2.

Analyses of the phospholipid compositions after 8-108 h of culture with 10% O_2 (stationary phase) identified LysoPE, lysoPG, PG, and PE as dominant phospholipid classes, whereas a small increase in aPG was observed (Fig. 5A). The strong rise in lysoPE (from 15% to 33% of lipids) and the concomitant decrease in PE (from 36% to 18% of lipids) compared to the (4 h) log phase bacteria was already apparent after 8 h of growth at the entry of the stationary growth phase. The percentage of lysoPG increased more gradually from 5% at 8 h to about 27% of the total lipids after 108 h of culture. Together, the percentage of lysophospholipids increased from 17% in the log phase to 45% of total lipids in the stationary phase bacteria. After growth for 24-108 h with 0.3% O_2, PG, PE, lysoPE and, to a lesser extent, lysoPG were also the most abundant lipid species (Fig. 5B). A small but significant increase was also noticed for aPG, PS and PX. The increase in lysophospholipids in the stationary growth phase was much less profound at 0.3% O_2 than after growth at 10% O_2 but still was about 25% of the total lipids.

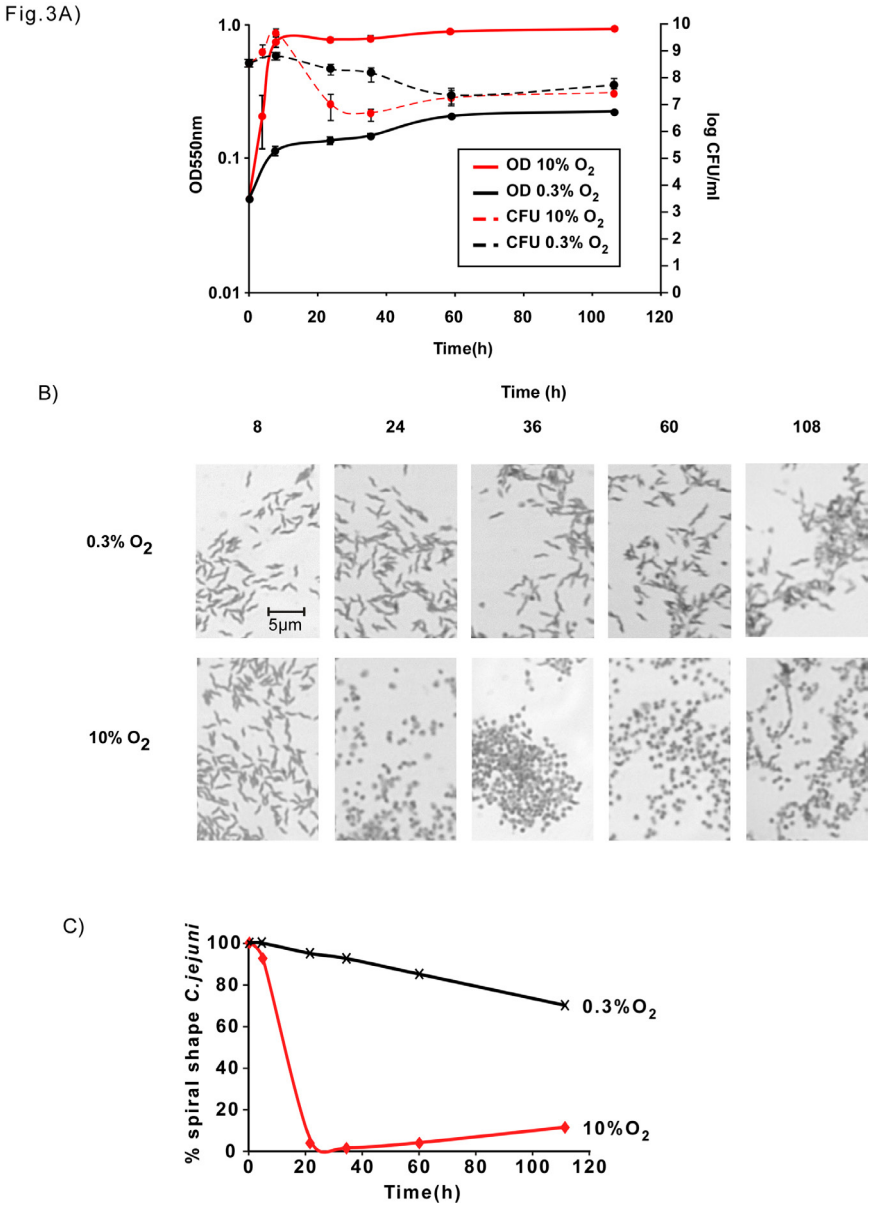


Fig. 3. Effect of environmental conditions on *C. jejuni* behavior. (A) Growth of *C. jejuni* at 42^{0}C in HI medium under microaerobic (10% O_2, 10% CO_2, 70% N_2, 10% H_2) and oxygen limited (0.3% O_2, 10% CO_2, 79,7% N_2, 10% H_2) conditions. The optical density (left Y-as) as well as the colony forming units (right Y-as) at the indicate time points are shown. The experiments were repeated three times in duplicate. (B) Morphology of *C. jejuni* after 8, 24, 36, 60 or 108 h growth in HI at 10% O_2 or 0.3% O_2 as determined by phase contrast light microscopy. (C) Percentage of spiral shape bacteria present in the samples taken at 8, 24, 36, 60 or 108 h of growth under the above-mentioned conditions. Data are represented as mean ± SEM.

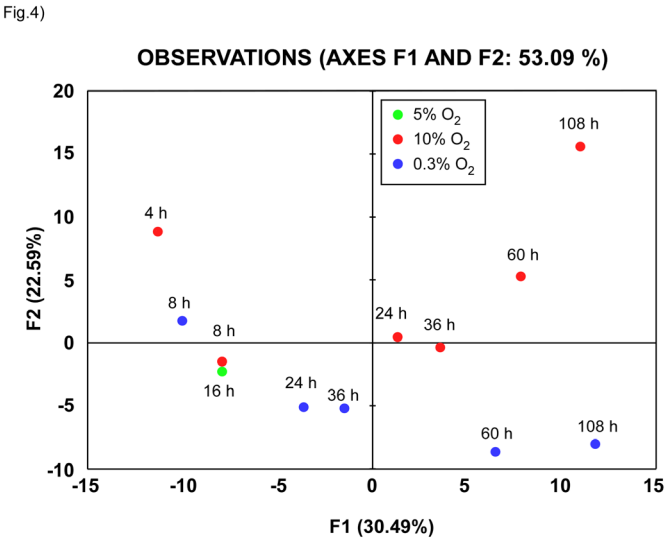


Fig. 4. Phospholipidome dynamics in response to environmental conditions. Principal component plot showing the differences between the *C. jejuni* phospholipidome after 4, 8, 24, 36, 60 or 108 h growth in HI under the indicated different growth conditions. Each point represents the average of the data obtained for phospholipidomes of three separate growth curve experiments performed in duplicate.

Effect of oxygen and age on the *C. jejuni* lipid composition

Comparison of the total phospholipid composition of *C. jejuni* grown with either 10% or 0.3% oxygen for 4 and 8 h, respectively (log phase) identified PG, PE and lysoPE as most abundant phospholipid classes under both conditions (Fig. 5A & 5B). At 10% O_2, the percentage of lysoPE in the log phase was slightly lower than after growth with 0.3% O_2.

Analyses of the phospholipid compositions after 8-108 h of culture with 10% O_2 (stationary phase) identified LysoPE, lysoPG, PG, and PE as dominant phospholipid classes, whereas a small increase in aPG was observed (Fig. 5A). The strong rise in lysoPE (from 15% to 33% of lipids) and the concomitant decrease in PE (from 36% to 18% of lipids) compared to the (4 h) log phase bacteria was already apparent after 8 h of growth at the entry of the stationary growth phase. The percentage of lysoPG increased more gradually from 5% at 8 h to about 27% of the total lipids after 108 h of culture. Together, the percentage of lysophospholipids increased from 17% in the log phase to 45% of total lipids in the stationary phase bacteria. After growth for 24-108 h with 0.3% O_2, PG, PE, lysoPE and, to a lesser extent, lysoPG were also the most abundant lipid species (Fig. 5B). A small but significant increase was also noticed for aPG, PS and PX. The increase in lysophospholipids in the stationary growth phase was much less profound at 0.3% O_2 than after growth at 10% O_2 but still was about 25% of the total lipids.

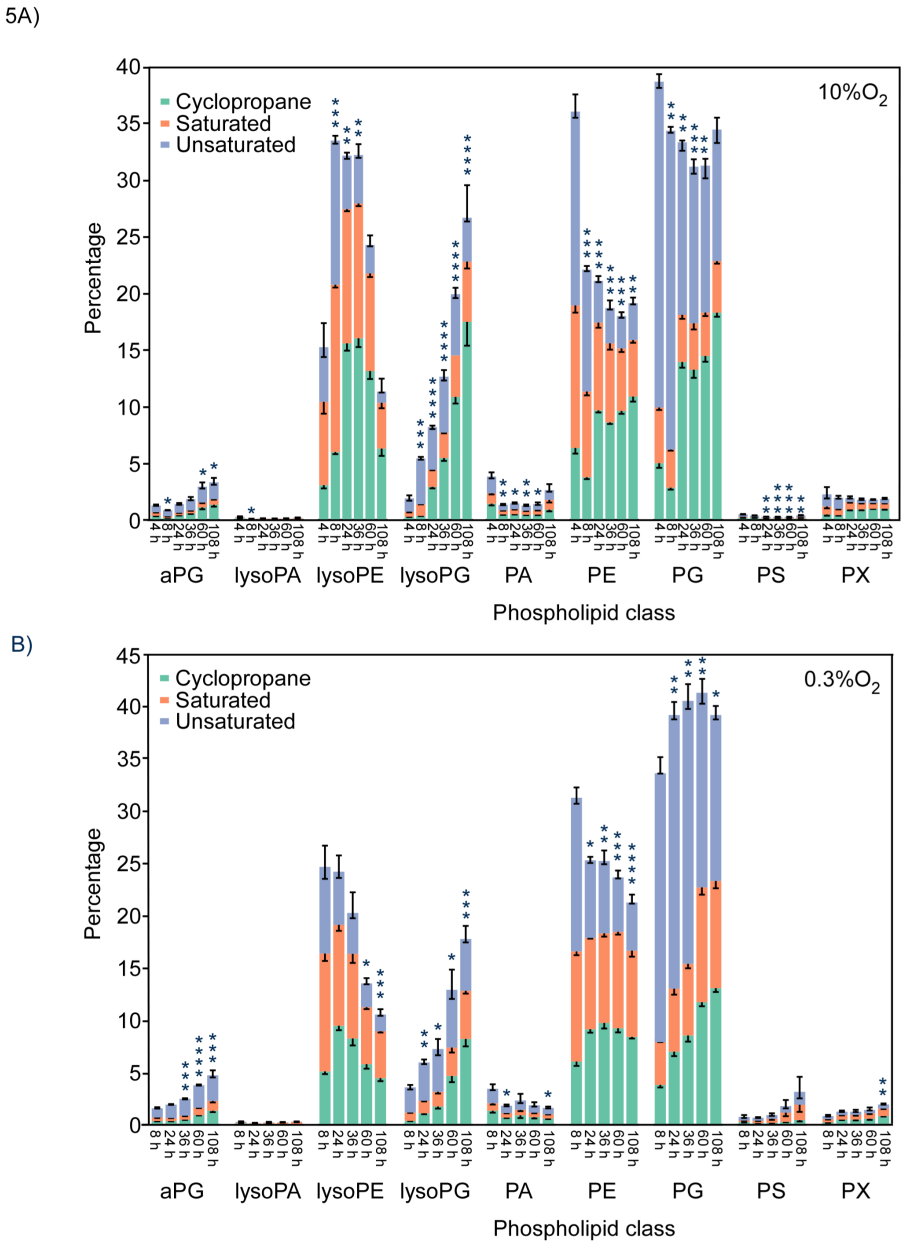


Fig. 5. Influence of the age and oxygen concentration on the phospholipid classes of *C. jejuni*. Percentage phospholipid classes and the percentage unsaturated, cyclopropane bonds and saturated minus cyclopropane bond containing fatty acids per phospholipid class estimated by LS-MS during the growth of *C. jejuni* at 10% O_2 (A) and at 0.3% O_2 (B). Data are represented as mean ± SEM. The displayed P values are calculated by comparing the first time point (4 or 8h) with the other time points. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are representative of three independent experiments performed in duplicate.

Analysis of the phospholipid head groups revealed that in all logarithmic (4 or 8 h) growing cultures ethanolamine (>50%) was the predominant phosphohead group followed by glycerol with 40% (Fig. 6A). An increase of ethanolamine containing phospholipids was observed at 8 h at 10% O_2 compared to 4 h of growth, which matched with a faster bacterial growth rate at 4 h (Fig. 3A & 6A). With aging of the bacterial culture, phospholipids with glycerol as phospholipid head group became the most abundant phospholipid species, making up >60% of the total phospholipid head groups, while ethanolamine containing phospholipids accounted for only 30%. Under low oxygen conditions this ethanolamine to glycerol shift was faster than at 10% O_2.

Closer analysis of the identified phospholipid acyl chains revealed that at 4-8 h of culture (log phase) the majority of phospholipid classes (~55%) carried unsaturated acyl chains both after growth with 10% O_2 (4 h) and 0.3% (8 h) of O_2 (Fig. 6B and C). There was however a clear difference between the acyl chains of the phospholipid's PG and PE, while PG mainly consists of unsaturated acyl chains, PE contain more saturated and cyclo-propane acyl chains (Fig.5A and 5B). In late stationary phase cultures with 10% O_2 (24-108 h) the majority (~55%) of phospholipid species contained cyclopropane-bonds, whereas the number of unsaturated acyl chain had dropped to <30%. At 0.3% O_2, the 24-108 h stationary phase cultures changed their percentage of lipids with unsaturated acyl chains slowly from 55% to 30%, with a gradual increase in lipids with cyclopropane-bonds from 18% to 35%. At this phase under both oxygen conditions phospholipids with ethanolamine as the phospholipid main group contain more acyl chains with saturated and cyclopropane compounds than phospholipids with glycerol as the head group (Fig 5A & 5B). The percentage of lipids with saturated acyl chains remained relatively constant at about 25% under both oxygen conditions (Fig. 6B and 6C). Overall, the noted changes in acyl chains were much faster and more pronounced in bacteria grown with 10% O_2 than 0.3% O_2. In general, prolonged growth of *C. jejuni* with 10% oxygen results in more lysophospholipids and more cyclo-propane bonds containing acyl groups compared to bacteria grown with 0.3% oxygen.

Impact of the enzymes CfA and PldA on the *C. jejuni* phospholipidome

In an attempt to identify factors that influence the composition and dynamics of the *C. jejuni* phospholipidome, we genetically inactivated the *cfa* and *pldA* genes. Inactivation of the *cfa* gene resulted in a 97% reduction in cyclopropane containing fatty acids (Fig. 7A). This indicates that the Cfa enzyme is responsible for the incorporation of the cyclopropane ring in *cis* double bond containing unsaturated fatty acids. This finding is consistent with the Cfa function in other bacteria and underpins the link between the observed upregulation of *cfa* transcript (Fig. 1B) and the increase in cyclopropane rings in the late stationary phase fatty acids (Fig. 6B and C). The *cfa* mutant also showed a 50% reduction of the saturated phospholipids

compared to the parent strain.

Mutation of the *pldA* gene decreased the relative amount of the *C. jejuni* lysophospholipids by 80% (Fig. 7B), consistent with the assumed function of PldA as a phospholipase. The *pldA* mutant still contained a small percentage (6%) of lysophospholipids (Fig. 7B), indicating the presence of alternative pathways of lysophospholipid formation. The reduction of lysophospholipids in the *pldA* mutant was accompanied by a relative increase of the phospholipid classes PE and PG as well as PX which may be the preferred targets of the PldA enzyme.

To exclude that the identified lysophospholipids were the result of PldA activity during the phospholipid extraction procedure, the phospholipids PE31: 1 and PC31: 1 were externally added to the WT and *pldA* mutant pellets. Similar amounts of these lipids were recovered intact after the phospholipid extraction (Fig. 7c). These data show that the large amounts of lysophospholipids detected in wild-type *C. jejuni* cells are a true reflection of the unusual dominance of this lipid class in this pathogen.

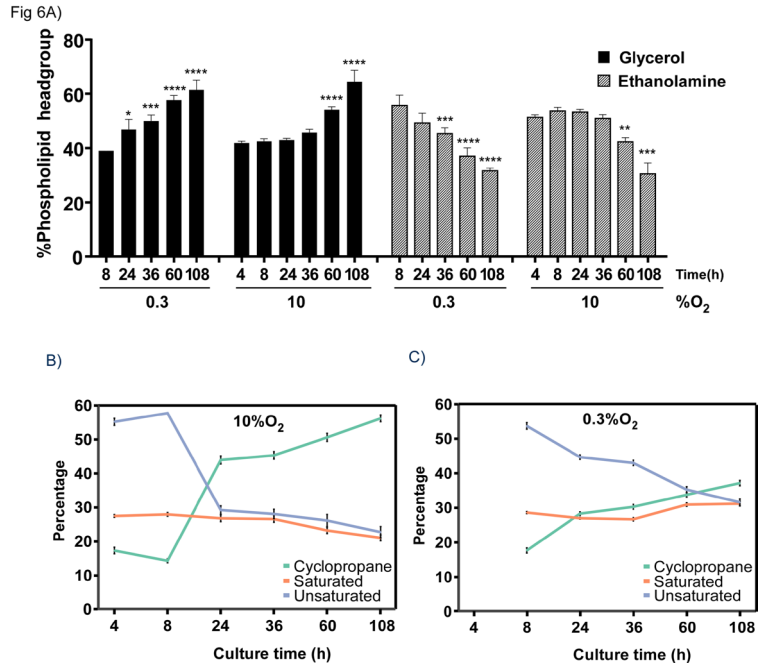


Fig. 6. Influence of the age and oxygen concentration on the phospholipid headgroup and acyl chains. (A) Percentage of the phospholipids containing ethanolamine or glycerol as phospholipid headgroup at 10% O_2 and at 0.3% O_2. Percentage unsaturated, cyclopropane bonds and saturated minus cyclopropane containing fatty acids of all phospholipids at 10% O_2 (B) and at 0.3% O_2 (C). Data are represented as mean ± SEM. The displayed P values are calculated by comparing the first time point (4 or 8h) with the other time points. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are representative of three independent experiments performed in duplicate.

Effect of the *C. jejuni* phospholipid composition on the bacterial motility phenotype

To learn more about the functional consequences of the observed changes in phospholipid composition, we tested the defined *cfa* and *pldA* mutant strains for their several phenotypic characteristics. The growth curve of both mutants was similar as that of the wildtype under both oxygen conditions (data not shown), however the viability of the *pldA* mutant in the stationary phase especially at 10 % O_2 was 1 log lower compared to the Wt and cfa mutant (Fig. S2). Next we tested the *cfa* mutant for pH sensitivity knowing that cyclopropane fatty acids have been shown to protect bacteria such as *E. coli* from low pH [25, 26]. Exposure of the *C. jejuni cfa* mutant to a pH shock (2 h, pH 4.0) or prolonged growth in medium at low pH (5) resulted in similar bacterial survival as noted for the parent strain (data not shown). As older cultures with coccoid morphology carry more cyclopropane fatty acids (Fig. 3D, 6B & C), we also compared the bacterial morphology of the *cfa* mutant and the wildtype strain using microscopy. No differences in the formation of the *C. jejuni* coccoids were observed. This suggest that cyclopropane-bond formation is not a prerequisite for coccoid formation. Inspection of the mutants for bacterial motility revealed that the *cfa* mutant was significantly more motile than the wildtype under both oxygen conditions (Fig. 7D). The motility of the *pldA* mutant and complemented *pldA* mutant and parent strain were similar after growth in HI medium at 10% O_2. However, when grown at 0.3% O_2, the *pldA* mutant appeared virtually non-motile in contrast to the complemented *pldA* strain, and the parent strain (Fig. 7D). This indicates that the *pldA* enzyme is needed for flagella-mediated *C. jejuni* motility and thus that the presence of lysophospholipids may positively influence bacterial flagella function.

Discussion

Bacteria need to change their membrane lipid composition in response to changes in the environment to allow them to survive unfavorable conditions. By using a high-throughput method for lipid analysis, we show that the C. *jejuni* phospholipid composition is highly dynamic and unique as it may consist of up to 45% of lysophospholipids. We could detect 203 different phospholipid species across 9 different lipid classes in *C. jejuni*, which is much more than the 63 species of 7 classes in *Enterococcus faecalis*, but comparable to the hundreds of lipids identified for *E. coli* [27, 28]. Seven phospholipid classes previously not reported in *C. jejuni* were detected: PA, PS, PX, lysoPG, LysoPE, LysoPA and acyl-PG besides the previously identified PG and PE (Fig. 2) [18]. PX, with a molecular weight of 133.075, might be a novel phospholipid class with a probable structural formula $C_5H_{11}NO_3$. MS2 data analysis and prediction by METLIN Mass Spectral Database (http://metlin.scripps.edu) suggests that this might be L-pentahomoserine, but further work will be required to elucidate its structure. The most abundant lipid classes in *C. jejuni* are PG, PE,

Lyso-PG and Lyso-PE (Fig. 5A & 5B). Lyso-PE is not very common, and so far, Lyso-PG has never been identified as a major lipid class in bacteria [2]. Phospholipid-to-lysophospholipids conversion may be a common response to stressful growth conditions than has currently been reported in the literature, since phospholipidome studies under different growth conditions have not yet been performed in many species.

The composition of the phospholipids classes in the phospholipidome of *C. jejuni* is continuously changing in response to altered environmental conditions. While under low oxygen conditions (0.3% O_2) the amount of lysophospholipids was stable at around 28% independent of the growth phase and age of culture, at 10% O_2 the amount of lysophospholipids increased and reached a maximum of 45% of the total phospholipids. Aging of the culture also caused a change in the composition of the lysophospholipids: more lysoPE was detected in the logarithmic growing bacteria, while in the late stationary phase lysoPG was more abundant. During the aging of the culture, phospholipids containing an ethanolamine as phosphohead group decreased to 25%, while lipids with glycerol containing headgroups increased by 25% (Fig. 6A). This variation in lipids deviates from observations in the model organism, *E. coli*, where the composition of the phospholipids (75% PE, 20% PG and 5% CL) is relatively constant under a broad spectrum of growth conditions [1]. Only CL, an important phospholipid class in most bacteria, including the closely related *Helicobacter pylori*, increases in *E. coli* to 10% when the cells enter the stationary phase. *C. jejuni* is unable to make cardiolipin [1, 29]. We noticed that the growth rate of *C. jejuni,* although dependent on the oxygen concentration, also correlates with the ethanolamine content of the phospholipidome. Dividing bacteria possess more phospholipids with ethanolamine as headgroup (compare Fig. 3A, 6A). A similar phenomenon is seen in *E. coli* mutants that are unable to make PE and have a strongly reduced doubling time [30]. The transition from PE to PG in *C. jejuni* could not be explained by transcriptional changes of the phospholipid head genes (*pssA, psd, pgsA* and *pgpA*) as the number of transcripts of these genes were all lower in stationary phase compared to logarithmic phase (Fig. 1B). How the transition from PE to PG is taken place merits further investigation.

In other bacteria, most phospholipids contain two acyl chains. Our results show that up to 49% of phospholipids of *C. jejuni* contain not two, but more or fewer acyl chains. Acyl-PG containing three acyl chains is a minor lipid class in *C. jejuni,* although it still increases to more than 5% of the total lipids in the late stationary phase (Fig. 5B). The enzyme(s) responsible for the addition of the third acyl chain to PG as well as the biological function of acyl-PG are unknown [2]. So far acyl-PG has only been identified in a few bacterial species; among them are *Caulobacter crescentus* and several marine bacterial species [31]. Like in *C. jejuni,* the amount of acyl-PG in *C. crescentus* increases in stationary growth phase [32]. The most

surprising finding in our study was that a large proportion of the phospholipids in all samples were lysophospholipids (up to 30-45%) that contain only one acyl chain (Fig. 5A, B & 7B). In most other bacterial species, lysophopholipids normally make up less than 1% of the bacterial membrane [11]. Only in *Vibrio cholerae,* high amounts of lysophospholipids (~30% of the total lipid composition) are found after exposure to bile salts in the early stages of infection or during growth in the presence of ocean sediment [33]. We have shown that the production of the majority of the *C. jejuni* lysophospholipids is accomplished by the PldA enzyme (Fig. 7B). In other bacteria, PldA remains dormant as an inactive monomer in growing cells [34], which is definitely not the case in *C. jejuni,* as the *pldA* mutant contained 80% less lysophospholipids compared to wild-type. One of reasons that *C. jejuni* might possess a high content of lysophospholipids is because its lacks the LplT-aas phospholipid repair mechanism as no homologues of the lysophospholipidtransporter LplT exist in *C. jejuni* which, translocate lyso forms of glycerophospholipids across the cytoplasmic membrane in Gram-negative bacteria [4, 11, 35].

As judged by swarm-plate assays, the *pldA* mutant was far less motile at 0.3% O_2 than the parent strain, while at 10% O_2, there was little difference (Fig. 7D). The reduced motility seen under low-oxygen conditions may explain why a *pldA* mutant in *C. jejuni* is impaired in colonizing the cecum of chickens [36]. The reduced motility phenotype indicates that lysophospholipids are (directly or indirectly) needed to allow *C. jejuni* to be fully motile under low oxygen conditions. The role of lysophospholipids is poorly characterized in bacteria, but lysophospholipids are essential in eukaryotic cells, as they possess pro- and anti-inflammatory properties [11]. In the pathogenic bacteria *V. cholerae*, *Helicobacter pylori* and *Yersinia pseudotuberculosis*, lysophospholipids accumulate in accordance with their pathogenic or survival potential when confronted with new hostile environmental conditions [11-13, 37].

In all phospholipid classes we detected shifts in the percentage of lipids with unsaturated and an even number of carbon containing fatty acids to cyclopropane ring containing lipids upon change of the growth conditions. At 10% O_2 the percentage of unsaturated fatty acids decreased from 60% at 8 h to 23% at 108 h of growth, while the percentage of cyclopropane fatty acids increased from 14% to up to 50% with aging of bacterial culture (Fig. 6B). In many bacteria, an increase of cyclopropane bonds in lipids during the stationary phase is a common feature [38]. Responsible for this is the enzyme cyclopropane fatty acid (CFA) synthase, which uses S-adenosylmethionine and three molecules of ATP to induce a cyclopropane ring in *cis*-unsaturated fatty acids [39]. We mutated the *cfa* gene in *C. jejuni* and showed that the cyclopropane fatty acids were almost completely absent in the *cfa* mutant (Fig. 7A). In *E. coli*, the transcription of the *cfa* gene is maximal during the transition from the late log phase to stationary phase of cell growth, because it is regulated by

the stationary-phase sigma factor RpoS [40]. Although RpoS is missing in *C. jejuni* we observed that, like in *E. coli,* the *cfa* transcription is increased in the stationary phase (Fig. 2B), suggesting that there must be a stationary phase transcription factor in *C. jejuni*. We observed that when there were more cyclopropane fatty acids present than unsaturated phospholipids, this coincided with a change in bacterial morphology from the spiral-shaped to the coccoid form (Fig. 3C & 6B & C). However, this change in phenotype was also observed for the *cfa* mutant, indicating that cyclopropane fatty acids are not required for coccoid transformation. Mutation of *cfA* gene affected the motility as the *cfa* mutant was more motile than the wildtype under both oxygen conditions (Fig. 7D). The stable cyclopropane bond instead of reactive double bond has been shown in other bacteria to protect the reactive double bond from adverse reactions during stationary phase such as high osmotic pressure, high temperature, low pH, nutrient deprivation and high alcohol concentrations [41]. No phenotypic differences could be observed between the wt and *cfa* mutant grown at different oxygen conditions or different pH conditions. This indicates that in *C. jejuni* mutation of *cfa* gene does not result in more sensitivity towards low pH, nutrient uptake or osmotic stress.

Depending on the type of headgroup and fatty acid composition, phospholipids have different effective shapes, which influence the curvature of the membrane [42, 43]. Our results indicate that the fast-growing, spiral-shaped *C. jejuni* possesses predominantly the phospholipid head group ethanolamine, unsaturated fatty acids and a reduced amount of lysophospholipids, while the coccoid form mainly possesses the head group glycerol, a large amount of cyclopropane lipids and a vast amount of lysophospholipids (Fig. 3C, 6A, B & C). Molecules with ethanolamine as headgroup induce a negative curvature while lysophospholipids introduce a positive curvature in the membrane. PG has a more rectangle shape and therefore has no influence on the curvature of membranes [44]. The geometric packing properties of PG and lysophospholipids therefore may together with the change in the peptidoglycan structure [45] allow the forming of spherical cells or coccoid forms.

In conclusion, *C. jejuni* possesses a unique phospholipidome compared to other bacteria. The phosholipidome is highly dynamic with changes occurring rapidly during the aging of the culture and coccoid formation. Large amounts of lysophospholipids are present in the phospholipidome due to the PldA enzyme, which may allow *C. jejuni* to be motile under low oxygen conditions. We showed that virtually all phospholipid species in the phospholipidome of *C. jejuni* are influenced by environmental changes which are probably needed to allow these bacteria to be optimally adapted to its environment.

Materials and methods

General growth conditions. *C. jejuni* strain 81116 and its derivatives were routinely grown on saponin agar plates (Biotrading, The Netherlands) at 42°C in a microaerophilic atmosphere (5% O_2, 10% CO_2, 10% H_2 and 75% N_2). Chloramphenicol (20 μg/ml) was added to the plates when appropriate. *E. coli* strains were grown on Luria-Bertani (LB) agar plates or in LB broth (Biotrading, The Netherlands) at 37°C.

Mutagenesis of the *pldA* and *cfa* genes. To disrupt the *pldA and cfa* genes, the genes and ~1 kb of their flanking regions were amplified from *C. jejuni* strain 81116 DNA by PCR using the primer pairs PldA-F/PldA-R and Cfa-F/Cfa-R, respectively (Table. S1). The ~3-kb PCR products were ligated into the pJET1.2/blunt cloning vector, resulting in the plasmids pJET*pldA* and pJET*cfA*. Inverse PCR was performed on these plasmids using the primers sets pldABamHI-F/pldABamHI-R and cfaBamHI-F/cfaBamHI-R, respectively (Table. S1). This resulted in PCR products with ~900- and ~1050-bp deletions in the *pldA* and *cfa* genes respectively and a newly introduced *Bam*HI restriction site. Both inverse PCR products were ligated to a *Bam*HI fragment containing the chloramphenicol resistance gene of pAV35 resulting in the knock-out constructs pJET*pldA*::Cm and pJET*cfa*::Cm. The constructs were verified by sequencing and subsequently used to mutate *C. jejuni* 81116 wildtype using natural transformation. Homologous recombination resulting in double cross-over events was verified by PCR. The mutants and parent strain showed similar bacterial growth rates in HI broth.

Construction of the *pldA* complementation plasmid. To complement the *pldA* mutant, the *pldA* gene was amplified of the chromosomal DNA of *C. jejuni* 81116 with *pfu* polymerase (Promega) and the primers SacIpldafor and XbaIpldarev (Table. S1). The product was digested with SacI and XbaI and ligated into the shuttle plasmid pMA5 [46]. The resulting complementation plasmid pMA5-*pldA* was first transformed into *E. coli* S17 and then conjugated [47] to the *pldA* mutant.

Bacterial growth assay. A *C. jejuni* starter culture was grown in Hearth Infusion medium (HI-medium, Biotrading, The Netherlands) for 16 h at 42°C in a microaerophilic atmosphere and then diluted to an OD_{550} of 0.05 in T25 flasks containing 5 ml of HI medium. Cultures were shaken (150 rpm) inside a hypoxic chamber (Coy labs, Grass Lake, USA) under high-oxygen (10% O_2, 10% CO_2, 70% N_2, 10% H_2) or under oxygen-limited conditions (0.3% O_2, 10% CO_2, 79,7% N_2, 10% H_2) at 42°C. The optical density (550 nm) as well as the viable counts (CFU/ml) of the cultures was measured at 0, 8, 24, 36, 60 and 108 h of growth. Growth of bacteria at 10% O_2 was also determined at 4 h. Given values are the mean of three experiments performed in duplicate.

Bacterial morphology. The morphology of *C. jejuni* (spiral vs coccoid shape) of every sample was determined by scoring 100 bacteria located in one field of a 100X phase contrast Olympus BH-2 microscope. Images were made using a CMEX DC 5000 camera and Euromex Image Focus V.30 software.

Phospholipid extraction and analysis. At the same time points as used for measurement of the optical density, medium samples (500 µl) were taken from six independent cultures and immediately frozen at -80°C. After all samples were obtained, they were thawed and centrifuged at 20,000 g for 5 min at 4°C. The pellets were washed once with PBS and resuspended in PBS to an OD_{550nm} of ~0.2 corresponding to 1.10^9 bacteria. Approximately 2.10^8 bacteria were transferred to glass coated 96-well plates with a conical bottom (ThermoFisher Scientific, Waltham, MA), and the plates were centrifuged (1,800 g, 20 min, 4°C). When indicated 10 µl of 15 µM phospholipids PE31:1 and PC31:1 (Sigma) both containing the acyl chains 14:1 and 17:0 were added to the pellet. Phospholipid extraction was performed as described by Juencken et al. [48], in brief pellets were resuspended in 150 µl of chloroform/methanol (1:1 v/v), extracted for 1 h at 4°C, and centrifuged (1,800 g, 20 min, 4°C). Supernatants were added to a glass-coated 96-well plate that was covered with aluminum foil and placed in the autosampler. Ten microliters of the phospholipid extract were separated using a HILIC column (Kinetex, 2.6 µm) at a flow rate of 1 ml/min to resolve different phospholipid classes. External phospholipid standards (PI, PS, PG, PC, PA and PE) with known concentrations were analyzed together with all other samples, and were used to determine the response factors of the corresponding lipid class. These response factors were used to correct for the difference in detection efficiency of the different lipid classes. The lysophospholipids were assumed to have the same response factors as the corresponding diacyl lipid. The lyso-phospholipids were assumed to have the same response factors as the corresponding diacyl-phospholipid. The response factor of PG was also used for the unknown classes: PX and acyl-PG. Column effluent was injected into a LTQ-XL mass spectrometer (ThermoFisher Scientific, Waltham, MA) and analyzed in the negative ion mode using electrospray ionization. Data was analyzed using R version 3.4.2 (R Development Core Team, 2016). Data were converted to mz(X)ML format and analyzed using XCMS version 1.52.0 [49, 50].

RNA-seq analysis. *C. jejuni* strain 81116 cultures were diluted to an OD_{550} of 0.05 in HI broth and grown under microaerophilic conditions (5% O_2, 10% CO_2, 75% N_2, 10% H_2) for 6 h or 12 h at 42°C. RNA was extracted from *C. jejuni* using RNA-Bee kit (Tel-Test). RNA samples were treated with RNAse-free DNAse I (Invitrogen) according to the manufacturer's manual. RNA-seq analysis was performed as previously described [51].

Motility assay. Overnight cultures of *C. jejuni* strain 81116, its isogenic *pldA* and *cfa* mutant strains and the complemented PldA mutant were diluted in 5 ml of HI to an OD_{550} of 0.05. At the mid-exponential growth phase 1.0 µl of each culture was injected into semi-solid medium (thioglycolate medium containing 0.4% agar) and incubated under 0.3% or 10% oxygen conditions at 42°C for 20 and 24 h, respectively. Motility was scored of four independent experiments by measuring the diameter of the colonies.

Statistical analysis. Statistical significance was determined using two-way ANOVA using Prism software (GraphPad, San Diego, CA). Results are shown as mean ± SEM.

Accession numbers. Gene expression ratios from the RNA-seq experiments are deposited in the GEO database under accession number GSE104231.

Conflict of interest
Authors declare no conflict of interest.

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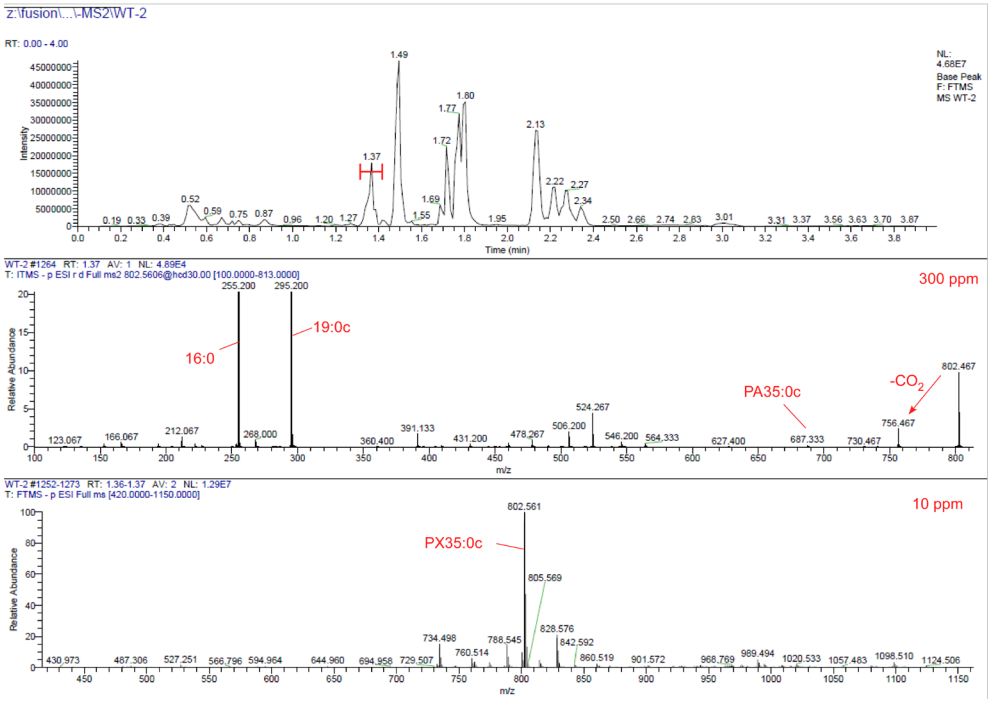
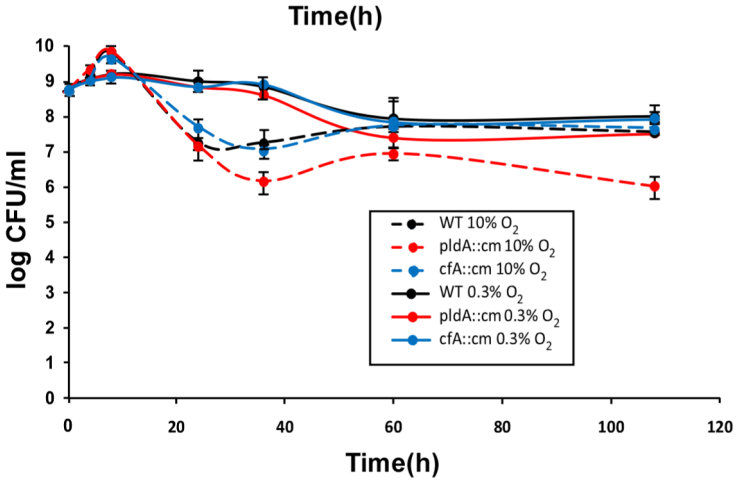
Supplemental materials



Supplementary Fig. S1. MS-2 data of phospholipid PX35:0c. PX35:0c with mass 802,561 contains the acyl chains 16:0 (255,2) and 19:0c (295,2). Phospholipid head group X has mass of 133,075 (802,561 – exact mass of PA35:0c (687,497) + H_20 (18,0105).



Supplementary Fig. S2. Viability counts (CFU/ml) of *C. jejuni* wildtype and the *cfa* and *plda* mutants at 42^{0}C in HI medium under microaerobic (10% O_2, 10% CO_2, 70% N_2, 10% H_2) and oxygen limited (0.3% O_2, 10% CO_2, 79,7% N_2, 10% H_2) conditions. The experiment was repeated three times. Data are represented as mean ± SEM.

Supplementary Table. S1. List of the used primers.

Primer	5'-3'
PldA-F	TTTACACTAAAACATAAAATACGA
PldA-R	CCTATCATACCAAGTGTTAAAA
PldABamHI-F	AGGATCCAAACACTTGCAAAGACTTTCC
PldABamHI-R	AGGATCCAGCCCATATAAAAACACATAAG
SacIPldAFor	AGAGCTCAAAGGAGATAAGATGAGAAAAATTGCTT
XbaIPldARev	ATCTAGAAATTGTAATGAAATTTGTTTTAAG
Cfa-F	GTACTGCTTTATCAAAATCTCC
Cfa-R	AGCAAAAGAAGAATTAAGCAAAG
CfaBamHI-F	AGGATCCTAAGTCTTACCAAGGACTAC
CfaBamHI-R	AGGATCCTCTTGATCCCAAAATACAATTCTA

4

Campylobacter jejuni benefits from the bile salt deoxycholate under low-oxygen condition in a PldA dependent manner

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Manuscript in preparation

Abstract

Enteric bacteria need to adapt themselves to endure the antibacterial activities of bile salts in the gut of animals and humans. One mechanism of adaptation involves alteration of their membrane composition. Bacterial phospholipase A (PldA) is a key enzyme in maintaining bacterial membrane homeostasis. *Campylobacter jejuni* is the most common bacterial cause of human gastroenteritis in the western world. However, how *C. jejuni* adapts and survives in the gut environment is still not fully understood. Here, we discovered that the PldA of this organism is needed for optimal growth of *C. jejuni* in the presence of the bile salt sodium deoxycholate (DOC). At high oxygen conditions DOC is toxic for *C. jejuni*, but under low oxygen conditions as exist in the gut, *C. jejuni* benefits of DOC. RNA-seq analyses and growth curves revealed that *C. jejuni* probably benefits from DOC because it upregulates iron metabolism in a PldA dependent manner, while the oxidative stress defense is less active in the wildtype compared to the *pldA* mutant. We show that in the presence of bile salts under low oxygen conditions, the PldA of C. jejuni is indispensable to acquire iron needed to maintain itself in the host.

Introduction

Successful bacteria can adapt their physiology in response to changing environmental conditions. The climate in the mammalian intestine clearly differs from that of the outside world. For example, in the intestine the oxygen concentration can be extremely low, and the bacteria are continuously exposed to bile. Bile salts play an important role in food digestion but also act as effective natural antimicrobials [1]. In order to survive and colonize the human gastrointestinal tract, commensal and pathogenic bacteria must deal with high concentration (0.2 to 2% (wt/vol)) of bile salts [2, 3]. Bile salts are bile acids that are conjugated with taurine or glycine residues [4]. Bile acids can be formed in two ways: primary bile acids are intermediate products of cholesterol degradation and synthesized by the liver, while secondary bile acids such as deoxycholate and litocholate result from the resident microbiota in the gut [5, 6]. Many intestinal bacteria can transform primary bile salts into secondary bile salts by removing the hydroxyl group at C7 [7]. This significantly increases bile salt pool diversity [8].

The release of bile salts into the intestine is one of the factors that the host utilizes to induce gut microbiome alterations [9]. Bile salts like sodium deoxycholate (DOC), can be used by bacteria as carbon source and electron acceptor [7, 10, 11] and can be an environmental signal to switch on virulence factors in *Shigella*, *Salmonella* and *Vibrio* species [11-15]. Bile salts can however also act as antibacterial compounds as they are able to disrupt bacterial membranes, denature proteins, chelate iron and calcium, and induce an SOS response resulting in DNA damage [11]. Well-known mechanisms of enteric bacteria to cope with bile salts are bacterial cell envelope modification (like LPS O-antigen length) [16]; CmeABC multidrug efflux pumps [17]; DNA repair [18, 19] and the RpoS-dependent stress response [20]. Bile exposure is also known to cause significant alterations into bacterial phospholipid profiles [21].

The Gram-negative bacterium *C. jejuni* is the leading cause of bacterial foodborne human enteritis throughout the world. Contaminated chicken meat is believed to be the main source of infection [22]. *C. jejuni* is microaerophilic and needs reduced oxygen concentrations for growth. In the human gut *C. jejuni* penetrates the intestinal mucus layer, colonizes the crypts, and disrupts the epithelial barrier. The molecular basis of *C. jejuni* infection however is still poorly understood [23]. In earlier studies, we determined the phospholipidome of *C. jejuni.* This revealed a very high (>30%) lysophospholipid (LPL) content [24]. The LPLs result for the most part from the activity of the phospholipase A (PldA) enzyme that cleaves fatty acid tails form phospholipids. We also showed that the LPLs are toxic to erythrocytes and epithelial cells [24, 25]. Furthermore, a functional *pldA* gene is needed to allow *C. jejuni* to colonize the cecum of chickens [26]. Of potential importance, the activity of the human PldA is increased in the presence of bile salts [27].

Considering the important roles of low oxygen availability, bile salts, and PldA

in *C. jejuni* infection, we here investigated the potential impact of these factors on *C. jejuni* by transcriptome analysis and assessment of bacterial growth. Wildtype and PldA-defective bacteria were grown under microaerobic and low oxygen conditions and in the absence and presence of DOC to mimic *in vivo* conditions at the site of infection.

Results

Effect of oxygen and PldA on *C. jejuni* colony morphology

To investigate the effects of oxygen and PldA on *C. jejuni* biology, we first compared the growth of *C. jejuni* strain 81116 and a generated isogenic *ΔpldA* derivative (*C. jejuni ΔpldA*) in different O_2 environments. Both strains seemed to grow equally well on blood free *Campylobacter* selective agar base under oxygen limited (0.3% oxygen) conditions with no differences in colony morphology. However, under low oxygen conditions (0.3%), the colony morphology of *pldA* mutant clearly differed from that of the wildtype. The *pldA* mutant had a wet / glossy colony appearance in contrast to the rather dry / dull colonies of the *C. jejuni* wildtype (Fig 1A). The aberrant morphology of the mutant could be restored by the introduction of a *pldA* complementation plasmid, yielding *C. jejuni ΔpldA +pldA*. Interestingly, all three strains formed wet / glossy colonies when grown on saponin agar charcoal plates (Fig 1B). One of the major differences between the two media is the presence of bile salts (0.1%) in blood free *Campylobacter* selective agar base. Indeed, the addition of DOC (0.1%) to saponin agar charcoal plates resulted in dry / dull colonies of the wildtype *C. jejuni* and *pldA* complemented strain, whereas the colonies of the *pldA* mutant kept their wet / glossy appearance (Fig 1C), all consistent with the colony phenotypes observed on blood-free *Campylobacter* selectivity agar. These data showed that bile salts can alter *C. jejuni* morphology in a PldA-dependent manner under conditions of low oxygen. Transmission electron microscopy (EM) on *C. jejuni* grown on the blood free *Campylobacter* selective agar base did not reveal differences in bacterial shape between the wildtype and *pldA* mutant (Fig S1A & B).

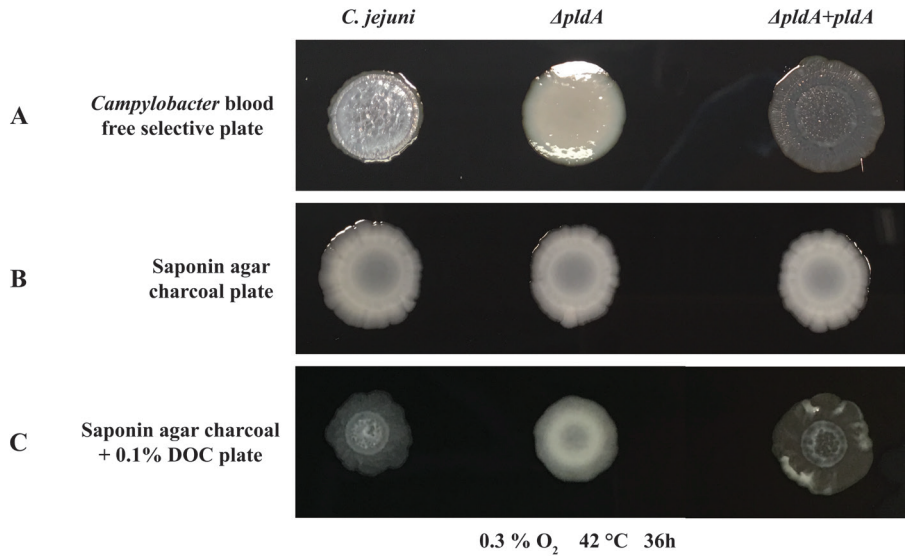

Figure 1. DOC modifies *C. jejuni* wild type colony morphology on *Campylobacter* blood free selective plate. Colonies of *C. jejuni* 81116 wildtype, *C. jejuni ΔpldA* and *C. jejuni ΔpldA +pldA* were grown on (A) Blood Free *Campylobacter* Selective Agar, (B) Saponin agar charcoal, and (C) Saponin agar charcoal+ 0.1% DOC. Bacterial morphology was visualized after 36 h of incubation under oxygen limited (0.3% O_2, 10% CO_2, 79.7% N_2, 10% H_2) conditions at 42°C.

Effects of PldA, DOC and oxygen on planktonic growth of *C. jejuni*

To investigate whether exposure to bile salts influences *C. jejuni* growth kinetics, we followed the planktonic growth of *C. jejuni* wildtype 81116, *C. jejuni ΔpldA*, and the complemented strain *C. jejuni ΔpldA +pldA* by measuring the culture optical density. Growth was monitored in the presence or absence of 0.1 % DOC and at 0.3% and 10% O_2, which mimic the oxygen concentrations in the intestinal mucosa [28] and surface water [29], respectively. These experiments revealed similar growth kinetics for the three strains when grown in the absence of DOC and irrespective of the oxygen concentration (Fig 2A & B). In the presence of 0.1% DOC, a clear inhibition of growth was seen for all three *C. jejuni* strains when grown under 10% O_2 concentration (Fig 2A). But unexpectedly, under oxygen-limited conditions (0.3% O_2), the addition of DOC stimulated the growth of the *C. jejuni* strains. The doubling times of the wild type increased from 2.97 h to 1.82 h and of the *ΔpldA* strain from 3.49 h to 2.85 h (Fig 2B). These results suggest that the oxygen concentration determines whether DOC promotes or inhibits the growth of *C. jejuni*.

It was also noted that in the presence of 0.1% DOC, the growth rate of *C. jejuni ΔpldA* was clearly reduced compared to the wildtype at both 0.3% and 10% O_2 (Fig. 2). The maximum doubling time at 0.3% O_2 was 1.82 h for the wildtype *versus* 2.85 h for *ΔpldA* mutant. At 10% O_2, the doubling time dropped from 1.41 h for the

wildtype strain to 2.31 h for the *ΔpldA* mutant. The reduced growth of the *pldA* mutant was rescued after complementation of the gene defect (Fig 2). Comparison of the lipid oligosaccharide (LOS), capsule patterns, biofilm formation, and protein composition after growth in HI or HI plus 0.1% DOC, showed no differences between the wildtype and *pldA* mutant (Fig S2). Overall, the results indicate that the PldA protein of *C. jejuni* is needed for optimal bacterial growth in the presence of DOC, irrespective of oxygen availability.

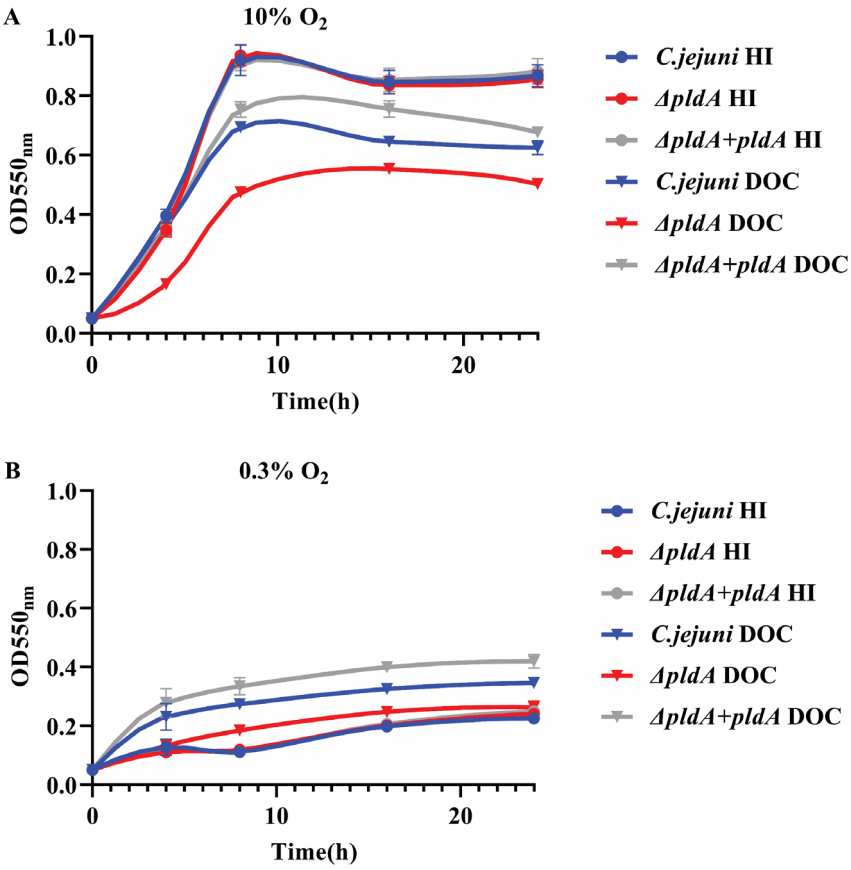

Figure 2. *C. jejuni* PldA is needed for optimal growth in the presence of DOC but the oxygen concentration determines whether DOC improves or inhibits *C. jejuni* growth. Growth curves of *C. jejuni* wildtype, *C. jejuni ΔpldA* and *C. jejuni ΔpldA +pldA* were generated in HI with or without 0.1% DOC under (A) microaerobic (10% O_2, 10% CO_2, 70% N_2, 10% H_2) or (B) oxygen limited (0.3% O_2, 10% CO_2, 79.7% N_2, 10% H_2) conditions at 42 °C. The optical density was measured at the indicated time points. Experiments were repeated three times in duplicate. Data are presented as mean ± SEM.

Effects of DOC on *C. jejuni* gene expression

To better understand how DOC and PldA can promote *C. jejuni* growth at low O_2, we performed RNA-seq on the wildtype and *pldA* mutant grown (6 h) in HI broth or HI broth plus 0.1% DOC. First, we searched for DOC regulated genes in wildtype *C. jejuni*. Hereto, we analyzed our RNA-seq results using the formula [(*C. jejuni* wildtype - *C. jejuni* wildtype DOC), a positive value indicates that DOC increased the transcription of the gene (upregulation), whereas a negative value points to a reduction of the transcription (downregulation). DOC induced a >3-fold change in the transcription of 66 genes compared to bacteria grown without DOC. Real-time PCR on 13 highly regulated and previously reported DOC dependent genes [5, 17, 30] yielded no gross differences in results between the real-time PCR and the RNA-seq data (Table. S1A), verifying the RNA-seq results. The majority of the identified transcripts (44 out of 66) were upregulated. They encode proteins involved in DNA repair (RecO), iron transport (C8J_0168-C8J 0169, C8J_1563, C8J_1564, p19) [31], tryptophan catabolism (TrpABF), the multidrug efflux pump (CmeBC), RNA polymerase (RpoC), purine biosynthesis (PurH), the LIV amino acid transport system (LivFGM), porphyrin metabolism (HemD), peptide translocation (C8J_1479), protein transport (SecY), and leucine biosynthesis pathway (LeuBCD) (Fig. 3). The products of the 22 downregulated transcripts function in electron transport (C8J_0040, NapA, NrfA, NrfH), chemotaxis (CheA), non-heme iron metabolism (C8J_1167), motility (C8J_1245), selenocysteine biosynthesis pathway (SelAB) and arsenic resistance (ArsC and ArsR). These results suggest that exposure of *C. jejuni* to DOC at low O_2 induces major alterations in bacterial physiology which may favor bacterial growth.

Comparison of the transcript levels in the *pldA* mutant grown in the absence or presence of DOC revealed 7 genes that were also highly upregulated in *C. jejuni* wildtype. These were iron transport genes (*C8J_0167-C8J_0169*, *p19*), tryptophan catabolism genes (*trpB* and *trpF*) and the hypothetical gene *C8J_0822*. Surprisingly, transcript levels of all other 59 genes that changed upon exposure to DOC in the wildtype were barely affected by DOC in the *pldA* mutant. This suggests that the expression of most of the DOC-sensitive genes is PldA dependent.

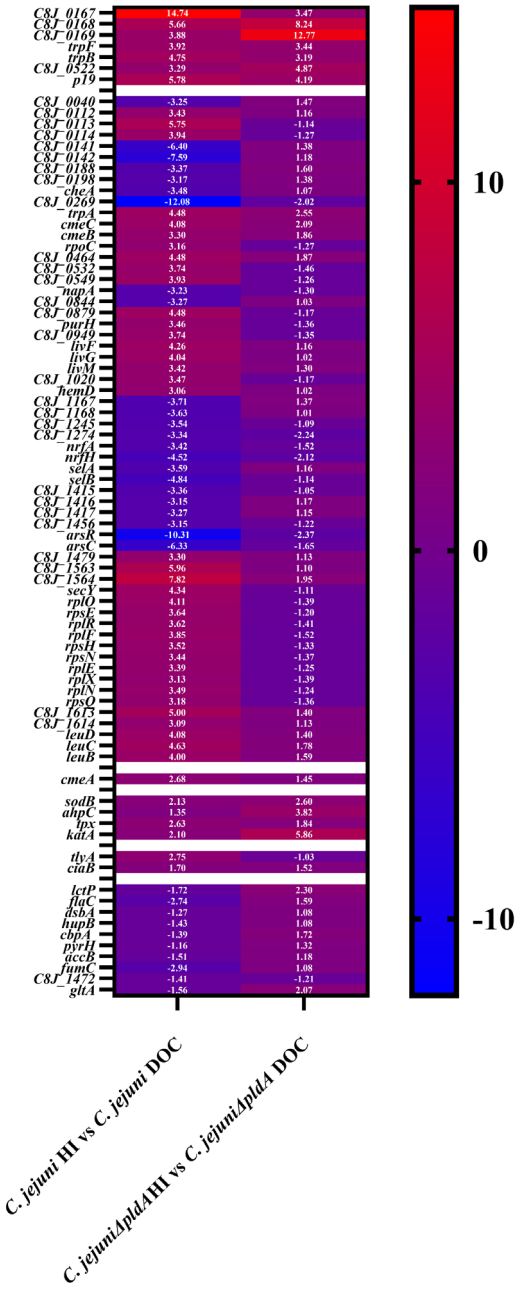

Figure 3. Genes affected by the addition of DOC in *C. jejuni* wildtype and its isogenic *ΔpldA* mutant. Heatmap and hierarchical clustering of the highly regulated genes and reported reference genes are presented. The experiments were repeated three times with similar results.

Effects of PldA on *C. jejuni* gene expression

Comparison of the gene transcripts levels in *C. jejuni* wildtype and *C. jejuni ΔpldA* grown in the absence and presence of 0.1% DOC (Figure 4), yielded 19 genes that showed a > 3-fold difference, irrespective of the presence of DOC. Besides the inactivated *pldA* gene itself, 11 of the remaining 18 transcripts were downregulated in the *pldA* mutant. These genes encode the hypothetical proteins C8J_0029, C8J_0030, C8J_0241 and C8J_0703, a putative cytochrome C-type haem-binding periplasmic protein C8J_0242, an aconitase AcnB [32, 33], two disulfide bridge introduction proteins DsbAB [34], arylsulfate sulfotransferase C8J_0813, a sodium/proline symporter PutP and PutA a proline dehydrogenase/delta-1-pyrroline-5-carboxylate dehydrogenase [35]. The six genes that were upregulated in the *pldA* mutant independent of DOC, are the three hypothetical genes *C8J_0877*, *C8J_1306* and *C8J_1307*, a putative iron-binding gene *C8J_0219*, an enterochelin ABC transporter substrate-binding protein encoded by *ceuB*, and an outer membrane hemin and hemoglobin receptor encoded by *chuA*. The upregulation of iron acquisition gene transcripts in the *pldA* mutant can be a sign of a shortage of iron availability, but this did not affect bacterial growth (Fig. 2).

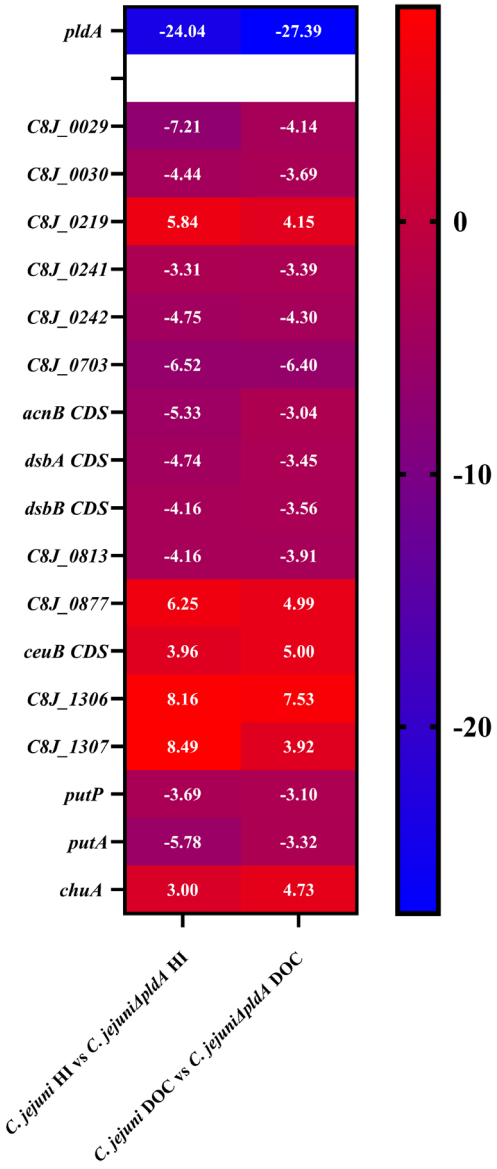

Figure 4. PldA expression dependent but DOC-insensitive genes. The heatmap and hierarchical clustering of PldA highly regulated genes are presented. The experiments were repeated three times with a similar outcome.

Combined effects of PldA and DOC on *C. jejuni gene* expression

Since in HI medium, there is no growth rate difference between *C. jejuni* wildtype and the *ΔpldA* mutant (Fig 2A & B), we assumed that the identified *pldA*-dependent but DOC-insensitive genes were not responsible for the reduced growth of the *pldA* mutant compared to the wildtype in medium with DOC. To find possible genes responsible for the growth defect of the *pldA* mutant in the presence of DOC, we analyzed our RNA-seq results using the formula [(*C. jejuni* wildtype DOC - *C. jejuni ΔpldA* DOC) - (*C. jejuni* wildtype HI - *C. jejuni ΔpldA* HI)]. Because differences in gene expression between the two strains which cultured in HI medium (*C. jejuni* wildtype HI - *C. jejuni ΔpldA* HI) did not directly affect bacterial growth rates, this difference was removed as background. A positive value indicates here that DOC in a PldA dependent manner increases the transcription of the gene (upregulation), whereas a negative value points to reduction of transcript (downregulation).

This analysis revealed a number of strongly (> 5-fold) downregulated genes. These include *C8J_0113*, *C8J_0532*, *C8J_0879*, *C8J_0949*, *C8J_1310*, *C8J_1563*, *C8J_1591*, *C8J_1593*, *C8J_1595*, *C8J_1596* and *C8J_1620* (Figure 5). The gene *C8J_0113* encoded a putative recombination protein RecO which is utilized by *C. jejuni* to defend against bile in the intestinal environment by repairing DNA gaps (single strand breaks) [36]. The genes *C8J_0532*, *C8J_0879*, *C8J_0949*, *C8J_1591* and *C8J_1620* all encode hypothetical proteins. The genes *C8J_1593 C8J_1595* and *C8J_1596* encoded three ribosomal proteins. The gene *C8J_1310* encoded a MmgE/PrpD family protein which is involved in propionate catabolism [37]. The gene *C8J_1563* encoded an iron ABC transporter permease. Besides *C8J_1563*, three iron metabolism gene (*C8J_0167, C8J_1564, C8J_1565*) are observed to be downregulated as well, another three genes (*C8J_1548, C8J_1549*, *C8J_1550*) are not involved in iron metabolism directly but were proposed to be downregulated by iron limitation [38, 39]. Together, these data may indicate that in the presence of DOC, *C. jejuni* PldA might be especially important for the optimal expression of genes involved in iron metabolism.

The analysis also identified number of genes that were (> 5-fold) upregulated by DOC. These include *C8J_0141*, *C8J_0142*, *C8J_0188*, *C8J_0269*, *C8J_1167*, *C8J_1272*, *C8J_1302* and *C8J_1487* (Figure 5). *C8J_0141*, *C8J_0142*, *C8J_0188*, *C8J_0269 and C8J_1302* encode four hypothetical proteins. *C8J_1167* is a hemerythrin-like non-heme protein [40]. *C8J_1272* encoded a MGC82361 protein, *C8J_1487* encoded a flavohemoprotein which is important to reduce oxidative stress. Based on previous studies, *C8J_0141* and *C8J_0142* play a role in peroxide stress defense [41, 42], *C8J_1167* is involved in oxygen storage and transport [40] and gene *C8J_1487* encodes a single-domain hemoglobin involved in oxidative stress defense. In addition to those highly upregulated genes, *ahpC*, *hspR*, *katA-C8J_1304* and *C8J_1514* which are involved in oxidative/aerobic stress response, are upregulated

as well [43-47]. The putative functions of the upregulated genes suggest that DOC exposure elicits an oxidative response, especially in the absence of PldA.

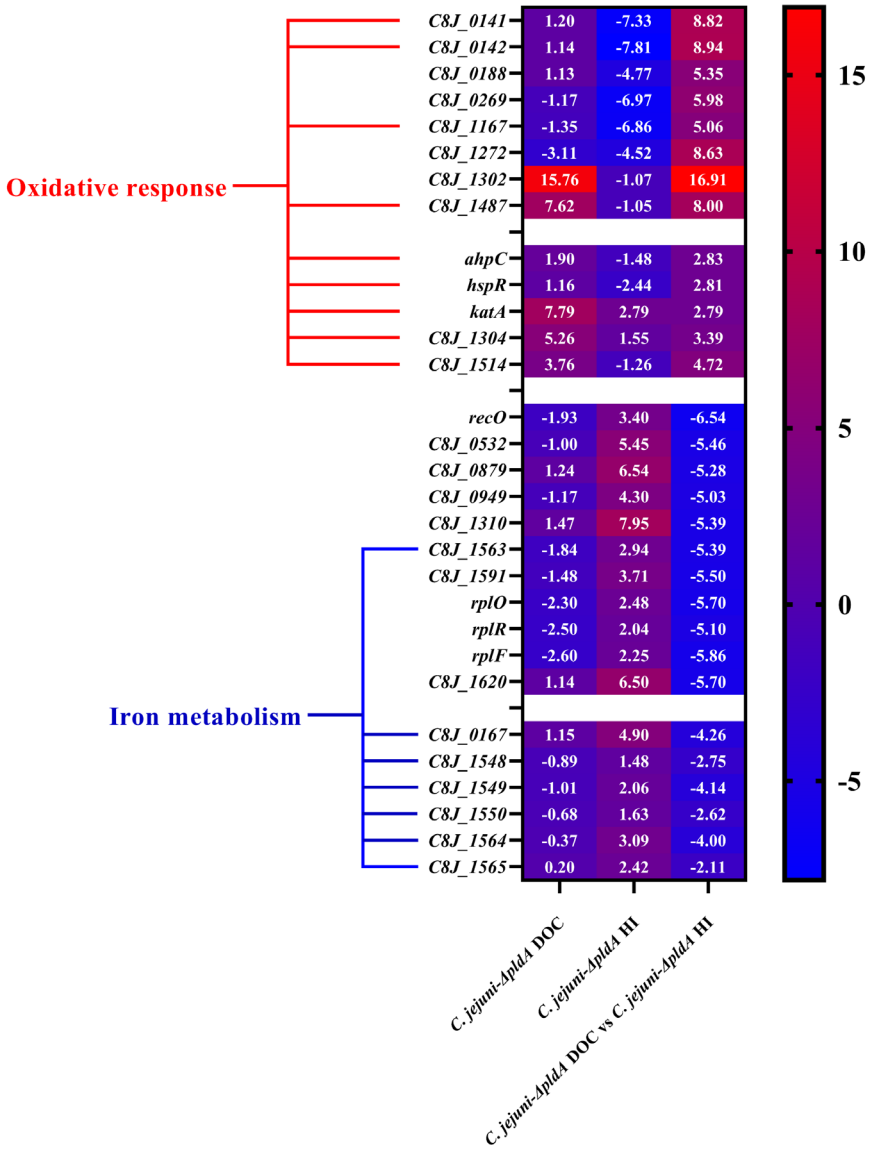

Figure 5. Transcription of genes dependent on the availability of DOC and a functional *pldA* gene. The heatmap and hierarchical clustering of the highly regulated genes are presented. The RNA-seq experiments were repeated three times with similar results.

DOC reduces efficient iron utilization by the PldA mutant

Based on our RNA-seq analyses, PldA appears to play a crucial role in iron metabolism, therefore we investigated whether we could rescue the reduced growth of the *pldA* mutant in the presence of DOC by adding additional ferric or ferrous iron sources. Growth curves were generated for the wildtype and *pldA* mutant strain in HI with or without DOC and with or without 50 µM Fe^{2+} or Fe^{3+} at 0.3% O_2. In the absence of DOC, no clear growth differences between the strains grown in HI or HI plus 50 µM Fe^{2+} or Fe^{3+} were seen (Fig. 6A). In the presence of DOC, the growth of the wildtype bacteria increased when 50 µM Fe^{2+} or Fe^{3+} was added to the media. This effect was not observed for the *pldA* mutant (Fig. 6B). This is in line with the observed down-regulation of iron-regulated genes in the DOC-exposed *pldA* mutant and indicates that iron utilization in the *pldA* mutant is strongly limited in the presence of DOC, inhibiting bacterial growth of the *pldA* mutant.

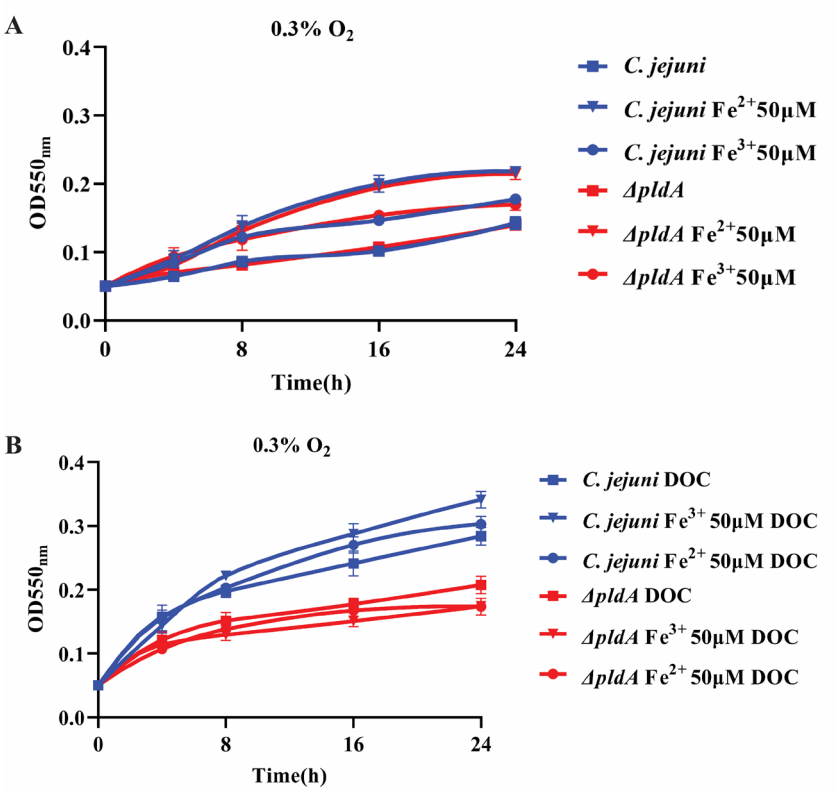

Figure 6. Growth curves of wildtype and *pldA* mutant with or without DOC and the addition of ferric or ferrous iron. *C. jejuni* wildtype and *ΔpldA* were grown (A) without or (B) with DOC with or without Fe^{2+} or Fe^{3+} at 42°C in HI, under oxygen limited (0.3% O_2, 10% CO_2, 79.7% N_2, 10% H_2) conditions. The optical density (Y- as) at the indicated time points are shown. The experiments were repeated three times in duplicate. Data are presented as mean ± SEM.

PldA reduces *C. jejuni* resistance to oxidative stress in the presence of DOC

The apparent upregulation of the oxygen stress defense systems by DOC in *C. jejuni ΔpldA*, led us to speculate that *C. jejuni ΔpldA* may have a stronger capacity to resist oxygen stress. To verify this, the *C. jejuni* wild type and *pldA* mutant were exposed to 80 nM hydrogen peroxide (H_2O_2) in HI and HI plus 0.1% DOC under microaerobic (5% O_2) conditions. Following 30 min exposure to H_2O_2 in 0.1% DOC, *C. jejuni ΔpldA* exhibited a significantly greater resistance to oxidative stress killing compared to *C. jejuni* wildtype, as determined by CFU counting (Fig. 7A). Without H_2O_2 treatment, in HI medium with or without 0.1% DOC no difference in CFU between *C. jejuni* wildtype and *C. jejuni ΔpldA* strain was observed (data not shown). These results are consistent with the RNA-seq data that suggest that in the presence of DOC the *PldA* mutant upregulates oxygen stress systems much better than the wildtype *C. jejuni*.

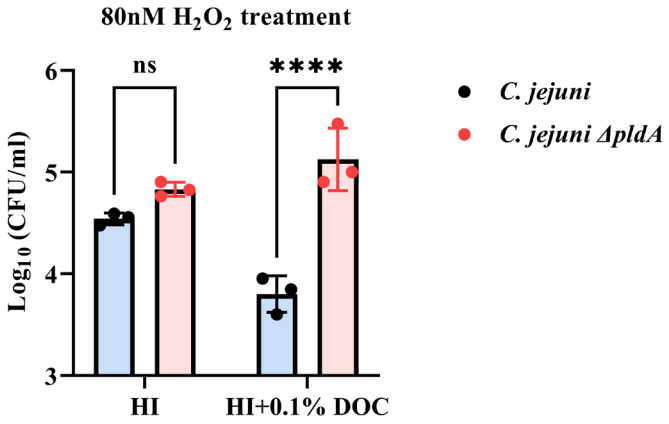

Figure 7. Survival assay to evaluate the susceptibility of *C. jejuni* wildtype and *C. jejuni ΔpldA* to oxidative stress. Survival assays of *C. jejuni* wildtype and *C. jejuni ΔpldA* strains were performed in HI with or without 0.1% DOC exposed to 80 nM H_2O_2 under microaerobic conditions for 30 min. After serial dilutions and incubation, CFUs/ml were counted. Data of three independent experiments with three independent preparations of bacterial samples and presented as mean values ± SEM, ****P < 0.0001, ns P > 0.1.

Discussion

Colonization of the (chicken) intestine by *C. jejuni* requires, amongst others, bacterial adaptation to a low oxygen environment, resistance to bile salts, and a functional PldA enzyme which catalyzes the formation of lysolipids. In the present study, we investigated the roles of PldA, the bile salt DOC, and low (0.3%) oxygen availability on *C. jejuni* biology, mimicking the *in vivo* situation. We provide evidence that DOC stimulates *C. jejuni* growth under low oxygen conditions and alters colony morphology in a PldA-dependent fashion. Transcriptomics and functional

assays indicate PldA-dependent and DOC-induced changes in gene expression that influence bacterial physiology. More specifically, under limited oxygen conditions *C. jejuni* PldA seems to enable the use of iron needed for optimal growth in the presence of DOC but makes the bacterium more vulnerable for oxidative stress.

Bile salts have an important role in the digestion of fat but are also important as antimicrobial molecules to control the different bacterial species in our gut [11]. Bacteria have evolved all kinds of mechanisms to avoid the toxicity of bile salts [11]. The Gram-negative bacterium *C. jejuni* inhabits the intestine of many mammals where it is exposed to bile salts in an oxygen-limited environment [48, 49]. The adaptation of *C. jejuni* to bile has previously been investigated under the microaerobic (5% O_2) conditions [5, 17, 30, 50, 51]. The main niche of *C. jejuni* has oxygen concentrations between 0.1-1% oxygen [52]. Our results indicate that, at low O_2, DOC changes *C. jejuni* colony morphology dependent on the expression of PldA, suggesting that the phospholipase influences the effects of the bile salt. We previously showed that PldA activity results in high levels of lysophospholipids in the *C. jejuni* [24]. This may influence the colony phenotype in the presence of bile salts. It can be imagined that exposure to DOC influence bacterial viability dependent on the presence of LPLs, resulting in a change in colony morphology. Alternatively, the high percentage of LPLs may cause changes in the surface characteristics that become apparent in the presence of DOC. Membrane proteins are known to be important for bacterial colony morphology as they facilitate transport the intracellular components to the cell surface [53]. We previously showed that PldA influences biological functions like flagella-driven motility, even without apparent changes in capsule, LOS or protein profiles. Importantly, the altered colony morphology was only observed at low O_2 and not when the bacteria were kept under the commonly used microaerobic conditions. This suggests that the effect may be related to alterations in *C. jejuni* metabolism. Our results stress the relevance of culturing *C. jejuni* in the laboratory under more natural environmental conditions.

To further investigate the influence of DOC in relation to PldA activity, we monitored the growth of the wildtype and *pldA* mutant during planktonic growth. Although bile salts are toxic for many bacteria due to their amphiphilic character, *C. jejuni* is considered to be bile resistant as it can been isolated from the gallbladder and even directly from bile [7, 54-56]. In contrast to being toxic, we found that DOC even improved the growth of *C. jejuni* under oxygen limited conditions (0.3% O_2) (Fig 1B), while it reduced growth in an environment with 10% oxygen (Fig 1A). To our knowledge, this is the first bacterium that benefits from the presence of DOC in an oxygen dependent fashion. A large number of aerobic bacteria are known to degrade bile salts, but only a few are known that can use bile salts as electron acceptor and carbon source [7, 10, 57]. For these bacteria, bile salts are a useful compound and a stress factor at the same time. How *C. jejuni* benefits from DOC needs to be

further explored.

In contrast to the improved growth of *C. jejuni* wildtype in the presence of DOC (at low O_2), we noticed a significant growth inhibition for the *pldA* mutant in the presence of DOC. This inhibition was independent of the oxygen concentration (Fig 1A & 1B). It is known that bile salts at high concentrations can rapidly dissolve membrane lipids and cause dissociation of integral membrane proteins [58, 59]. Our results suggest that PldA or its products, the LPLs, prevent the toxicity of DOC exposure, i.e., confer bile salt resistance. This function may at least partially explain the critical role of PldA in the colonization of the chicken intestine.

We conducted transcriptomics to better understand how *C. jejuni* benefits from DOC and what role PldA plays in this process. RNA-seq revealed that DOC exposure at low O_2 induced major changes in gene transcript levels, of which many were PldA dependent. The addition of bile salts has previously been shown to induce transcriptional alterations in *C. jejuni* grown under microaerophilic (rather than low O_2) growth conditions [5, 17, 30]. Our results indicate that a number of these genes are also upregulated under the low oxygen conditions that we have tested. We could confirm that the bile salt resistance genes (CmeABC multidrug efflux pump, *cmeABC*) [17], oxidative stress genes (*sodB*, *ahpC*, *tpx*, *katA*) [5], virulence genes (*ciaB, tlyA*) [30] are upregulated by DOC, and *lctP*, *flaC*, *dsbA*, *hupB*, *cbpA*, *pyrH*, *accB*, *fumC*, *C8J_1472*, *gltA* are downregulated by DOC, however less than 4-fold. Nine of these ten downregulated genes were not downregulated by DOC in the *pldA* strain, suggesting that the expression of these genes is also PldA dependent. We also noted solely DOC- or PldA-dependent alterations in gene expression. A general finding was that, at low O_2, DOC exposure increased the transcript levels of a number of the iron-regulated genes. This is likely due to the well-known iron-chelating effect of bile salts [60] which may cause iron starvation resulting in increased transcription of iron-regulated genes to restore bacterial growth. In *Escherichia coli,* bile salts have also been reported to induce expression of genes involved in iron acquisition and metabolism and to promote bacterial growth in iron-deficient conditions [61].

In the presence of DOC, we found that the transcripts levels of several iron transport genes were significantly downregulated in *C. jejuni ΔpldA* (by using the formula (*C. jejuni* wildtype DOC - *C. jejuni ΔpldA* DOC) - (*C. jejuni* wildtype HI - *C. jejuni ΔpldA* HI)). This indicates that in the presence of DOC, *C. jejuni ΔpldA* (at low O_2) suffered from iron starvation and PldA activity might aid efficient absorption of iron by *C. jejuni*. This hypothesis is supported by the growth curve experiments. These showed that the addition of extra ferrous or ferric iron does not rescue the growth defect of *C. jejuni ΔpldA* (Fig. 6) while it does significantly improve the growth performance of *C. jejuni* wildtype. This suggests, that iron metabolism in the *pldA* mutant (at low O_2) is strongly limited. The ability to acquire and utilize iron may thus also be a factor that contributes to the observed PldA-dependent increase

in *C. jejuni* growth promotion in the presence of DOC.

The RNA-seq analyses also showed alterations in transcript levels of genes involved in the bacterial oxidative stress response. The results suggest that, in the presence of DOC, *C. jejuni* wildtype is more susceptible to oxidative stress than the *pldA* mutant (Fig. 5). This hypothesis was supported by results of the oxidative stress survival assay (Fig. 7). It should be noted that all the highly regulated oxidative response genes are known to be directly regulated by Fur family proteins. These proteins are known to play an essential role in the regulation of the *C. jejuni* oxidative stress defense and iron transport systems [41, 62]. Oxidative stress induced by iron deficiency is a common feature of bacteria [63-65]. We suggest that the above-mentioned iron shortage in *C. jejuni ΔpldA* leads to the activation of Fur-regulated oxidative stress defending genes, which causes the *pldA* mutant to be less vulnerable for oxidative stress than the wildtype. Previous studies by both Palyada et al. [42] and Holmes et al. [41] also identified that *C. jejuni* iron starvation causes upregulation of the expression of oxidative stress defenses genes including *ahpC*, *katA*, *C8J_1304*. The finding that we did not observe growth differences at 10% O_2 levels (Fig. 2A) may indicate that Fur independent iron transporters like the FeoAB [66] are more active at microaerophilic conditions, but this needs to be further explored.

A third physiological system of *C. jejuni* that may be influenced by exposure to DOC is tryptophan and branched-chain amino acids (leucine, isoleucine and valine) metabolism. Currently, it is unknown how *C. jejuni* benefits from the upregulation of tryptophan and high-affinity branched-chain amino acids gene pathways during the exposure of bile salts. The bacterial tryptophan catabolite indole together with bile acids can regulate epithelial inflammation and gut immunity and branched-chain amino acid support the evasion of host defenses [67, 68]. In eukaryotic cells, elevated bile acid levels have been implied to be relevant to an abnormal tryptophan metabolism and bacteria which produce branched-chain amino acid like leucine are known to effect bile hemostasis by conjugating bile acids [69, 70]. Thus, it can be imagined that an increase in these amino acids is important for pathogenesis of *C. jejuni* in host gut.

PldA has long been considered to be an essential factor in the intestinal colonization of *C. jejuni* as well as other enteric pathogens [26, 71]. However, the underlying mechanism has not been fully revealed. We previously reported that in the absence of PldA, *C. jejuni* is less motile under limited oxygen conditions [24]. Our RNA-seq analyses revealed decreased transcripts of the thiol-disulfide oxidoreductase forming gene *dsbA* in the *pldA* mutant. DsbA has been reported to be regulated by iron in a Fur-dependent manner and to play a crucial role in *C. jejuni* motility as it influences the activity of the paralyzed flagella gene *pflA* [34, 72, 73]. The reduced DsbA expression thus might be the explanation for the motility defect in *C. jejuni ΔpldA* at low O_2. The bacterial respiratory electron transfer chain participates in the formation of DsbA [74] but whether the transcription of *dsbA* is regulated by

oxygen availability awaits future study.

In conclusion, bile resistance, oxidative stress defense, and iron acquisition contribute significantly to *C. jejuni* colonization of the gastrointestinal tract [51, 66, 75]. The present study demonstrates that these processes are tightly linked and that bile salts in conjunction with PldA promote *C. jejuni* growth and survival in a low oxygen environment. Other enteric pathogens might utilize similar mechanisms to defend against the toxic components of bile and to optimally adapt to the intestinal niche.

Material and methods

Bacteria culture

C. jejuni wildtype strain 81116, a human isolate originally isolated from a waterborne outbreak [76], its isogenic *pldA* mutant (*C. jejuni ΔpldA*), and the complemented *pldA* mutant (*C. jejuni ΔpldA +pldA*) [24] were routinely grown on saponin agar plates containing 4% lysed horse blood or in Hearth Infusion (HI) medium (Biotrading, Mijdrecht, The Netherlands) under microaerophilic conditions (5% O_2, 10% CO_2, 75% N_2, 10% H_2) at 42 °C. When appropriate the media were supplemented with DOC (0.1%), chloramphenicol (15 μg/ml) or kanamycin (25 μg/ml).

C. jejuni morphology detection

C. jejuni, *C. jejuni ΔpldA* and *C. jejuni ΔpldA +pldA* were routinely grown under microaerophilic conditions at 42°C in HI medium for 24 h. Bacteria were collected by centrifugation (10 min, 3,000×g) and resuspended in HI medium to a final OD_{550} of 1. Five microliter of bacterial suspension was loaded on the surface of 1) *Campylobacter* blood-free selective agar base plates (Thermo Fisher Scientific), 2) saponin agar charcoal plates containing 4% lysed horse blood and 4% bacteriological charcoal, or 3) saponin agar charcoal plates containing 4% lysed horse blood, 4% bacteriological charcoal and 0.1% DOC (Merck). Plates and incubated for 36 h under oxygen-limited conditions at 42 °C.

Electron Microscopy

The *C. jejuni* wildtype and *C. jejuni ΔpldA* were grown in HI broth plus 0.1% DOC under oxygen-limited conditions (0.3% O_2, 10% CO_2, 79.7 %N_2, 10% H_2) for 36 h at 42 °C. Bacteria were collected by centrifugation (10 min, 3,000×g), washed three times with phosphate-buffered saline (PBS, Thermo Fisher Scientific) and finally resuspended in DPBS to a final OD_{550} of 1. Carbon activated copper grids were incubated with 10 μL of the DPBS resuspended bacterial culture for 10-30 min and washed three times with PBS). The bacteria were fixed on the grids using 1% glutaraldehyde (Sigma-Aldrich) in DPBS for 10 min, washed two times

with DPBS and, subsequently, four times with Milli-Q water. The grids were then briefly rinsed with methylcellulose/uranyl acetate (pH 4) and incubated for 5 min with methylcellulose/uranyl acetate (pH 4) on ice. Grids were looped out of the solution and air dried. Samples were imaged on a Tecnai-12 electron microscope (FEI, Hillsboro, Oregon, USA).

LOS detection

LOS isolation and staining was performed as described before [77]. In brief, *C. jejuni* strains were grown under oxygen-limited conditions at 42°C in 5 ml HI or HI plus 0.1% DOC medium for 24 h. Bacteria were collected by centrifugation (10 min, 3,000×g) and resuspended in DPBS to a final OD_{550} of 1. Samples were boiled for 5 min and then treated with 10 µL protease K (20 mg/mL) overnight at 55 °C. Three times Laemmli buffer was added, and the samples were loaded on a 16% Tris-Tricine gel. After electrophoresis the gel was fixed for 30 min with 40% ethanol and 5% acetic acid, oxidized 5 min with 0.7% sodium periodic acid with 40% ethanol and 5% acetic acid, washed 5 min for 3 times in distilled water, stained with distilled water containing 19% 0.1 M NaOH, 1.3% ammonium hydroxide (>28%) and 3.3% 20% w/v silver nitrate, washed, and developed with distilled water containing 0.1% formaldehyde (37%) and 0.1% citric acid (100 mg/mL) until bands appeared. The reaction was stopped by washing with distilled water containing 7% acetic acid.

Capsule detection

The polysaccharide capsule of *C. jejuni* was visualized with the cationic dye Alcian blue, as previously reported [78]. In short, *C. jejuni* strains were grown under oxygen-limited conditions condition at 42°C in 5 ml HI or HI plus 0.1% DOC medium for 24 h. Bacteria ($1x10^8$) were collected by centrifugation (10 min, 3,000×g) and resuspended in 100 µL lysis buffer (3.2% 1 M Tris-HCl pH 6.8, 0.14 M SDS, 0.37 mM bromophenol blue, 20% glycerol, and 76.8% distilled water), samples were boiled for 10 min at 100 °C and then treated with 10 µL protease K (20 mg/mL) overnight at 55°C. After heating for 10 min at 100 °C, 20 µL of sample was loaded on 10% SDS-PAGE gel. The gel was stained with Alcian blue solution (2% acetic acid, 40% methanol and 0.5% Alcian blue 8GX (Sigma-Aldrich)) for 30-60 min and destained with 2% acetic acid and 40% methanol until bands appeared.

Biofilm formation

C. jejuni biofilms were detected by using crystal violet staining assay, as reported [79]. In summary, *C. jejuni* strains were grown in 15 ml polypropylene tubes containing 5 ml HI broth or HI broth plus 0.1% DOC under oxygen-limited conditions at 42°C. After 24 h the culture media were removed from 15 ml tubes and 10 ml of fixing solution (0.05% w/v crystal violet, 1% formaldehyde (37%), 10%

DPBS and 1% methanol) was added to the tube. Biofilms were stained for 20 min at room temperature, washed with H_2O, and air dried.

SDS-PAGE

The *C. jejuni* wildtype and *C. jejuni ΔpldA* were grown in HI medium with or without DOC for 36 h at 42°C under microaerophilic conditions and then diluted to an OD_{550} of 1 with DPBS. Bacterial samples were mixed with 3x Laemmli Sample Buffer, lysed, and denatured at 95°C for 15 min. Samples were loaded in equal volumes (10 µL) onto a 12% acrylamide gel. Gels were run for 30 min at 50 V and then another 60 min at 150 V. Gels were stained with 20 ml PageBlue Protein Staining Solution (Thermo Fisher Scientific) for 1 h and destained overnight in 80:10:10 MQ: methanol: acetic acid. Gels were imaged with a Universal Hood III (Biorad).

Bacterial growth assay

C. jejuni wildtype, *C. jejuni ΔpldA* and *C. jejuni ΔpldA +pldA* starter cultures were grown in HI medium for 24 h at 42°C under microaerophilic conditions and then diluted to an OD_{550} of 0.05 in T25 flasks containing 5 ml of HI broth or HI broth plus 0.1% DOC. Cultures were shaken (160 rpm) under high-oxygen (10% O_2, 10% CO_2, 70% N_2, 10% H_2) or under oxygen-limited conditions (0.3% O_2, 10% CO_2, 79.7 %N_2, 10% H_2) at 42°C. Ferrous sulfate or ferric sulfate (50 µM) was added to the medium when appropriate. The optical density (OD_{550}) of the culture was measured at 0, 4, 8, 16 and 24 h of growth. Given values are the mean of three independent experiments performed in duplicate.

RNA-seq analysis

C. jejuni wildtype and *C. jejuni ΔpldA* start cultures were diluted to an OD_{550} of 0.05 in HI broth or HI broth plus 0.1% DOC, and then grown under oxygen-limited conditions (0.3% O_2, 10% CO_2, 79.7 %N_2, 10% H_2) for 6 h at 42°C. RNA was extracted from *C. jejuni* and *C. jejuni ΔpldA* using RNA-Bee kit (Tel-Test). RNA samples were treated with RNAse-free DNAse I (Invitrogen) according to the manufacturer's manual. RNA-seq analysis was performed as previously described [80].

Quantitative real-time RT-PCR (RT-PCR) analyses

Real-time RT-PCR analysis was performed as previously described [81]. Primers used in this study are listed in Table. S1B. The calculated threshold cycle (Ct) for each detected gene amplification was normalized to the Ct value for the housekeeping gene *rpoD* amplified of the corresponding sample, before calculating fold change using the arithmetic formula ($2^{-\Delta\Delta Ct}$) [82]. Each sample was repeated with three independent preparations of RNA.

Oxidative stress assay

C. jejuni wildtype and *C. jejuni ΔpldA* mutant starter cultures was grown in HI medium for 24 h at 42°C in microaerophilic conditions and then diluted to an OD_{550} of 0.05 in T25 flasks containing 5 ml of HI medium or HI medium plus 0.1% DOC. Cultures were shaken (160 rpm) under oxygen-limited conditions (0.3% O_2, 10% CO_2, 79.7 %N_2, 10% H_2) at 42°C for 24 h. Bacteria were collected by centrifugation (10 min, 3,000×g) and resuspended in DPBS to a final OD_{550} of 0.1. Bacterial suspensions were incubated with or without 80 nM H_2O_2 for 30 min at 42°C under microaerobic conditions. Serial dilutions were performed and plated onto saponin agar plates. Plates were incubated at 42°C under microaerophilic conditions and colonies were counted after 24 h.

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Supplementary materials

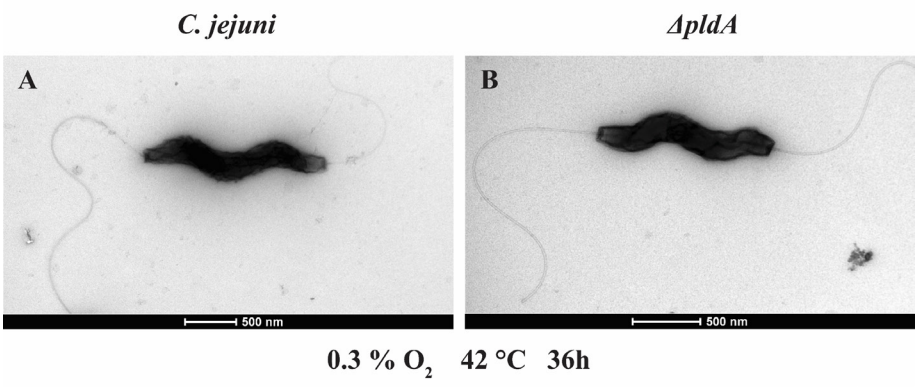

Figure S1. Electron microscopy images of (A) *C. jejuni* wildtype and (B) *C. jejuni ΔpldA* grown in HI plus 0.1% DOC. Bacterial morphology was visualized by transmission electron microscopy after 36 h incubation under oxygen limited (0.3% O_2, 10% CO_2, 79.7% N_2, 10% H_2) conditions at 42 °C. Scale bars are 500 nm.

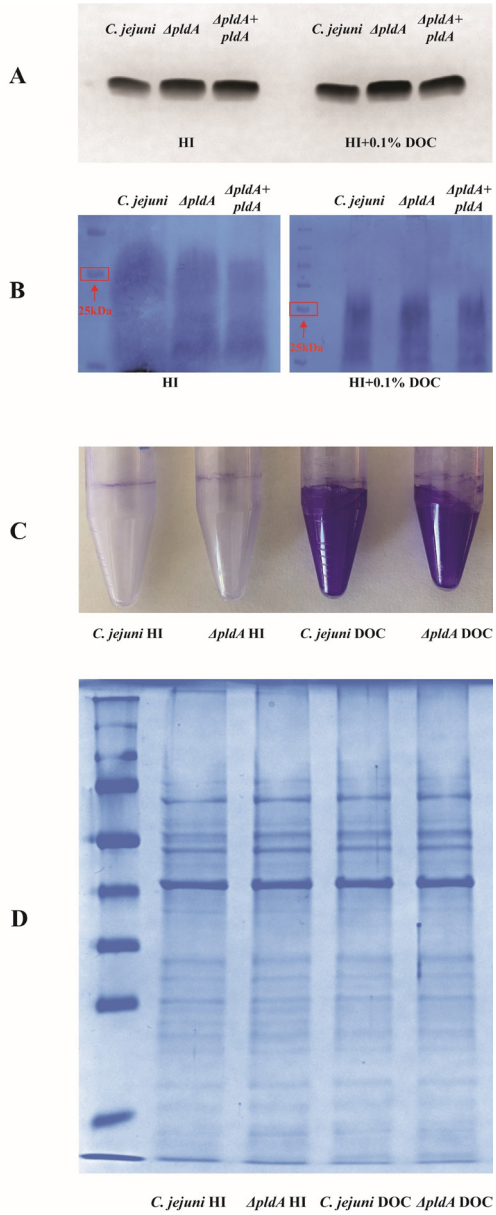

Figure S2. LOS, capsule and biofilm formation of the wildtype *C. jejuni* and *pldA* mutant grown under oxygen limited (0.3% O_2, 10% CO_2, 79.7% N_2, 10% H_2) conditions at 42 °C. **(A)** LOS were separated after 36 h of growth by SDS-PAGE and stained by silver nitrate**,** **(B)** Capsule was separated also after 36 h of growth by SDS-PAGE and stained with Alcian blue **(C)** Biofilm formation was measured after 24 h of growth in HI or HI plus 0.1% DOC in glass tubes stained with crystal violet. (D) Bacteria protein composition was determined after 36 h of growth by SDS-PAGE and staining with Coomassie brilliant blue.

Supplementary Table. S1A. List of RNA-seq and real-time PCR results of genes of *C. jejuni* 81116 regulated by DOC.

Gene	Common name	RNA-seq change (fold)	RT change (fold)
C8J_0269	*C8J_0269*	-12.08	-9.83
C8J_0325	*trpB*	4.75	4.92
C8J_0341	*cmeC*	4.08	7.13
C8J_1276	*C8J_1276*	-4.52	-2.97
C8J_1303	*katA*	2.10	3.14
C8J_1457	*C8J_1457*	-10.31	-4.68
C8J_1563	*C8J_1563*	5.96	7.80
C8J_1592	*secY*	4.34	9.13
C8J_1593	*rplO*	4.11	3.24
C8J_1613	*C8J_1613*	5.00	1.96
C8J_1622	*leuD*	4.08	1.98
C8J_1623	*leuC*	4.63	1.52
C8J_1624	*leuB*	4.00	12.45

Supplementary Table. S1B. List of used primers.

Primer	5'-3'
C8J_0269-F	AATTTGCCATACTCACACCCAAT
C8J_0269-R	GAGGCGGGATAGTTGCTCCTA
C8J_0325-F	CAAGGTTTTACAAGATGATTTAGGCAAT
C8J_0325-R	GCAAAGGACCAATTCCTGGAT
C8J_0341-F	TTAAAGCGCTCTTCCATTGCAAA
C8J_0341-R	TTGTAACAGCTGTACCTTTTGCG
C8J_1276-F	GGCAAATGACAATCCACACAAG
C8J_1276-R	GGCAGGACCACACTCTCAAAA
C8J_1303-F	CTCCTCTTTTCCTACGCTTTTCTACT
C8J_1303-R	TCTCACATCGCGTTCAGCAT
C8J_1457-F	CAGGATTTTTATATGTCAAGCGTAAGG
C8J_1457-R	TTCAAAAAGGTCAGAATGCAGTTT
C8J_1563-F	AGGGCTTTACTCAAGTGCAGAAGTA
C8J_1563-R	AGCCTACATAAGCTGAGCAATACCA
C8J_1592-F	ATTTGTTCGCCAAGCCACATTAA
C8J_1592-R	TTGGTTCAAAGTATAGGCGTTGC
C8J_1593-F	TTTCTAGCAGTTTGACCTTTACCCC
C8J_1593-R	ATTTAACAAAAGCAGCGGGTTCAAC
C8J_1613-F	ATTTTGCATGGCTTCGATGACTT
C8J_1613-R	TTGGCTTAGCGTAGGTAGATTGG
C8J_1622-F	GGTAACGATGTAGCAAGCAACTT
C8J_1622-R	AATGCATCTTTTGGGCGACTTTT
C8J_1623-F	ACAAGCACAAGAACTTTATGTGT
C8J_1623-R	GCATTTTGTTTGACAGCTAAACGG
C8J_1624-F	CCATAATCAACCAAAGCCCAAGG
C8J_1624-R	TGGCAAGCATTTGTTCCATGATT

5

Campylobacter jejuni permeabilizes the host cell membrane by short chain lysophosphatidylethanolamines

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Abstract

Lysophospholipids (LPLs) are crucial for regulating epithelial integrity and homeostasis in eukaryotes, however the effects of LPLs produced by bacteria on host cells is largely unknown. The membrane of the human bacterial pathogen *Campylobacter jejuni* is rich in LPLs. Although *C. jejuni* possesses several virulence factors, it lacks traditional virulence factors like type III secretion systems, present in most enteropathogens. Here we provide evidence that membrane lipids lysophosphatidyl-ethanolamines (lysoPEs) of *C. jejuni* are able to lyse erythrocytes and are toxic for HeLa and Caco-2 cells. Lactate dehydrogenase (LDH) release assays and confocal microscopy revealed that lysoPE permeabilizes the cells. LysoPE toxicity was partially rescued by oxidative stress inhibitors, indicating that intracellular reactive oxygen species may contribute to the cell damage. Our results show that especially the short-chain lysoPEs (C:14) which is abundantly present in the *C. jejuni* membrane may be considered as a novel virulence factor.

Introduction

Lysophospholipids (LPLs) are bioactive signaling molecules containing a single fatty acid tail. In eukaryotic cells, LPLs exhibit diverse biological properties such as promoting cell growth, acting as potent lipid mediators or reducing bacterial infections [1,2]. LPLs are generated as metabolic intermediates in phospholipid synthesis or during membrane degradation [3]. The formation of LPLs from phospholipids is due to activation of phospholipase A1 or A2. Phospholipase A1 ($PldA_1$) and phospholipase A2 ($PldA_2$), hydrolyzing the stereospecific numbering (*Sn*)-1 and -2 acyl chain, respectively [4]. (*Sn*)-1 LPLs possess more shorter, saturated acyl chains than (*Sn*)-2 LPLs while (*Sn*)-2 LPLs possess more unsaturated acyl chains [5]. (*Sn*)-1 LPLs and (*Sn*)-2 LPLs might have different biological functions as only (*Sn*)-1 LPLs can act as mediators of antimicrobial activity towards Gram-positive bacteria [6]. Lysophosphatidic acid (lysoPA) is important in controlling and signaling cancer [7]; lysophosphatidylcholine (lysoPC) evokes cellular injury by oxidative events that involve formation of low-density lipoprotein. Both lysoPA and lysoPC of the host trigger the release of the proinflammatory flagellin from *Salmonella* thereby enhancing the innate and inflammatory responses towards this bacterium [8]. The role of other LPLs like lysophosphatidylethanolamine (lysoPE) has not been elucidated to such a high degree.

Bacteria usually contain small amounts (<1%) of LPLs in their membrane [3], mostly found in the form of lysoPE [9]. It has been mentioned that lysoPEs isolated from *Bacteroidetes Chitinophaga* spp. have antimicrobial activities against certain Gram-positive bacteria [10]. LysoPA and its precursor lysoPC derived from *Lactobacillus plantarum* has been considered being toxic for humans and could disturb the signaling networks in host cells [11]. The biological function of LPLs in bacteria is still poorly understood, but they may play a role in bacterial survival or invasion [2]. LPLs may be an underestimated factor in bacterial pathogenesis and inflammation response of the host.

We previously showed that the bacterial pathogen *Campylobacter jejuni* possesses a wide spectrum of LPLs that varies dependent on the environmental conditions [12]. *C. jejuni* is the leading cause of bacterial foodborne human gastroenteritis in developed countries [13]. Symptomatic infection typically involves intestinal inflammation, fever, and bloody diarrhea [13]. *C. jejuni* is supposed to penetrate the intestinal mucus layer, colonize the crypts, and disrupt the epithelial barrier [14]. Although *C. jejuni* possesses a number of virulence factors such as flagella, proteases, adhesins, type VI secretion system and cytolethal distending toxin, it lacks traditional virulence factors like type III secretion systems [15], present in most enteropathogens and therefor the molecular basis of *C. jejuni* infection is still poorly understood [16].

In the present study we investigated the biological role(s) of the LPLs of *C. jejuni* as potential virulence determinant. We demonstrate that *C. jejuni* PldA generates

both (*Sn*)-1 and (*Sn*)-2 LPLs. The generated short chain fatty acids lysoPE was found to exert hemolytic activity and effectively damage different types of eukaryotic cells, indicating that it may act as a virulence factor.

Results

C. jejuni PldA produces (*Sn*)-1 as well as (*Sn*)-2 LPLs

To ensure that *C. jejuni* produced lysolipids under the conditions employed, we extracted LPLs from 16 h old cultures of *C. jejuni* wildtype 81116 and its isogenic *ΔpldA* mutant strain growth under microaerophilic conditions at 42°C. Liquid chromatography tandem mass spectrometry (LC-MS/MS) of extracted lipids clearly demonstrated the presence of both (*Sn*)-1 and -2 acyl chain LPLs in wildtype *C. jejuni*. For example, the majority of lysoPG 18:0 was present as (*Sn*)-1 lysoPG (0/18:0), while (*Sn*)-2 lysoPG (18:0/0) was roughly 4 times less detected (Fig. 1A). Both lysoPG species were virtually absent in a mutant strain lacking a functional PldA (Fig. 1B). Similar results were observed for other LPLs. This indicates that PldA is the primary enzyme involved in LPL formation in *C. jejuni* and that this enzyme is able to cleave both (*Sn*)-1 and -2 acyl chains but prefers the (*Sn*)-1 site.

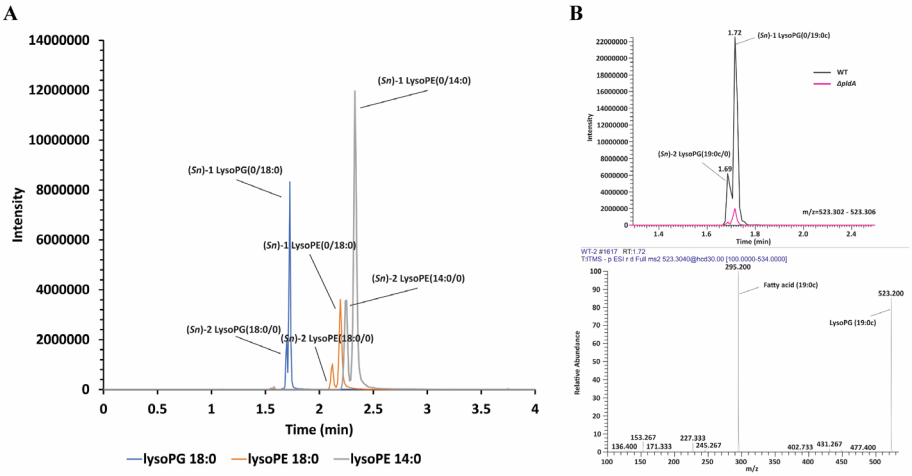

Fig 1. Phospholipid cleavage by *C. jejuni* PldA. LC-MS spectra of (A) the main LPLs in wildtype *C. jejuni* strain 81116. (B) lysoPG (19:0c) in *C. jejuni* wildtype and *C. jejuni ΔpldA*.

C. jejuni LPLs cause erythrocytes to lyse

To test the effect of bacterial LPLs on eukaryotic cells, we first determined the hemolytic activity of wildtype *C. jejuni* and the *ΔpldA* mutant. Wildtype *C. jejuni* caused strong hemolysis of horse erythrocytes in contrast to *C. jejuni ΔpldA*. Complementation of the mutant (*C. jejuni ΔpldA +pldA*) restored the strong hemolytic activity (Fig. 2A). No hemolysis was observed by using *C. jejuni* cell-free

culture supernatant (Fig. 2B) nor when the pellet fraction of ultracentrifugated cell free culture supernatant was used, suggesting that the hemolysis required bacteria-host cell contact. In other bacterial species phospholipase A itself has been shown to induce hemolysis. These enzymes generally prefer phosphatidylcholine (PC) as substrates [17]. *C. jejuni*-mediated hemolysis was observed for both PC-rich (horse, chicken and human) and PC-deficient (sheep) cells indicating that the hemolysis was PC independent (Fig. 2A). Furthermore, the *C. jejuni*-induced hemolysis was maintained after heating of the bacteria (75°C, 30 min) (Fig. 2B) , indicating that the activity was insensitive to denaturation. Hemolysis was also still present when horse erythrocytes were incubated with isolated membranes of *C. jejuni*, even after proteinase K treatment followed by heat inactivation (Fig. 2C). These results together strongly suggest that *C. jejuni* LPLs are causing hemolysis.

Short lysoPEs are responsible for hemolysis

In order to determine the phospholipid species responsible for the red blood cell lysis, we separated the major phospholipid classes PG, PE, lysoPG and lysoPE from *C. jejuni* wildtype. The purity of the phospholipid classes samples are shown figure S1. Minimal lysis was observed when PG, PE or lysoPG were incubated with horse erythrocytes, whereas the lysoPE fraction lysed more than 90% of the cells (Fig. 2D). This indicates that *C. jejuni* lysoPE is the primary cause for hemolysis. Next we investigated the effect of the length of the fatty acid tail using commercial lysoPE species. All lysoPE species induced hemolysis but the shortest fatty acid tail containing lysoPE were most effective (Fig. 2E). Of note, lysoPE14 and lysoPE16 make up 50% of the *C. jejuni* lysoPE molecules [12]. Together, these results indicate that short lysoPE species disrupt the integrity of the cell membrane of horse erythrocytes.

Short lysoPEs are also toxic for epithelial cells

During the natural infection, *C. jejuni* is in close contact with mucosal epithelial cells. To determine whether lysoPE may also damage epithelial cells we measured the LDH release. Wildtype *C. jejuni* caused considerable LDH release from the human HeLa and Caco-2 cells after 5 h of incubation. This effect was much less for *ΔpldA* mutant, while the complemented *C. jejuni ΔpldA +pldA* mutant regained the harmful wildtype behavior (Fig. 3A). Strong LDH release was also observed after exposure to purified *C. jejuni*-derived lysoPE (Fig. 3B). In agreement with the hemolysis, the short fatty acid tail containing lysoPE 14:0 caused the highest LDH release (Fig. 3C). Together, the results indicate that short chain lysoPE as present in *C. jejuni* not only displays hemolytic activity but also causes damage to epithelial cells.

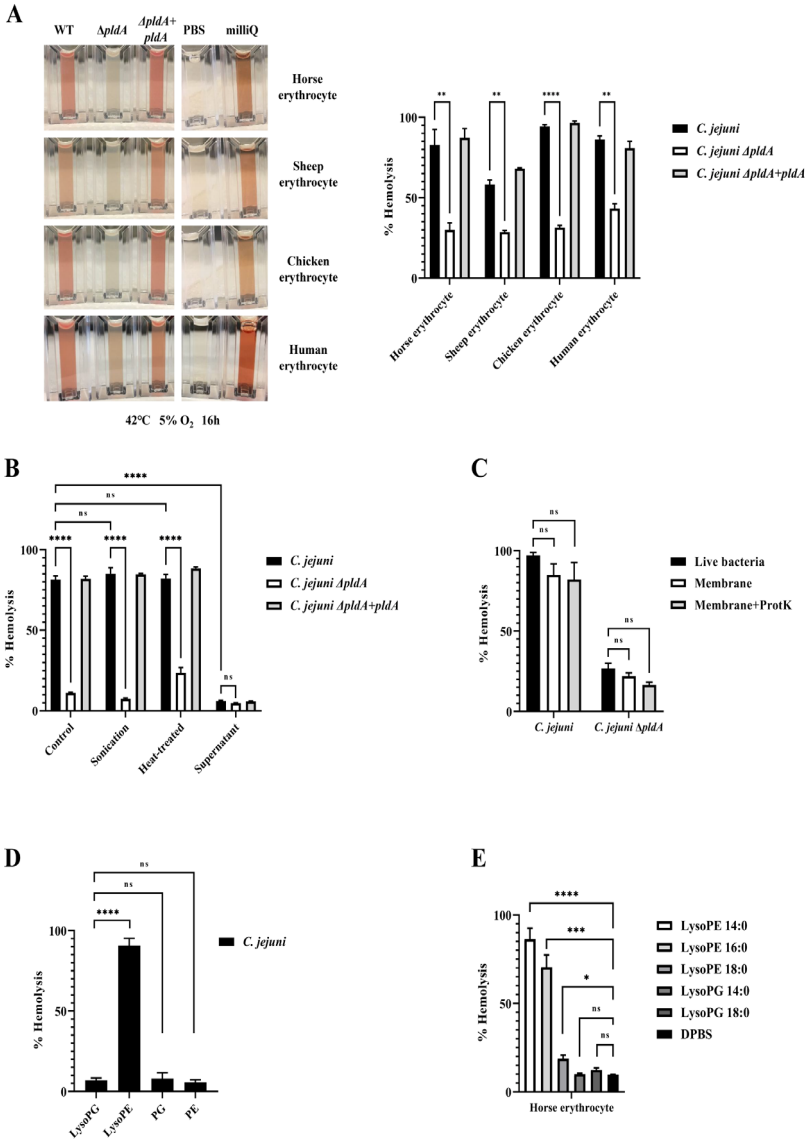

Fig 2. *C. jejuni* lysoPE induced hemolysis. (A) *C. jejuni* strains were incubated with erythrocytes from different species; the hemolysis results depicted in the left panel were quantified by measuring absorbance at 420 nm. (B-E) Hemolysis of horse erythrocytes after incubation with: (B) live, heat-treated or sonicated *C. jejuni*, or with the cell-free supernatant of *C. jejuni*; (C) live bacteria, whole membrane or protease K-treated membranes of *C. jejuni*; (D) purified major phospholipid classes; (E) commercially available LPLs. MilliQ water and DPBS were used as positive (100% value) and negative (0% value) control in the hemolysis assay, respectively. Data of three independent experiments with three independent preparations of bacterial samples are presented as mean values ± standard deviation, *P < 0.1, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns P > 0.1.

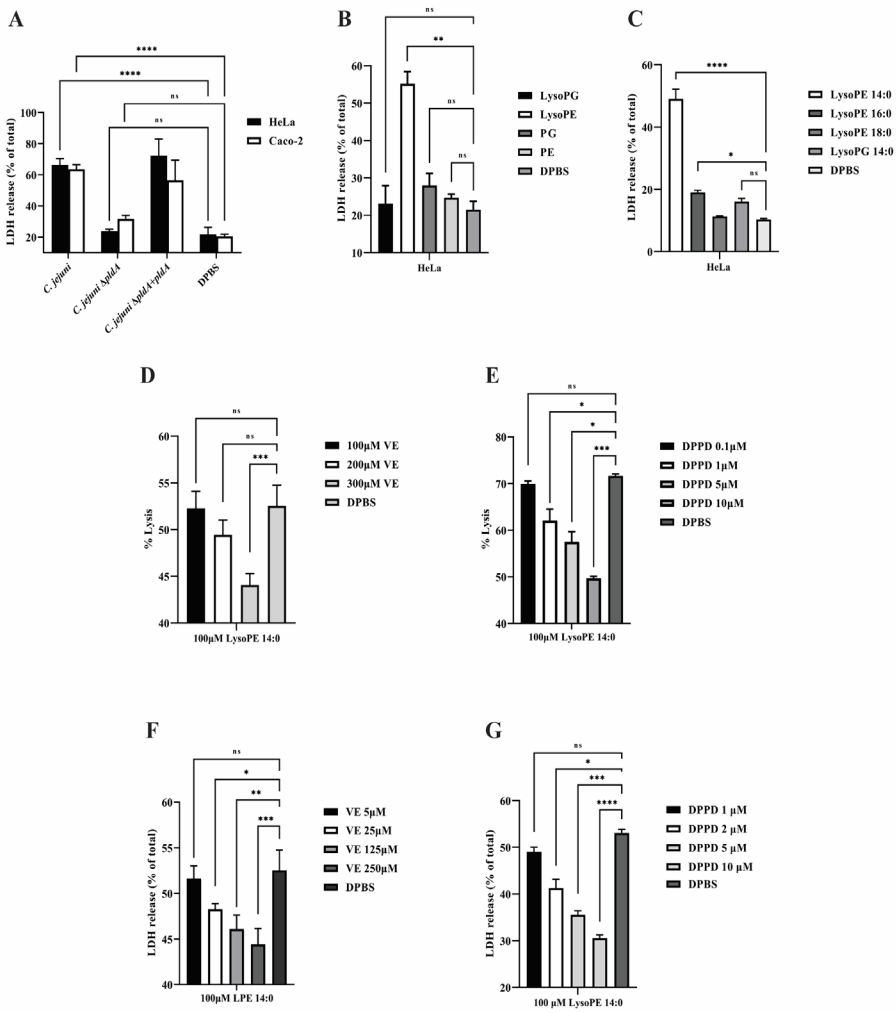

Fig 3. *C. jejuni* lysoPE-induced toxicity for host cells rescued by vitamin E and DPPD. (A) LDH release of HeLa and Caco-2 cells treated with *C. jejuni* strains. (B) HeLa cells treated with purified phospholipid fractions. (C) HeLa cells treated with commercially available LPLs. (D-E) Horse erythrocytes and (F-G) HeLa cells were treated without or with vitamin E or DPPD, washed and then exposed to lysoPE 14:0. Data are from three independent experiments with three independent preparations of bacterial samples and presented as mean values ± standard deviation, *P < 0.1, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns P > 0.1.

LysoPE induces cell damage by oxidative stress

To investigate whether the lysoPE-induced cell damage is due to oxidative stress as seen for lysoPC [18], the effect of two antioxidants, vitamin E and DPPD, was examined. Horse erythrocytes were pre-exposed to antioxidant, and then incubated with lysoPE 14:0. For both antioxidants a clear concentration dependent inhibition of

lysoPE-induced hemolysis was observed (Fig. 3D & E). Experiments with epithelial cells yielded similar results with significantly less damage after pre-treatment of cells with vitamin E and DPPD (Fig. 3F & G). To corroborate these findings we applied confocal microscopy. In the absence of LPLs, epithelial cell membranes were impermeable to the green fluorescent lectin WGA (Fig. 4A & S2A). However, after incubation with lysoPE 14:0 also nuclear membranes became WGA-positive (red arrows in Fig. 4B & S2B), but not for lysoPG 14:0 treatment (Fig. 4C & S2C), indicating that lysoPE enabled the lectin to pass the plasma membrane and enter the cells. Pretreatment of the cells with antioxidant prior to lysoPE 14:0 treatment prevented nuclear membrane staining (Fig. 4D & S2D) consistent with the LDH release results. Together, these results point to oxidative stress as a major factor in the short chain lysoPE-induced cell damage.

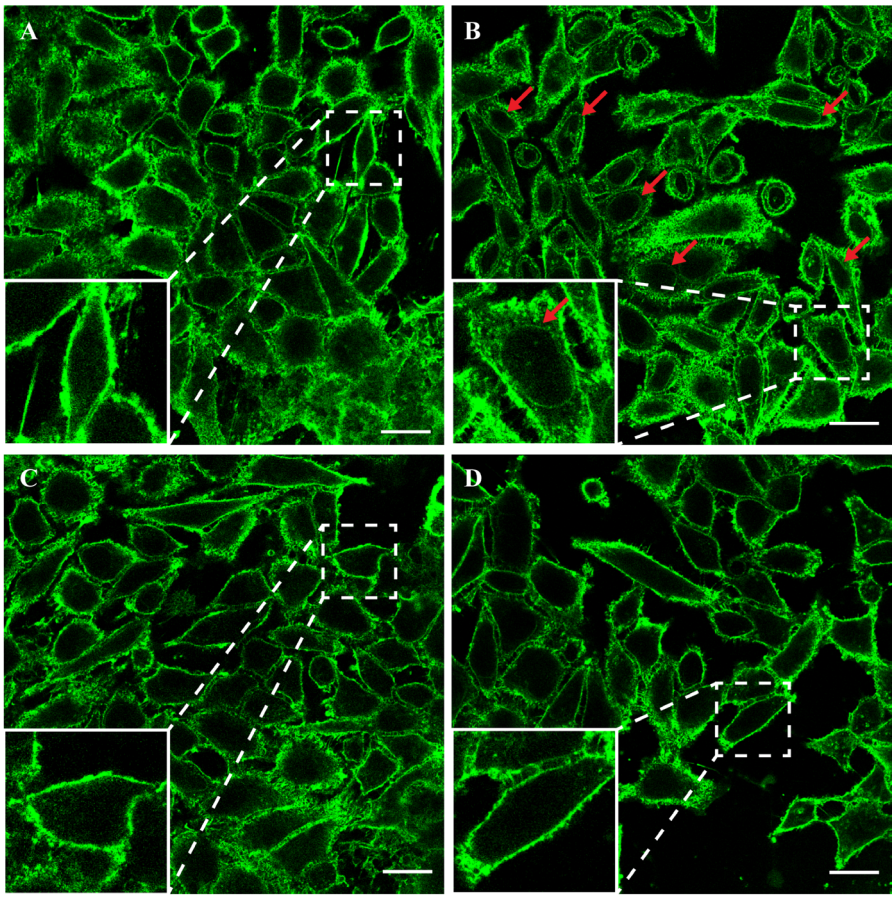

Fig 4. LysoPE permeabilizes the epithelial cells membrane. HeLa cells incubated with (A) DPBS, (B) lysoPE 14:0, (C) lysoPG 14:0, or (D) pre-treated with vitamin E, washed and incubated with lysoPE 14:0 were stained with the membrane stain fluorescent WGA (green) and/or nuclear DAPI stain (blue) and visualized by confocal microscopy. Red arrows point to nuclear membrane stained with WGA. White scale bars represent 5 μm.

Discussion

In eukaryotic cells, LPLs play an essential role in a broad variety of biological processes [1]. Recently, the human gut microbiota has been shown to contribute to the production of lysoPC, which causes damage of the epithelial barrier [19]. Some bacterial pathogens including *C. jejuni* can produce large amounts of LPLs but their effect on host cell biology is largely unknown. Here we show that the PldA of *C. jejuni* possesses phospholipase activity that generates (*Sn*)-1 and (*Sn*)-2 LPLs which is more typical for phospholipase class B proteins [20]. We also for the first time provide evidence that the produced lysoPE phospholipids can lyse erythrocytes and damage epithelial cells. This effect is especially evident for short chain lysoPE species and can be rescued by oxidative stress inhibitors. These results indicate that *C. jejuni* lysoPE may be an important unforeseen bacterial virulence factor that causes cell damage (at least partially) via an oxidative stress-sensitive mechanism.

The finding that *C. jejuni* PldA generates both (*Sn*)-1 and (*Sn*)-2 LPLs was unexpected as the amino acid sequence characteristics suggest that the enzyme belongs to the phospholipid class A family of proteins [21]. The observed virtual absence of LPLs in *C. jejuni ΔpldA* indicates that no other phospholipases are active. However, our LC-MS/MS results clearly indicate that the *C. jejuni* PldA enzyme prefers to cleave at the (*Sn*)-1 site. The position of (*Sn*) cleavage is relevant as $PldA_1$ generates mostly saturated LPL, while $PldA_2$ generates mostly unsaturated or cyclo phospholipids [5], with different biological effects on membrane function [22]. The finding that the *C. jejuni* PldA enzyme prefers to cleave at the (*Sn*)-1 site implies the formation of a large amount of membrane integrity reducing LPLs [22].

The first evidence of a cytotoxic effect of *C. jejuni* LPLs was the observed hemolysis caused by *C. jejuni* wildtype but not *C. jejuni ΔpldA*. Complementation of the *pldA* defect confirmed the crucial role of LPL formation in the toxicity. *C. jejuni*-induced hemolytic activity has previously been reported for both type VI secretion system-positive and negative *C. jejuni* strains, but the causing factor is still unclear [23, 24]. It has been speculated that the hemolysis was due to an intracellular component released after cell death or lysis [25], or by the PldA directly targeting host cell membranes. Here we provide evidence that the PldA products, the LPLs, exert strong hemolytic activity.

Fractionation of the major phospholipid classes of *C. jejuni* identified lysoPE as prime hemolysis inducing factor (Fig. 2D). So far only lysoPA and lysoPC have been reported to affect erythrocytes [26]. We found that besides the head group, the length of the tail of the lysoPE is also important for hemolysis as especially short lysoPEs were toxic (Fig. 2E). This resembles observations with lysoPC where increasing the chain length of the hydrophobic tail decreases the rate of the hemolytic reaction [27]. According to our previous results the phospholipidome of *C. jejuni* can consists of more than 33% lysoPE of which almost 50% is present as lysoPE 14 and 16 [12]. This

likely explains why the membranes of live or dead *C. jejuni* bacteria are toxic for erythrocytes.

Interestingly, the cytotoxicity of *C. jejuni* membranes and purified lysoPE was also observed for epithelial cells as evident from the strong PldA dependent increase of LDH release and the staining of intracellular membranes with WGA in lysoPE-treated cells only (Fig. 4B & S2B). Maximum LDH release and intracellular staining were observed after exposure to short chain fatty acid containing lysoPE (Fig. 3C). In humans short chain fatty acids have been identified as signaling molecules between the gut microbiota and the host, and are regarded as toxic at high concentration [28]. *C. jejuni* has been shown to induce LDH release in human neutrophils and dendritic cells (less than 10%), but in epithelial cells the LDH release is relatively low [15, 29]. We were able to strongly increase the LDH release from the epithelial cells by replacing the tissue culture medium with DPBS during the incubation with *C. jejuni* (Fig. S3). We noticed that calcium excess in the culture medium reduces the *C. jejuni* cytotoxicity as has been noted for *C. coli* PldA [30].

What is causing lysoPE-induced cell damage? It has been shown that the incorporation of even a small amount (1 mol.%) of fatty acids or lysolipids in lipid membranes creates instabilities in the lipid bilayer [31]. One theory for LPL induced cell damage is that LPLs such as lysoPC can evoke an oxidant stress-dependent transient membrane permeabilization in cells [32]. Our results support this hypothesis as two antioxidants, vitamin E and DPPD protected the cells from the LPLs damage. Both inhibitors reduced the lysoPE 14:0 induced cytotoxicity and inhibited the intracellular membrane staining (Fig. 4D). The mechanism of toxicity of lysoPE 14:0 may thus resemble the effect of as lysoPC leading to a stress-dependent transient membrane permeabilization [32].

In conclusion, we for the first time identified *C. jejuni* lysolipids, especially lysoPE, as cytotoxic factor. The toxic short-tailed lysoPE induces hemolysis and induces oxidant stress-dependent membrane leakage in epithelial cells. Bacterial lysoPE can thus be considered as a novel virulence factor of *C. jejuni* and possibly other bacterial pathogens that generate large amounts of toxic lysoPE.

Materials and Methods

Bacteria and mammalian cell culture

C. jejuni wildtype strain 81116, originally isolated from a human waterborne outbreak [33], its isogenic *pldA* mutant (*C. jejuni ΔpldA*), and the complemented *pldA* mutant (*C. jejuni ΔpldA +pldA*) [12] were routinely grown on saponin agar as described [12]. HeLa cells [34] and Caco-2 cells (ATCC-HTB-37) were grown in 25 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) at 37˚C and 10% CO_2.

Membrane isolation, lipid LC-MS/MS analysis and extraction

C. jejuni membranes were collected by *N*-lauroylsarcosine assay [35], using sonication and Tris (PH 8.0) buffer instead of French pressure cell press and HEPES buffer, respectively. Lipid extraction and analysis were done as described before [12]. For more information on membrane isolation and lipid extraction and analysis see supplementary materials (Supplementary Material 1). Commercial (lyso) phospholipids, lysoPE 14:0, lysoPE 16:0, lysoPE 18:0, lysoPG 14:0, lysoPG 18:0 and PE 16:0 were purchased from Avanti Polar Lipids Inc. (Alabama, USA) to investigate the effect of the length of LPL fatty acid tail on biological functions.

Hemolysis and cytotoxicity assays

Hemolysis and cytotoxicity were determined as described [24] using heat-treated (75°C, 30 min) bacteria [36], sonicated (3×60 s) bacteria, isolated membranes, 10 µmol *C. jejuni* purified LPLs or 50 µmol commercial LPLs. Hemolysis was expressed as percentage of cell lysis (absorbance OD_{420}) compared to the positive control (cells lysed with milliQ water). Host cell cytotoxicity was determined by measurement of the lactate dehydrogenase (LDH) release from 10^6 tissue culture cells at 5 h after addition of *C. jejuni* at a bacteria to host cell ratio of 100:1, or of the indicated amount of LPL. When appropriate, host cells were pre-treated (16 h) with one of the antioxidants, vitamin E and *N,N'*-diphenyl-1,4-phenylenediamine (DPPD) (Sigma-Aldrich) [32]. For more detailed information on hemolysis and cytotoxicity assays see Supplementary Material 1. Data are expressed as the mean ±SEM of at least three independent experiments. Statistical significance was determined using two way ANOVA analysis with Geisser-Greenhouse correction using Prism software (GraphPad, San Diego, CA).

Confocal microscopy

Confocal microscopy [37] was performed on cells (10^6) incubated (5 h) with commercial lysoPE 14:0 or lysoPG 14:0 to visualize the lysoPE induced cell damage. When appropriate antioxidants were added 16 h before LPL treatment and washed away before lysoPE exposure. Cells were fixed and membranes were stained with plasma membrane counterstain Wheat Germ Agglutinin (WGA) Alexa Fluor™ 488 Conjugate (W11261, Invitrogen). Nucleic acids were stained with DAPI (D21490, Invitrogen) without permeabilization. Images were collected on a Leica SPE-II confocal microscope.

Conflict of interest

The authors have declared no conflict of interest.

Funding

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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Supplemental materials

Membrane isolation

C. jejuni membranes were collected by *N*-lauroylsarcosine assay [1]. In short, bacteria were grown in 100 ml HI medium for 16 h under microaerophilic conditions at 42°C. Bacterial pellets were collected by centrifugation (3,000×g, 10 min) and resuspended in 1 ml of 20 mM Tris-HCl, 5 mM EDTA, 20% sucrose, 0.15 mg lysozyme, pH 8.0 and incubated on ice for 40 min. Next, 40 µl of 0.5 M $MgCl_2$ was added to the sample, which was then centrifugated (9,600×g, 20 min) to collect the supernatant containing the periplasm fraction. The remaining pellet was resuspended in 2 ml of ice cold 10 mM Tris-HCl, pH 8.0. Next, the cells were lysed by sonication for 3×60 second on ice. After centrifugation (6,200×g, 10 min) the membrane containing supernatant was collected. Finally, bacterial whole membranes were collected by ultracentrifugation at 100,000 g for 1 h at 4°C. The pellet was washed with 10 mM Tris-HCl, pH 8.0 and resuspended in 1 ml DPBS.

Lipid LC-MS/MS analysis and extraction

Lipid extraction and analysis were done as described before [2]. In brief, bacteria were grown in 5 ml HI medium for 16 h under microaerophilic conditions at 42°C. The pellets were washed once with Dulbecco's Phosphate Buffered Saline (DPBS) and resuspended in 0.8 ml DPBS and mixed with 3 ml chloroform/methanol (1:1 v/v). Supernatants were collected by centrifugation for 10 min at 4,600 rpm and mixed with 3 ml Milli-Q water. Phospholipids were collected and separated using a HILIC column (Kinetex, 2.6 µm) to resolve different phospholipid classes.

Hemolysis assays

Campylobacter cultures (5ml) were shaken (160 rpm) in T25 cell culture tested flasks (10790113, Corning) for 16 h at 37°C under microaerophilic conditions. The cultures were pelleted and resuspended in DPBS to OD_{550} of 1. Depending on the experimental design, resuspended bacteria were untreated, heat-treated for 30 min at 75°C, or sonicated for 3×60 second (VC-40, Sonics & Materials Inc, Newtown, USA). The bacterial culture, bacterial membranes or commercial (lyso) phospholipids (1 ml) were mixed with 0.25 ml of a 5% (v/v) horse, sheep, human or chicken erythrocyte suspension (Biotrading) in DPBS in a 1.5 ml Eppendorf tube. When live bacteria were used the caps of the Eppendorf tubes were perforated to allow gas exchange. After 16 h of incubation at 37°C under microaerophilic conditions, the bacteria and cells were resuspended and centrifuged (1,000×g, 5 min). The OD_{420} of the supernatants was measured as indicator of the degree of hemolysis. Negative (with 1 ml DPBS) and positive (with added 1 ml milliQ water) controls were included in all assays. Hemolysis was scored as percentage of cell lysis of the positive control. Data are expressed as the mean ±SEM of at least three independent experiments.

Data were analyzed using Graphpad Prism software.

Cytotoxicity assays

The extent of membrane permeabilization was determined by measurement of the LDH release from the cells. Cytotoxicity toward tissue culture cells were determined using HeLa- and Caco-2 cells grown for 24 h in a 96-well plate in DMEM+10% FCS at 37°C in 10% CO_2 atmosphere. The cells were washed with DPBS and the medium was replaced by 50 µl of DMEM (without FCS) or DPBS. *C. jejuni* strains were grown 16 h in HI broth, collected by centrifugation (3,000×g, 10 min), resuspended in DPBS, and added to the host cells at a bacteria to host cell ratio of 100:1. Ten µmol of lysoPE extracted from *C. jejuni* or 50 µmol of commercial lysoPE 14:0, lysoPE 16:0, lysoPE 18:0 or lysoPG 14:0 was added to 10^6 tissue culture cells and kept for 5 h at 37°C in 10% CO_2 before total cellular and secreted LDH from the cells were determined with the Cytotoxicity Detection Kit^{PLUS} (LDH) according to the manufacturer's protocol. Antioxidants vitamin E and *N*,*N*'-diphenyl-1,4-phenylenediamine (DPPD) purchased from Sigma-Aldrich (St. Louis, MO) were used to investigate whether they could inhibit the LPLs cytotoxicity. Hereto tissue culture cells were treated with one of the antioxidants for 16 h. Prior to the addition of the LPLs, the cells were washed with DPBS to eliminate noncellular interactions between the antioxidants and LPLs. The percentage of cytotoxicity after 5 h incubation was calculated as the percentage of LDH release compared to the total LDH concentration (% of cytotoxicity= 100*(LDH released/total LDH)). Presented results are from three individual assays performed in triplicate. Data were analyzed using Graphpad Prism software.

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Supplementary figures

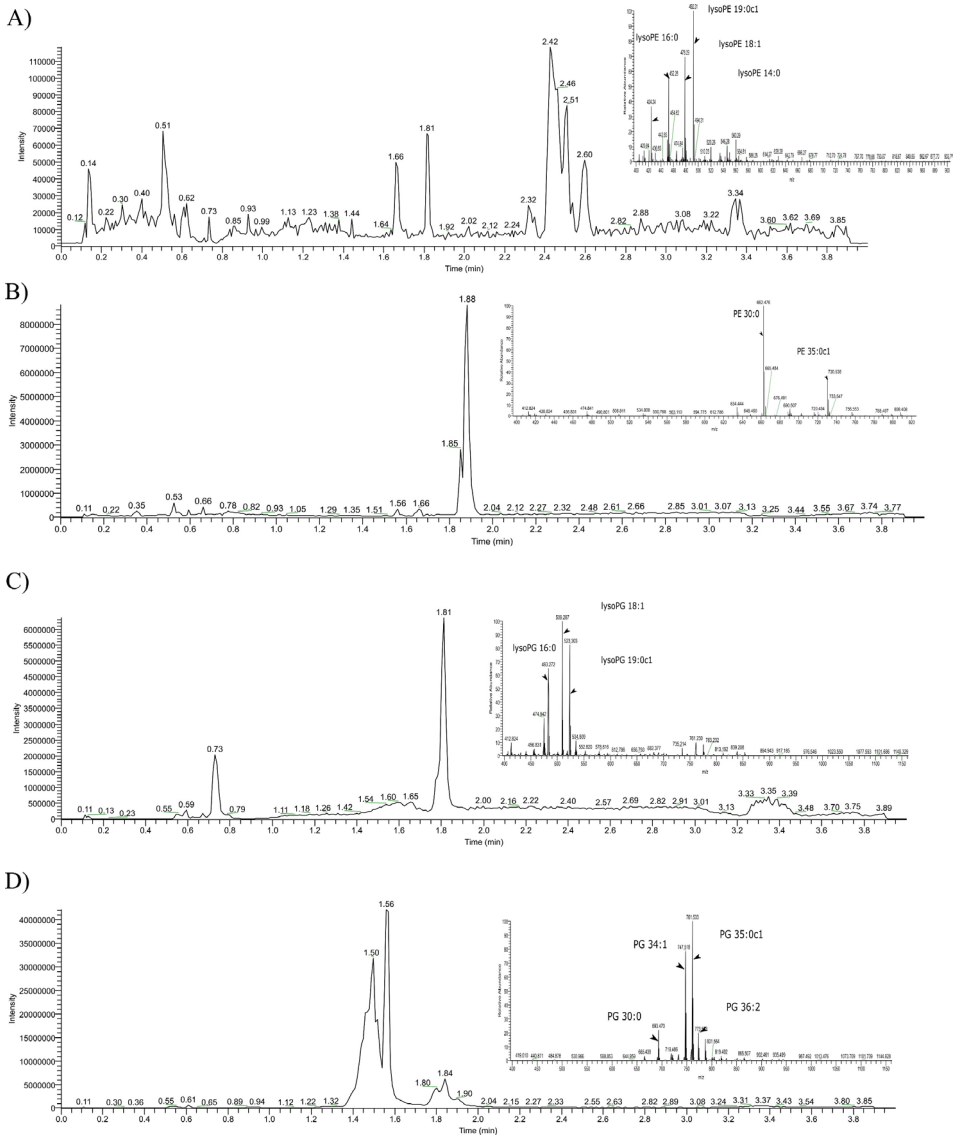

Fig S1: A base peak chromatogram with MS-2 data (inlay) of the separated major phospholipid classes from wildtype *C. jejuni* strain 81116. (A) lysoPE = lysophosphatidylethanolamine, (B) PE = phosphatidylethanolamine, (C) lysoPG = lysophosphatidylglycerol, (D) PG = phosphatidylglycerol.

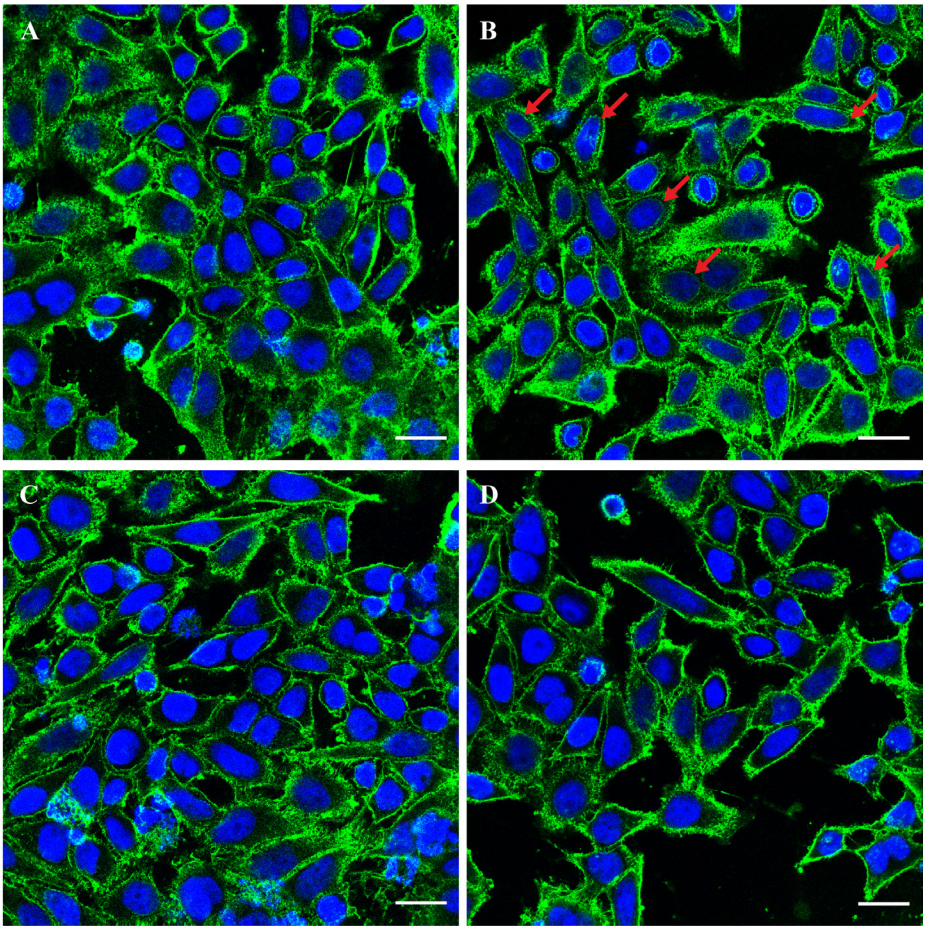

Fig S2: Cells membrane and nucleus staining. Confocal microscopy of HeLa cells (10^6) stained with WGA Alexa Fluor™ 488 Conjugate (green) to visualize the cell membrane and DAPI (blue) to stain the nuclei. HeLa cells incubated (A) with DPBS for 5 h, (B) with 500 µM lysoPE 14:0 for 5 h, (C) with 500 µM lysoPG 14:0 for 5 h, (D) pre-treated with 250 µM vitamin E for 24 h followed by incubation with 500 µM lysoPE 14:0. Red arrows point to nuclear membrane stained with WGA. White scale bars represent 5 µm.

6

General Discussion

General Discussion

All living cells are surrounded by a membrane that separates the interior of the cell from the outside environment. The cell membrane also allows selective bidirectional passage of nutrients and waste products. Cells need and can rapidly adapt their membrane properties to survive changing environmental conditions. The cytoplasmic (inner) membrane of bacteria is composed of approximately equal proportions of lipids and proteins. The lipids form a bilayer made up of phospholipid molecules. Bacterial phospholipid homeostasis provides the proper membrane fluidity and charge to stabilize interactions with membrane-associated proteins [1]. Modifications of the bacterial membrane lipidome may occur as part of the defense against certain types of environmental stress. In addition to phospholipids, bacteria often have lipids with attached glycan residues. Such glycolipids including lipopolysaccharide (LPS), lipoarabinomannan (LAM), phthiocerol dimycocerosates (PDIM) are not located in the inner membrane but often play a role in bacterial pathogenesis [2]. The most dominant type of lipids in the bacterial inner membrane are glycerophospholipids. The potential role of these lipids in the bacteria-host interaction and bacterial stress resistance is still largely unclear. The work described in this thesis was designed to determine the phospholipidome of the food-borne pathogen *Campylobacter jejuni* under different environmental conditions and to assess the role of distinct (classes of) phospholipids in bacterial pathogenesis and/or stress adaptation. This knowledge should help us to understand how this pathogen adapts to human host and subverts host cell biology which may result in novel strategies to prevent bacterial infection. In this final chapter we summarize our findings and discuss them in a broader context of interactions at the microbial-host interface and bacterial pathogenesis.

Characteristics of the *C. jejuni* phospholipidome

In **Chapter 3**, we elucidated the phospholipidome of *C. jejuni* strain 81116. Two hundred and three species of phospholipids were detected belonging to nine different lipid classes including phosphatidic acid (PA), phosphatidylserine (PS), lysophosphatidylglycerol (lysoPG), lysophosphatidylethanolamine (lysoPE), lysophosphatidic acid (lysoPA), acyl-phosphatidylglycerol (acyl-PG). One novel lipid class PX was found. The composition of the phospholipidome underwent alterations in response to changes in the environment, indicating that the lipidome composition is dynamic and subject to regulation. Especially the lysophospholipids (LPLs) lysoPE, lysoPG and lysoPA can reach an astonishing maximum of 45% of the total phospholipids under certain environmental conditions. The accumulation of LPLs appeared to be crucial for *C. jejuni* motility under the low-oxygen conditions.

The phospholipidome of C. jejuni

Prior to the work described in this thesis, the knowledge of the major membrane *C. jejuni* lipid repertoire was limited to the detection of the phospholipids PE and PG with a acyl chains C14:0, C16:0, C16:1, C18:1 and C19:0, with or without the substitution of cyclopropane [3, 4]. Gene function prediction of the phospholipid biosynthesis pathway of *C. jejuni* suggested that theoretically many more phospholipids could be synthesized. Our phospholipidome analysis, described in **Chapter 3,** led to the discovery of >200 different *C. jejuni* phospholipids but it can be imagined that even additional lipids are produced under more extreme growth conditions. Under the tested microaerophilic growth conditions, PG was the most abundant phospholipid class, followed by PE, and the previously undetected lipid classes lysoPE, the unknown lipid PX, lysoPG, PA, acyl-PG, PS and lysoPA. The conventional lipid class cardiolipin (CL) which is present in most bacteria was not detected in *C. jejuni* [4], which corresponds to the apparent lack of the enzyme CL synthase (ClsABC) in *C. jejuni*. In *Escherichia coli*, the elimination of CL resulted in decreased stress susceptibility as several stress sigma factors can be activated [5]. The natural defect of CL formation might suggest that *C. jejuni* is less stress sensitive. The length of *C. jejuni* phospholipid fatty acid tails varied from 12 to 21 carbon atoms, and saturated, unsaturated, cyclopropane structures were detected for all phospholipids species. The discovered membrane phospholipid diversity likely contributes to the survival of the bacterium under conditions of environmental stress. However more research is needed to pinpoint which changes in the lipidome repertoire are needed to deal with specific types of stress. Further analysis of the fatty acid tails showed that approximately 20% of the *C. jejuni* phospholipids were LPLs. This was unexpected as LPLs are usually present at relatively low levels (less than 1%) in most other bacterial species [6]. Although the biological function of LPLs in bacteria has hardly been studied, LPLs have been identified as important signaling molecules in eukaryotic cells [7-9]. In bacteria, the accumulation of LPLs is considered to be linked to stress responses [6, 10-12]. Up to 80% of *C. jejuni's* LPLs are lysoPEs, indicating that these molecules play a vital role in this organism.

What could be the advantage to *C. jejuni* of producing a high level of LPLs? LPLs are considered to be nonbilayer-forming lipids and their accumulation in the membrane has been shown to increase the membrane permeability and induce cell membrane curvature [13, 14]. To prevent and repair these potential membrane-destabilizing effects, most bacterial species have evolved LPLs transport systems (LplT) that rapidly translocate membrane LPLs, especially lysoPE and lysoPG, to the cytoplasmic side of the inner membrane [6]. Subsequently, the imported LPLs are reacylated by peripheral LPLs acyltransferase or acyl-acyl carrier protein synthetase (Aas) to regenerate nontoxic phospholipid [15]. In some bacterial species, the LplT and Aas proteins are physically connected as a fusion protein [16]. Interestingly, *C. jejuni*

lacks the classical LplT-Aas phospholipid repair system as no homologs of the LplT appear to be present in the genome. This may explain the high levels of LPLs in *C. jejuni*. So far it is not known whether other high LPL-containing bacteria such as *Vibrio cholerae*, *Yersinia pseudotuberculosis*, and *Helicobacter pylori* [12, 17-19], also lack an efficient LPLs transport system. *V. cholerae* has been reported to have a LPLs transport system in which the LplT and PlsC proteins are fused to a single protein but that does not contain Aas [16]. This may influence the LPLs elimination rate. In search for the biological function of *C. jejuni* LPLs in bacterial pathogenesis we discovered that lysoPEs and especially short-chain (C:14) lysoPE, may be considered as a novel virulence factor as described in **Chapter 5** (see below).

The dynamics of the C. jejuni phospholipidome

For a long time, the composition of bacterial membrane phospholipids was widely believed to be relatively constant [20]. Today, however, the ability to alter the membrane phospholipid composition is considered a crucial physiological process for bacterial survival and adaptation in response to environmental stress [5]. The detected alterations in *C. jejuni* fatty acid gene transcripts, described in **Chapter 3**, indicate that *C. jejuni* modifies its phospholipid repertoire in response to changes in the environment. One profound change was the increase in the percentage of LPLs in the stationary growth phase. While a phospholipid molecule is assumed to form a cylindrical shape, LPL is considered to have a conical shape [21]. This change in structure may facilitate the formation of the coccoid form of *C. jejuni* that is typically seen at the late stationary phase. The *C. jejuni* cell membrane also becomes more fluid in the stationary phase which might impact the function of other membrane constituents such as proteins [22, 23]. When the alterations phospholipids reach the inner layer of the bacterial outer membrane of Gram-negative bacteria, it may also influence the properties of the outer membrane [5, 24]. Phospholipid synthesis is also coupled to peptidoglycan metabolism [25, 26]. Peptidoglycan is considered to be the main structural component of the wall and crucial in maintaining the shape of bacteria. It would be of interest to investigate the scenario that the increased LPL formation results in altered peptidoglycan synthesis which may contribute to the transition from a spiral shape to coccoid *C. jejuni* morphology [27, 28]. In *E. coli*, the composition and distribution of membrane lipids, especially PE have been shown to be important for the size and shape of bacteria [5, 29]. It was observed that spiral shaped *C. jejuni* grown at 10% O_2 rapidly change to a coccoid shape after entering the stationary growth phase, whereas most bacteria kept their spiral shape up to 108 h of growth at 0.3% O_2. Although, we found that the amount of PE in *C. jejuni* was relatively constant during growth at a 10% or 0.3% oxygen concentration, the exact localization of PE as well as other (lyso)phospholipids in the outer and inner membrane is still unknown. Investigation of the (change in) localization of LPLs

under different growth conditions may teach us more about their role in peptidoglycan synthesis and bacterial morphology. One approach to explore the role of (lyso) PE or other phospholipid species in *C. jejuni* morphologis to inactivate the genes encoding the relevant enzymes. For example, elimination of the majority of (lyso) PE or (lyso)PG may be achieved by mutating the phosphatidylserine decarboxylase (Psd) or phosphatidylglycerol phosphate phosphatase (PgpP) genes, respectively. To investigate the effect of the distribution of (lyso)phospholipids in the cell envelope on *C. jejuni* morphology, the inner membrane and outer membrane or even the individual lipid layers could be separated and then subjected to phospholipidome analysis.

Transcriptional regulation of C. jejuni phospholipid biosynthesis genes

A still open question is how *C. jejuni* can alter its phospholipids in different growth environments. *C. jejuni* whole genome sequence analysis revealed 22 genes related to phospholipid biosynthesis [30, 31]. Our RNA-seq analysis revealed upregulation of the fatty acid chain elongation genes and the cyclopropane fatty acid synthase genes in the logarithmic and stationary growth phase, respectively. This suggests regulation at the level of gene transcription or mRNA stability. It is known that the expression of bacterial lipid biosynthesis genes can vary with growth rate, nutrient availability, and environmental stimuli [32], however, the correlation between bacterial physiological features and lipid pathways has not been clearly elucidated. With the construction of mutants in phospholipid biosynthesis genes of diverse bacterial species, we can specifically and comprehensively elucidate the function of phospholipid biosynthesis genes and gain knowledge of how they alter specific bacterial biological characteristics leading to the adaptation to the environment.

The role of *C. jejuni* phospholipids in adaptation to environmental stress

Environmental adaptation of C. jejuni

As a zoonotic food-borne pathogen, *C. jejuni* encounters a multitude of growth environments ranging from animals, amoebas, and the human gut. These niches differ in many environmental factors including the concentration of oxygen and other electron acceptors, nutrients, iron availability, and exposure to bile salts. Bile salts like deoxycholate (DOC) have previously been claimed to act as a detergent for *C. jejuni* as well as other enteric pathogens [33-35]. In other species, bile salts have been shown to induce the production of reactive oxygen species (ROS) which are extremely lethal for bacteria [36, 37]. As *C. jejuni* can clearly resist the exposure to bile salts in the gut environment, mechanisms should exist that counteract the harmful detergent activity of DOC. Changes in the *C. jejuni* phospholipid composition in response to bile salts may contribute to the bile salt resistance. In fact, we observed in **Chapter 4** that under the limited oxygen conditions, DOC even promoted the growth

rather than the killing *C. jejuni*. This may be related to the detected upregulation of iron and amino acids transport genes in the presence of DOC. Bile salts have been demonstrated to deprive the environment from available iron which induces upregulation of iron acquisition systems in certain bacterial species and facilitates bacterial growth of e.g. *E. coli* [38]. The upregulation of iron uptake genes might be a survival strategy for *C. jejuni* in host gut. Our *C. jejuni* transcriptome analysis also indicated that besides iron metabolism, tryptophan and branched-chain amino acids (BCAAs) catabolism is upregulated by DOC. Although *C. jejuni* cannot utilize tryptophan directly as a nutrient and the acquisition of BCAAs doesn't appear to be required for growth of *C. jejuni*, the upregulation of these two metabolic pathways might facilitate bacterial infection or colonization [39-41]. The BCAAs are considered to be vital nutrients for bacterial physiology, as they contributes to protein synthesis and are converted to a precursor for fatty acid synthesis [42, 43]. As major constituent of membrane fatty acids in some bacteria, BCAAs are believed to be crucial for regulating bacterial membrane fluidity [43, 44]. The changes on BCAAs metabolism also likely influence bacterial membrane homeostasis as they control membrane phospholipid structure [42]. Whether the altered BCAAs biosynthesis in DOC-exposed *C. jejuni* and the concomitant changes in fatty acid biosynthesis and membrane properties contribute to the bile resistance remains to be elucidated. It may also be worthwhile to investigate whether the altered membrane composition in turn alters the activities of the amino acid transporters in the membrane and in this way even reinforces the effect.

The role of membrane phospholipid hydrolysis in bile salt adaptation

To better understand the significance of the changes in membrane lipids in the resistance to bile salts, we took advantage of a generated mutant defective in the phospholipase PldA. This enzyme hydrolyzes the fatty acid tail from phospholipids, resulting in the formation of lysolipids which are a main constituent of the *C. jejuni* membrane. The PldA mutant showed significantly reduced bacterial growth in the presence of DOC under oxygen limited conditions (**Chapter 4**). This shows that the PldA activity is a key element in *C. jejuni* growth in the presence of DOC and likely an indispensable factor in the adaptation and resistance to bile salts.

The interplay between bile salts and PldA activity is not clear. It has been reported that bile salt can enhance PldA activity by the formation of a bile salt-PldA complex [45]. In eukaryotic cells, inactivation of PldA results in upregulation of ROS production [46]. It is tempting to speculate that in *C. jejuni* the PldA enzyme also plays a role in the neutralization of the (often lethal) DOC-induced ROS production observed in other bacterial species. We noted that the *C. jejuni ΔpldA* mutant showed upregulation of the oxidative resistance system, suggesting the presence of higher levels of ROS in the absence of PldA.

LPLs have previously been claimed to play a vital role in the induction of oxidative stress in eukaryotic cells [47-49]. However, we noticed that *C. jejuni* could survive very well with high amounts of LPLs in its cell membrane. This may indicate that *C. jejuni* contains defense mechanisms to detoxify ROS or inhibit ROS generation. In a recent study LPLs have been shown to reduce the formation of ROS, they found that by reducing the basal level of oxidative-related protein, NADPH oxidase 2 (Nox2), lysoPE exerts a significant protective effect against oxidative stress in the certain type eukaryotic cells [47]. We hypothesize that the PldA mediated ROS neutralization in *C. jejuni* might also be related to the presence of LPLs. To further confirm this, we can try to eliminate or reduce specific LPL species from *C. jejuni* by mutating the key enzymes which are involved in LPLs biosynthesis and then quantify the ROS production of the different mutant strains grown in media containing bile salts by using the dihydroethidium (DHE) assay [47].

PldA and motility

Besides its effect on *C. jejuni* growth and stress adaptation, we noticed that PldA activity also influences bacterial motility. Flagella-mediated movement of the bacterium is essential for bacterial colonization and infection. Our discovery may explain why a *C. jejuni ΔpldA* was found to be key for the colonization of chicken [50]. As described in **Chapter 3,** we detected the loss of motility only when the bacteria were grown under low oxygen conditions as likely exist *in vivo*. The question is how PldA i.e., the formation of lysolipids influences flagella-driven bacterial movement. In **Chapter 4**, we compared the RNA transcripts of the wildtype and *ΔpldA* mutant and noted that the motility-related gene *dsbA* is significantly downregulated in *C. jejuni ΔpldA* mutant. In addition, the expression of the *flgI* gene transcript which encodes a flagella P-ring protein, was decreased in the *ΔpldA* mutant. DsbA has previously been reported to play a crucial role in *C. jejuni* motility under microaerobic conditions [51], and FlgI has been identified as the substrate for DsbA in *E. coli* [52]. The downregulation of DsbA and FlgI may thus be key factors contributing to motility-defect in *C. jejuni ΔpldA* under the low oxygen condition. Why the motility change was observed under low oxygen conditions only remains to be investigated but may be related to the alterations in lysolipid content.

C. jejuni lysophospholipids as potential virulence factor

It is evident that phospholipids are key determinants in bacterial physiology and survival. The possible effects of bacterial phospholipids and lipid-modifying enzymes such as PldA on host cell physiology are much less clear. Especially the possible function of LPLs in bacterial pathogenesis is still largely unknown. In **Chapter 2** of this thesis, we reviewed current knowledge and the emerging awareness of the role of LPLs in bacterial adaptation, survival, invasion, and

host-microbial interactions. LPLs have thus far largely been considered as minor (<1%) lipid-derived metabolic intermediates in bacterial phospholipid biosynthesis and as negligible, nonbilayer-forming molecules with detergent-like properties [6, 53, 54]. Bacterial LPLs can be produced in three different ways: (1) by endogenous phospholipase A (PldA) which hydrolysis phospholipids [55], (2) as side-products in lipid A or lipoprotein *de novo* biosynthetic pathways (as side-products) [56, 57], and (3) by exogenous phospholipase action (host secretary phospholipase A_2). In **Chapters 3** and **5** we showed that the majority of *C. jejuni* LPLs are formed by PldA-dependent enzymatic transformation of phospholipids. Genetic inactivation of *pldA* resulted in more than 80% reduction of *C. jejuni* LPLs. The residual LPLs may be derived during lipoprotein formation. The key enzyme in lipoprotein *de novo* biosynthesis, the apolipoprotein *N*-acyltransferase Lnt, is present in *C. jejuni* in contrast to the key enzyme in lipid A *de novo* biosynthesis, the lipid A palmitoyltransferase (PagP).

As mentioned above, we discovered that PldA and thus likely the formation of LPLs is crucial for maintaining the flagella-driven motility of *C. jejuni* under the low oxygen conditions and influence the colonization of chickens [58]. To further decipher the possible role of PldA and LPLs in *C. jejuni* pathogenesis, we investigated the effect of inactivation of PldA and of purified LPLs on host cells. As described in **Chapter 5**, this approach revealed a difference in the ability of *C. jejuni* and its *ΔpldA* derivative to lyse erythrocytes. Although hemolysis has previously been attributed to hydrolysis of the host cell membranes by bacterial PldA [55, 59, 60], we unequivocally demonstrate in this thesis that *C. jejuni*-derived short chain lysoPE can lyse erythrocytes. LysoPE is the most dominant class of LPL in *C. jejuni* but is considered incompatible with the typical membrane bilayer structure because of its induction of a positive membrane curvature. LysoPE is ubiquitous in bacteria but usually present at too low levels to consider a role in bacterial pathogenesis. However, hemolysis has been reported to be induced by bacteria-derived lysoPC. An increase in the chain length of the hydrophobic tail of lysoPC decreased the rate of the hemolytic reaction [61]. Along these lines, short chain PC (10:0, 12:0, 14:0) can be spontaneously transferred into erythrocyte membranes[74]. The accumulation of PC in the erythrocyte membrane causes hemolysis at a rate dependent on the length of PC (PC 10:0>PC 12:0>PC 14:0) [62]. This trend is consistent with our findings for lysoPE-induced hemolysis. The phospholipidome of *C. jejuni* contains a high percentage (>33%), of lysoPE of which almost 50% consists of lysoPE 14 and 16 [63]. We suggest that short chain lysoPE triggers hemolysis by insertion and destabilization of the erythrocyte cell membrane. The lysoPE-induced hemolysis may be advantageous to the pathogen as a strategy of the release iron and nutrients from the red blood cells. Despite the strong evidence of lysoPE-induced lysis of red blood cells, we cannot exclude that bacterial PldA can subvert host cell physiology as well. At present, we have been unable to either purify the enzyme in an active form, or to clone

and express recombinant functional *C. jejuni* PldA, perhaps due the the absence of the appropriate chaperone. Also, the localization of the enzyme in the bacterial cell envelope is still unknown. Future studies are needed to exclude or resolve the possible role of the enzyme in bacterial pathogenesis. This is important as PldA-deficient *C. jejuni* lose their ability to colonize the gut, suggesting that it may be a target for infection intervention.

Importantly, *C. jejuni* lysoPE did not only lyse red blood cells but (as described in **Chapter 5)** also caused permeabilization of epithelial cell membranes which are a primary target during natural infection. Previous research has shown that incorporation of even a small amount (1 mol.%) of fatty acids or lysolipids in lipid membranes can cause instabilities in the lipid bilayer [64]. We propose a similar mode of action for C. *jejuni*-derived lysoPE. It can be imagined that after insertion into the cell membrane, non-cylindrical lysolipids, such as lysoPEs, introduce a positive curvature into an existing lipid membrane thereby leading to instability and disruption of epithelial cell membrane permeability [64, 65, 66]. Whether and how the short chain bacterial lysoPEs insert into the host cell membrane still needs to be resolved. One approach to address this issue may be to label short-chain lysoPEs with a fluorescence signal or C13, and then check whether the related signal can be captured from the target cell membrane. Our results thus far suggest that lysoPE 14:0 induces cell damage by evoking an oxidant stress-dependent transient membrane permeabilization in cells, which resembles observations made with lysoPC [67]. Together, our RNA-seq analyses and results from the oxidative stress survival assay and bacterial growth curves described in **Chapter 4**, suggests that bacterial LPLs may have a bipartite function of neutralizing bacterial ROS but triggering oxidative stress in host cells. Further experiments could be done to measure the intracellular ROS in *C. jejuni* when they are cultured in low oxygen condition with bile salts.

Conclusion and perspectives

The aim of this thesis was to investigate the dynamics of the phospholipidome of *C. jejuni* and to identify biological functions of specific phospholipids in bacterial pathogenesis and environmental stress adaptation. Our results show that the lipid composition of the *C. jejuni* membrane is highly dynamic with an unusually high content of LPLs due to the activity of the enzyme PldA. PldA and the produced LPLs influence bacterial morphology and biological properties like the the resistance to bile salts, but also damage host cells which may provide access to substrates leaked from permeabilized cells or lysed cells. Our latest RNA-seq experiments (not part of this thesis) suggest that lysoPE 14:0 even causes increased expression of several host cell genes involved in biosynthesis of cholesterol, an important component in maintaining the stability of the host cell membrane [68]. Cholesterol in mammalian cell membranes can act as binding site for many microbial pathogens, including *H.*

pylori, which is phylogenetically closely related and shares many phenotypic traits with *C. jejuni* [69, 70]. Membrane cholesterol is also known to affect the function of surface receptors targeted by these pathogens and to be required for microbial entry into host cell intracellular compartments [70]. Furthermore, membrane cholesterol has been shown to be a important for the internalization and cytotoxicity of Cytolethal Distending Toxin (CDT) [71, 72], and to be essential for *C. jejuni* CDT-induced host cells apoptosis and intestinal inflammation [72]. Thus, *C. jejuni* lysoPEs might contribute to pathogenesis not only by disrupting host cell membrane hemostasis but also acting as a signaling molecule to facilitate or amplify CDT intoxication. Intracellular pathogens have been speculated to target cholesterol to obtain nutrients or manipulate cellular signaling [73]. Whether *C. jejun*i exploits this strategy is unknown but may be shown in experiments that e.g. deplete host cell membranes from cholesterol or disrupt the involved pathways.

In this thesis we provide evidence that lysoPE induces oxidant stress-dependent membrane leakage in epithelial cells. This may be highly relevant considering the epithelial cell damage observed during natural infection. Detailed understanding of the mechanism underlying the cell damage awaits further study. In certain type of breast cancer cells, lysoPE induces an increase in intracellular calcium via the lysophosphatidic acid receptor type 2 ($LPAR_2$) [74, 75]. This change in calcium levels may be the key factor in lysoPC-induced endothelial oxidative stress [76]. It is possible that LPAR also acts as a receptor for *C. jejuni* lysoPE to induce cytotoxicity. Currently, six different kinds of LAPR have been detected in eukaryotic cells. The expression of type 1, 2, 3, 4 and 6 LPAR is extremely low in HeLa cells and erythroid cells, but the expression of $LPAR_5$ is relatively high according to the Human Protein Atlas. Specific knockdown or overexpression of the receptors may teach us more about their possible involvement in lysoPE recognition.

As important as its effects on host cells, we found that PldA and LPLs have a role in the resistance to bile salts and thus in gut colonization. Our RNA-seq analyses, growth curves, and survival assay indicate that *C. jejuni* not only resists but even benefits from bile salts via a PldA-dependent higher iron transport efficiency and lower susceptibility to oxidative stress. The PldA-dependence of these processes is not seen when bacteria are grown in the absence of bile salts. In eukaryotic cells, the catalytic rate of PldA is significantly modified by bile salts due to binding of polar hydroxyl and sulfate/carboxy groups to PldA [77]. However, this influence is biphasic, the activity of the PldA-bile salt complex can increase as well as decrease [45, 77]. Considering the specific PldA-dependent growth performance of *C. jejuni* in the presence of bile salt, we speculate that the activity of *C. jejuni* PldA is also altered by bile salts. Assessment of the percentage of LPLs in the phospholipidome of *C. jejuni* may resolve whether this interaction results in further activation or inhibition of PldA activity. The presence of bile salt also significantly affected the transcripts encoding

transport systems involved in iron and amino acids as well as the oxidative stress response in *C. jejuni* when the bacteria are grown under low oxygen. This effects were also strongly dependent on a functional PldA. Other enteropathogens that have a high percentage of LPL in their membranes might share the same mechanism to adapt and survive in host intestine. Our work thus may lead to a more general understanding of the biological significance of LPLs in bacterial membranes, host adaptation, and bacterial pathogenesis.

Nowadays, *Campylobacter* infection is a major burden to human health and a primary cause of enterocolitis in the developed countries. Yet, the pathogenesis of the diseases is still not well understood. In this thesis we discovered that *C. jejuni*-derived lysoPEs as a novel potential virulence factor. PldA activity and lysoPE are important for bacterial motility in a low oxygen environment, promote bacterial growth, contribute to bile resistance and nutrient acquisition, and display cytotoxic effects towards host cells. Considering this, inhibition of *C. jejuni* LPLs generation might be considered as a novel strategy to limit *Campylobacter* infection. Many PldA inhibitors including cyclooxygenase (COX), beta-sitosterol, γCdcPLI have already been developed and utilized as anti-inflammatory, anti-parasite, and anti-angiogenic agent [78-80]. A targeted therapeutic approach using clinically available sPLA2-IIA inhibitors was recently suggested to reduce COVID-19 mortality [81]. Considering the research findings described in this thesis and the availability of tested PldA inhibitors, we recommend further investigation of the potential use of these compounds in the prevention and elimination *C. jejuni* infection in humans and in chicken.

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C. jejuni 81116:: pldA 22-12-2022

Appendices

English Summary

Nederlands Samenvatting

Acknowledgement

Curriculum Vitae

Publications

Summary

The integrity of the bacterial envelope is crucial for bacteria to survive. The bacterial membrane separates the intracellular environment from the harsh external surroundings. The cytoplasmic membrane is composed of approximately equal proportions of proteins and lipids allowing bacteria to absorb nutrients and expel waste. The main lipids in the bacterial membrane are phospholipids which provide a structural basis for the many membrane proteins. The architectural features of membrane phospholipids can significantly influence the functions of membrane proteins. In response to changes in their environment, bacteria need to change not only their protein repertoire, but also their phospholipid composition. The capacity to alter the composition of the phospholipidome is recognized as a crucial physiological process to allow bacteria to survive and adapt in response to environmental stress. A small fraction of the phospholipids in the bacterial membrane are lysophospholipids (LPLs). Although LPLs are minor phospholipid species of the bacterial cell membrane, in eukaryotic cells they are important signaling molecules. In some pathogenic bacteria, LPLs can be strongly increased under certain stress conditions, however, the biological function of LPLs in bacteria has not been investigated.

C. jejuni is a Gram-negative motile, slim spiral shaped bacterium, which can transform into a coccoid form. Adaptation of *C. jejuni* to different environmental situations has mainly been studied at the metabolic and proteome level. Knowledge of the *C. jejuni* phospholipid repertoire has so far been limited to the detection of phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). *C. jejuni* is the leading cause of bacterial foodborne illness especially in developed countries. The symptoms of the infection typically involve intestinal inflammation, fever, and bloody diarrhea, but the molecular basis of infection is still cryptic. *C. jejuni* is known to express a complex array of virulence factors to overcome host defenses but lacks traditional virulence factors such as a type III secretion system (T3SS) present in most enteropathogens.

The goal of the research described in this thesis was to determine the phospholipidome of *C. jejuni* under different environmental conditions and to assess the role of distinct (classes of) phospholipids in bacterial pathogenesis and/or stress adaptation. In **Chapter 2**, we provide an overview of the different LPLs species present in bacteria and summarize the knowledge about their role in bacterial adaptation, survival, invasion and host-microbe interaction. A most notable feature of LPLs is that they accumulate in the cell membrane when bacteria are stressed. LPLs are lipid-derived metabolic intermediates in bacterial phospholipids biosynthesis. They are normally considered as negligible and nonbilayer-forming ingredients (<1%) of the bacterial membrane due to their detergent-like properties. Bacterial LPLs can

be produced in three different ways: (1) by endogenous phospholipase A (PldA) which hydrolyze phospholipids, (2) by palmitoyltransferase (PagP) which adds an acyl chain of phospholipids to hexa-acylated lipopolysaccharides (LPS), and (3) by the *N*-acyltransferase (Lnt) which transfer a fatty acid moiety from phospholipids to the lipoprotein precursor. Due to the membrane-destabilizing feature of LPLs in Gram-negative bacteria, LPLs are usually rapidly transported by the bacterial LPLs transport system (LplT) to the cytoplasm where they are neutralized. In Gram-positive bacteria LPLs are rare as they are toxic for these bacteria. LPLs influence the integrity of the membrane lipid bilayer; therefore, they are supposed to affect cellular structure and function. In eukaryotes LPLs are known as potent bioactive messengers which are involved in immune responses and can act as multifunctional cellular growth factors. The biological role of LPLs in bacteria might have been underestimated for a long time. In the limited bacterial LPLs studies available, it has been reported that (1) LPLs can act as a chaperone which directly affect the structure and function of membrane proteins; (2) can be used by some bacterial species to compete with other microbes or to facilitate bacterial infection. Host-derived LPLs have been shown to be exploited by pathogenic bacteria to promote cellular invasion and inflammation but also benefit the host by defending against bacterial infection and alleviating bacteria-induced intestinal inflammation.

In **Chapter 3**, we for the first time investigated the nature of the phospholipids produced by *C. jejuni* under different growth conditions. The genome of *C. jejuni* contains 22 genes implicated in phospholipid biosynthesis. These include genes encoding the acyl-phosphate transfer system (*plsX*, *plsY*) needed for the synthesis of lysophosphatidic acid (lysoPA). LysoPA can be further acylated by PlsC to yield phosphatidic acid (PA), the precursor for the other phospholipids phosphatidylserine (PS), PE, and PG, encoded by the genes *pssA*, *psd*, *pgsA* and *pgpA*, respectively. Comparison of transcript abundance of *C. jejuni* phospholipid biosynthesis genes in bacteria in the logarithmic and stationary growth phase pointed to strong regulation of (especially) the fatty acid chain elongation genes and the cyclopropane fatty acid synthase genes. Phospholipidome analyses revealed two hundred and three different phospholipid species belonging to nine different lipid classes. Besides PE and PG, seven new phospholipid classes were detected: PA, PS, lysophosphatidylglycerol (lysoPG), lysophosphatidylethanolamine (lysoPE), lysoPA, acyl-phosphatidylglycerol (acyl-PG) and a novel lipid class PX. The length of phospholipid fatty acid tails was found to vary between 12 and 21 carbon atoms. Furthermore, saturated, unsaturated and cyclopropane structures were detected in all different phospholipid classes. Percentagewise, the *C. jejuni* membrane contained an unusually high number (up to 40%) of LPLs.

To learn more about the adaptive changes of the phospholipid repertoire in different growth environments, we compared the phospholipidome composition after

growth under different oxygen conditions and with aging of the cells. *C. jejuni* can survive in surface water with oxygen concentrations of up to 10% but also in the gut of mammals at oxygen concentrations of 0.3%. Principal component analysis of the phospholipidome clearly showed major shifts in bacterial phospholipid composition under different oxygen conditions. At high (10%) oxygen conditions, the composition of unsaturated fatty acids decreased from 60% to 23% with aging of the culture, while cyclopropane fatty acids accumulated from 14% to up to 50%. Remarkably, the percentage of LPLs also increased reaching a maximum of 45% of the total phospholipids when the culture was 36 h of age. The increase in percentage of LPLs (mainly lysoPE) resulted in a significant decrease of PE. With the accumulation of LPLs, the shape of *C. jejuni* converted from the typical spiral shape to the coccoid form. At low (0.3%) oxygen conditions as exist in the gut, the percentage of unsaturated fatty acids during growth slowly dropped from 55% to 30%, while the cyclopropane-bonds containing phospholipids gradually rose from 18% to 35%. The percentage of LPLs at 0.3% O_2 was relatively stable at around the high level of 28% of total phospholipids, independent of the growth phase of culture. Most *C. jejuni* bacteria preserved their spiral shape under this condition. In addition to the variation of phospholipid classes, the length of phospholipid acyl chains shifted in response to age and oxygen concentration. Functionally, the high level of LPLs appeared to be essential for maintaining the motility of *C. jejuni* at low oxygen conditions.

In the intestine, *C. jejuni* has not only to adapt to low oxygen availability but also to the presence of bile. Bile salts are known to play a role in intestinal homeostasis by controlling the size and the composition of the intestinal microbiota as they work as antimicrobials in the gut. Like many other enteric pathogens, *C. jejuni* thus needs to rapidly adapt itself when it encounters bile salts in the intestine. In **Chapter 4**, we investigated the role of the *C. jejuni* membrane LPLs during bile salt exposure using an isogenic mutant defective in the phospholipase PldA. This enzyme cleaves phospholipid fatty acid tails and is required for the formation of LPLs. Growth of *C. jejuni* under low oxygen conditions in the presence of the bile salt sodium deoxycholate (DOC) showed a bile salt-dependent difference in bacterial colony morphology between the wildtype and the *pldA* mutant. We also noted that DOC significantly promoted the growth of wildtype *C. jejuni* under the limited low oxygen condition (0.3% O_2), although it inhibited *C. jejuni* growth in a 10% O_2 environment. In contrast, exposure of the *pldA* mutant to DOC at 0.3% O_2 resulted in reduced bacterial growth, indicating that PldA (or its products) is important to adapt to DOC. Transcriptome analyses and biological assays showed that wildtype *C. jejuni* exposure to DOC at low O_2 increased the transcript levels of a number of iron-regulated genes. This effect could result from the well-known iron-chelating effect of bile salts which reduces iron availability. In contrast, exposure of *C. jejuni ΔpldA* to DOC caused a significantly downregulation of several iron transport gene

transcripts. This may indicate that PldA is needed to adapt to iron starvation duuring bile salt exposure and may explain the different effect of DOC exposure on bacterial growth of the wildtype and the *pldA* mutant. This hypothesis was supported by growth assays that showed that the addition of ferrous or ferric iron was not able to rescue the growth defect of *C. jejuni ΔpldA*. A shortage of iron in the absence of PldA was further suggested by the observed upregulation of oxidative stress response genes in *C. jejuni ΔpldA* exposed to DOC. A common feature of bacteria in response to iron starvation is the upregulation of transcription of oxidative stress response genes. Indeed, our oxidative stress survival assays showed that the *pldA* mutant was less vulnerable to oxidative stress than the wildtype.

Besides changes in iron and oxidative stress response gene transcripts during DOC exposure (at low O_2), our transcriptomics indicated changes in tryptophan and branched-chain amino acids (leucine, isoleucine and valine) metabolism. How *C. jejuni* benefits from the upregulation of tryptophan and high-affinity branched-chain amino acids gene pathways remains to be elucidated. It can be imagined that an increase in these amino acids is important for *C. jejuni* pathogenesis as the bacterial tryptophan catabolite indole can regulate epithelial inflammation and gut immunity in the presence of bile acids and branched-chain amino acid supports the bacterial evasion of host defenses.

In **Chapter 5**, we focused on the potential role of LPLs as a novel virulence factor of *C. jejuni*. LPLs have been found to be essential in eukaryotic cells as growth factor or potent lipid mediators in a broad variety of processes such as carcinogenesis, immunity, or regulation of metabolic diseases. Based on the amino acid sequence characteristics *C. jejuni* PldA belongs to the phospholipid class A. But high-performance LC-MS/MS showed that *C. jejuni* contains both (*Sn*)-1 and -2 LPLs and that both were almost absent in the PldA mutant. Thus *C. jejuni* PldA can cleave on the (*Sn*)-1 as well as (*Sn*)-2 position and therefore possesses both $PldA_1$ and $PldA_2$ phospholipase activity. Although the amino acid sequence between PldA and PldB family of proteins is quite different, the function of the *C. jejuni* PldA enzyme is more similar to the phospholipid B class of enzymes.

In search for a possible role of LPLs in *C. jejuni* virulence, we discovered that the PldA is required to cause hemolysis. We demonstrated that it is not the PldA enzyme itself but its LPL products that cause the red blood cells to lyse. Testing of individual phospholipid classes of *C. jejuni* revealed that the hemolysis is due to the activity of lysoPE molecules. We found that not only the phospholipid head group but also the length of the fatty acid tail of the lysoPE is important for this toxic effect. Especially the short lysoPE 14:0 and 16:0 damaged the red blood cells. Interestingly, our phospholipidome analysis showed that *C. jejuni* membranes can consists of more than 33% of lysoPE, of which almost 50 % is present as lysoPE 14 and 16. This likely explains why the membranes of live or dead *C. jejuni* bacteria are toxic for red

blood cells. The lysis of the red blood cells may release iron sources and thus aid the establishment of infection.

Importantly, isolated *C. jejuni* membranes were not only toxic for red blood cells but also for epithelial cells as evidenced by a strong PldA-dependent increase of LDH release. Maximum LDH release was observed when the epithelial cells were incubated with lysoPE 14:0. Confocal microscopy revealed that treatment with lysoPE resulted in staining of nuclear membranes consistent with an increased plasma membrane permeability. It has been shown that lysoPC can evoke an oxidant stress-dependent transient membrane permeabilization in cells. To investigate whether lysoPE (like lysoPC) has a similar effect, we tested two inhibitors of the oxidative stress response, vitamin E and DPPD. Both inhibitors reduced the lysoPE 14:0 induced hemolysis and LDH release from epithelial cells and inhibited the nuclear membrane staining. Thus, the cytotoxicity of lysoPE 14:0 resembles the mechanisms described for eukaryotic lysoPC and mainly involves an oxidant stress-dependent transient membrane permeabilization. The discovered cytotoxicity of *C. jejuni* lysoPE may be an important novel determinant of *C. jejuni* virulence.

Overall, we described in this thesis our efforts to dissect the biological features of bacterial membrane phospholipids, with special emphasis on the function of LPLs in bacterial infection and environmental stress adaptation. We discovered that *C. jejuni* has a unique phospholipidome compared to other bacteria. The phosholipidome is highly dynamic with changes occurring rapidly during the aging of the culture and in response to oxygen availability. *C. jejuni* membranes contain a high percentage of LPLs due to activity of the phospholipase PldA. This enzyme is required for bacterial motility under low oxygen conditions as exist in the intestine and is important for iron acquisition during exposure to bile salts. Other enteric pathogens may utilize similar mechanisms to defend against the toxic components of bile and to optimally adapt to the intestinal niche. In addition to stress adaptation, the cytotoxicity of the high percentage of LPLs present in the membranes of *C. jejuni* may contribute to bacterial pathogenesis. In particular, the short-tailed lysoPE is toxic and induces an oxidant stress-dependent transient membrane permeabilization in eukaryotic cells. Bacterial lysoPE can thus be considered as a novel virulence factor. Our results about the role of bacterial PldA and LPLs during *C. jejuni* colonization and pathogenesis provide a strong foundation for future research aimed at better understanding and prevention of infection caused by *C. jejuni* and other lysoPE producing bacterial pathogens.

Nederlandse samenvatting

De integriteit van de bacteriële envelop is cruciaal voor bacteriën om te overleven. De bacteriële membraan scheidt het cytoplasma af van de externe omgeving. De cytoplasmatische membraan bestaat uit ongeveer gelijke hoeveelheden eiwitten en lipiden, waardoor bacteriën voedingsstoffen kunnen opnemen en afval kunnen uitscheiden. De belangrijkste lipiden in het bacteriële membraan zijn fosfolipiden die een fundament vormen voor de vele membraaneiwitten. De membraanfosfolipiden kunnen de functies van membraaneiwitten aanzienlijk beïnvloeden. Als reactie op veranderingen in hun omgeving moeten bacteriën niet alleen hun eiwitrepertoire veranderen, maar ook hun fosfolipidesamenstelling. Het vermogen om de samenstelling van het fosfolipidoom te veranderen, wordt erkend als een cruciaal fysiologisch proces om bacteriën in staat te stellen te overleven en zich aan te passen als reactie op omgevingsstress. Een klein deel van de fosfolipiden in de bacteriële membraan zijn lysofosfolipiden (LPLs). Onder bepaalde stress omstandigheden kunnen sommige pathogene bacteriën de hoeveelheid LPLs sterk verhogen. In eukaryote cellen zijn LPLs belangrijke signaalmoleculen, echter in bacteriën zijn de functies van LPLs nog niet bestudeerd.

C. jejuni is een Gram-negatieve beweeglijke, slanke, spiraalvormige bacterie, die kan veranderen in een coccoïde vorm. Aanpassing van *C. jejuni* aan verschillende omgevingssituaties is voornamelijk bestudeerd op metabool en eiwitniveau. Kennis van het *C. jejuni* fosfolipidenrepertoire is tot nu toe beperkt tot het voorkomen van fosfatidylethanolamine (PE) en fosfatidylglycerol (PG). *C. jejuni* is de belangrijkste bacteriële oorzaak van door voedsel overgedragen ziekten, vooral in ontwikkelde landen. De symptomen van de infectie omvatten meestal darmontsteking, koorts en bloederige diarree, maar de moleculaire basis van infectie is nog steeds onduidelijk. *C. jejuni* bezit een reeks aan virulentiefactoren die de afweer van de gastheer kunnen omzeilen, maar traditionele virulentiefactoren zoals een type III-secretiesysteem (T3SS) die aanwezig zijn in de meeste enteropathogenen ontbreken.

Het doel van het onderzoek beschreven in dit proefschrift was om het fosfolipidoom van *C. jejuni* onder verschillende omgevingscondities op te helderen en om de rol van verschillende (klassen van) fosfolipiden in bacteriële pathogenese en/of stressadaptatie te achterhalen. In **Hoofdstuk 2** geven we een overzicht van de verschillende LPLs-soorten die aanwezig zijn in bacteriën en hebben we de huidige kennis over hun rol in bacteriële adaptatie, overleving, invasie en gastheer-microbe interactie samengevat. De meest opvallende eigenschap van LPLs is dat ze zich ophopen in de celmembraan wanneer bacteriën worden gestrest. LPLs zijn metabole tussenproducten in de biosynthese van bacteriële fosfolipiden. Ze worden normaal gesproken beschouwd als verwaarloosbare en niet-dubbellaagvormende

componenten (<1%) van het bacteriële membraan. Bestaande fosfolipiden kunnen echter op drie verschillende manieren worden getransformeerd tot LPLs: (1) door endogene fosfolipase A (PldA) dat fosfolipiden hydrolyseert, (2) door palmitoyltransferase (PagP) dat een acylketen van fosfolipiden toevoegt aan hexageacyleerde lipopolysacchariden (LPS), en (3) door de *N*-acyltransferase (Lnt) dat een vetzuurgroep van fosfolipiden naar de lipoproteïne-precursor overbrengt. Vanwege de membraan-destabiliserende eigenschap van LPLs in Gram-negatieve bacteriën, worden LPLs meestal snel getransporteerd door het bacteriële LPL's-transportsysteem (LplT) naar het cytoplasma, waar ze worden geneutraliseerd. Bij Gram-positieve bacteriën zijn LPLs zeldzaam, omdat ze toxisch zijn voor deze bacteriën. LPLs beïnvloeden de integriteit van de fosfolipide lipide dubbellaag; daarom wordt verondersteld dat ze de cellulaire structuur en functie beïnvloeden. Bij eukaryoten staan LPLs bekend als krachtige bioactieve moleculen die betrokken zijn bij immuunreacties en kunnen werken als multifunctionele cellulaire groeifactoren. De biologische rol van LPLs in bacteriën is mogelijk lange tijd onderschat. Uit de schaarse literatuur over bacteriële LPL is gebleken dat (1) LPLs kunnen fungeren als een chaperonne die de structuur en functie van membraaneiwitten direct beïnvloeden; (2) sommige bacteriesoorten LPL gebruiken om te concurreren met andere microben of om bacteriële infectie te vergemakkelijken. Van de gastheer afgeleide LPLs is aangetoond dat ze de cellulaire invasie en ontsteking veroorzaakt door pathogene bacteriën kunnen bevorderen, maar ook andersom dat ze de gastheer kunnen helpen zich te verdedigen tegen bacteriële infecties of door bacteriën geïnduceerde darmontsteking kunnen verlichten.

In **Hoofdstuk 3** hebben we voor het eerst de aard van de fosfolipiden die door *C. jejuni* worden geproduceerd onder verschillende groeiomstandigheden opgehelderd. Het genoom van *C. jejuni* bevat 22 genen die betrokken zijn bij de biosynthese van fosfolipiden. Deze omvatten genen die coderen voor het acylfosfaat overdrachts systeem (*plsX*, *plsY*) dat nodig is voor de synthese van lysoPA. LysoPA kan verder worden geacyleerd door PlsC, wat leidt tot fosfatidinezuur (PA). PA is de voorloper van de andere fosfolipiden PS, PE en PG, die worden gecodeerd door respectievelijk de genen *pssA*, *psd*, *pgsA* en *pgpA*. Transcriptoom analyse van logaritmische en stationaire opgegroeide *C. jejuni* bacteriën wees op een sterke regulatie van (vooral) de vetzuurketen verlengende en cyclopropaan synthese genen. Opheldering van het *C.jejuni* fosfolipidoom resulteerde in de identificatie van 203 verschillende fosfolipiden die behoren tot negen verschillende lipidenklassen. Naast PE en PG werden zeven nieuwe fosfolipidenklassen gedetecteerd: fosfatidinezuur (PA), fosfatidylserine (PS), lysofosfatidylglycerol (lysoPG), lysofosfatidyl-ethanolamine (lysoPE), lysofosfatidinezuur (lysoPA), acyl-fosfatidylglycerol (lysoPG), en een nieuwe lipideklasse PX. De lengte van fosfolipide vetzuurstaarten bleek tussen 12 en 21 koolstofatomen te variëren. Daarnaast werden in alle verschillende

fosfolipidenklassen verzadigde, onverzadigde en cyclopropaanlipide gedetecteerd. Zeer opvallend was het ongewone hoge aantal LPL (tot wel 40% van de totale fosfolipiden) dat voorkomt in de *C. jejuni*-membraan.

Om meer te weten te komen over de veranderingen die kunnen voorkomen onder invloed van verschillende groeicondities in het fosfolipidoom van *C.jejuni*, hebben we de fosfolipidoom samenstelling gevolgd in de tijd onder verschillende zuurstofcondities (10 en 0.3% O_2). *C. jejuni* komt voor in oppervlaktewater met een zuurstofconcentratie van rond de 10%, maar kan zich ook prima vermenigvuldigen in de darm van zoogdieren bij zuurstofconcentraties van 0.3%. Analyse van het fosfolipidoom toonde duidelijk grote verschuivingen aan in de bacteriële fosfolipidesamenstelling onder verschillende zuurstofcondities. Bij hoog zuurstof (10%) nam de samenstelling van onverzadigde vetzuren af van 60% naar 23% en bij het verouderen van de bacteriecultuur bleek dat de cyclopropaanvetzuren zich ophoopten van 14% tot maximaal 50%. Opmerkelijk was de toename van de LPLs die na 36 uur te hebben gegroeid, 45% van de totale hoeveelheid fosfolipiden uitmaakte. De toename van het percentage LPLs (voornamelijk lysoPE) resulteerde in een significante afname van PE. Met de opeenhoping van LPLs veranderde de vorm van *C. jejuni* van de typische spiraalvorm naar de coccoïde vorm. Bij laag zuurstofcondities (0.3%) zoals die in de darm voorkomen, daalde het percentage onverzadigde vetzuren tijdens de groei langzaam van 55% naar 30%, terwijl de cyclopropaan bevattende fosfolipiden geleidelijk stegen van 18% naar 35%. Het hoge percentage LPLs was relatief stabiel tijdens de groei bij 0.3% O_2 en maakte ongeveer 28% van het totale fosfolipiden uit. De meeste *C. jejuni*-bacteriën behielden onder deze omstandigheden hun spiraalvorm. Naast de variatie van fosfolipideklassen, verschoof de lengte van fosfolipide-acylketens als reactie op groeifase en zuurstofconcentratie. Functioneel bleek het hoge niveau van LPLs essentieel te zijn voor het handhaven van de beweeglijkheid van *C. jejuni* bij lage zuurstofcondities.

In de darm moet *C. jejuni* zich niet alleen aanpassen aan een lage zuurstofbeschikbaarheid, maar ook aan de aanwezigheid van gal. Van galzouten is bekend dat ze een rol spelen bij de homeostase van de darm, omdat ze als antimicrobiële stoffen de omvang en de samenstelling van de darmmicrobiota kunnen regelen. Net als veel andere darmpathogenen, moet *C. jejuni* zich dus snel aanpassen wanneer het galzouten in de darm tegenkomt. In **Hoofdstuk 4** hebben we de rol van de *C. jejuni* LPL's tijdens blootstelling aan galzout onderzocht door gebruikt te maken van een niet LPLs producerende fosfolipase A (PldA) mutant. Het PldA enzym knipt vetzuurstaarten af van fosfolipiden waardoor LPLs worden gevormd. Onder lage zuurstofcondities in aanwezigheid van het galzout natriumdeoxycholaat (DOC) werd een verschillende galzoutafhankelijk morfologie waargenomen tussen de wildtype en de *pldA*-mutant kolonies. Onder de zuurstofarme conditie (0.3% O_2) bevorderde DOC de groei van wildtype *C. jejuni* significant, maar DOC remde de

groei in een 10% O_2-omgeving. Daarentegen resulteerde blootstelling van de *pldA*-mutant aan DOC bij 0,3% O_2 in verminderde bacteriegroei, wat aangeeft dat PldA (of zijn producten) belangrijk is om zich aan te passen aan DOC. Transcriptoom data en biologische tests toonden aan dat blootstelling van wildtype *C. jejuni* aan DOC bij lage O_2 de transcriptniveaus van een aantal door ijzer gereguleerde genen verhoogde. Dit effect kan het gevolg zijn van het bekende ijzer chelerende effect van galzouten dat de beschikbaarheid van ijzer vermindert. Daarentegen veroorzaakte blootstelling van *C. jejuni ΔpldA* aan DOC een significante neerwaartse regulatie van verschillende transcripten van ijzertransportgenen. Dit zou erop kunnen wijzen dat het PldA belangrijk is voor een gedegen fundament voor membraaneiwitten betrokken bij het ijzermetabolisme om het ijzergebrek veroorzaakt door de blootstelling aan galzouten te doen verminderen. Mogelijk kan dit ook de verschillende effecten van blootstelling aan DOC op de bacteriegroei van de wildtype en de *pldA*-mutant verklaren.

Deze hypothese werd ondersteund door groeitesten, die aantoonden dat toevoeging van Fe^{2+} of Fe^{3+} het groeidefect van *C. jejuni ΔpldA* niet kon doen verbeteren. Tevens werd een verhoogde transcriptie van oxidatieve stress respons genen in *C. jejuni ΔpldA* blootgesteld aan DOC waargenomen. Een gemeenschappelijk kenmerk van bacteriën in reactie op ijzergebrek is de verhoogde transcriptie van oxidatieve stress respons genen. Oxidatieve stress-overlevingstests toonden inderdaad aan dat de *pldA*-mutant minder kwetsbaar was voor oxidatieve stress dan het wildtype.

Naast veranderingen in het mRNA van ijzer- en oxidatieve stress respons genen tijdens DOC-blootstelling (bij lage O_2), gaven onze transcriptomics veranderingen aan in het metabolisme van tryptofaan en vertakte aminozuren (leucine, isoleucine en valine). Hoe *C. jejuni* profiteert van de verhoogde transcriptie van tryptofaan genen en hoge affiniteit voor aminozuren met vertakte ketens, moet nog worden opgehelderd. Men kan zich voorstellen dat een toename van deze aminozuren belangrijk zijn voor de pathogenese van *C. jejuni*, aangezien het bacteriële tryptofaan-kataboliet-indol, epitheel ontstekingen en darmimmuniteit kan reguleren in aanwezigheid van galzuren en vertakte aminozuren de bacteriële ontwijking van de afweer van de gastheer ondersteunen.

In **Hoofdstuk 5** hebben we ons gericht op de mogelijke rol van LPLs als een nieuwe virulentiefactor van *C. jejuni*. Er was al gevonden dat LPLs essentieel zijn in eukaryote cellen en betrokken zijn bij een breed scala aan processen zoals carcinogenese, immuniteit of regulering van metabole ziekten. Op basis van de kenmerken van de aminozuursequentie behoort *C. jejuni* PldA tot de fosfolipidenklasse A. Maar hoogwaardige LC-MS/MS toonde aan dat *C. jejuni* zowel (*Sn*)-1 als (*Sn*)-2 LPLs bevat en dat beide bijna afwezig waren in de PldA-mutant. *C. jejuni* PldA kan dus fosfolipiden op zowel op de (*Sn*)-1- als op de (*Sn*)-2-positie splitsen en bezit daarom zowel PldA1- als PldA2-fosfolipase-activiteit.

Hoewel de aminozuursequentie tussen de PldA- en de PldB-familie van eiwitten behoorlijk verschilt, lijkt de functie van het *C. jejuni* PldA-enzym meer op de fosfolipide B-klasse van enzymen.

Op zoek naar een mogelijke rol van LPLs in de virulentie van *C. jejuni*, ontdekten we dat de PldA nodig is om hemolyse te veroorzaken. We hebben aangetoond dat niet het PldA-enzym zelf, maar de LPL-producten ervoor zorgen dat de rode bloedcellen lyseren. Het testen van individuele fosfolipidenklassen van *C. jejuni* onthulde dat de hemolyse het gevolg was van de activiteit van lysoPE-moleculen. We vonden dat niet alleen de fosfolipide hoofdgroep maar ook de lengte van de vetzuurstaart van het lysoPE belangrijk is voor dit toxische effect. Vooral de korte lysoPE 14:0 en 16:0 beschadigde de rode bloedcellen. Onze fosfolipidoom analyse toonde aan dat *C. jejuni*-membranen voor meer dan 33% uit lysoPE kunnen bestaan, waarvan bijna 50% aanwezig is als lysoPE 14 en 16. Dit verklaart waarschijnlijk waarom de membranen van levende zowel als van dode *C. jejuni*-bacteriën toxische zijn voor rode bloedcellen. Door de lysis van de rode bloedcellen kan ijzer vrijkomen wat de *C.jejuni* infectie ten goede kan komen.

Belangrijk is dat geïsoleerde *C. jejuni*-membranen niet alleen toxisch waren voor rode bloedcellen, maar ook voor epitheelcellen, zoals blijkt uit een sterke PldA-afhankelijke toename van LDH-afgifte. Maximale LDH-afgifte werd waargenomen wanneer de epitheelcellen werden geïncubeerd met lysoPE 14:0. Confocale microscopie onthulde dat behandeling met lysoPE resulteerde in kleuring van kernmembranen wat overeenkomt met een verhoogde permeabiliteit van het plasmamembraan. Er was aangetoond dat lysoPC een tijdelijke oxidatieve stress-afhankelijke membraan permeabiliteit in cellen kan oproepen. Om te onderzoeken of lysoPE (net zoals lysoPC) een soortgelijk effect heeft, hebben we twee remmers van de oxidatieve stress respons, vitamine E en DPPD, getest. Beide remmers verminderden de door lysoPE 14:0 geïnduceerde hemolyse en LDH-afgifte uit epitheelcellen en remden de kleuring van het kernmembraan. De cytotoxiciteit van lysoPE 14:0 lijkt dus op het mechanisme dat is beschreven voor lysoPC en omvat voornamelijk een tijdelijke oxidatieve stress-afhankelijke membraan permeabiliteit. *C. jejuni* lysoPE kan zo een belangrijke nieuwe virulentie factor zijn van *C. jejuni*.

In dit proefschrift hebben we onze inspanningen beschreven om de biologische kenmerken van bacteriële membraanfosfolipiden te ontleden, met speciale nadruk op de functie van LPLs bij bacteriële infectie en aanpassing aan omgevingsstress. We ontdekten dat *C. jejuni* een uniek fosfolipidoom heeft in vergelijking met andere bacteriën. Het fosfolipidoom is zeer dynamisch met veranderingen die snel optreden tijdens de veroudering van de cultuur en als reactie op de beschikbaarheid van zuurstof. De membranen van *C. jejuni* bevatten een hoog percentage LPLs vanwege de activiteit van het fosfolipase PldA. Dit enzym is onder zuurstofarme omstandigheden zoals aanwezig in de darm, nodig voor bacteriële motiliteit

en is belangrijk voor de ijzeropname tijdens blootstelling aan galzouten. Andere darmpathogenen kunnen bezitten wellicht vergelijkbare mechanismen om zich te verdedigen tegen de toxische componenten van gal en om zich optimaal aan te passen aan de darm. Naast stress adaptatie kan de cytotoxiciteit van het hoge percentage LPL's dat aanwezig is in de membranen van *C. jejuni* bijdragen aan bacteriële pathogenese. In het bijzonder is het korte vetzuurstaart bevattende lysoPE toxisch en induceert het een tijdelijke oxidatieve stress-afhankelijke membraan permeabiliteit in eukaryote cellen. Bacteriële lysoPE kan dus worden beschouwd als een nieuwe virulentiefactor. Onze resultaten over de rol van bacteriële PldA en LPL's tijdens kolonisatie en pathogenese van *C. jejuni* bieden een sterke basis voor toekomstig onderzoek gericht op een beter begrip en preventie van infectie veroorzaakt door *C. jejuni* en andere lysoPE-producerende bacteriële pathogenen.

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Xuefeng Cao (雪峰)

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Curriculum vitae

Xuefeng Cao was born on November 6, 1991 in Chengdu, Sichuan province, China. After graduating from high school in 2010, he started his undergraduate study in Veterinary Medicine at Sichuan Agricultural University. Since 2011, he initiated his internship in the laboratory of Prof. Zhijun Zhong, studying the diagnosis and treatment of dogs with intractable vomiting and potassium deficiency. After receiving his bachelor's degree in 2014, he started his master in Sichuan Key Laboratory of Conservation Biology for Endangered Wildlife, Clinical Veterinary Medicine, Sichuan Agricultural University. Under the continued supervision of Prof. Zhijun Zhong, he finished his master degree with a thesis, entitled "Diagnosing Canine Parvovirus and Canine Distemper Viral without Nucleic Acid Extraction and Genetic Evolution Analysis of Canine Parvovirus in Dogs". In October 2017, with the support of China Scholarship Council (CSC), he got the opportunity to start his Ph.D. research at Infection Biology Division, Department of Biomolecular Health Science, Faculty of Veterinary Medicine, Utrecht University, the Netherlands. Under the supervision of Dr. Marc Wösten and Prof. dr. Jos van Putten, his research focused on the determination of *C. jejuni* phospholipidome under different environmental conditions and to assess the role of distinct (classes of) phospholipids in bacterial pathogenesis and/or stress adaptation. The results of this work are described in this thesis. From February 2023, Xuefeng will follow his passion for bacteria-host interation as Assistant Professor at College of Veterinary Medicine, Southwest University, Chongqing, China.

Publications

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