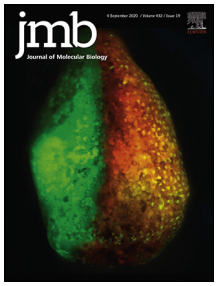

The Unique Phospholipidome of the Enteric Pathogen *Campylobacter jejuni*: Lysophosholipids Are Required for Motility at Low Oxygen Availability

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https://doi.org/10.1016/j.jmb.2020.07.012
Edited by Bert Poolman

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 *C. jejuni* using RNA-seq revealed differential expression of putative phospholipid biosynthesis genes. By applying high-performance liquid chromatography tandem–mass spectrometry, we identified 203 phospholipid species representing the first determination of the phospholipidome of this pathogen. We identified nine different phospholipid classes carrying between one and three 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 mol 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 ($\leq$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 42 °C. 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 81,116 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) (Figure 1(a)). 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 PA. This phosholipid may be further converted by the putative *cdsA, pssA, psd, pgsA* and *pgpA* gene products into PS, PE and 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].

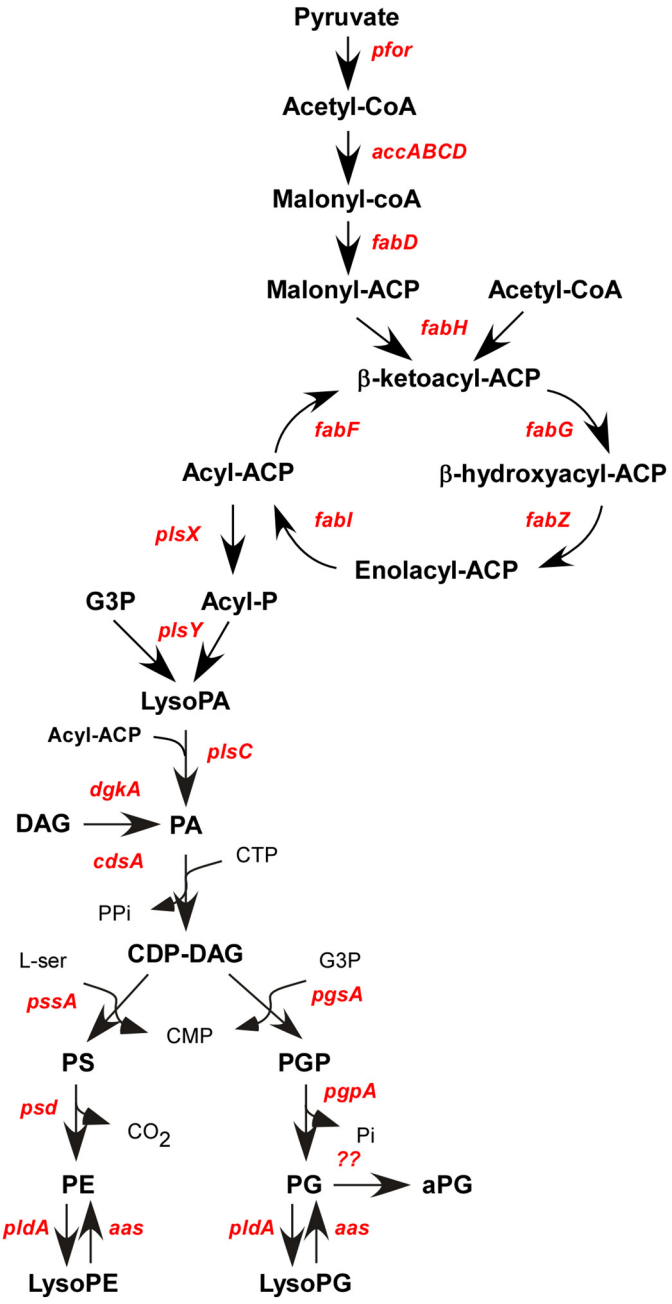
In the present study, we applied a high-throughput LC–MS approach to determine total lipid composition of *C. jejuni* under different environmental conditions. Our results indicate that the phospholipid composition of *C. jejuni* is highly dynamic and varies in response to the aging of the culture, and 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.

Results

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

To investigate whether the transcription of the annotated phospholipid biosynthesis genes of *C. jejuni* strain 81,116 (Figure 1(a)) 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

(a)



(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

Figure 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 81,116. 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 81,116 grown for 6 h (Log) *versus* 12 h (Stat) in HI at 42 °C under microaerophilic conditions (5% O_2, 10% $C0_2$, 10% H_2 and 75% N_2).

logarithmic phase compared to the stationary phase (Figure 1(b), Table S1). 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 81,116 grown in Heart Infusion (HI) medium (16 h, 42 °C) under microaerophilic conditions (5% O_2, 10% CO_2, 10% H_2 and 75%

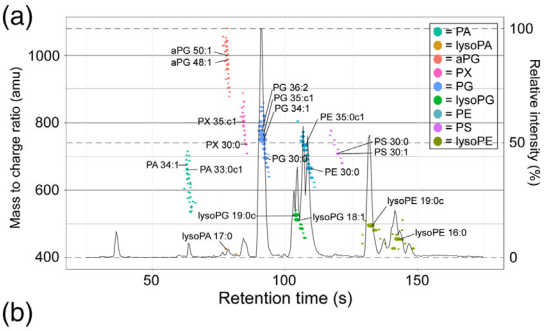
N_2). Bacterial chloroform/methanol extracts were passaged over a HILIC column and analyzed by mass spectrometry. This resulted in the detection of 203 different phospholipids with the phospholipid head group ethanolamine, glycerol, hydrogen, serine, or nonidentifiable factor X. The lipids belonged to 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%) (Figure 2(a), Table S2). 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 possesses a headgroup of a molecular mass of 133.075 (Figure 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 CL, LPG and 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 (Figure 2(b)). The complete list of lipid species that constitute the *C. jejuni* lipidome is presented in Table S2.

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 8 h, 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 *versus* 8 h at 10% O_2) and higher number of viable bacteria (Figure 3(a)).

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 (Figure 3(b)), 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 (Figure 3(c)).

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 (Figure 4). These changes occurred under both oxygen conditions tested but with clear differences. To better understand



(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							

Figure 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 is 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).

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 (Figure 5(a) and (b)). At 10%

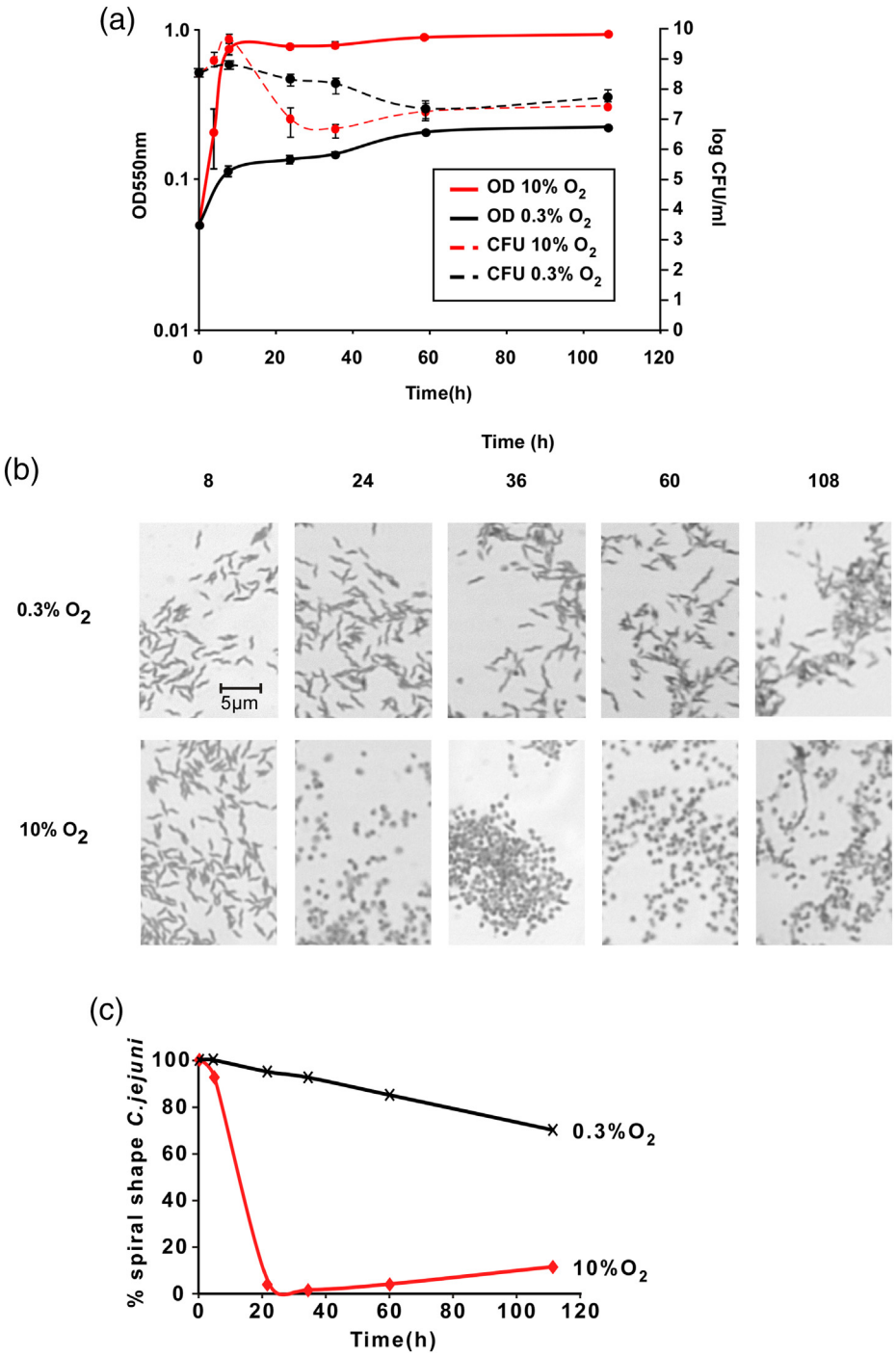


Figure 3. Effect of environmental conditions on *C. jejuni* behavior. (a) Growth of *C. jejuni* at 42 °C in HI medium under microaerobic (10% O_2, 10% CO_2, 70% N_2, 10% H_2) and oxygen limited (0.3% O_2, 10% CO_2, 79.7% N_2, 10% H_2) conditions. The optical density (left Y-as) as well as the colony 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.

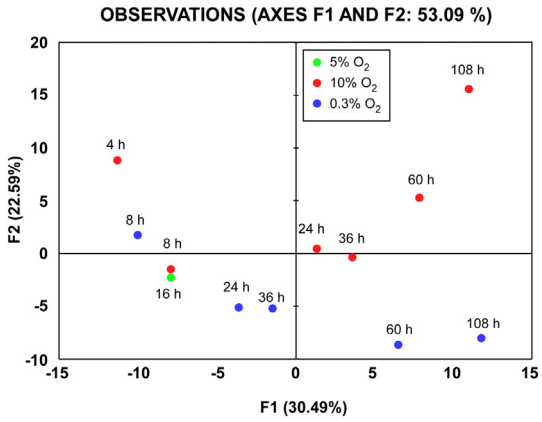


Figure 4. Phospholipidome dynamics in response to environmental conditions. Principal component plot showing the differences between the *C. jejuni* phospholipidome after 4, 8, 24, 36, 60 or 108 h growth in HI under the indicated different growth conditions. Each point represents the average of the data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

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 (Figure 5(a)). 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 (Figure 5(b)). 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.

Analysis of the phospholipid head groups revealed that in all logarithmic (4 or 8 h) growing cultures ethanolamine (>50%) was the predominant phospho-head group followed by glycerol with 40% (Figure 6(a)). 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 (Figure 3(a) and 6(a)). 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 (Figure 6(b) 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 (Figure 5(a) and (b)). 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- to 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 (Figure 5(a) and (b)). The percentage of lipids with saturated acyl chains remained relatively constant at about 25% under both oxygen conditions (Figure 6(b) and (c)). 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 (Figure 7(a), see also Table S3). 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 (Figure 1(b)) and the increase in cyclopropane rings in the late stationary phase fatty acids (Figure 6(b) 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% (Figure 7(b), see also Table S3), consistent with the

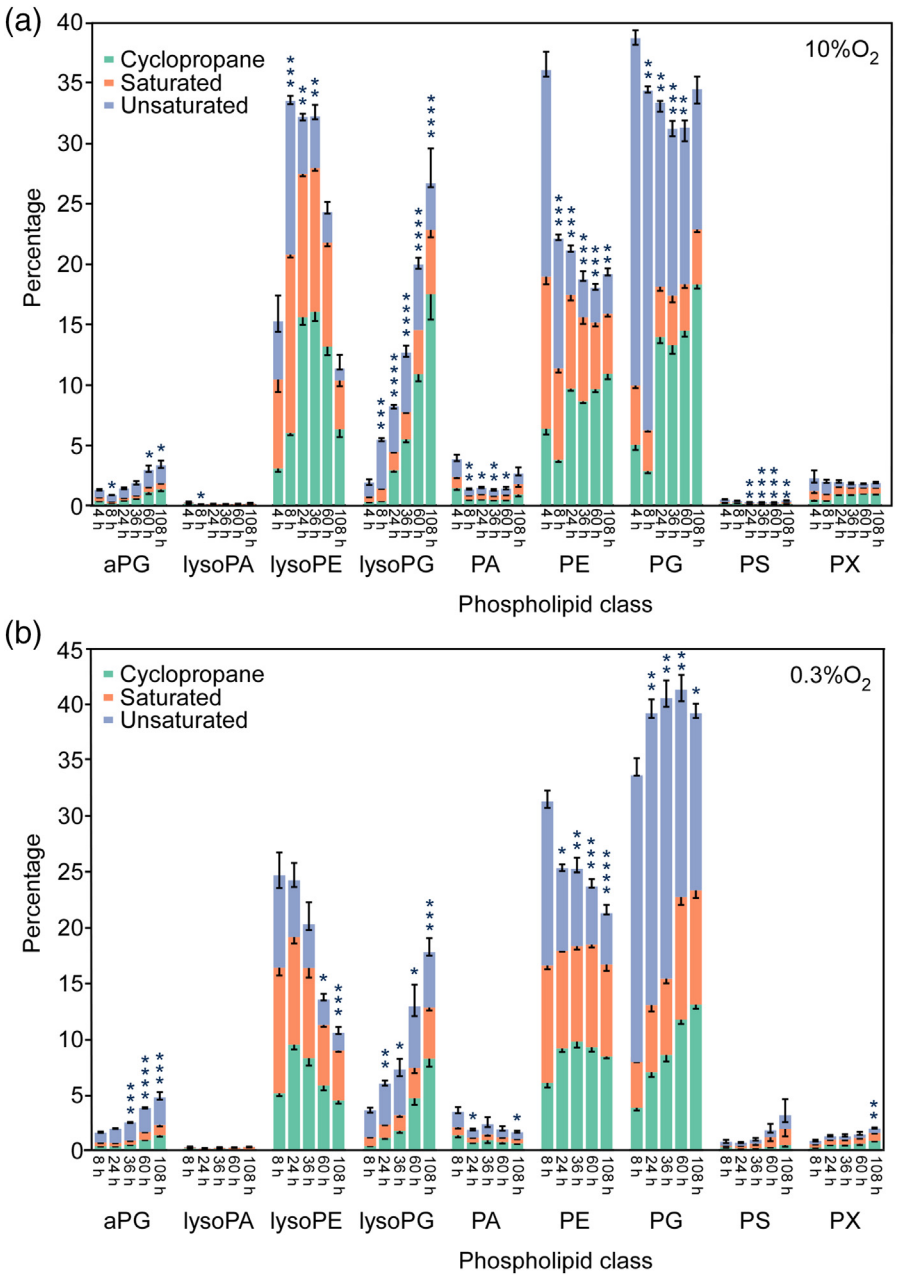


Figure 5. Influence of the age and oxygen concentration on the phospholipid classes of *C. jejuni.* Percentage phospholipid classes and the percentage unsaturated, cyclopropane bonds and saturated minus cyclopropane bond containing fatty acids per phospholipid class estimated by LS-MS during the growth 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 8 h) with the other time points. *$p < .05$, **$p < .01$, ***$p < .001$,****$p < .0001$. Data are representative of three independent experiments performed in duplicate.

assumed function of PldA as a phospholipase. The *pldA* mutant still contained a small percentage (6%) of lysophospholipids (Figure 7(b)), 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

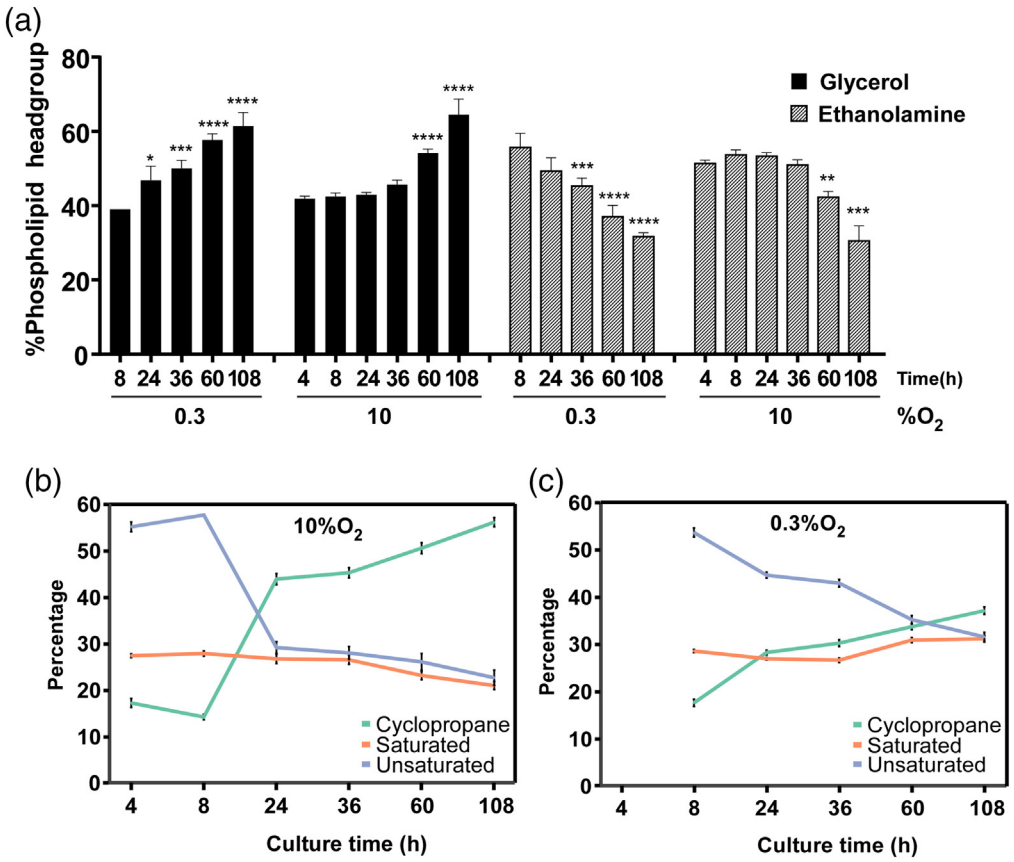


Figure 6. Influence of the age and oxygen concentration on the phospholipid headgroup and acyl chains. (a) Percentage of the phospholipids containing ethanolamine or glycerol as phospholipid headgroup at 10% O_2 and at 0.3% O_2. Percentage unsaturated, cyclopropane bonds and saturated minus cyclopropane 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 8 h) with the other time points. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$. Data are representative of three independent experiments performed in duplicate.

recovered intact after the phospholipid extraction (Figure 7(c)). 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.

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 wild-type 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 (Figure 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 (Figures 3(d) and 6(b) and (c)), we also compared the bacterial morphology of the *cfa* mutant and the wild-type strain using microscopy. No differences in the formation of the *C. jejuni* coccoids were observed. This suggests that cyclopropane-bond formation is not a prerequisite for coccoid formation. Inspection of the mutants for bacterial motility revealed that the *cfa* mutant was significant more motile than the wild-type under both oxygen conditions (Figure 7(d)). 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 (Figure 7(d)). This indicates that the *pldA* enzyme is

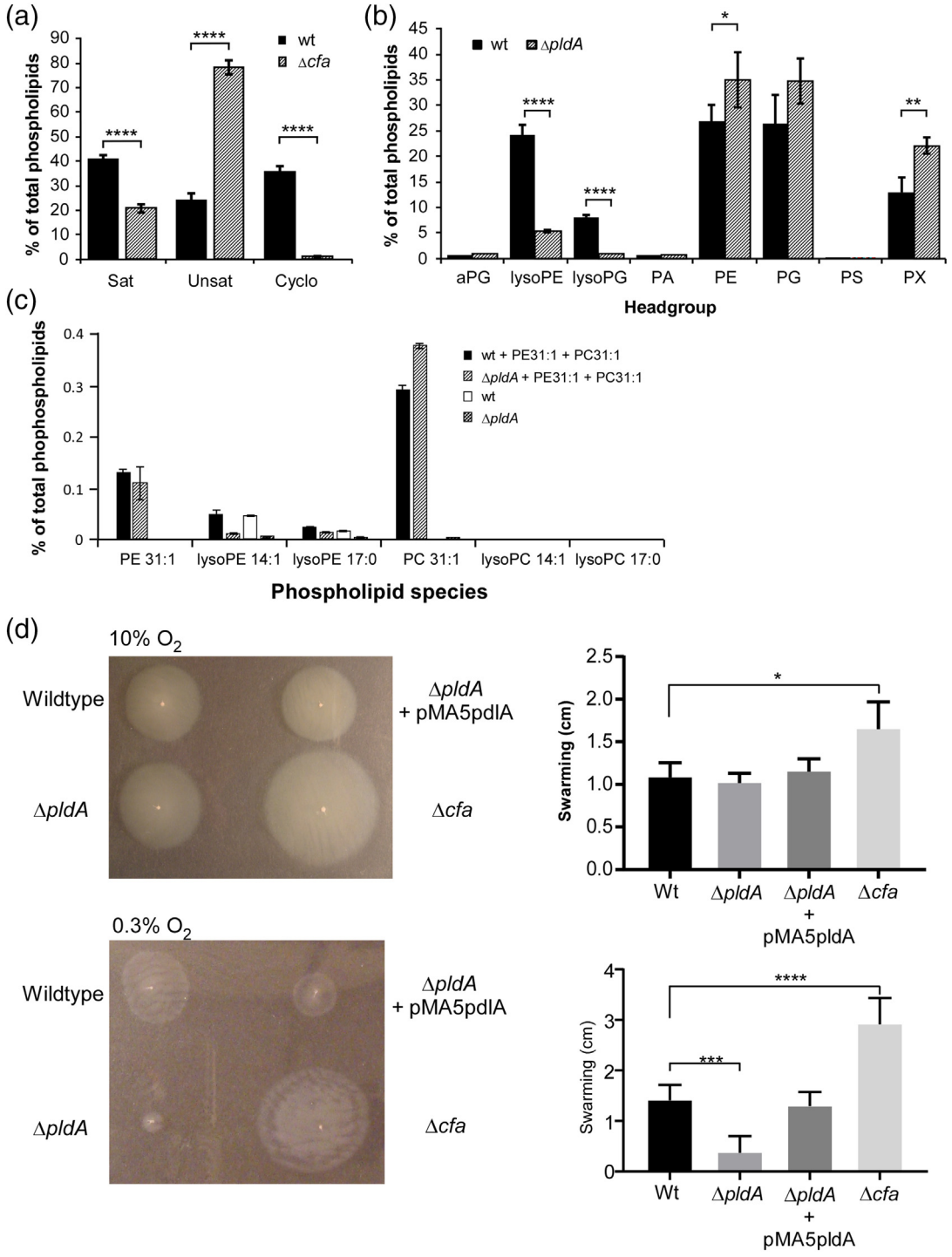


Figure 7. Phenotypic changes in *cf*a and *pldA* mutants. (a) Percentages of saturated, unsaturated and cyclo-propane bonds containing phospholipids in the *cfa* mutant and the parental *C. jejuni* strain 81,116 (wt). Data are the mean of three independent experiments. (b) Percentage of lipid classes in *pldA* mutant and wt *C.jejuni*. Data are mean ± SEM (n = 3) independent experiments. *$p < .05$, **$p < .01$, ****$p < .0001$. (c) Degradation of externally added phospholipids PE 31:1 and PC 31:1 during the phospholipid isolation procedure of the wild-type and *pldA* mutant. (d) Motility of the wt, *cfa, pldA* and complemented *pldA* mutant after stabbing into semi-solid medium and incubation under 0.3% or 10% oxygen conditions at 42 °C. Motility was scored of four independent experiments by measuring the diameter of the colonies.

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 nine 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 (Figure 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 (Figure 5 (a) and (b)). 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 (O.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 aging of the culture, phospholipids containing an ethanolamine as phosphohead group decreased to 25%, while lipids with glycerol containing headgroups increased by 25% (Figure 6(a)). This variation in lipids deviates from observations in the model organism, *Escherichia 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 CL [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 Figures 3(a) and 6(a)). 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 numbers of transcripts of these genes were all lower in stationary phase compared to logarithmic phase (Figure 1(b)). 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 (Figure 5(b)). 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 (Figures 5(a) and (b) and 7(b)). 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 (Figure 7(b)). 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 homologs of the lysophospholipidtransporter lplT exist in *C. jejuni,* which translocate lyso forms of glycerophospholipids across the cytoplasmic membrane in Gram-negative bacteria [4,35,11]

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 (Figure 7 (d)). 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*, *H. 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 (Figure 6(b)). 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 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 (Figure 7(a)). 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 (Figure 2(b)), 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 (Figures 3 (c) and 6(b) and (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 wild-type under both oxygen conditions (Figure 7(d)). 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 toward 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 (Figures 3(c) and 6(a)–(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 81,116 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 agar plates or in Luria-Bertani 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 81,116 DNA by PCR using the primer pairs PldA-F/PldA-R and Cfa-F/Cfa-R, respectively (Table S4). The ~3-kb PCR products were ligated into the pJET1.2/blunt cloning vector, resulting in the plasmids pJET*pldA* and pJET*cf*a. Inverse PCR was performed on these plasmids using the primers sets pldABamHI-F/pldABamHI-R and cfaBamHI-F/cfaBamHI-R, respectively (Table S4). 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* 81,116 wild-type 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* 81,116 with *pfu* polymerase (Promega) and the primers SacIpldafor and XbaIpldarev (Table S4). 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 *versus* coccoid shape) of every sample was determined by scoring 100 bacteria located in one field of a 100× 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 (1800***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 Jeucken *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 (1800***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 was 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 lyso-phospholipids 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 were 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]. Resulting data are given in Table S2 (lipidomic changes induced by age and oxygen concentration).

RNA-seq analysis

C. jejuni strain 81,116 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 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 81,116, 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.

Author Credits

X.C., J.P., and M.W. conceived the project. X.C. and M.W. designed the experiments.

L.H. prepared phospholipid samples and microscopic pictures. C.P. and S.H..

performed the RNA-seq studies. J.B. and C.H. performed all phospholipid data. X.C. engineered the mutants. X.C., J.P., D.K. and M.W. wrote the manuscript with contribution from all of the authors.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2020.07.012.

Acknowledgement

This work was supported by the China Scholarship Council grant 201706910078 to Xuefeng Cao.

Received 22 May 2020;
Received in revised form 14 July 2020;
Accepted 20 July 2020
Available online 22 July 2020

Keywords:
Campylobacter jejuni;
phospholipidome;
lysophospholipids;
PldA;
Cfa

Abbreviations used:
PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; LPG, lysyl-phosphatidylglycerol; PI, phosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine; PS, phosphatidylserine; Cfa, cyclopropane fatty acid synthase; PldA, phospholipase A; LysoPA, lyso-phosphatidic acid.

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