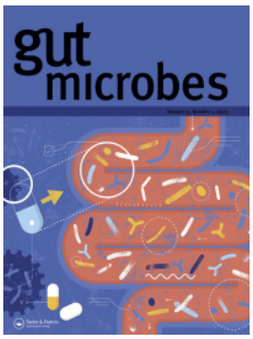

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Campylobacter jejuni benefits from the bile salt deoxycholate under low-oxygen condition in a PldA dependent manner

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ABSTRACT

Enteric bacteria need to adapt to endure the antibacterial activities of bile salts in the gut. Phospholipase A (PldA) is a key enzyme in the maintenance of bacterial membrane homeostasis. Bacteria respond to stress by modulating their membrane composition. *Campylobacter jejuni* is the most common cause of human worldwide. However, the mechanism by which *C. jejuni* adapts and survives in the gut environment is not fully understood. In this study, we investigated the roles of PldA, bile salt sodium deoxycholate (DOC), and oxygen availability in *C. jejuni* biology, mimicking an *in vivo* situation. Growth curves were used to determine the adaptation of *C. jejuni* to bile salts. RNA-seq and functional assays were employed to investigate the PldA-dependent and DOC-induced changes in gene expression that influence bacterial physiology. Survival studies were performed to address oxidative stress defense in *C. jejuni*. Here, we discovered that PldA of *C. jejuni* is required for optimal growth in the presence of bile salt DOC. Under high oxygen conditions, DOC is toxic to *C. jejuni*, but under low oxygen conditions, as is present in the lumen of the gut, *C. jejuni* benefits from DOC. *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 to oxidative stress. In conclusion, DOC stimulates *C. jejuni* growth under low oxygen conditions and alters colony morphology in a PldA-dependent manner. *C. jejuni* benefits from DOC by upregulating iron metabolism in a PldA-dependent manner.

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Introduction

Successful bacteria can adapt their physiology to changing environmental conditions.[1] The climate in the mammalian intestine varies from relative high oxygen availability near the epithelial border to almost zero in the lumen[2] while bile salts range from 0.2 to 2% (wt/vol).[3] Bile salts play an important role in food digestion but also act as effective natural antimicrobials.[4] To survive and colonize the human gastrointestinal tract, commensal and pathogenic bacteria must deal with high concentration of bile salts.[5,6] Bile salts are bile acids conjugated with taurine or glycine residues.[7] Bile acids can be formed in two ways: primary bile acids are intermediate products of cholesterol degradation and are synthesized by the liver, while secondary bile acids such as deoxycholate and litocholate result from the resident microbiota in the gut.[8] Many intestinal bacteria can transform primary bile salts into secondary bile salts by removing the hydroxyl group at C7.[9] This significantly increases the bile salt pool diversity.[10]

The release of bile salts into the intestine is one of the factors that the host utilizes to induce gut microbiome alterations.[11] Bile salts such as sodium deoxycholate (DOC) in the intestine can be used by bacteria as carbon source or electron acceptor[9,12,13] and can be an environmental signal to switch on virulence factors such as in *Shigella*, *Salmonella* and *Vibrio* species.[13–17] 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.[13] The well-known mechanisms by which enteric bacteria cope with bile salts are bacterial cell envelope modification (such as LPS O-antigen length);[18] CmeABC multidrug efflux pumps;[19] DNA repair[20,21] and the RpoS-dependent

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stress response.[22] Bile exposure also causes significant alterations in bacterial phospholipid profiles.[23]

The Gram-negative bacterium *C. jejuni* is the leading cause of foodborne enteritis in humans worldwide.[24] Contaminated chicken meat is believed to be the main source of infections.[24] *C. jejuni* is microaerophilic and requires reduced oxygen concentration to grow as exist in the intestine. In the human gut, *C. jejuni* penetrates the intestinal mucus layer, colonizes crypts, and disrupts the epithelial barrier. However, the molecular basis of *C. jejuni* infection remains poorly understood.[25] Recently, we determined the phospholipidome of *C. jejuni* grown under different conditions.[26] *C. jejuni* appears to make hundreds of different phospholipids, which display a high variation dependent on the environmental oxygen concentration and the age and of the Campylobacter culture. Very high amounts (30–50%) of the phospholipids of *C. jejuni* are lysophospholipids (LPLs).[26] The LPLs results for the most part, from the activity of the phospholipase A (PldA) enzyme that cleaves fatty acid tails from phospholipids. We and others have shown that micromolar concentrations of distinct bacterial LPLs are toxic to erythrocytes and epithelial cells.[26–28] Furthermore, a functional *pldA* gene is required to allow *C. jejuni* to colonize the cecum of chickens.[29] In the gut *C. jejuni* is exposed to bile salts which leads to the production of reactive oxygen species (ROS) which have been shown to cause DNA lesions in *C. jejuni* [30].

Considering the important role of oxygen availability, bile salts, and PldA in *C. jejuni* infection, we investigated the potential impact of these factors on *C. jejuni* using transcriptome analysis and assessment of bacterial growth. Wild-type and PldA-defective bacteria were cultured in the absence and presence of DOC under microaerobic (10% O_2) or low-oxygen conditions (0.3%) mimicking intestinal oxygen concentrations near the epithelial cell border versus in the lumen, respectively.

Results

Effect of PldA mutation on C. jejuni colony morphology

Independent of the oxygen concentration we noticed that the colony morphology of PldA mutant grown on blood free *Campylobacter* selectivity agar base, differs from that of the wild-type (Figure 1 and S1A). The *pldA* mutant had a wet/glossy colony appearance in contrast to dry/dull colonies of the *C. jejuni* wild-type (Figure 1a). The aberrant morphology of the mutant was restored by the introduction of a *pldA* complementation plasmid, yielding *C. jejuni ΔpldA + pldA*. Interestingly, all three strains formed wet or glossy colonies when grown on saponin agar charcoal plates (Figure 1B). One of the major differences between the two media is the presence of bile salts (0.1%) in the blood-free *Campylobacter* selectivity agar base. Indeed, the addition of DOC (0.1%) to saponin agar charcoal plate resulted in dry/dull colonies of the wild-type *C. jejuni* and *pldA* complemented strain, whereas the colonies of the *pldA* mutant retained their wet/glossy appearance (Figure 1c), all consistent with the colony phenotypes observed on blood-free *Campylobacter* selectivity agar. These data show that bile salts can alter *C. jejuni* morphology in a PldA-dependent manner. Transmission electron microscopy (TEM) and lipid oligosaccharide (LOS), of *C. jejuni* grown on blood-free *Campylobacter* selectivity agar base did not reveal differences between the wild-type and *pldA* mutant (Fig S1B, C & D).

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

To investigate whether exposure to bile salts influenced *C. jejuni* growth kinetics, the planktonic growth of *C. jejuni* wild-type 81,116, *C. jejuni ΔpldA*, and the complemented strain *C. jejuni ΔpldA + pldA* has been followed by measuring the culture optical density and by CFU counting. Growth was monitored in the presence or absence of 0.1% DOC and at 0.3% and 10% O_2. These experiments revealed similar growth kinetics for the three strains when grown in the absence of DOC, irrespective of oxygen concentration (Figure 2a, b and S2A & B). In the presence of 0.1% DOC, a clear inhibition of growth was observed for all three *C. jejuni* strains when grown under 10% O_2 concentration (Figure 2a & S2A). However, unexpectedly, under oxygen-limited conditions (0.3% O_2), the addition of DOC stimulated the growth of the *C. jejuni* strains

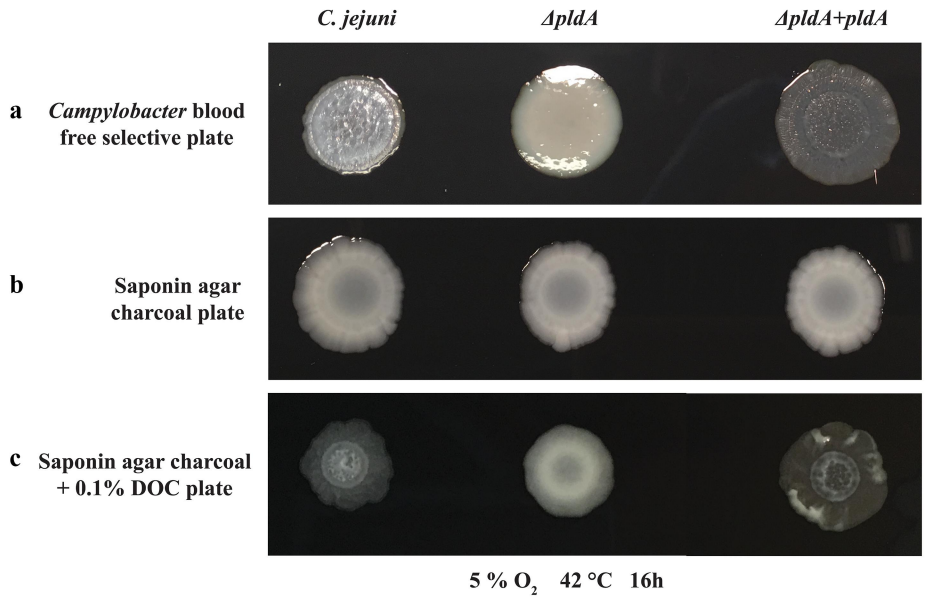

Figure 1. DOC modified *C. jejuni* wild-type colony morphology on *Campylobacter* blood free selective plate. Colonies of *C. jejuni* 81116 wild-type, *C. jejuni ΔpldA* and *C. jejuni ΔpldA+pldA* were grown on (a) Blood Free *Campylobacter* Selectivity Agar Base plate, (b) Saponin agar charcoal plate, and (c) Saponin agar charcoal + 0.1% DOC plate. Bacterial morphology was visualized after 16 h of incubation under oxygen limited (5% O_2, 10% CO_2, 75% N_2, 10% H_2) conditions at 42°C.

(Figure 2b & S2B). The maximum doubling times of the wild type increased from 5.5 h to 2 h and of the *ΔpldA* strain from 7,5 h to 7 h (Figure 2b) and the number of CFU/ml of the wild-type and complemented PldA strain was almost 3 times higher in the presence of DOC then without DOC (Fig S2B). These results suggest that oxygen concentration could influence 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 and CFU/ml of *C. jejuni ΔpldA* was clearly reduced compared to the wild-type at both 0.3% and 10% O_2 (Figure 2a, b, S2A &B). The maximum doubling time at 0.3% O_2 was 2 h for the wild type *versus* 7 h for *ΔpldA* mutant (Figure 2b). After 8 h, the number of CFU/ml of the wild-type strain was more than 2 times higher than *ΔpldA* strain (Fig S2B). At 10% O_2, the doubling time dropped to 2 h for the wild type strain and 2.2 h for the *ΔpldA* mutant (Figure 2a). The growth defect was restored after complementation of the mutant with an intact copy of the *pldA* gene (Figure 2a, b, S2A &B). Comparison of the LOS, capsule patterns, biofilm formation, and protein composition after growth in Heart-Infusion (HI) or HI plus 0.1% DOC, showed again no differences between the wild-type and *pldA* mutant (Fig S3). Overall, these results indicate that the PldA protein of *C. jejuni* is needed for optimal bacterial growth in the presence of DOC, irrespective of oxygen availability.

Effect of DOC on the membrane integrity

As it is known that DOC reduces the membrane integrity,[31] we stained equal amount of bacteria grown with or without DOC at 10 or 0.3% O_2 with a 1:1 mixture of the nucleic acid staining dyes SYTO-9 (Green) and propidium iodide (red) (Figure 3). SYTO-9 labels all bacteria, while propidium iodide penetrates only bacteria with a damaged membrane, causing a reduction in the SYTO-9 stain fluorescent. Most wild-type and *pldA* mutant bacteria grown at 10% O_2 had an intact membrane as they were labeled well with CYTO-9. When DOC was present a large number of bacteria were stained red and no bright green bacteria were observed, indicating that they all possess a damaged membrane. No difference could be observed between the strains cultured with or without DOC at 0.3% O_2, indicating that DOC does not affect the membrane integrity of these bacteria under oxygen-limited conditions.

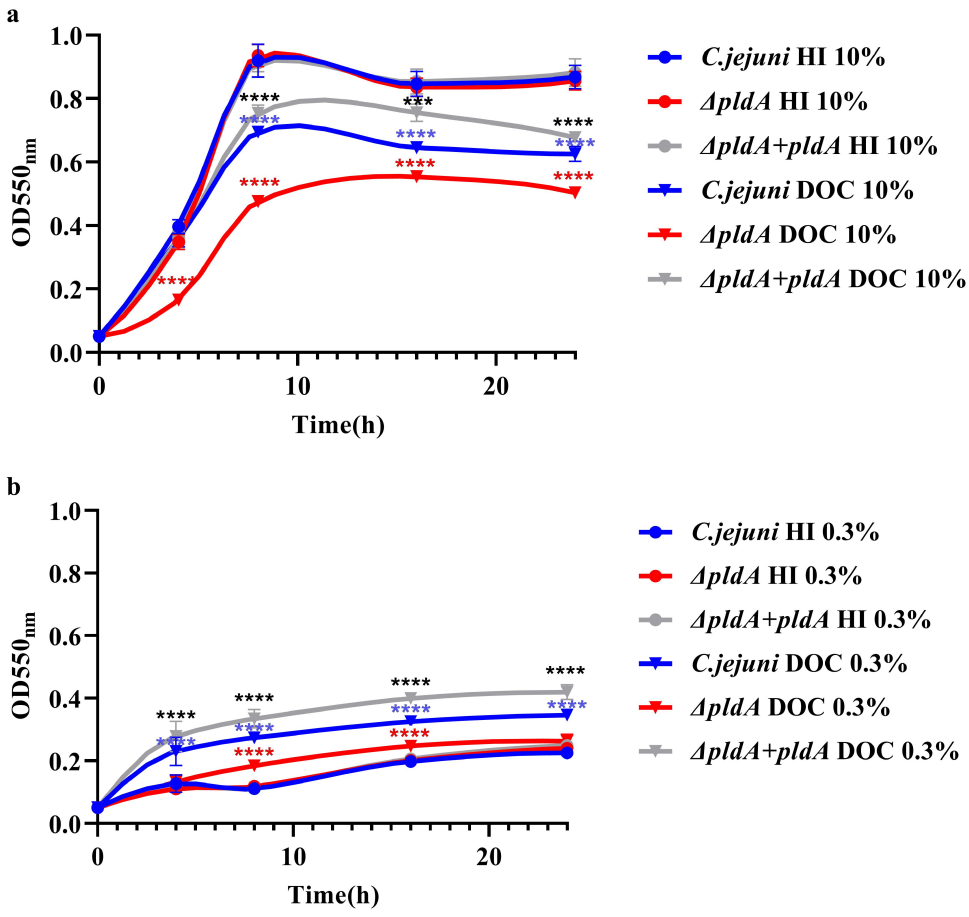

Figure 2. *C. jejuni* PldA is needed for optimal growth in the presence of DOC, but the oxygen concentration determines whether DOC improves or inhibits *C. jejuni* growth. Growth curves of *C. jejuni* wild-type, *C. jejuni ΔpldA* and *C. jejuni ΔpldA +pldA* were generated in HI with or without 0.1% DOC under (a) microaerobic (10% O_2, 10% CO_2, 70% N_2, 10% H_2) or (a) 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 represented as mean ± SEM. The displayed *P* values are calculated by comparing the HI vs HI + DOC treatment of the particular strain.****P < .0001, ***P < .001,*P < .1.

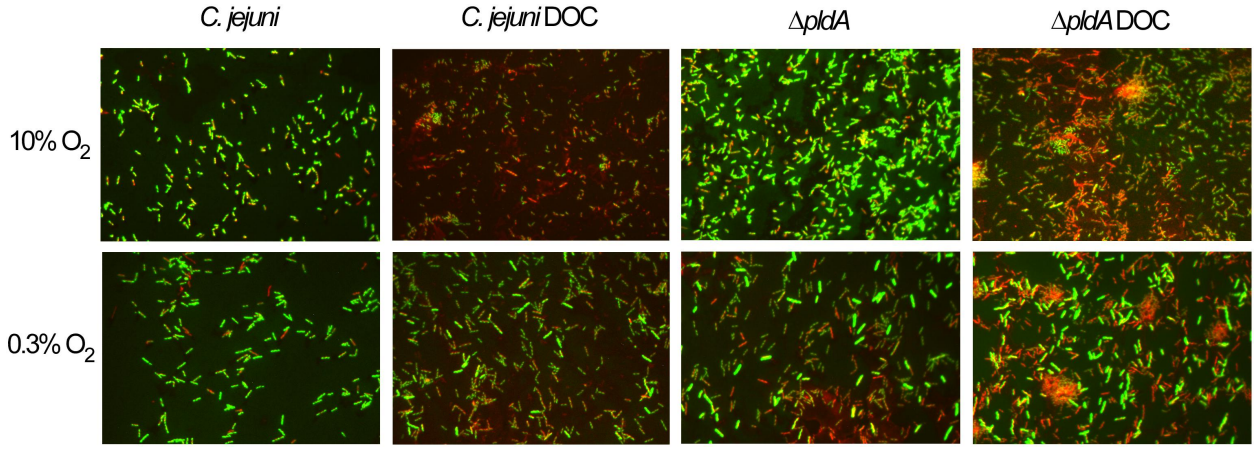

Figure 3. Influence of DOC on the membrane integrity of the *C. jejuni* wild-type and PldA mutant. Equal amount of bacteria grown with or without DOC at 10 or 0.3% O_2 were stained with a 1:1 mixture of the nucleic acid staining dyes SYTO-9 (Green) and propidium iodide (red). Slides with the stained bacteria were captured using EVOS fluorescent microscope (Thermo Scientific).

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 wild-type and *pldA* mutant grown for 6 h in HI broth or HI broth plus 0.1% DOC. First, we searched for DOC regulated genes in wild-type *C. jejuni* (Table S1A & B). Here, the RNA-seq results has been analyzed using the formula (*C. jejuni* wild-type - *C. jejuni* wild-type DOC), with a positive value indicating that DOC increases the transcription of the gene (upregulation), whereas a negative value indicates a reduction in transcription (downregulation). DOC induced a > 3-fold change in the transcription of 66 genes compared to bacteria grown without DOC (Table S1C). Real-time PCR of 13 highly regulated and previously reported DOC dependent genes[19,30,32] yielded no gross differences in results between the real-time PCR and RNA-seq data (Table S2A), verifying the RNA-seq results. The majority of the identified genes (44 of 66) were upregulated. These genes encode proteins involved in DNA repair (RecO), iron transport (C8J_0168-C8J 0169, C8J_1563, C8J_1564, p19),[33] tryptophan catabolism (TrpABF), multidrug efflux pump (CmeBC), RNA polymerase (RpoC), purine biosynthesis (PurH), LIV amino acid transport system (LivFGM), porphyrin metabolism (HemD), peptide translocation (C8J_1479), protein transport (SecY), and the leucine biosynthesis pathway (LeuBCD) (Figure 4, Table S1A & S1C). The products of the 22 downregulated genes were involved 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 the exposure of *C. jejuni* to DOC at low O_2 levels 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 seven genes that were also highly upregulated in *C. jejuni* wild-type (Table S1C). 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 the other 59 genes that changed upon exposure to DOC in the wild type were barely affected by DOC in the *pldA* mutant. This finding suggests that the expression of many DOC-sensitive genes is PldA-dependent.

Effects of PldA on C. jejuni gene expression

Comparison of the gene transcripts levels in *C. jejuni* wild-type and *C. jejuni ΔpldA* grown in the absence and presence of 0.1% DOC (Fig S5; Table S1D and S1E), yielded 19 genes that showed a > 3-fold difference, irrespective of the presence of DOC (Table S1F). In addition to the inactivated *pldA* gene, 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 heme-binding periplasmic protein C8J_0242; an aconitase AcnB;[34,35] two disulfide bridge introduction proteins DsbAB;[36] arylsulfate sulfotransferase C8J_0813; a sodium/proline symporter PutP and PutA, a proline dehydrogenase/delta-1-pyrroline-5-carboxylate dehydrogenase.[37] The six genes that were upregulated in the *pldA* mutant independent of DOC were 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 could be a sign of a shortage of iron availability, but this did not affect bacterial growth (Figure 2).

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

In HI medium, there was no growth rate difference between *C. jejuni* wild-type and the *ΔpldA* mutant (Figure 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 wild-type in medium with DOC. To identify the 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* wild-type DOC - *C. jejuni ΔpldA*

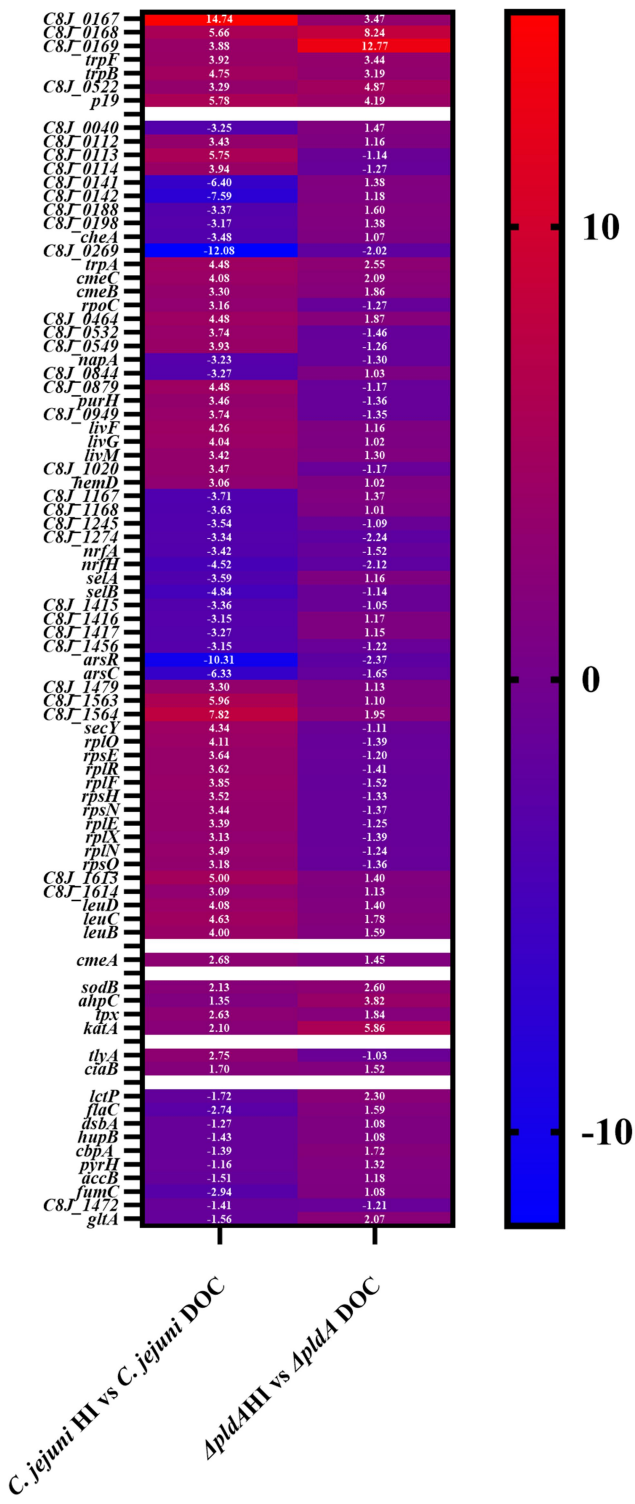

Figure 4. Genes affected by the addition of DOC in *C. jejuni* wild-type and its isogenic *ΔpldA* mutant. Heatmap and hierarchical clustering of the highly regulated genes and reported reference genes are presented. The experiments were repeated three times.

DOC)- (*C. jejuni* wild-type HI - *C. jejuni ΔpldA* HI)]. Because differences in gene expression between the two strains cultured in HI medium (*C. jejuni* wild-type HI - *C. jejuni ΔpldA* HI) did not directly affect bacterial growth rates, this difference was removed as a background. A positive value indicated that DOC in a PldA-dependent manner increased the transcription of the gene (upregulation), whereas a negative value indicated a reduction in transcription (downregulation).

This analysis revealed several 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; Table S1H). The gene *C8J_0113* encodes 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).[38] The genes *C8J_0532*, *C8J_0879*, *C8J_0949*, *C8J_1591* and *C8J_1620* encode hypothetical proteins. Genes *C8J_1593 C8J_1595* and *C8J_1596*, encoded three ribosomal proteins. The gene *C8J_1310* encodes an MmgE/PrpD family protein which is involved in propionate catabolism.[39] *C8J_1563* encodes an iron ABC transporter permease. Besides *C8J_1563*, three iron metabolism genes (*C8J_0167, C8J_1564, C8J_1565*) (Table S1H) were also downregulated and another three genes (*C8J_1548, C8J_1549*, *C8J_1550*) (Table S1H) were not directly involved in iron metabolism but were proposed to be downregulated by iron limitation.[40,41] Together, these data 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 several numbers of genes that were upregulated by DOC (>5-fold). These include *C8J_0141*, *C8J_0142*, *C8J_0188*, *C8J_0269*, *C8J_1167*, *C8J_1272*, *C8J_1302* and *C8J_1487* (Figure 5; Table S1H). *C8J_0141*, *C8J_0142*, *C8J_0188*, *C8J_0269 and C8J_1302* encoded four hypothetical proteins. *C8J_1167* is a hemerythrin-like non-heme protein.[42] *C8J_1272* encodes an MGC82361 protein, *C8J_1487* encodes a flavohemoprotein which is important for reducing oxidative stress. Based on previous studies, *C8J_0141* and *C8J_0142* play a role in peroxide stress defense.[43,44] *C8J_1167* is involved in oxygen storage and transport[42] and gene *C8J_1487* encodes a single-domain hemoglobin involved in oxidative stress defense. In addition to the highly upregulated genes, *ahpC*, *hspR*, *katA-C8J_1304* and *C8J_1514*

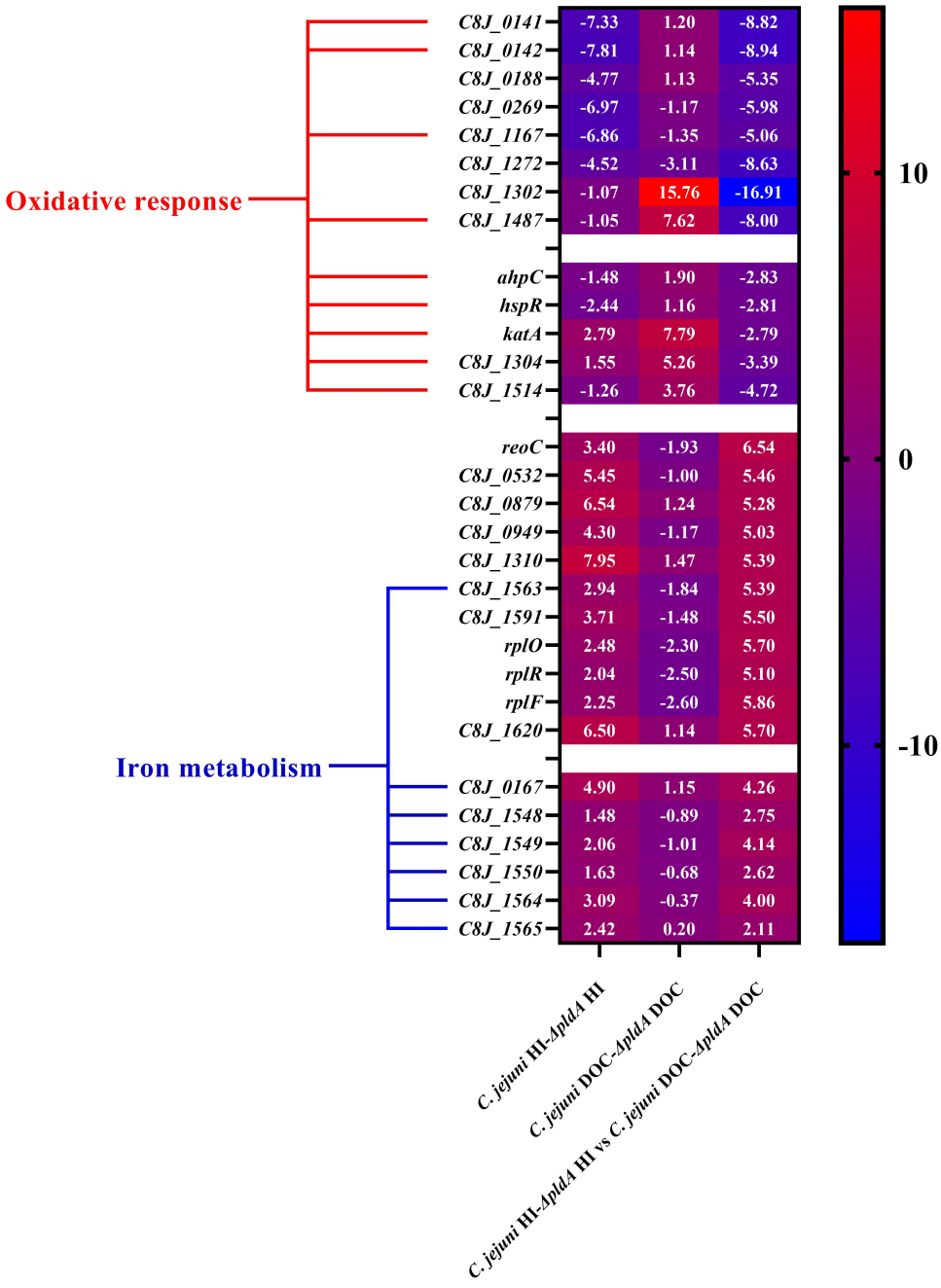

Figure 5. Transcription of genes dependent on the availability of DOC and a functional *pldA* gene. The heatmap and hierarchical clustering of the highly regulated genes are presented. The RNA-seq experiments were repeated three times with similar results.

which are involved in the oxidative/aerobic stress response are upregulated as well.[45–49] Other well-studied oxidative stress resistance genes including *ahpC*, *hspR*, *katA*, *C8J_1304* and *C8J_1514* are also upregulated (Table S1H). The putative functions of the upregulated genes suggests that DOC exposure elicits an oxidative response, particularly in the absence of PldA.

DOC reduces efficient iron utilization by the PldA mutant

Based on our RNA-seq analysis, PldA appears to play a crucial role in iron metabolism; therefore, we investigated whether the reduced growth of the *pldA* mutant in the presence of DOC could be rescued by providing additional ferric or ferrous

iron sources. Hereto growth curves were generated for the wild-type 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 were observed between the strains grown in HI or HI with 50 µM Fe^{2+} or Fe^{3+} (Figure 6a). In the presence of DOC, the growth of wild-type bacteria improved when 50 µM Fe^{2+} or Fe^{3+} was added to the media. In contrast DOC together with the addition of iron lowered the optical density of the *pldA* mutant (Figure 6b). This is consistent with the observed down-regulation of iron-regulated genes in the DOC-exposed *pldA* mutant, indicating that iron uptake in the *pldA* mutant is highly restricted in the presence of DOC, thereby limiting the bacterial growth of the *pldA* mutant.

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

The apparent upregulation of 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* wild type, as determined by CFU counting (Figure 7a). Without H_2O_2 treatment, in HI medium with or without 0.1% DOC no difference in CFU between *C. jejuni* wild-type and *C. jejuni ΔpldA* strains was observed (data

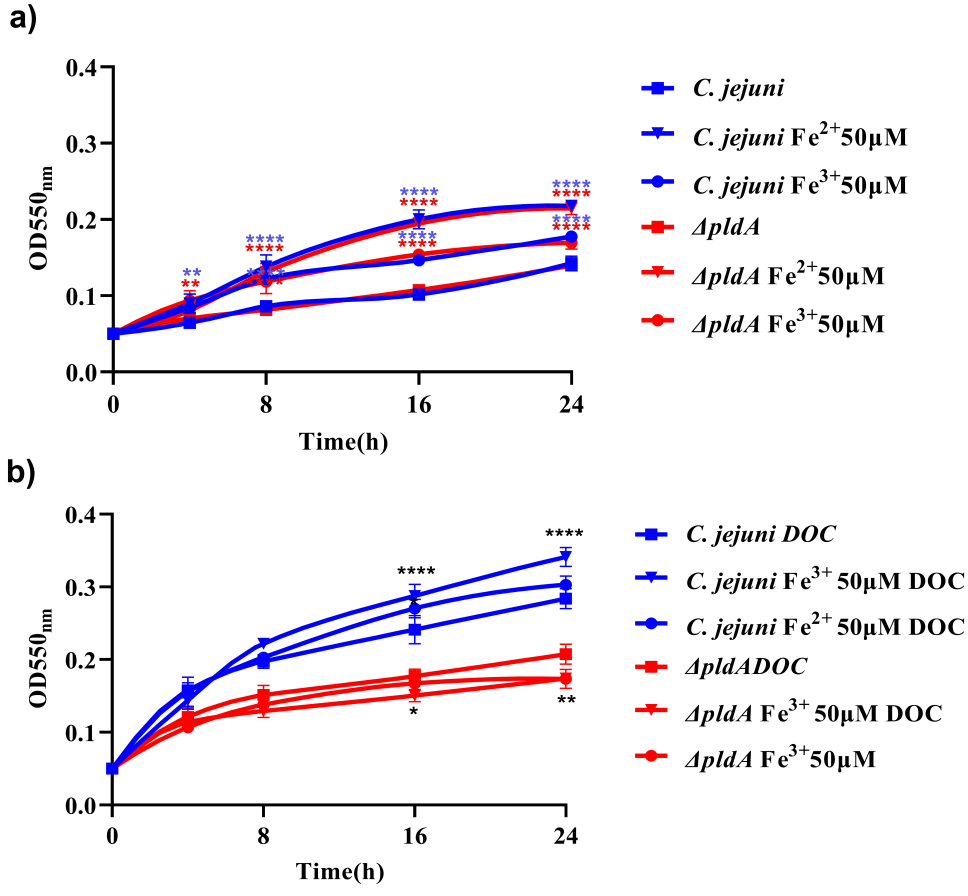

Figure 6. Growth curves of wild-type and *pldA* mutant with or without DOC and the addition of ferric or ferrous iron. *C. jejuni* wild-type and *ΔpldA* were grown (a) without or (b) with DOC with or without Fe^{2+} or Fe^{3+} at 42°C in HI, under oxygen limited (0.3% O_2, 10% CO_2, 79.7% N_2, 10% H_2) conditions. The optical density (Y- as) at the indicated time points are shown. The experiments were repeated three times in duplicate. Data are represented as mean ± SEM. ****$P < .0001$, **$P < .01$, *$P < .1$.

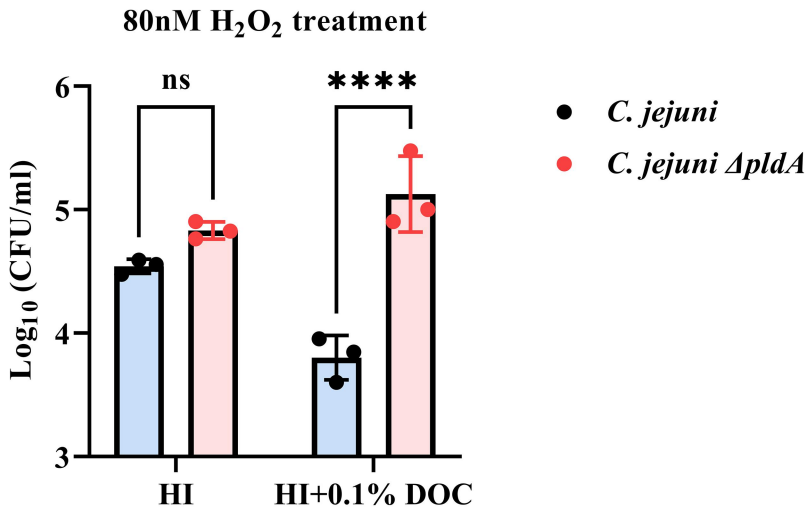

Figure 7. Survival assay to evaluate the susceptibility of *C. jejuni* wild-type and C. *jejuni ΔpldA* to oxidative stress. Survival assays of *C. jejuni* wild-type and *C. jejuni ΔpldA* strains were performed in HI with or without 0.1% DOC exposed to 80 nM H_2O_2 under microaerobic conditions for 30 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, ****$P < .0001$, ns $P > 0.1$.

not shown). These results are consistent with the RNA-seq data, suggesting that in the presence of DOC the PldA mutant upregulates oxygen stress systems much better than wild-type *C. jejuni*.

Discussion

Bile salts play an important role in the digestion of fat, but are also important as antimicrobial molecules to control different bacterial species in the gut.[13] Bacteria have evolved various mechanisms to avoid the toxicity of bile salts.[13] The Gram-negative bacterium *C. jejuni* inhabits the intestine of many mammals where it is exposed to bile salts in an oxygen-limited environment.[50,51] The adaptation of *C. jejuni* to bile has previously been investigated under the microaerobic (5% O_2) conditions as present close to the epithelial cell layer, however in the lumen the oxygen concentration is between 0.1–1%.[19,30,32,52–54] In this study we provide evidence that DOC stimulates *C. jejuni* growth under low-oxygen conditions and alters colony morphology in a PldA-dependent manner. Transcriptomic and functional assays indicate that PldA-dependent and DOC-induced changes in gene expression 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 to oxidative stress.

We have previously shown that PldA activity results in high levels of lysophospholipids in *C. jejuni*. [26] This may influence the colony phenotype in the presence of bile salts. It can be inferred that exposure to DOC influences bacterial viability depending on the presence of LPLs, resulting in a change in colony morphology. Alternatively, at high percentage of LPLs may cause changes in the surface characteristics that become apparent in the presence of DOC. Outer membrane proteins are known to be important for bacterial colony morphology, as they facilitate the transport of intracellular components to the cell surface.[55] We previously showed that PldA influences biological functions such as flagella-driven motility, even without apparent changes in the capsule, LOS, or protein profiles.[26] This suggests that this effect may be related to alterations in *C. jejuni* metabolism.

To further investigate the influence of DOC on PldA activity, the growth of the wild-type and *pldA* mutant during planktonic growth was monitored. DOC improved the growth of *C. jejuni* under oxygen limited conditions (0.3% O_2) (Figure 2b & S2B), whereas it reduced the growth in an environment with 10% oxygen (Figure 2a & S2A). At 10% O_2 the membrane integrity was strongly reduced by DOC, but at 0.3% O_2 DOC has no influence on membrane integrity (Figure 3). Bile salts are toxic to many bacteria owing to their amphiphilic character.[9] Under microaerobic conditions DOC

has been shown as we also have observed to reduce the growth of *C. jejuni* probably due to the accumulation of reactive oxygen species (ROS) and the occurrence of DNA lesions.[30] It might be that bile salts are less toxic for certain gut bacteria under anaerobic conditions as has been observed for *Listeria* monocytogenes.[9,56] *C. jejuni* is considered to be bile resistant, as it can be isolated from the gallbladder and even directly from bile.[57–59] Many aerobic bacteria are known to degrade bile salts, but only a few are known to use bile salts as electron acceptors or carbon sources.[9,12,60] For these bacteria bile salts are useful compounds and stress factors at the same time. To the best of our knowledge, this is the first bacterium to benefit from the presence of DOC in an oxygen-dependent manner.

Transcriptomics were performed to better understand how *C. jejuni* benefits from DOC and the role of PldA in this process. 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.[19,30,32] Results in this study indicate that a number of these genes were also upregulated under the low oxygen conditions that were tested (Table S1C). A general finding was that at low O_2, DOC exposure increased the transcript levels of a number of iron-regulated genes. This is likely due to the well-known iron-chelating effect of bile salts[31] 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 the expression of genes involved in iron acquisition and metabolism, and to promote bacterial growth under iron-deficient conditions.[61]

In the presence of DOC, the transcript levels of several iron transport genes have been found were significantly downregulated in *C. jejuni ΔpldA*. This indicated that in the presence of DOC, *C. jejuni ΔpldA* (at low O_2) suffered from iron starvation and PldA activity might aid the efficient absorption of iron by *C. jejuni*. This hypothesis is consistent with the previous findings that bacterial pathogens can gain access to additional intracellular iron pools through the upregulation of phospholipase expression.[62] The involvement of PldA in the iron acquisition has recently also been observed in *Pseudomonas aeruginosa* where phospholipase PlaF regulates the iron uptake.[63] Growth curve experiments results showed that the addition of extra ferrous or ferric iron did not rescue the growth defect of *C. jejuni ΔpldA* (Figure 6) but significantly improved the growth performance of *C. jejuni* wild-type, indicating that iron metabolism in the *pldA* mutant (at low O_2) is strongly limited. Thus, the ability to acquire iron may also contribute to the PldA-dependent increase in *C. jejuni* growth promotion in the presence of DOC.

RNA-seq analysis also showed alterations in the transcript levels of genes involved in the bacterial oxidative stress response. The results suggest that, in the presence of DOC, the *C. jejuni* wild-type was more susceptible to ROS than *pldA* mutant (Figure 5). This hypothesis is supported by the results of the oxidative stress survival assay (Figure 7). It should be noted that all of 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 *C. jejuni* oxidative stress defense and iron transport systems.[43,64] Oxidative stress induced by iron deficiency is a common feature in bacteria.[65–67] The results suggest iron starvation in *C. jejuni ΔpldA* leads to the activation of Fur-regulated oxidative stress-defending genes, which causes the *pldA* mutant to be less vulnerable to oxidative stress than the wild-type. Previous studies by both Palyada et al.[44] and Holmes et al.[43] also identified that when *C. jejuni* are starved with iron, the expression of oxidative stress defense genes including *ahpC*, *katA*, *C8J_1304* are upregulated. Consistent with our data, but also confirmed that in the presence of DOC, *C. jejuni ΔpldA* suffers from intracellular iron deficiency. Fur independent iron transporters like the FeoAB,[68] may be more active at microaerophilic conditions as we did not observe growth difference at 10% O_2 levels (Figure 2a) but this needs to be further explored.

A third physiological system in *C. jejuni* that may be influenced by exposure to DOC is tryptophan and branched-chain amino acids (leucine, isoleucine and valine) metabolism. It is currently unknown how *C. jejuni* benefits from the upregulation of tryptophan and high-affinity branched-chain amino acids gene pathways during exposure to bile salts. The bacterial

tryptophan catabolite indole together with bile acids can regulate epithelial inflammation and gut immunity, and branched-chain amino acids support evasion of host defenses.[69,70] In eukaryotic cells, elevated bile acid levels have been implied to be relevant to abnormal tryptophan metabolism, and bacteria that produce branched-chain amino acids such as leucine are known to affect bile hemostasis by conjugating bile acids.[71,72] Thus, it can be inferred that an increase in these amino acids is important for the pathogenesis of *C. jejuni*.

PldA has long been considered an essential factor in the intestinal colonization of *C. jejuni* as well as other enteric pathogens.[29,73] However, the underlying mechanisms have not been completely elucidated. Previously we reported that, in the absence of PldA, *C. jejuni* is less motile under limited oxygen conditions.[26] RNA-seq analysis revealed decreased transcripts of the thiol-disulfide oxidoreductase forming gene *dsbA* in the *pldA* mutant (Table S1F). 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*.[36,74,75] Thus, reduced DsbA expression might explain the motility defect in *C. jejuni ΔpldA* at low O_2. The bacterial respiratory electron transfer chain participates in the formation of DsbA[76] however whether the transcription of *dsbA* is regulated by oxygen availability awaits future study.

Conclusions

The present study demonstrates that the processes of bile resistance, oxidative stress defense, and iron acquisition of *C. jejuni* are tightly linked and that bile salts in conjunction with PldA promote *C. jejuni* growth and survival in a low oxygen environment. Other enteric bacteria might utilize similar mechanisms to defend against the toxic components of bile and optimally adapt to the intestinal niche.

Limitations

In this study, we utilized the RNA-seq assay to investigate how *C. jejuni* benefits from the DOC under low-oxygen condition in a PldA dependent manner and concluded that bile resistance, oxidative stress defense, and iron acquisition might played essential roles in this process. However, the mutant strains of key genes which were highly regulated by DOC in a PldA dependent manner were not constructed to validate the inference. In the further studies, it is important to verify the hypothesizes in this study by determine the planktonic growth character of those mutant strains.

Material and methods

Bacteria culture

C. jejuni wild-type strain 81,116, is a human isolate originally isolated from a waterborne outbreak,[77] its isogenic *pldA* mutant (*C. jejuni ΔpldA*), and the complemented *pldA* mutant (*C. jejuni ΔpldA +pldA*)[26] was 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 medium was 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 were incubated for 16 h under microaerophilic conditions at 42°C. Morphology of bacteria colonies were visual examined by size, shape, color, opacity, and consistency and imaged using a were obtained with a 10-megapixel Nikon D200 camera.

Electron microscopy

Electron Microscopy was performed as described before.[78] In brief, *C. jejuni* wild-type and *C. jejuni ΔpldA* were grown in HI broth with 0.1% DOC under microaerophilic conditions (5% O_2, 10% CO_2, 75%N_2, 10% H_2) for 16 h at 42°C. Bacteria were collected by centrifugation (10 min, 3,000×g), washed three times with Dulbecco's phosphate-buffered saline (DPBS, Thermo Fisher Scientific) and resuspended in DPBS to a final OD_{550} of 1. Carbon activated copper grids were incubated with 10 µL of the bacterial culture resuspended for 10–30 min and washed three times with DPBS. 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. The grids were looped out of the solution and air dried. Samples were imaged using a Tecnai-12 electron microscope (FEI, Hillsboro, Oregon, USA).

LOS detection

LOS isolation and staining were performed as described before.[79] In brief, *C. jejuni* strains were grown under oxygen-limited conditions at 42°C on Campylobacter blood-free selective agar base plates or in 5 ml HI or HI plus 0.1% DOC medium for 24 h. Bacteria colonies were taken up in 1 ml DPBS. 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 Proteinase K (20 mg/mL) overnight at 55°C. Three times Laemmli buffer was added, and the samples were loaded onto 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 with 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 using the cationic dye Alcian Blue, as previously reported.[80] 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 (1×10^8 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 onto 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 using a crystal violet staining assay, as reported[81]. 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 condition 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. The biofilms were stained for 20 min at room temperature, washed with H_2O, and air dried. Stained biofilms were imaged on a white paper using a Sony Alpha 6700 camera.

SDS-PAGE

The *C. jejuni* wild-type and *C. jejuni ΔpldA* were grown in HI medium with or without DOC for 16 h at 42°C under microaerophilic conditions and then diluted to an OD_{550} of 1 with DPBS. Bacterial samples were mixed with 3× Laemmli Sample Buffer, lysed, and denatured at 95°C for 15 minutes.

Samples were loaded into equal volumes (10 µL) onto a 12% acrylamide gel. Gels were run for 30 min at 50 V and then for 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 using Universal Hood III (Bio-Rad).

Bacterial growth assay

C. jejuni wild-type, *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}) as well as viable counts (CFU/ml) of the cultures was measured after 0, 4, 8, 16 and 24 h of growth. The values are the means of three independent experiments performed in duplicate.

Membrane integrity

C. jejuni wild-type,and the *ΔpldA* mutant were grown in HI medium with or without 0.1% DOC for 16 h at 42°C under high or oxygen-limited conditions. Equal amount of bacteria were three times washed with physiological salt (0.9% NaCl) and bacteria were taken up in 300 µl 0,9% NaCl. Bacteria were incubated for 15 minutes with 1 µl 1:1 mixture SYTO-9 dye (2,34 mM) and propidium iodide (20 mM) (Live/dead Baclight Bacterial viability kits, Thermo fisher Scientific). Slides with the stained bacteria were captured using EVOS fluorescent microscope (Thermo Scientific) at 100X magnification and equal light intensity between the slides quantified using EVOS software.

RNA-seq

C. jejuni wild-type 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 an RNA-Bee kit (Tel-Test). RNA samples were treated with RNAse-free DNase I (Invitrogen) according to the manufacturer's instructions. RNA-Seq was performed as previously described[82]. Briefly, the total RNA for each sample was rRNA depleted by utilizing the Ribo-zero Magnetic Kit for Gram-negative bacterial (Illumina) following instructions of option 1 manufacturer. Libraries for the Illumina MiSeq were processed using the llumina® cDNA synthesis & the illumina® RNA Prep Ligation kit (Illumina) and the IDT for Illumina RNA UD indexes Set A, following instructions of manufacture. Pooled libraries were sequenced on a HiSeq4000, read length 150bp by Macrogen Europe (Amsterdam, The Netherlands).

The fastq sequences were trimmed to remove inferior bases and assembled into reference genome CP000814 using Bowtie within Geneious 11.1. Geneious software was used to calculate the normalized transcripts per million (TPM) and DEseq2 method to compare expression levels between control growth conditions.

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

Real-time RT-PCR was performed as previously described.[83] Primers used in this study are listed in Table S2B. The calculated threshold cycle (Ct) for each detected gene amplification was normalized to the Ct value for the housekeeping gene *rpoD* amplified from the corresponding sample, before calculating the fold change using the arithmetic formula ($2^{-\Delta\ \Delta Ct}$).[84] Each sample was subjected to three independent preparations RNA.

Oxidative stress resistance assay

Bacteria oxidative stress resistance was measured as described before.[85] *C. jejuni* wild-type and *C. jejuni ΔpldA* mutant 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 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 prepared and plated onto saponin agar plates. Plates were incubated at 42°C under microaerophilic conditions and colonies were counted after 24 h.

Statistical analysis

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

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