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Cj1411c Encodes for a Cytochrome P450 Involved in *Campylobacter jejuni* 81-176 Pathogenicity

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Abstract

Cytochrome P450s are *b*-heme-containing enzymes that are able to introduce oxygen atoms into a wide variety of organic substrates. They are extremely widespread in nature having diverse functions at both biochemical and physiological level. The genome of *C. jejuni* 81-176 encodes a single cytochrome P450 (*Cj1411c*) that has no close homologues. *Cj1411c* is unusual in its genomic location within a cluster involved in the biosynthesis of outer surface structures. Here we show that *E. coli* expressed and affinity-purified *C. jejuni* cytochrome P450 is lipophilic, containing one equivalent Cys-ligated heme. Immunoblotting confirmed the association of cytochrome P450 with membrane fractions. A *Cj1411c* deletion mutant had significantly reduced ability to infect human cells and was less able to survive following exposure to human serum when compared to the wild type strain. Phenotypically following staining with Alcian blue, we show that a *Cj1411c* deletion mutant produces significantly less capsular polysaccharide. This study describes the first known membrane-bound bacterial cytochrome P450 and its involvement in *Campylobacter* virulence.

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Introduction

During the past few decades *Campylobacter* has been identified as the most common cause of bacterial diarrheal illness in the European Union and worldwide [1]. This pathogen is also responsible, in some patients, for severe neurological diseases including Guillain-Barré syndrome [2]. Over 95% of campylobacteriosis cases are endemic, the rest attributable to outbreaks, usually due to contaminated private water supplies or unpasteurised milk [3]. *Campylobacter jejuni* 81-176 was originally isolated from a 9-year-old girl with diarrhea, and it was shown to cause severe disease in human patients [4].

Cytochromes P450s are a superfamily of proteins with a maximum absorption at 450 nm and are characterized by the presence of a conserved Cys residue [5]. This cysteine distinguishes cytochrome P450s from other oxygen activating enzymes such as globins and peroxidases that utilize histidine during the reaction with hydroperoxide [6]. The number of cytochrome P450 proteins encoded in bacteria is variable. *Escherichia coli*, for example, has no cytochrome P450 encoded in its genome, whereas *Mycobacterium tuberculosis*

contains twenty and *Streptococcus coelicolor* eighteen P450 cytochromes [7]. The bacterial cytochrome P450 enzymes have functions that include camphor degradation [8] and biotin synthesis [9]. The genome of *C. jejuni* 81-176 encodes a single cytochrome P450, *Cj1411c* (CYP1411c).

The CYP1411c coding sequence is 1359 bp long (453 amino acids). It is located in a genomic region that mainly encodes proteins known to be involved in bacterial outer surface biosynthesis, and amino-acid transferases. The function of the *Campylobacter* enzyme cannot be inferred from sequence comparisons, but the location of the coding sequence at the downstream end of the large *Kps* gene region (capsule biosynthesis cluster) indicates a possible function in the biosynthesis of cell surface components [10].

Outer surface structures are important in *Campylobacter* pathogenesis. They have been shown to be responsible for adherence and invasion, colonization and disease, maintenance of cell surface charge and serum resistance [11-14]. In this study we show that CYP1411c is a membrane-bound cytochrome P450, that when mutated significantly impairs *C. jejuni* 81-176 pathogenicity. In addition, CYP1411c

efficiency was calculated as the average of the total number of CFU/total initial inoculum. *C. jejuni* 81-176 passaged in RPMI 1640 (without cells) was also tested for the ability to adhere to and invade HCT-8 cells. All assays were conducted in triplicate and repeated independently three times. The significance of differences in adhesion and invasion between samples was determined using the Student *t* test. A *P* value of <0.05 was defined as significant.

Cell infection for RNA extraction

HCT-8 cells were seeded on 12 well plates at a density of 10^8 /well and allowed to grow overnight to 60-70% confluence. Agar-grown bacteria were used to prepare the inocula for infection experiments. Bacteria were harvested from plates and suspended in RPMI 1640 medium. Six well plates, containing HCT-8 cells, were inoculated with 1 ml of bacterial suspension with OD_{600} of 0.4. HCT-8 cells were infected at 37°C under microaerobic conditions (5% O₂, 5% CO₂, and 90% N₂) for 1.5 and 3h. Viable counts were determined for inocula at each passage (both with and without host cells) to ensure that similar numbers of live bacteria were present at each stage of infection and for non-infected controls.

RNA extraction and qPCR

Total RNA was isolated from internalized bacteria by using the RNeasy Kit (Qiagen). The RNA was reverse transcribed using the High Capacity cDNA reverse transcription kit (Applied Biosystems, CA) according to the manufacturer's protocol. The mRNA levels were determined by quantitative RT-PCR using SYBR Green (Applied Biosystems, CA) on a 7900 Fast Real-Time System (Applied Biosystems, CA) and the primers qP450F (5'-atgagtgatgccctttttcca-3') and qP450R (5'-ctccctaaaagcggactaaaag-3'). Relative quantity of p450 mRNA was calculated using the ΔCt method. Universal eubacterial primers for rRNA 16S were used as an endogenous control (UniF340-5'-actcctacgggagcagcagc-3' and UniR514-5'-attaccgctgctgctggc-3'). For each experiment the RNA was extracted from 6 wells of infected cells and also from the bacteria in control experiments in the absence of cells.

CPS staining

CPS was prepared from bacteria as described by Hitchcock [17]. Bacteria were harvested by centrifugation and suspended in 100 μ l of lysis buffer containing 31.25 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate, 0.025% bromophenol blue, and 20% glycerol. After heating to 100°C for 5 min, 20 μ l of the sample was transferred into a fresh tube, 1 μ l of 20 mg/ml proteinase K was added to the solution, and the tubes were incubated for 1 h at 50°C. The samples were fractionated on NuPage Novex 10% bis-Tris gels (Invitrogen, United Kingdom). Following electrophoresis, gels were stained with Alcian blue (0.1% Alcian blue in 40% ethanol, 5% acetic acid) [18].

Serum resistance assay

The sensitivity of bacteria to human serum was measured [19]. *C. jejuni* 81-176 wild type and *Cj1411c* deletion mutant were plate-grown and resuspended in fresh tissue culture

medium (RPMI 1640) to give an OD_{600} of 0.1. Five microliters of bacterial suspension was added to duplicate wells of a six-well plate containing 800 μ l of Mueller-Hinton broth and 200 μ l of active pooled human serum or to separate wells containing 800 μ l of Mueller-Hinton broth and 200 μ l heat-inactivated human serum to give a total volume of 1ml. The plates were incubated for 1 h at 37°C under microaerobic conditions. Bacteria from each well were diluted 10-fold and 50 μ l plated on dry Mueller-Hinton agar plates. The plates were incubated at 37°C under microaerobic conditions. Colony counts were performed after an incubation time of 2 days. All assays were conducted in triplicate and repeated independently three times.

Western Blotting

Proteins were separated on 10% SDS polyacrylamide gels and transferred to 0.45 μ m nitrocellulose membrane (Millipore). Following WB the membranes were blocked in 3% BSA + 0.05% Tween for 30 minutes. Primary antibodies against *Cj1411c* P450 (rabbit polyclonal), anti-His (sc-53073, Santa Cruz Biotechnology) were used. The anti-CadF and anti-Fur antibodies (rabbit polyclonal) confirmed the purity of our membrane fractionations. Following washing in PBS-TWEEN the membranes were incubated with the secondary antibodies (goat anti-rabbit - #7074S, Cell Signaling; goat anti-mouse sc-2031, Santa Cruz). Detection was performed with the PIERCE chemiluminescence kit (Thermo, Fisher Scientific). Densitometry was performed using Fiji ImageJ software.

Subcellular fractionation

Bacterial cellular fractionation was performed as previously described [20]. Bacteria were harvested by centrifugation in 20mM Tris (pH 7.5) at 12000g for 20 minutes at 4°C followed by washing 3 times in 20mM Tris (pH 7.5). The bacteria were then disrupted by sonication in 20mM Tris (pH 7.5) with protease inhibitors (Sigma, UK). In order to collect unbroken cells we have centrifuged the sonicated material for 10 minutes at 2000 rpm and kept the supernatant. In order to collect the total membranes the sonicated material was centrifuged at 40000g for 1 hour at 4°C and the supernatant containing the cytosolic fraction was collected. In order to separate the inner membranes from the outer membranes the resulting pellet was resuspended in 20mM Tris (pH 7.5) containing 2% sodium lauryl sarcosine and incubated for 30 minutes at 4°C. Following centrifugation at 40000g for 1 hour at 4°C the supernatant fraction contains the inner membranes and the outer membranes are in the pellet. The outer membranes were resuspended in 20mM Tris (pH 7.5). The purity of our membrane fractionations was controlled using an anti-Fur antibody (a cytosolic protein) and anti-CadF antibody (an outer membrane protein).

UV-visible spectroscopy

The visible absorption spectra for the purified cytochrome P450 protein were recorded by using a Shimadzu UV-1650 dual-wavelength/double beam spectrophotometer. The measurement was performed in a 1 cm path length quartz cuvette containing 1 μ M purified protein in 25mM Tris (pH 7.5) and 200mM KCl. The spectra was recorded between 800-300

nm. For the carbon monoxide (CO) bound spectra CO was bubbled through the sample for one minute and the spectrum was recorded as mentioned above.

Results

1. Spectroscopic characterization of the purified protein

To analyze the cellular localization of *Cj1411c* and its biochemical characteristics purified active enzyme and anti-CYP1411c antibody were essential. The CYP1411c protein was overexpressed in *E. coli* Tuner DE3 and purified by 2-step chromatography. Figure 1(a) shows the successfully purified cytochrome P450 protein following Coomassie blue staining of SDS gels. Following successful overexpression and purification we raised an antibody against CYP1411c for further characterization by Western blotting, (Figure 1c). Spectroscopy showed a heme Soret band at 422 nm and a spectrum characteristic of a low spin ferric active cytochrome P450 (Figure 1b). Addition of 1 mg sodium dithionite caused no change to the spectrum, indicating that reduction of the heme to the ferrous form is unfavourable under these conditions [6]. The fact that the heme is unable to be reduced in the purified enzyme suggests that substrate binding will act as a trigger for reduction, as is common in cytochrome P450s, by inducing a reduction potential shift [6]. This can only be tested once the substrate has been identified. Reduction potential can also be influenced by environment, and it is possible that the enzyme needs to be associated with a membrane to be functional in this respect.

2. The role of *Cj1411c* in *Campylobacter jejuni* 81-176 pathogenicity

In order to investigate the role of CYP1411c in *C. jejuni* 81-176 virulence we constructed a deletion mutant (*C. jejuni* 81-176 Δ *Cj1411c*). The deletion strain was reconstituted using the PYR107 shuttle plasmid (*C. jejuni* 81-176 Δ *Cj1411c*::*Cj1411c*) The successful *Cj1411c* gene deletion was confirmed by Western blotting using an anti-P450 antibody (Figure 1c).

Because cytochrome P450s are associated with pathogenicity in other organisms we investigated the role CYP1411c in virulence by comparing the adhesion (Figure 2a), invasion (Figure 2b) of the wild type strain with the CYP1411c deletion mutant and also with the reconstituted mutant. Following gentamicin protection assay, the deletion mutant showed a significant reduction in invasion ($P = 0.005$), with the virulence being restored following reconstitution. Levels of adhesion were unaffected.

We also tested the effect of CYP1411c gene deletion on serum resistance as it is directly connected to virulence. The wild type strain had an average rate of survival of 60%. However, there was a dramatic decrease (23%) in the rate of survival of the cytochrome P450 deletion strain ($P < 0.003$). The reconstituted strain showed restored survival when compared to the wild type strain (Figure 2c). These results demonstrate that the CYP1411c is involved in *C. jejuni* 81-176

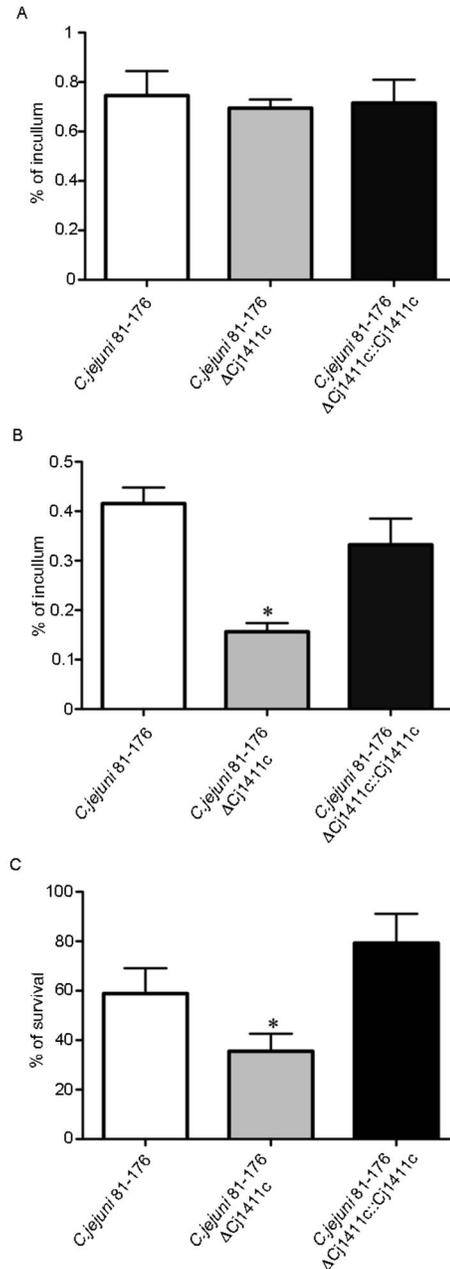


Figure 2. Virulence and serum resistance. (a) adhesion to HCT-8 cells, (b) invasion of HCT-8 cells of *C. jejuni* 81-176 wild type, *C. jejuni* 81-176 Δ *Cj1411c* and *C. jejuni* 81-176 Δ *Cj1411c*::*Cj1411c*. (c) serum resistance - the survival rate is defined as the number of *C. jejuni* 81-176 colonies isolated following exposure to human serum divided by the number of colonies surviving in heat inactivated serum, expressed as a percentage. Statistical significance (Student's *t* test) relative to the level of wild type strain is indicated. * $P=0.001$. The experiments were done in triplicate and on three separate occasions. The error bars represent standard deviations for six separate wells.

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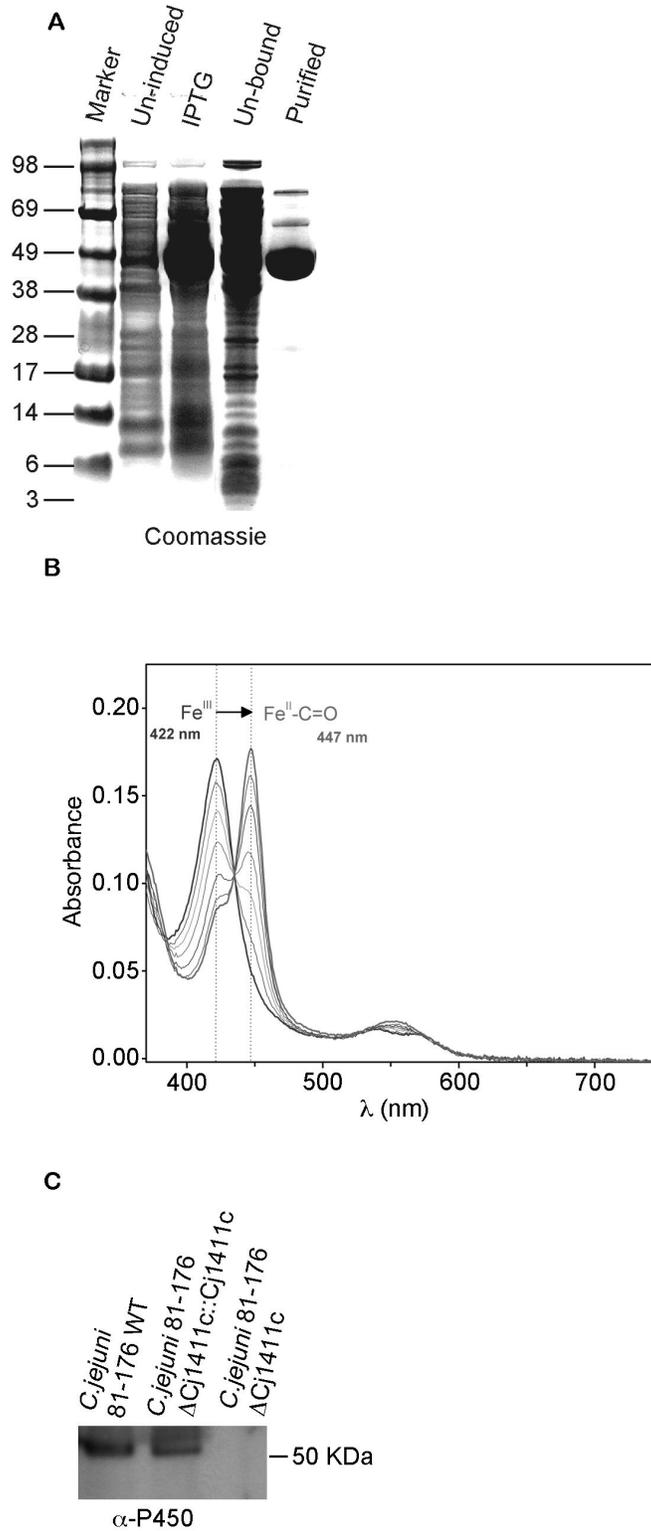


Figure 1. *Cj1411c* protein and mutant characterization. (a) Visualization of protein expression using 10% SDS-PAGE and stained with Coomassie Blue (b) Identification of the 450 nm absorption in the Soret band with CO bound on recombinant CYP1411c. Successive spectra show the oxidation transition from a 420 nm peak to a 450 nm peak in CYP1411c following exposure to CO. (c) Western blot analysis with an anti-CYP1411c antibody following gene deletion showing the absence of the cytochrome P450 protein in *C. jejuni 81-176 ΔCj1411c*.

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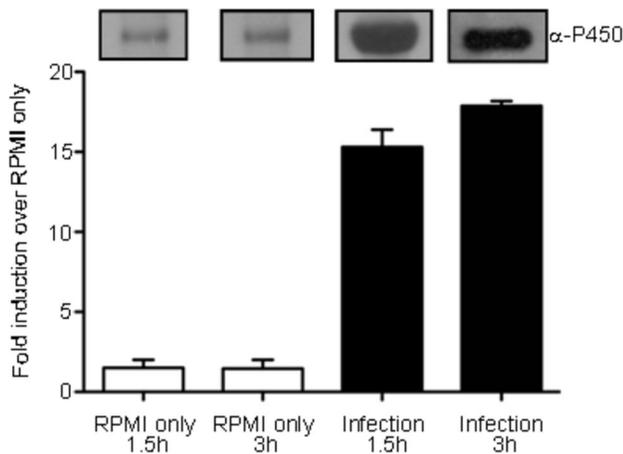


Figure 3. qRT-PCR analysis of *Cj1411c* gene expression during infection. *Cj1411c* expression analysis in *C. jejuni* 81-176 following infection of HCT-8 cells. The gene expression level for 1.5h in RPMI was set to 1 as the basal level. All other levels are expressed as fold change over the basal gene expression. Error bars represent \pm S.D. of 3 independent experiments. The corresponding protein expression levels assessed by Western blot with the anti-P450 antibody are shown.

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pathogenicity by contributing to its virulence and serum resistance.

3. CYP1411c gene expression is up-regulated following invasion of epithelial cells

Next we have investigated the hypothesis that *Cj1411c* is important during survival inside the host following internalization. In order to test this hypothesis we isolated RNA from bacteria that had infected human epithelial HCT-8 cells for intervals of 1.5 and 3 hours. We show, using q-PCR, that the CYP1411c gene expression levels were significantly increased when *C. jejuni* attached to or internalized in human intestinal epithelial cells. In the absence of HCT-8 cells the gene expression levels remained low whereas the 16S rRNA levels remained constant (data not shown) (Figure 3). Investigation of protein levels by Western blotting using the anti-CYP1411c antibody confirmed the q-PCR results (Figure 3). These results show that CYP1411c is up-regulated after internalization, suggesting that this enzyme is metabolically required when the pathogen invades the host. This could explain the significant decrease in invasiveness of the deletion mutant (Figure 2b).

4. CYP1411c a membrane-associated cytochrome P450

In order to investigate the subcellular localization of CYP1411c we have separated cytosolic, inner membrane and outer membrane proteins from *C. jejuni* 81-176. Western blotting on the individual fractions was performed using the anti-P450 antibody (Figure 4A). CYP1411c was present in all three fractions, including the inner and outer membrane. Densitometry on blots showed that 83% of the total CYP1411c

present in *C. jejuni* 81-176 was membrane bound. As the majority of CYP1411c is membrane bound, its redox partners or possible substrates should be localized within the same subcellular fraction. In order to check the purity of bacterial fractionations we have used an anti-Fur antibody, as Fur protein is a known cytosolic protein (Figure 4B) and an anti-CadF antibody detection of CadF, a known outer membrane protein (Figure 4C). Following bacterial fractionation we investigated by spectroscopy the effect of membrane proteins on purified CYP1411c absorption spectra. The purified enzyme was incubated with *C. jejuni* 81-176 inner membrane fractions for overnight, followed by the addition of dithionite, which caused a slow shift in the Soret band from 422 to 400 nm and the merging of the α and β bands. Such an effect is characteristic of a low to high spin transition in cytochrome P450s, often observed on substrate binding (Figure 5). The spin-state change facilitates reduction of the protein, which was also observed in this case. Our results show that *C. jejuni* 81-176 inner membrane proteins can cause an alteration in spectra, suggesting an interaction with purified CYP1411c. Such changes in spectra were not observed when probed with cytosolic fractions from *C. jejuni* 81-176. This result confirms that either the redox partner or the substrate of CYP1411c is localized within this bacterial fraction and is important for enzyme function. This data identifies the CYP1411c as the first bacterial cytochrome P450 to be membrane-associated.

5. CYP1411c is involved in outer surface biosynthesis

In light of the unique position of this cytochrome P450 in the capsule biogenesis locus, and the effect of deleting *Cj1411c* on pathogenicity, we hypothesized that the cytochrome P450 protein is involved in or contributes to CPS (capsule polysaccharide) production. We extracted CPS from *C. jejuni* 81-176 wild type, $\Delta Cj1411c$ deletion mutant and $\Delta Cj1411c::Cj1411c$ reconstituted mutant. Following separation in SDS gels, staining with Alcian blue was performed. As depicted in Figure 6 the CYP1411c mutant (Figure 6, lane 3) displayed significantly reduced CPS and LOS staining when compared to the wild type strain (Figure 6, lane 2). The recovery of staining in the reconstituted mutant (Figure 6, lane 4) indicates clearly that CYP1411c impacts on the biosynthesis of these structures.

Discussion

Cytochrome P450s comprise an important group of proteins involved in the synthesis of physiologically active compounds, drug metabolism and bioconversion of xenobiotics. Although the *C. jejuni* 81-176 genome database annotation identifies only one cytochrome P450, no data are available on the possible functions of this protein. Overexpression of the protein in *E. coli* and antibody production allowed us to identify this cytochrome not only in cytosolic fractions but also in membrane fractions. This is consistent with data obtained from bioinformatics that place this cytochrome within a genomic cluster involved in biosynthesis of outer surface structures (<http://www.sanger.ac.uk>). While other known prokaryotic cytochrome P450s are cytosolic proteins, the eukaryotic

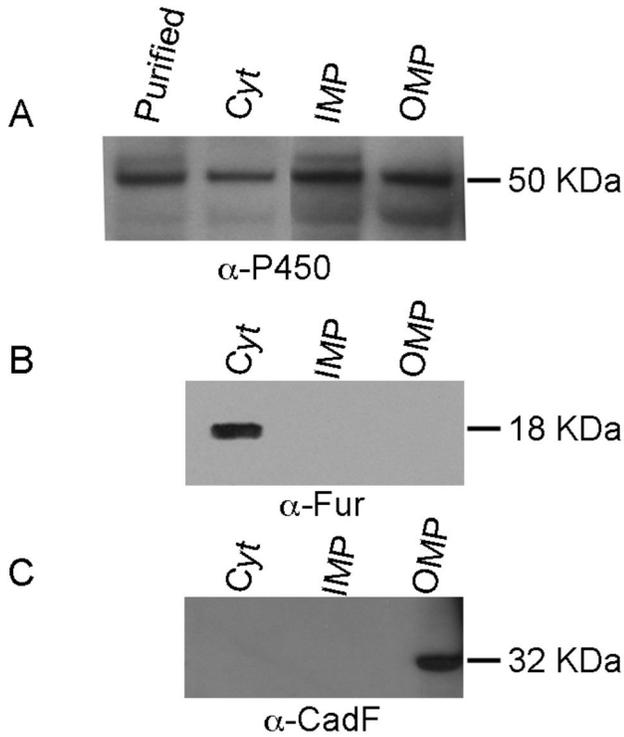


Figure 4. CYP1411c cellular localization. (A) Anti-P450 immunoblotting on bacterial fractions (cytosolic fraction, Cyt; inner membrane protein, IMP and outer membrane protein fraction, OMP). Recombinant CYP1411c protein (purified) was used as a positive control. Fractions were validated with an antibody recognizing the cytosolic Fur protein (B) and the outer membrane protein CadF (C).

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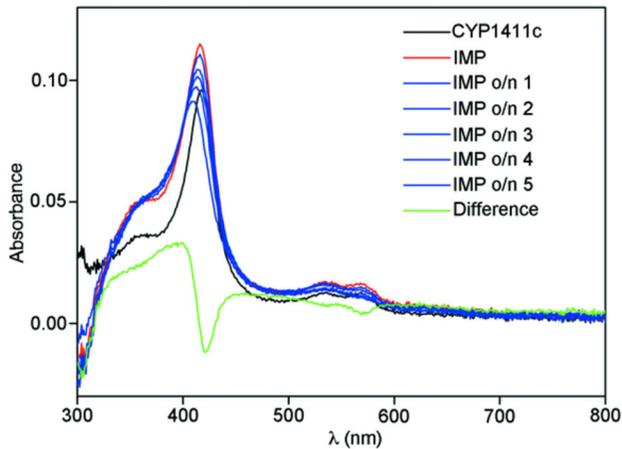


Figure 5. CYP1411c spectral change following incubation with the inner membrane fraction. IMP o/n 1-5 represent successive spectral recordings following incubation of purified CYP1411c protein with *C. jejuni* 81-176 inner membrane proteins. Spectra were recorded at 1 min intervals.

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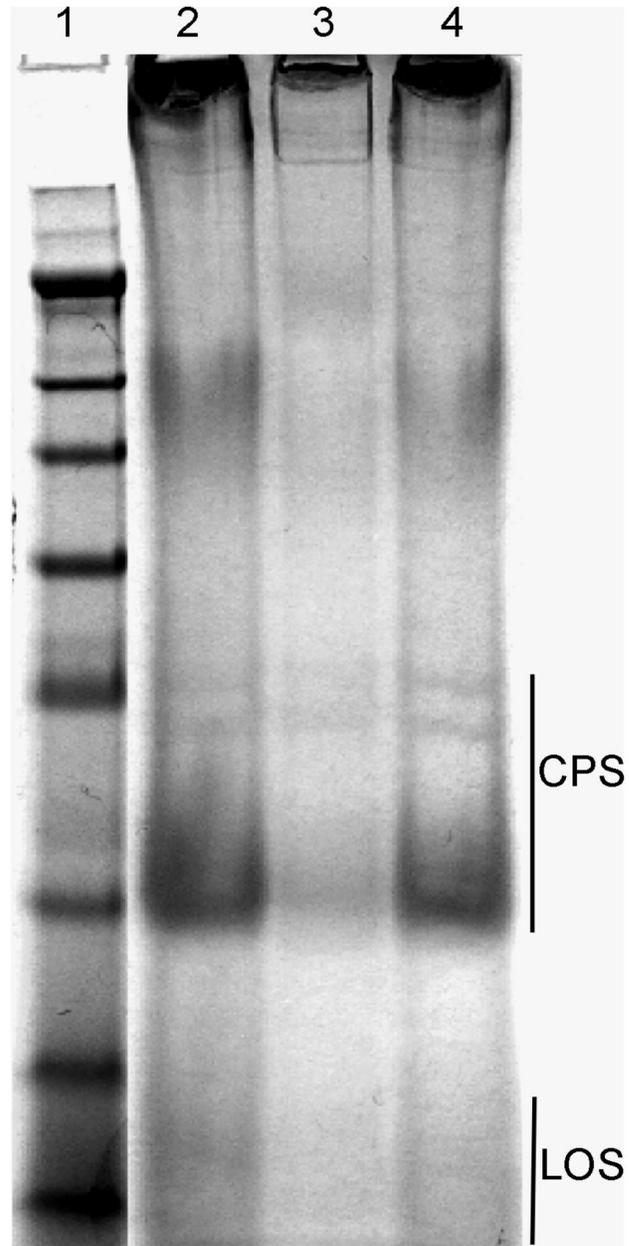


Figure 6. The effect of *Cj1411c* gene deletion on CPS production. Alcian blue stained CPS of *C. jejuni* 81-176 wild type (lane 2) was compared with the CPS extracted *C. jejuni* 81-176 $\Delta Cj1411c$ (lane 3) and *C. jejuni* 81-176 $\Delta Cj1411c::Cj1411c$ (lane 4).

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enzymes are known to be integral membrane proteins [21]. This common feature of mammalian cytochrome P450s is important, because their catalytic domain is facing the cytosol allowing them to bind liposoluble substrates [22]. In our experiments we have identified *Cj1411c* as being predominantly localized in the outer membrane fraction. While not unambiguously confirming this location, the control

experiments, using known cytosolic and membrane proteins, support this finding. We speculate that the cytochrome P450 enzyme substrate is membrane localized in *Campylobacter* and that the cytosolic fraction of the CYP1411c enzyme might only be required during the catalytic cycle in electron scavenging. These hypotheses require further investigation.

The biochemical studies performed on purified protein suggest that the reduction potential of the heme is very low. A similar situation is observed with the F393W mutant of P450BM3 [6]. A slow shift in the Soret band to 447 nm indicated formation of the cytochrome P450 ferrous CO complex characteristic of Cys-thiolate ligated heme when CO gas was added to the sample (Figure 1B). Thus, CO is able to induce the heme into the ferrous form, not accessible in the presence of dithionite only. Observation of a Cys-ligated ferrous heme CO complex confirms that CYP1411c is a typical cytochrome P450, and was purified with the protein-heme ligand complex intact. The change in absorption following interaction with inner membrane fractions represents a key result for its functionality. It is important for cytochrome P450s to have redox partners in the vicinity because they require two electrons for their catalytic function and these are usually supplied in two consecutive steps from NAD(P) H via the redox partners. Interestingly one of the predicted redox partners for CYP1411c is an iron-sulfur protein (*Cj0074c*), known to be localized within the inner membrane fraction [23].

In *C. jejuni* 81-176, outer surface components are actively involved in mediating its pathogenicity. The unique localization of CYP1411c at the end of the surface biosynthesis cluster suggested its possible association with bacterial membranes and involvement in the formation of surface components. The CYP1411c deletion mutant is significantly less virulent than wild type *C. jejuni* 81-176. The decrease in virulence was accompanied by loss of resistance to human serum. One of the outer surface features associated with attenuated virulence is the capsule polysaccharide (CPS). The CYP1411c deletion mutant produced significantly less CPS than the wild type *C. jejuni* 81-176 strain. Previous studies demonstrated that loss of

CPS can impair the ability of *C. jejuni* to invade human cells providing a possible explanation for the attenuation of *C. jejuni* Δ *Cj1411c* invasiveness [14,20,24,25].

The significant increase in gene and protein expression of CYP1411c after invasion of the host, associated with the loss in pathogenicity of the deletion mutant, highlights the importance of this cytochrome P450 in host-pathogen interaction. Involvement of CYP1411c in pathogenicity has been described also in other pathogens. For example *M. tuberculosis* CYP125 is involved in catabolism of host cholesterol generating immunomodulatory compounds to increase survival in the host [26]. The role of cytochrome P450s in survival within the host goes beyond the bacterial regnum. A similar role was shown for CYP5122A1, a cytochrome P450 from the parasite *Leishmania donovani*. In this parasite CYP5122A1 was linked to processes including cell growth, infection and ergosterol biosynthesis [27].

Conclusion

In summary *C. jejuni* CYP1411c is uniquely located in the membrane fractions of this organism and plays a role in CPS production. Its role in pathogenicity points to its potential as a drug target. Further studies are required to elucidate the complex biological role of this protein.

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Author Contributions

Conceived and designed the experiments: NC UK BB SD. Performed the experiments: LA GP AG. Analyzed the data: NC SD BB. Contributed reagents/materials/analysis tools: NC SD. Wrote the manuscript: NC BB.

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