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The CprS sensor kinase of the zoonotic pathogen *Campylobacter jejuni* influences biofilm formation and is required for optimal chick colonization

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Summary

***Campylobacter jejuni*, a prevalent cause of bacterial gastroenteritis, must adapt to different environments to be a successful pathogen. We previously identified a *C. jejuni* two-component regulatory system (Cj1226/7c) as upregulated during cell infections. Analyses described herein led us to designate the system CprRS (*Campylobacter* planktonic growth regulation). While the response regulator was essential, a *cprS* sensor kinase mutant was viable. The Δ*cprS* mutant displayed an apparent growth defect and formed dramatically enhanced and accelerated biofilms independent of upregulation of previously characterized surface polysaccharides. Δ*cprS* also displayed a striking dose-dependent defect for colonization of chicks and was modestly enhanced for intracellular survival in INT407 cells. Proteomics analyses identified changes consistent with modulation of essential metabolic genes, upregulation of stress tolerance proteins, and increased expression of MOMP and FlaA. Consistent with expression profiling, we observed enhanced motility and secretion in Δ*cprS*, and decreased osmotolerance and oxidative stress tolerance. We also found that *C. jejuni* biofilms contain a DNase I-sensitive component and that biofilm formation is influenced by deoxycholate and the metabolic substrate fumarate. These results suggest that CprRS influences expression of factors important for biofilm formation, colonization and stress tolerance, and also add to our understanding of *C. jejuni* biofilm physiology.**

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Introduction

Campylobacter jejuni is a Gram-negative food-borne pathogen now recognized as the leading cause of bacterial gastroenteritis in the developed world, infecting approximately 1% of the Canadian and US populations annually (Altekruse *et al.*, 1999). *C. jejuni* is a commensal in many animal species, yet infection in humans commonly presents as acute gastroenteritis with symptoms of campylobacteriosis including nausea, vomiting, and intense, often bloody, diarrhoea. While usually self-limiting, this can proceed to bacteraemia in immunocompromised individuals, and *C. jejuni* infection is a common antecedent to serious medical sequelae such as Guillain–Barré syndrome, a sometimes fatal ascending bilateral paralysis (Young and Mansfield, 2005; Helms *et al.*, 2006). Strains resistant to antibiotics such as fluoroquinolones are becoming more common, and at present a vaccine is not available (Butzler, 2004); thus, new avenues for control are needed.

A key reservoir for *C. jejuni* infection is avian species, where it resides asymptomatically as a component of the intestinal microflora. This is likely responsible for its widespread presence in commercial poultry products (Jacobs-Rietsma, 2000). *C. jejuni* is also present in the intestinal mucosa of other animals; therefore, water contaminated with agricultural run-off and raw milk are also responsible for outbreaks. Consequently, as a zoonotic pathogen, *C. jejuni* encounters a variety of *in vivo* and transmission-related environmental challenges, and its presence as a serious public health concern suggests that it can overcome these challenges sufficiently (Chan *et al.*, 2001). This is intriguingly inconsistent with its fastidious

and fragile behaviour: culture of *C. jejuni* in the laboratory requires rich media, moderately increased (37–42°C) temperatures, and a low O_2/high CO_2 atmosphere. This suggests the existence of survival mechanisms that may not be immediately apparent from experiments performed solely in pure broth culture.

Compared with other enteric pathogens, relatively little is known about the molecular mechanisms underlying *C. jejuni* pathogenesis. This is due in large part to its recalcitrance to molecular genetics techniques developed in other bacteria and fastidious requirements for growth in the lab. Sequence analysis also suggests the absence of many classical enteric pathogen virulence factors, thus limiting the utility of comparative genomics (Parkhill *et al*., 2000; Hofreuter *et al*., 2006; Pearson *et al*., 2007). Work to date has demonstrated the importance of motility and surface carbohydrates, and has identified a cytolethal distending toxin involved in host cell cycle arrest (Young *et al*., 2007). Like *Salmonella* spp., the ability of *C. jejuni* to invade cells of the gastrointestinal epithelium is important for virulence; however, while the recently identified *C. jejuni*-containing vacuole (CCV) also avoids the canonical endocytic pathway and fusion with lysosomes, its maturation and trafficking appears to be unique in that it utilizes the microtubule network and resides near the Golgi apparatus (Watson and Galan, 2008). Furthermore, experiments have suggested that in the absence of a prototypical, dedicated type III secretion system such as those utilized by pathogenic *Escherichia coli*, *C. jejuni* uses the flagellar export apparatus to secrete effectors such as CiaB that aid host cell invasion and colonization (Konkel *et al*., 2004). Taken together, this suggests that *C. jejuni* uses distinct paradigms from prototypical enteric pathogens such as *E. coli* and *Salmonella*.

In addition to the environmental challenges of its zoonotic lifestyle, the absence of established enteric virulence factors in *C. jejuni* suggests that features contributing to its survival play a central role in its success as a pathogen. In support of this, several global regulatory and stress survival factors in *C. jejuni* (i.e. the stringent response and polyphosphate metabolism) also have been shown to control phenotypes directly linked to virulence (Gaynor *et al*., 2005; Candon *et al*., 2007). The importance of broad changes in physiology such as the viable but non-culturable state and, more definitively, biofilm formation to *C. jejuni* resilience have also been noted (Trachoo *et al*., 2002; Joshua *et al*., 2006). Upwards of 99% of bacterial species, including *C. jejuni*, exist outside of the laboratory primarily not as free-swimming, planktonic cells, but as part of communities called biofilms (Ehrlich *et al*., 2005), which exhibit marked differences in metabolism, cell physiology, and importantly, stress resistance from their broth-grown counterparts (O'Toole and Kolter, 1998). While analysis of biofilm formation in *C. jejuni* is in its infancy, work to date suggests that biofilms contribute to stress tolerance during pathogenesis (Joshua *et al*., 2006; Kalmokoff *et al*., 2006; McLennan *et al*., 2007; Fields and Thompson, 2008).

Adaptation of a bacterium to its environment requires regulation of gene expression. Two-component regulatory systems (TCRSs) are ubiquitous systems used by prokaryotes to respond to environmental changes (Eppinger *et al*., 2004) and achieve transduction of information from the environment via phosphorelay between two proteins, a membrane-bound sensor histidine kinase (SK) and its cognate cytoplasmic DNA-binding response regulator (RR). Activation of the RR results in expression changes that bring forth adaptation to the current challenge. The *C. jejuni* genome encodes a limited repertoire of regulatory elements (Parkhill *et al*., 2000), including a relatively small number of TCRSs (seven SKs and 12 RRs). TCRSs in many pathogens control phenotypes closely associated with virulence or survival; likewise, in *C. jejuni*, all TCRSs characterized to date (FlgRS, RacRS, DccRS and the orphan RR CbrR) (Bras *et al*., 1999; MacKichan *et al*., 2004; Wosten *et al*., 2004; Raphael *et al*., 2005) are required for optimal colonization of chicks except PhosSR (Wosten *et al*., 2006). Thus, TCRSs are attractive candidates for study of paradigms underlying *C. jejuni* survival and pathogenesis.

The availability of *C. jejuni* genome sequences has aided the development of new genetic tools to explore the molecular mechanisms mediating survival during pathogenesis. Microarray analysis of the transcriptional response of *C. jejuni* to INT407 epithelial cell monolayers (Gaynor *et al*., 2005) identified a TCRS encoded by Cj1226c and Cj1227c that may contribute to adaptation to pathogenesis-related environments. We have designated this system CprRS (*C*ampylobacter planktonic growth regulation). In this study, we provide genetic, molecular, proteomic and phenotypic evidence demonstrating that this TCRS likely co-ordinates survival- and pathogenesis-related phenotypes in *C. jejuni* through control of essential biological processes, stress tolerance and biofilm formation, thereby contributing to the success of this apparently fragile zoonotic pathogen.

Results

The C. jejuni *genes Cj1226c and Cj1227c encode a TCRS pair, CprRS; the CprR RR is essential for viability*

The annotated *C. jejuni* NCTC 11168 genome contains seven SKs and 12 RRs (Parkhill *et al*., 2000), and among these are five TCRS pairs in which the SK is encoded immediately downstream of the RR. One such pair, encoded by Cj1226c and Cj1227c, was previously identified as upregulated in the presence of live epithelial cells *in*

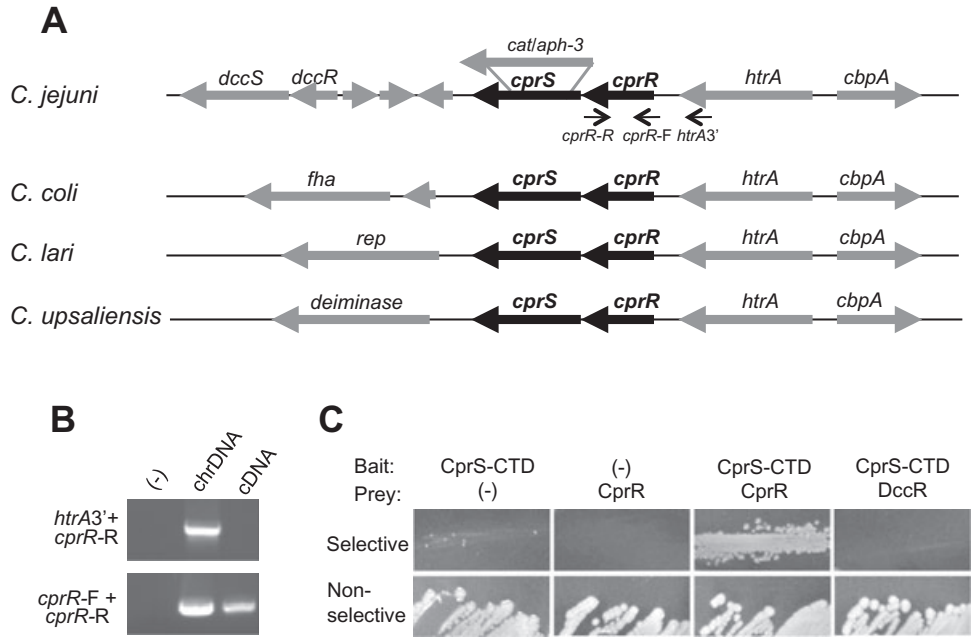


Fig. 1. Identification and genetic analysis of a *Campylobacter*-specific TCRS, *cprRS*.
A. Location of the *cprR* response regulator and *cprS* sensor kinase homologues (annotated as Cj1226c and Cj1227c in strain 11168) in the genomes of *Campylobacter* spp., identified using CampyDB (http://xbase.bham.ac.uk/campydb/). Approximate location of *cat*- and *aph-3*-marked deletions in *cprS* are shown, resulting in strains designated Δ*cprS* and Δ*cprS*::Km^R respectively.
B. *cprR* and *htrA* are transcribed independently. Chromosomal DNA (chrDNA) or cDNA was used as a template for PCR using the primer combinations indicated in (A).
C. Bacterial two-hybrid analysis demonstrates that the CprS C-terminal domain (CTD) interacts with CprR, but not a control *C. jejuni* response regulator, DccR.

vitro (Gaynor *et al*., 2005). CD-Search analyses of each component suggest that Cj1226c encodes an EnvZ-family SK, and the protein encoded by Cj1227c contains domains characteristic of the OmpR family of DNA-binding RRs (Marchler-Bauer and Bryant, 2004). The organization of the region surrounding Cj1226c and Cj1227c in various *Campylobacter* spp. is shown in Fig. 1A.

The TCRS encoded by Cj1226c and Cj1227c appears to be well conserved only among the campylobacters. BLAST analysis of ε-proteobacterial genomes sequenced to date identified putative orthologues of Cj1226c and Cj1227c in other Campylobacteraceae (*C. coli*, 100% and 97% amino acid similarity respectively; *C. upsaliensis*, 99% and 89% similarity; *C. lari*, 94% and 81% similarity; *C. fetus*, 82% and 69% similarity; *Arcobacter butzleri*, 71% and 52% similarity). In each case, the genes are encoded adjacent to the *htrA* gene, strongly suggesting orthology (Fig. 1A). In contrast, within the Helicobacteraceae, numerous TCRS genes within the same organism are all similarly unrelated to Cj1226c and Cj1227c (the closest Cj1227c and Cj1226c homologues are respectively: *Sulfuromonas denitrificans*, 73% and 56% similarity; *Helicobacter pylori*, 69% and 58% similarity; *Wolinella succinogenes*, 68% and 53% similarity). This suggests homology, but prevents distinction of clear orthologues from paralogues. In addition, both Cj1226c and Cj1227c showed higher similarity to other *C. jejuni* TCRS homologues, such as RacRS, than to potential orthologues in other taxa. Furthermore, none of the sequenced genera outside the Campylobacteraceae (*Helicobacter*, *Wolinella*, *Sulfuromonas*) have a TCRS encoded adjacent to *htrA* except for *Nitratiruptor*, a deep-sea vent ε-proteobacterium that appears to have diverged before the split of the Campylobacterales (Nakagawa *et al*., 2007), making solid evidence for Cj1226c and Cj1227c orthologues even more elusive. Based on conservation of this TCRS among members of the *Campylobacter* genus and potential divergence or absence of homologues in other ε-proteobacterial species, together with the phenotypes observed for Cj1226c mutants (see below), we propose renaming the system CprRS (*Campylobacter* planktonic growth regulation RR and SK).

To explore the physiological role of CprRS in *C. jejuni*, we set out to perform mutational analysis on each gene. Numerous efforts to delete the RR gene using different mutagenesis constructs were unsuccessful. Mutagenesis of Cj1227c (*cprR*) has been attempted by three other groups (Raphael *et al*., 2005; J. Ketley, pers. comm.; J.K. MacKichan, E.C. Gaynor and S. Falkow, unpublished), with Raphael *et al*. reporting that Cj1227c could only be

inactivated when a second copy was present at a heterologous location. These results thus strongly suggest that activity of this RR is essential for viability of *C. jejuni* under laboratory conditions. In contrast, deletion mutagenesis of Cj1226c (*cprS)* was achieved using two different constructs, one with a Cm^R cassette replacing the entire *cprS* coding region and one with a non-polar Km^R cassette, suggesting that the activity of the SK is dispensable. The resulting strains were designated Δ*cprS* and Δ*cprS*::Km^R; as they behaved similarly in subsequent experiments, they were used interchangeably.

The non-essential nature of the SK, together with the fact that the RR is essential, raised the question of whether these proteins in fact form a cognate system. In all sequenced *Campylobacter* species, the genes encoding CprRS are encoded next to each other with a 4-base-pair overlap, and in all cases, are encoded adjacent to *htrA*. Operon prediction (Price *et al*., 2005) and microarray expression analysis (E. Gaynor, unpublished; Gaynor *et al*., 2004) suggest that they are encoded in a stand-alone two-gene operon. Nonetheless, because of the conservation of this genomic organization within the campylobacters, we explored the transcriptional (and thus the possible functional) relationship between *cprRS* and *htrA*. RT-PCR was performed using primers annealing to the RR gene only, or the 3′ end of *htrA* and the 3′ end of the RR (Fig. 1A). While amplicons were observed for both primer sets when chromosomal DNA was used as template, PCR products using cDNA as a template were only obtained for the *cprR* primer set (Fig. 1B), suggesting that *cprR* is transcribed independently from *htrA*.

Although the above transcriptional and operon analyses strongly suggest that these genes encode a cognate TCRS, we also wished to demonstrate a direct physical interaction between CprR and CprS. We used a bacterial two-hybrid system to measure physical association between the two proteins as has been previously employed (Kulasekara *et al*., 2005). Following co-transformation of bait and prey constructs (see *Experimental procedures*), colonies on selective media were obtained for CprS and CprR, but not CprS and a control RR (DccR) (Fig. 1C), indicating that only interaction of CprS with CprR was strong enough to activate expression of the reporter genes.

ΔcprS *displays an apparent growth defect in broth culture*

The role of CprRS in *C. jejuni* biology and pathogenesis was explored via extensive phenotypic characterization of the Δ*cprS* mutant. In shaking broth culture, Δ*cprS* appeared to have a late-stage culturability defect (Fig. 2), with a 4-log decrease in recovery of culturable bacteria [colony-forming units (cfu) ml^{-1}] beyond 48 h of culture.

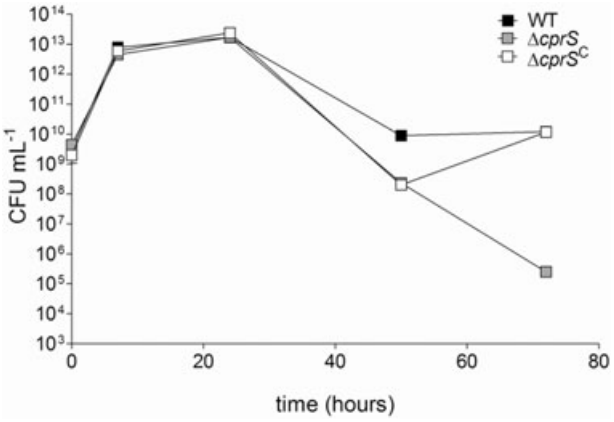


Fig. 2. A Δ*cprS* sensor kinase mutant displays a growth defect compared with the WT strain in rich broth culture. WT (black squares), Δ*cprS* (grey squares) and Δ*cprS*^{C} (white squares) were cultured in MH broth; at indicated time points, samples were taken to measure colony-forming units (cfu). Error bars are present but in most cases are too small to see.

These initial results were not surprising, given the essential nature of the cognate RR. The Km^R cassette used to make one Δ*cprS* strain is non-polar, and our data suggest that *cprRS* is a stand-alone two-gene operon (Fig. 1B); furthermore, the flanking genes are likely essential (tRNA-Asn, *cprR*) or are involved in distinct phenotypes such as heat sensitivity not observed in Δ*cprS* (*htrA*) (Brondsted *et al*., 2005) (Table S1). Nonetheless, we addressed the possibility that the growth phenotype was not linked to the targeted mutation by inserting a wild-type (WT) copy of *cprS* into a heterologous location in the chromosome of Δ*cprS* to create the complemented strain Δ*cprS*^{C}, which complemented the growth phenotype.

ΔcprS *exhibits enhanced and accelerated biofilm formation*

Upon closer observation of broth cultures prepared for further experiments, aggregates of bacteria were clearly visible in the flasks containing Δ*cprS*. We also noted a 30% increase in autoagglutination for Δ*cprS* compared with WT (data not shown), and crystal violet staining suggested that Δ*cprS* was adhering to shaking culture tubes (Fig. 3A). Taken together, this raised the possibility that the apparent growth defect shown in Fig. 2 may partially reflect aggregation of Δ*cprS* both with other bacteria and with abiotic surfaces, resulting in the loss of organisms from the media/planktonic fraction where samples were harvested for growth curve analysis.

An ensuing hypothesis to these observations is that Δ*cprS* may favour surface-attached biofilm growth rather planktonic growth. Indeed, standing culture biofilm crystal violet assays (O'Toole and Kolter, 1998; McLennan *et al*., 2007) clearly demonstrated that Δ*cprS* exhibited a visible

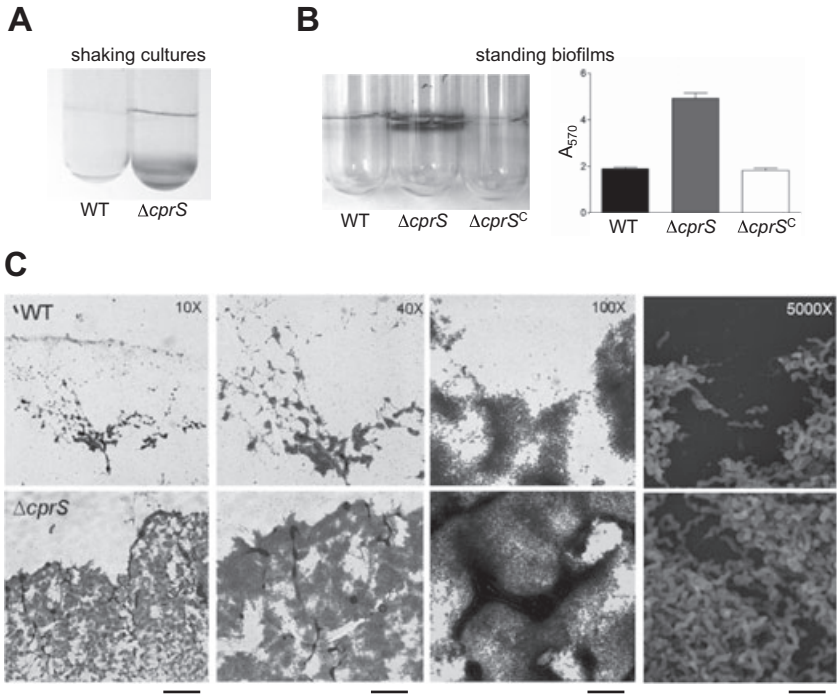


Fig. 3. Δ*cprS* exhibits increased surface growth characteristics compared with WT.
A. Δ*cprS* adheres to shaking broth culture tubes. WT and Δ*cprS* were grown overnight with shaking at 200 r.p.m. in MH broth, and adherent bacteria were visualized by staining cultures directly with 0.2% crystal violet.
B. Δ*cprS* shows enhanced biofilm formation. Overnight liquid cultures of WT, Δ*cprS*, and Δ*cprS*^{C} were diluted to an optical density of 0.0002 in MH broth, added to borosilicate glass tubes and incubated without shaking for 2 days. Biofilms were stained by the addition of crystal violet to a final concentration of 0.2% and tubes were photographed (left). Biofilms formed by WT (black bar), Δ*cprS* (grey bar) and Δ*cprS*^{C} (white bar) were quantified (right) by dissolving adhered crystal violet with 30% methanol/10% acetic acid and measuring the A_{570} of the resulting solution. Quantifications were performed in triplicate.
C. Δ*cprS* biofilms are enhanced and accelerated compared with WT and are comprised of spiral-shaped bacteria. Biofilms were grown on glass coverslips in tubes prepared as in Fig. 4, and the region at the air–liquid interface was either stained with crystal violet for bright field microscopy (10×, 40×, 100×; first three panels) or prepared for scanning electron microscopy (SEM) (5000×; far right panels). Pictures are representative of a large region of each slide. From left to right, the bars represent ~400 μm, 100 μm, 40 μm, and 10 μm.

enhancement of biofilm formation compared with WT after only 1 day of incubation (Fig. 3B, left). Not surprisingly, when adhered crystal violet was dissolved and quantified, a statistically significant ($P < 0.005$) difference for Δ*cprS* compared with the WT strain was seen (Fig. 3B, right). Again, the complemented Δ*cprS*^{C} strain exhibited biofilm formation at levels comparable to WT, supporting the hypothesis that CprS controls aspects of *C jejuni* physiology related to biofilm formation and/or planktonic growth.

We performed microscopy to confirm that the crystal violet-stained material represented healthy *C. jejuni* biofilms present at the air–liquid interface. Bright field microscopy and crystal violet staining (10×–100× magnifications) clearly identified ordered microcolony structures, suggesting active biofilm development rather than non-specific aggregation and adherence to the surface or acellular material (Fig. 3C). Biofilm formation appeared to be both accelerated and enhanced for Δ*cprS*, where much more biofilm material was observed compared with WT. Furthermore, a larger proportion of the Δ*cprS* biofilms exhibited mature biofilm characteristics such as mushroom-like architecture and water channels. There have been numerous reports that *C. jejuni* enters a viable but non-culturable state at later growth stages, which is often defined by a switch from spiral to coccoid physiology. As the Δ*cprS* mutant appeared to display a late-stage culturability defect (Fig. 2), we wanted to observe the morphology of the bacteria within the Δ*cprS* biofilms in order to solidify our conclusion that the growth 'defect' in liquid culture may at least in part be attributed to increased sessile growth rather than a loss in culturability *per se*. Scanning electron microscopy (SEM) (5000× magnification) confirmed spiral morphology (i.e. not coccoid) for both strains (Fig. 3C, far right panels).

Accelerated and enhanced ΔcprS *biofilms form independent of changes in surface carbohydrates;* C. jejuni *biofilms are DNase I-sensitive and contain fibres that react with DNA stains*

We performed further analyses to explore the molecular bases underlying the enhanced biofilm phenotype of

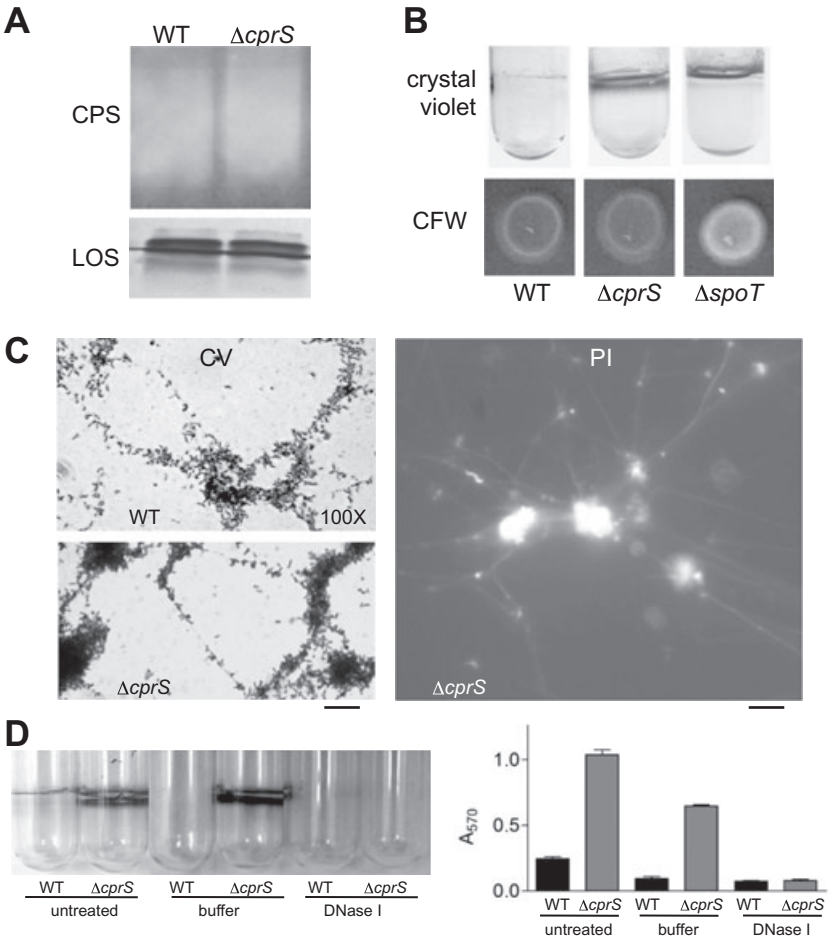


Fig. 4. Enhanced biofilm formation in Δ*cprS* is independent of changes in surface polysaccharides; the *C. jejuni* biofilm matrix exhibits a fibrous appearance and contains DNA.
A. Enhanced biofilm formation in Δ*cprS* is independent of changes in lipooligosaccharide (LOS) and capsular polysaccharide (CPS). Total carbohydrates were extracted from equal numbers of plate-grown bacteria and subjected to SDS-PAGE followed by silver staining to visualize LOS and Western blotting with Penner anti-O36 serotype antibody to visualize CPS.
B. Δ*cprS* does not overproduce a CFW-reactive surface polysaccharide. Overnight cultures of WT, Δ*cprS* and the positive control Δ*spoT* were spotted on BHI agar supplemented with 0.002% calcofluor white (CFW) for assessment of production of surface carbohydrates with β 1-3 and β 1-4 linkages. Crystal violet-stained biofilms for each strain are also shown for comparison.
C. Fibres can be seen in both Δ*cprS* (shown) and WT (not shown) biofilms. Biofilms were grown on glass coverslips as in Fig. 3C and stained with either crystal violet (CV, left) or propidium iodide (PI, right) and visualized by microscopy at 100× magnification. Bars represent ~25 μm (CV) and ~20 μm (PI).
D. *C. jejuni* biofilms can be disrupted by treatment with DNase I. WT and Δ*cprS* biofilms were grown for 2 days and either left untreated or rinsed with PBS, then incubated for 3 h in buffer alone or buffer with DNase I, followed by crystal violet staining.

Δ*cprS*. As the importance of surface carbohydrates in *C. jejuni* biofilms has been demonstrated (Kalmokoff *et al.*, 2006; McLennan *et al.*, 2007), we hypothesized that changes in such polysaccharides may be occurring in Δ*cprS*. We first extracted total carbohydrates from both WT and Δ*cprS* for separation by polyacrylamide gel electrophoresis, followed by silver staining to visualize lipooligosaccharide (LOS) and Western blotting with Penner antiserum to compare levels of capsular polysaccharide (CPS). The mutant strain had a similar LOS profile to the parental strain, both in amount and in species present; levels of CPS likewise appeared to be similar (Fig. 4A). Recent work has shown that a *C. jejuni* Δ*spoT* mutant forms enhanced biofilms commensurate with upregulation of a calcofluor white (CFW)-reactive polysaccharide (McLennan *et al.*, 2007). However, CFW reactivity for Δ*cprS* was nearly identical to the WT strain (Fig. 4B). Similar results were obtained with Congo Red, another carbohydrate-binding dye (data not shown), further suggesting that the enhanced biofilm phenotype of Δ*cprS* was independent of previously characterized surface carbohydrates.

Microscopy of crystal violet-stained biofilms consistently suggested that the bacteria and microcolonies were connected by fibrous-like structures as exemplified in Fig. 3C, WT panel, 40× magnification. Closer examination clearly showed the presence of fibres connecting bacteria and microcolonies (Fig. 4C, left). We also observed these fibres under non-fixed conditions, and noted that the fibres also stained with propidium iodide (PI) (Fig. 4C, right). Shown are Δ*cprS* samples; similar fibres were also observed for WT biofilms. As both crystal violet and propidium iodide can stain DNA, we hypothesized that the fibres were, at least in part, composed of DNA. To provide support for this, 2-day-old biofilms were rinsed with PBS and incubated with DNase I. We found that DNase I treatment resulted in a striking decrease in Δ*cprS* biofilms compared with Δ*cprS* biofilms treated with buffer alone ($P < 0.0001$). WT biofilms were also disrupted by DNase I treatment despite not reaching statistical significance compared with buffer-treated biofilms. No difference in survival of each strain in the presence of similar concentrations of DNase I was observed (data not shown). These data provide the first

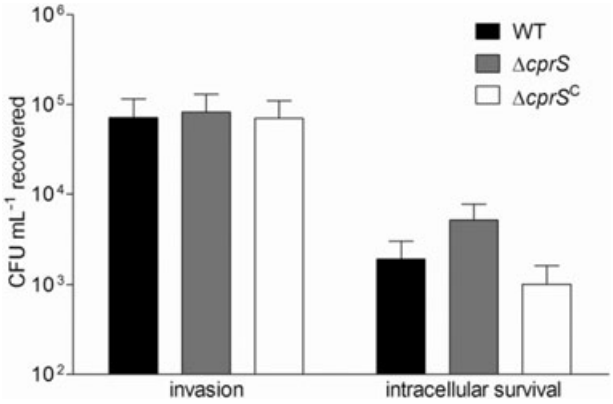


Fig. 5. Δ*cprS* invades INT407 cells at levels comparable to WT but exhibits a modest increase in intracellular survival. WT (black bars), Δ*cprS* (grey bars) and Δ*cprS*^{C} were grown biphasically overnight and used to infect semi-confluent monolayers of INT407 intestinal epithelial cells at a multiplicity of infection of ~100. Following 3 h of infection and 2 h of gentamicin treatment, monolayers were lysed and serial dilutions were plated to determine intracellular bacteria (invasion). Intracellular survival was determined after an additional 5 h of incubation.

evidence for the presence of DNA within the matrix surrounding *C. jejuni* biofilms.

ΔcprS *shows modestly increased intracellular survival but is defective for colonization of 1-day-old chicks*

Because of its initial identification in a cell infection screen, we next explored the role of CprS in host-related phenotypes. We first assessed invasion and intracellular survival of Δ*cprS* in human epithelial cell monolayers. INT407 cell monolayers were infected with WT, Δ*cprS* or Δ*cprS*^{C} bacteria, and there was no significant difference in the number of bacteria recovered at the invasion time point following 3 h of infection and 2 h of gentamicin treatment (Fig. 5). However, a surprising and reproducible two- to fivefold increase in the number of bacteria surviving an additional 5 h incubation within the INT407 cells was observed for Δ*cprS* compared with both WT and the complemented Δ*cprS*^{C} strain. Both WT and Δ*cprS* exhibited the same gentamicin susceptibilities and tolerance of the INT407 cell water and syringe lysis procedures; likewise, no differences were observed for bacterial survival in media above the cells during the infection period, or for bacterial adherence to INT407 monolayers (data not shown).

Next, to assess the contribution of CprS to *in vivo* fitness of *C. jejuni*, we tested the ability of Δ*cprS* to colonize 1-day-old chicks (Fig. 6). Groups of birds were infected with WT and Δ*cprS* at increasing inoculation levels; 6 days post infection, birds were sacrificed and caecal contents assayed for viable *C. jejuni*. Compared with WT, Δ*cprS* exhibited approximately 2.7-log, 5.7-log and 4.2-log lower average levels of colonization at doses of 10^2, 10^4 and 10^6 cfu, respectively, with corresponding statistically significant *P*-values of 0.008, 6.8×10^{-7} and 0.013. Furthermore, at doses of 10^4 and 10^6, several Δ*cprS*-inoculated chicks did not harbour detectable levels of *C. jejuni* whereas the WT strain colonized all chicks to very high levels. Both WT and Δ*cprS* colonized equally well at doses above 10^6 cfu (data not shown). These data suggest that Δ*cprS* is required for optimal colonization of chicks at lower doses, and that this defect can be overcome when chicks are administered higher doses of bacteria.

Proteomics identifies specific protein expression differences between WT and ΔcprS *strains*

We next hypothesized that specific and/or global protein differences may underlie the striking biofilm and chick colonization observations; thus, we explored global protein expression profiles of WT, Δ*cprS* and Δ*cprS*^{C}. Two-dimensional gel electrophoresis and mass spectrometry analyses revealed that numerous proteins varied significantly in expression between WT and Δ*cprS*, most of which are involved in stress tolerance, cell surface structures, regulation, and metabolic pathways (Fig. 7). Furthermore, the majority of expression changes were rescued in the Δ*cprS*^{C} complemented strain.

Several oxidative stress tolerance proteins were upregulated in Δ*cprS* compared with WT including catalase (KatA), thioredoxin reductase (TrxB) and alkyl hydroperoxide reductase (AhpC). In contrast, superoxide

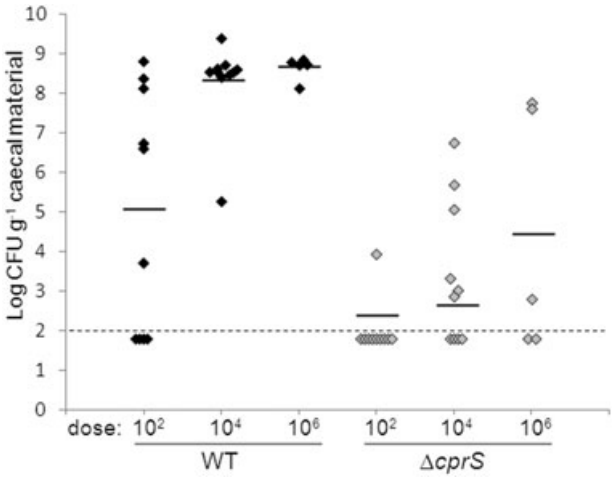
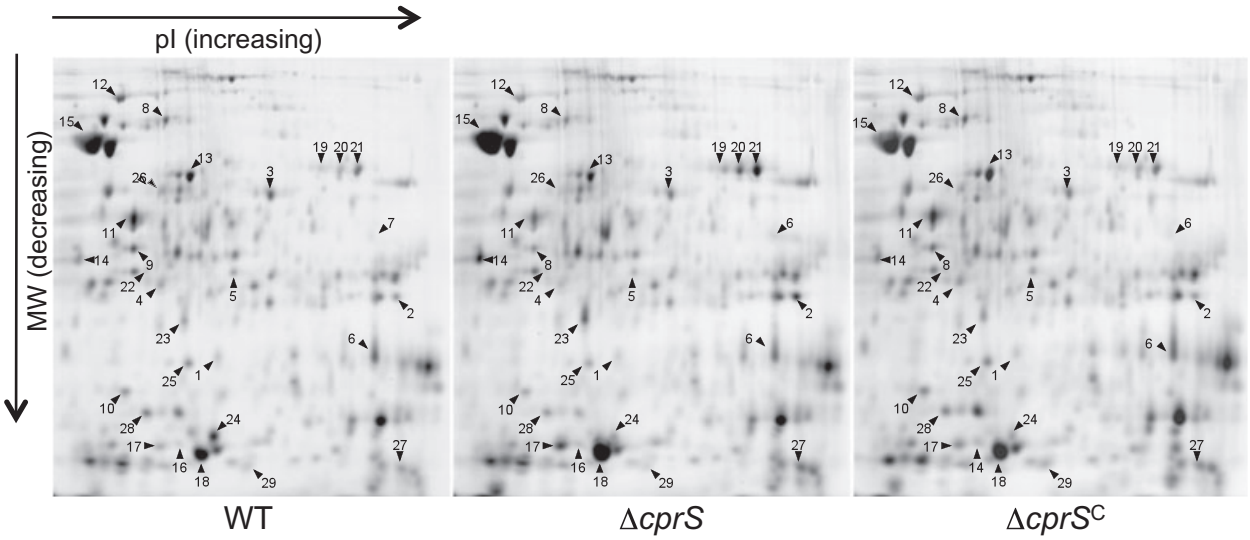


Fig. 6. Δ*cprS* displays a dose-dependent chick colonization defect. One-day-old chicks were orally challenged with the indicated number of cfu of WT (black diamonds) or Δ*cprS* (grey diamonds) bacteria. Six days post infection, birds were sacrificed, and caecal colonization levels were determined by plating on *C. jejuni*-selective MH agar. Each data point represents the log cfu g^{-1} recovered from an individual chick, with the average recovery for each dosage denoted by a black bar. The limit of detection (100 cfu) is denoted by the thin dashed line.

A



B

	Spot	Protein name	Putative function	Fold difference Δ*cprS* (vs. WT)	Fold difference Δ*cprS*^{C} (vs. Δ*cprS*)
Metabolic	1	FabI	enoyl-[acyl-carrier-protein] reductase	1.88	1.10
	2	FbpA	ferric binding protein	2.29	-2.71
	3	FumC	fumarate hydratase	-1.76	1.02
	4	Fba	fructose-bisphosphate aldolase	-1.78	1.53
	5	Asd	aspartate-semialdehyde dehydrogenase	-1.75	1.32
	6	SucD	succinyl-CoA synthetase alpha chain	-1.55	1.36
Protein synthesis	7	HisS	histidyl-tRNA synthetase	1.96	-1.91
	8	RpsA	30S ribosomal protein S1	-1.93	1.55
	9	EF-Ts	translation elongation factor TS	-2.63	1.64
	10	EF-P	translation elongation factor P	-1.99	1.71
	11	EF-Tu	translation elongation factor Tu	-2.68	1.74
	12	EF-G	translation elongation factor G	-2.31	1.28
	13	Tig	trigger factor	-1.65	1.35
Cell surface	14	MOMP	major outer membrane protein	2.23	-1.97
	15	FlaA	flagellar filament protein	1.61	-2.65
Stress response	16, 17, 18	AhpC	antioxidant, AhpC/Tsa family	1.75, 3.31, 2.29	-1.07, -2.19, -1.93
	19, 20, 21	KatA	catalase	1.78, 2.08, 2.13	-2.21, -2.78, -2.38
	22	Rrc	non-haem iron protein	-1.73	1.17
	23	TrxB	thioredoxin reductase	2.06	-2.37
	24	SodB	superoxide dismutase	-2.25	-1.15
Unknown	25	Cj0706	hypothetical protein	-1.92	1.83
	26	Cj0092	putative periplasmic protein	-1.86	1.15
	27	Cj0998	putative periplasmic protein, YceI-like	1.92	-1.45
Regulatory	28	Cj0355c	two-component response regulator	-2.04	1.53
	29	LuxS	autoinducer-2 synthase	-1.65	2.13

Fig. 7. Proteomic analysis of WT, Δ*cprS* and Δ*cprS*^{C} identifies expression differences that may underlie the phenotypes of Δ*cprS*. Cells were grown in MH broth to mid-log phase and harvested for proteomic analyses as described in *Experimental procedures*.
A. Two-dimensional SDS-PAGE analysis of the proteomes of WT, Δ*cprS* and Δ*cprS*^{C}.
B. Proteins showing significant increases or decreases in expression in Δ*cprS* compared with the WT strain. Note that in most cases, complementation of Δ*cprS* (Δ*cprS*^{C}) resulted in approximately WT levels of protein expression.

dismutase (SodB) and the non-haem iron protein Rrc were expressed at lower levels in Δ*cprS*, although the change in SodB expression was not complemented in Δ*cprS*^{C}. Interestingly, both the major outer membrane protein (MOMP) (encoded by *porA*) and the flagellar filament protein FlaA were upregulated in Δ*cprS* compared with WT and Δ*cprS*^{C} Cj0998 (YceI-like, isoprenoid transport and/or metabolism) was also upregulated. Many

metabolic proteins were downregulated in Δ*cprS*, including fructose bisphosphate aldolase (Fba), fumarate hydratase (FumC), succinyl-CoA synthetase alpha chain (SucD) and aspartate-semialdehyde dehydrogenase (Asd). Also showing lower expression in Δ*cprS* versus WT were proteins involved in translation, such as ribosomal protein S1, trigger factor, EF-Tu, EF-G, EF-P and EF-Ts; however, a histidyl-tRNA was more highly expressed in Δ*cprS*. The nutrient acquisition protein ferric binding protein (FbpA) and a putative enoyl-[acyl-carrier-protein] reductase (FabI) were also lower in Δ*cprS*, although expression of FabI was not complemented in Δ*cprS*^{C}. Also interesting was downregulation of Cj0355c, encoding an orphan RR, as well as the autoinducer-2 (AI-2) synthase, LuxS. Finally, two unknown proteins encoded by Cj0706 and Cj0092 were also disregulated in Δ*cprS*. The diversity of disregulated proteins in Δ*cprS* suggested that CprRS controls numerous aspects of *C. jejuni* biology, including essential metabolic functions, some of which may be involved in biofilm formation and/or the *in vitro* and host-related phenotypes of Δ*cprS*.

Consistent with several proteomics observations, ΔcprS *exhibits enhanced motility, protein secretion, and osmotic and oxidative stress susceptibilities*

To explore and strengthen connections between the proteomics data and phenotypes observed for Δ*cprS*, a number of follow-up experiments were performed. Because of the higher FlaA expression seen in Δ*cprS*, we assessed this strain for motility. Microscopy suggested that Δ*cprS* was highly motile, and agar stab assays likewise showed an increase in motility in this strain (Fig. 8A). Because the flagellar apparatus has been proposed to function in protein secretion in *C. jejuni* (Konkel *et al*., 2004), and given the FlaA and MOMP protein expression differences, we also investigated whether subcellular protein localization or secreted protein profiles were altered in Δ*cprS*. Interestingly, while subcellular fractionation did not reveal notable differences in the cellular distribution of proteins, we did observe an increase in several specific proteins in the media fraction of Δ*cprS* compared with WT (Fig. 8B).

Initial extensive assessments of the Δ*cprS* mutant strain for stress-related phenotypes surprisingly revealed no obvious differences between Δ*cprS* and the parental strain for many conditions tested (Table S1). However, in keeping with changes in the twofold increase in MOMP porin overexpression, Δ*cprS* exhibited a marked decrease in its ability to form colonies on media containing moderate (1%) levels of NaCl (Fig. 8C), as well as decreased survival in broth culture supplemented with NaCl (data not shown). Complementation restored the osmotic stress defect of Δ*cprS* to WT levels (Fig. 8C). These data indicate that Δ*cprS* is defective for osmotolerance, which was further supported by a two- to fourfold decrease in the minimum inhibitory concentration (MIC) for other salts such as $MgCl_2$ and KCl (data not shown). After noting changes in expression of oxidative stress proteins, we also tested survival of Δ*cprS* in broth culture supplemented with the oxidative stress agent t-butylhydroperoxide, and noted a small but reproducible increase in sensitivity ($P < 0.05$) in the Δ*cprS* mutant compared with WT (Fig. 8D). We have also observed a slight increase in sensitivity to both hydrogen peroxide and paraquat (data not shown).

Biofilm formation in C. jejuni *is suppressed by fumarate and promoted by deoxycholate*

In *Pseudomonas aeruginosa*, the availability of carbon sources such as glucose, succinate and glutamate directs the maturation of biofilms (Shrout *et al*., 2006). Because we identified metabolic changes in the Δ*cprS* hyperbiofilm-forming strain by proteomics, we tested biofilm formation in *C. jejuni* of media supplemented with different metabolic substrates (Fig. 9A). Whereas glutamine and glycine did not influence biofilm formation, *C. jejuni* grown in the presence of 50 mM fumarate visibly favoured growth in the planktonic fraction, with a twofold increase in the ratio of planktonic (OD_{600}) to biofilm (A_{570}) bacteria. In contrast, the C2-dicarboxylate pyruvate had no effect on biofilm formation (data not shown).

Finally, because of the expression changes in surface proteins in Δ*cprS*, we tested the effect of various detergents on biofilm formation (Fig. 9B). Biofilms were grown in the presence of sub-MIC levels of the bile salt deoxycholate (DOC), Triton X-100, Tween-20 and sodium dodecyl sulphate (SDS). Interestingly, DOC significantly increased biofilm formation, causing WT bacteria to make biofilms at levels similar to Δ*cprS*. In contrast, the other detergents had no effect. No differences were observed in MICs between strains for each of these compounds (data not shown). Propidium iodide staining of WT (Fig. 9C) and Δ*cprS* (not shown) biofilms formed in the presence of DOC showed that they exhibit normal biofilm architecture and form an extensive fibrous network, consistent with observations shown in Fig. 4C. No fibres were observed by propidium iodide staining of coverslips incubated overnight in MH broth with DOC alone (data not shown). WT biofilms formed in the presence of DOC were also visibly and quantitatively disrupted with DNase I (Fig. 9D; $P < 0.005$ for DNase I-treated versus buffer-treated biofilms), with A_{570} values for all samples similar to those shown in Fig. 4D for Δ*cprS* biofilms. Together, these data indicate that DOC enhances biofilm formation in *C. jejuni* and provides further evidence for DNA as a component of the *C. jejuni* biofilm matrix.

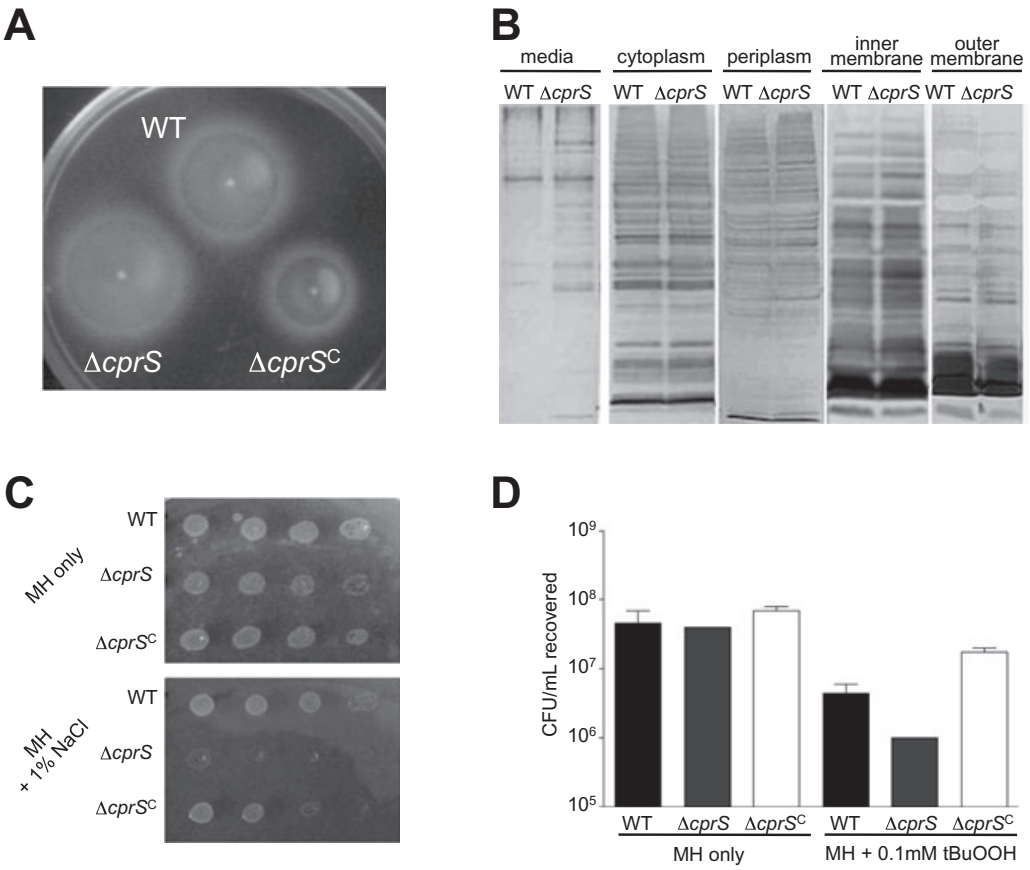


Fig. 8. Proteomics follow-up studies reveal enhanced motility, protein secretion, and osmotic and oxidative sensitivity differences between WT and Δ*cprS*.
A. Δ*cprS* shows an increase in motility compared with WT. Overnight cultures of WT, Δ*cprS* and Δ*cprS*^{C} were stabbed into MH plates with 0.4% agar and incubated microaerobically for 24 h.
B. Protein secretion is increased in Δ*cprS*. The media fraction was clarified by centrifugation from overnight cultures of WT and Δ*cprS* at approximately equal optical densities. The collected cells were then subjected to subcellular fractionation to allow analysis of the secreted (media), cytosolic, periplasmic, inner membrane and outer membrane protein profiles. Equal volumes of sample were separated by SDS-PAGE followed by silver staining.
C. The Δ*cprS* mutant displays a decreased ability to grow under osmotic stress. Overnight cultures of WT and Δ*cprS* in MH broth were diluted to an optical density of 0.05 in MH broth, serially diluted 10-fold, spotted on MH plates or MH plates supplemented with 1% (w/v) NaCl and incubated for 24 h microaerobically. Dilutions were spotted left to right, with the highest starting CFUs on the left most side of the plate.
D. The Δ*cprS* mutant shows decreased ability to survive in the presence of oxidative stress. Bacteria were suspended at an OD_{600} of 0.02 in MH broth or MH broth supplemented with 0.1 mM t-butylhydroperoxide (tBuOOH). Following 30 min of incubation at 37°C microaerobically, samples were serially diluted and plated for cfu counts.

Discussion

In *C. jejuni*, as in other zoonotic bacteria, comprehensive shifts in physiology may be required to adapt to either a transmission or host environment. The capacity for gene regulation in *C. jejuni* is limited; nonetheless, its prevalence suggests it must harbour mechanisms that allow such changes. Here we report the characterization of the *C. jejuni* CprS sensor kinase, which may influence such aspects of *C. jejuni* pathogenesis through control of biofilm or planktonic growth and modulation of essential biological functions. This work has also identified new paradigms for biofilm formation in *C. jejuni.*

Our preliminary genetic analyses indicated that the activity of the CprR RR, but not the CprS SK, was essential for viability of *C. jejuni* in the laboratory. It is possible that CprR mutants enter into a viable but non-culturable state and are not recoverable by colony isolation except under specific unknown conditions. Nonetheless, it is intriguing that only the RR appears to be required for viability. The predicted operonic structure of *cprRS* suggests a functional relationship, and while phosphotransfer could not be directly shown due to insolubility of CprS *in vitro* (S. Svensson and E. Gaynor, unpubl. obs.), the C-terminal domain of CprS interacted specifically with CprR by two-hybrid analysis. Based on work in other

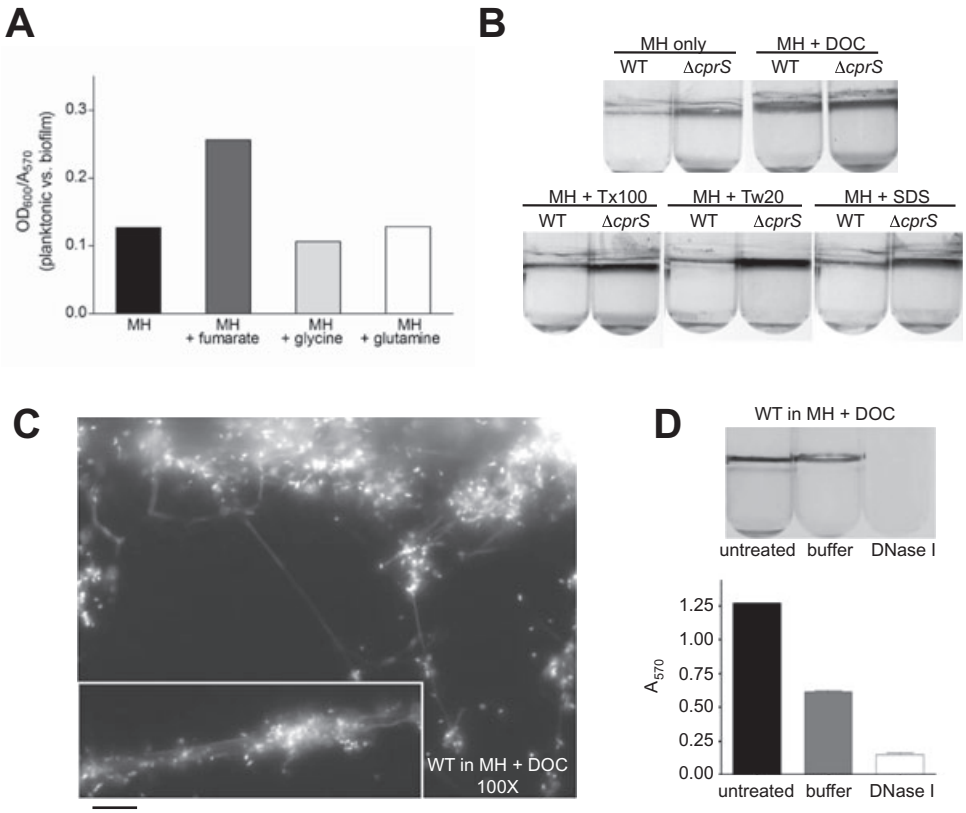


Fig. 9. *C. jejuni* biofilm formation is inhibited by fumarate and enhanced by deoxycholate (DOC).
A. Fumarate promotes planktonic growth of *C. jejuni*. Biofilms were grown in MH broth or MH broth supplemented with 50 mM fumarate, glycine or glutamine. After 2 days of microaerobic incubation, samples were carefully removed from the media (planktonic) fraction to measure OD_{600}, and biofilms were then quantified by crystal violet staining. Shown is an average of the planktonic (OD_{600}) to biofilm (A_{570}) ratio for each strain.
B. DOC enhances biofilm formation in *C. jejuni*. Biofilms were grown in MH broth supplemented with sub-MIC levels of various detergents. Concentrations used were as follows: DOC, 0.05%; Triton X-100 (Tx-100), 0.0005%; Tween-20 (Tw-20), 0.002%; and sodium dodecyl sulphate (SDS), 0.00025%.
C. DOC-induced biofilms exhibit normal architecture and fibre formation. WT (shown) and Δ*cprS* (not shown) biofilms were grown on glass coverslips in MH + 0.05% DOC, stained with propidium iodide, and visualized by microscopy at 100× magnification. The bar represents ~40 μm.
D. WT biofilms grown in the presence of DOC are visibly disrupted by DNase I treatment. WT (shown) and Δ*cprS* (not shown) biofilms were grown for 2 days in MH + 0.05% DOC and either left untreated or rinsed with PBS, then incubated for 3 h in buffer alone or buffer with DNase I, followed by crystal violet staining.

systems (Kulasekara *et al*., 2005) this interaction strongly supports a functional relationship between the two components.

The essential nature of CprR suggests that CprRS might control global aspects of physiology central to the biology of *C. jejuni*, similar to the essential *Caulobacter* RR CtrA (Quon *et al*., 1996) or the YycF RR in *Bacillus subtilis* (Szurmant *et al*., 2005). Consistent with control of essential processes, we observed apparent growth defects for Δ*cprS* in rich broth, and our data also suggest that CprS may influence global changes such as the biofilm-planktonic switch. The essential nature of only the RR is intriguing, but can be explained by a variety of scenarios. For instance, cross-talk between TCRSs has been demonstrated in the absence of the cognate SK in other bacteria (Verhamme *et al*., 2002). This may be especially significant for a TCRS (such as CprRS) that regulates genes required for viability, where non-cognate SK(s) may effect phosphotransfer to the RR when the native cognate SK is absent. Furthermore, acetyl phosphate can also phosphorylate RRs in *E. coli* (McCleary and Stock, 1994), and in *C. jejuni*, this may allow basal levels of RR phosphorylation that permit viability. Alternatively, phosphotransfer may not be required for CprR to modulate essential genes, similar to the *H. pylori* essential RRs HP1021 and HP1043 (Muller *et al*., 2007). *H. pylori* also encodes a TCRS, HP165-HP166, where like CprRS, only the RR is essential for growth (Schar *et al*., 2005). In this system, there are two species of RR-controlled promoters – one recognized by HP166 (which presumably regulates essential genes) and a second, lower-affinity class of promoters bound by

phospho-HP166 (which activates dispensable genes) only when environmental conditions stimulate RR phosphorylation. In a bacterium with limited regulatory capacity such as *C. jejuni*, this scenario is especially attractive, as it would allow different forms of the same RR to be devoted to separate regulons.

There is debate as to whether the biofilm lifestyle is the default lifestyle for bacteria, or whether planktonic growth is simply an artefact of laboratory culture. The hyperbiofilm phenotype of Δ*cprS* suggests that this phenomenon is a regulated process, and this work suggests that CprS may play a role in its regulation. Numerous TCRSs have been implicated in regulation of biofilm formation in other pathogens through control of phenomena such as adhesin expression (Kulasekara *et al*., 2005), exopolysaccharide production (Hussa *et al*., 2008) and quorum sensing (Dong *et al*., 2008). Direct involvement of a TCRS in *C. jejuni* biofilms is supported by observations of defective biofilm formation upon inactivation of Cj0688, encoding a likely phosphate acetyltransferase (Joshua *et al*., 2006) which may affect RR phosphorylation. Furthermore, the *C. jejuni* orphan RR CbrR contains a putative GGDEF domain (Raphael *et al*., 2005) which may be involved in production of c-di-GMP, a mediator of biofilm formation in other pathogens (Romling and Amikam, 2006). A role for CbrR in control of *C. jejuni* biofilm phenotypes has not been demonstrated; however, the CprS SK is an appealing candidate cognate SK for this RR. Finally, downregulation of the Cj0355c RR, which is also essential (Raphael *et al*., 2005; J.A. Fields and S.A. Thompson, unpublished), in the 81-176 Δ*cprS* mutant, while in apparent contrast to previous work where Cj0355c was upregulated in *C. jejuni* NCTC 11168 biofilms (Kalmokoff *et al*., 2006), is consistent with CprRS affecting transcription of and thereby interacting with other regulators of both key biological processes and biofilm formation.

Mechanistic insight into *C. jejuni* biofilm formation is relatively limited, and we hypothesized that identifying specific physiological changes in the hyperbiofilm-forming Δ*cprS* mutant could be used to understand *C. jejuni* biofilms in more detail. Biofilm bacteria typically secrete an extracellular matrix, often composed of polysaccharides (Branda *et al*., 2005). While *H. pylori* secretes an exopolysaccharide during biofilm growth (Stark *et al*., 1999) and other enteric pathogens such as *Salmonella* and *E. coli* also utilize carbohydrates extensively (Romling, 2005), the matrix surrounding *C. jejuni* biofilms remains ill-defined. A Δ*spoT* stringent response mutant overproduces a CFW-reactive exopolysaccharide, production of which correlates with biofilm formation (McLennan *et al*., 2007). In contrast, analyses of LOS profile, capsule production and CFW reactivity of Δ*cprS* revealed no differences from WT. Furthermore, analysis of the *C. jejuni* genome did not identify homologues of biofilm-associated proteins such as Bap or Esp (Cucarella *et al*., 2001; Toledo-Arana *et al*., 2001).

A component of the biofilm matrix in other bacteria is extracellular DNA (Whitchurch *et al*., 2002). We observed fibres extending between microcolonies in *C. jejuni* biofilms stained with crystal violet or propidium iodide, both of which stain DNA. The presence of DNA in the *C. jejuni* biofilm matrix was further supported by our observations that the extensive biofilms formed by Δ*cprS* could be visibly disrupted by treatment with DNase I, and that WT (and Δ*cprS*) biofilms formed in the presence of DOC likewise exhibited striking DNase I sensitivity. Interestingly, strain 81-176 forms more robust biofilms than strain 11168 (S. Svensson and E. Gaynor, unpublished) and also harbours a plasmid (pVIR) containing components of a putative type IV secretion system (T4SS) not found in strain 11168 (Bacon *et al*., 2000). It will be interesting, in future work, to explore a possible involvement of this plasmid in secretion of DNA to the biofilm matrix.

Because our interest in CprRS was initially piqued by its identification in a cell infection screen, we hypothesized that CprRS may control phenotypes specifically related to pathogenesis. In spite of several growth- and stress-related defects *in vitro*, the Δ*cprS* mutant showed no obvious defect in epithelial cell invasion. Interestingly, however, we consistently recovered more Δ*cprS* than WT bacteria from INT407 cells following several hours of intracellular survival. It remains to be seen whether this is due to increased bacterial or epithelial cell survival, either of which could result in enhanced recovery of intracellular bacteria. Nonetheless, to the best of our knowledge, this is the first report of a *C. jejuni* mutant exhibiting enhanced longer-term intracellular recovery, particularly when coupled with no invasion differences from WT. It has been reported that exposure of certain clinical strains of *C. jejuni* to an aerobic atmosphere prior to infection of epithelial cells *in vitro* enhances intracellular survival (Mihaljevic *et al*., 2007). In light of our proteomics data demonstrating upregulation of oxidative stress tolerance proteins such as AhpC and KatA in Δ*cprS*, the stress resulting from disregulation of essential processes in this mutant may 'prime' the bacteria for survival within the stressful intraepithelial cell environment.

In contrast to the *in vitro* infection data, the Δ*cprS* mutant was significantly impaired for chick colonization, consistent with several other *C. jejuni* TCRS mutants (Bras *et al*., 1999; MacKichan *et al*., 2004; Wosten *et al*., 2004; 2006; Raphael *et al*., 2005). Inactivation of *ppk1* in *C. jejuni* also results in a colonization defect that, like Δ*cprS*, is rescued by increasing the inoculating dose. A correlative dose-dependent increase in biofilm formation for the *ppk1* mutant was proposed as potentially responsible for restoration of WT colonization levels at higher

doses (Candon *et al*., 2007). In addition, a proteomics comparison of robust and poor chicken-colonizing strains of *C. jejuni* suggested that many expression trends in the robust colonizer mirrored those previously identified in biofilm- or agar-grown bacteria (Seal *et al*., 2007). Together, this suggests that at higher infective doses, the enhanced biofilm forming ability of Δ*cprS* may allow the mutant to overcome planktonic sensitivities (i.e. stress survival defects) that may occur with disregulation of essential genes.

We took a global proteomics-based approach to identify protein expression changes that might help explain the diverse pathogenesis-related phenotypes observed for Δ*cprS*. This identified approximately 20 differentially expressed proteins in the Δ*cprS* mutant compared with WT, with the vast majority of these proteins present at WT levels in Δ*cprS*[C]. We detected differences in proteins involved in several distinct aspects of physiology, including oxidative stress tolerance, metabolism and cell surface characteristics, as well as regulatory proteins. Many expression changes correlated well with previous biofilms studies. For instance, increased MOMP, FlaA, Cj0998 and succinyl-CoA synthetase expression was consistent with proteomics analyses of *C. jejuni peb4* mutants, which are defective for biofilm formation and have decreased expression of these proteins (Asakura *et al*., 2007), and those of agar-grown (sessile) *C. jejuni*, which also identified succinyl-CoA synthetase and trigger factor as upregulated (Sampathkumar *et al*., 2006). Increased expression of TrxB (Asakura *et al*., 2007), AhpC and FlaA in Δ*cprS* is also consistent with proteomics analyses of WT *C. jejuni* biofilms (Kalmokoff *et al*., 2006). MOMP and FlaA also serve as adhesins in *C. jejuni* and therefore have relevance to biofilm formation (Moser *et al*., 1997), and previous work suggests that unlike other bacteria such as *P. aeruginosa*, biofilm-residing *C. jejuni* maintain their flagella (Kalmokoff *et al*., 2006). In contrast, downregulation of LuxS in Δ*cprS* was a surprising finding. While a role for AI-2 in quorum sensing in *C. jejuni* has not been demonstrated, it has been reported that *C. jejuni luxS* mutants exhibit decreased motility, autoagglutination (Jeon *et al*., 2003) and biofilm formation (Reeser *et al*., 2007), which we have also noted (S. Svensson and E. Gaynor, unpublished). Our expression profiling thus supports an AI-2-independent mechanism for the biofilm phenotype of Δ*cprS*.

Altered expression of metabolic proteins is consistent with several of the phenotypes associated with deletion of *cprS*. For instance, the essential nature of many metabolic genes makes them ideal candidates for regulation by an essential RR, and proteins representing of a number of metabolic pathways such as glycolysis (Fba), the trichloroacetic acid (TCA) cycle (FumC, SucD), fatty acid biosynthesis (FabI), amino acid metabolism and biosynthesis (Asd), and protein synthesis (elongation factors, tRNA synthetase, ribosomal protein S1) were expressed differently in Δ*cprS*. Some of the metabolic differences observed might also reflect shifts that occur when *C. jejuni* enters or exits a biofilm. Likewise, metabolic alterations may help explain the modest increase in recovery of Δ*cprS* from within INT407 cells, as *C. jejuni* intracellular survival has recently been shown to involve adaptation to the likely very low-oxygen CCV (Watson and Galan, 2008) which is also likely to be a nutrient-poor environment (Gaynor *et al*., 2005; Candon *et al*., 2007). Consistent with this, microarray analyses suggest that *C. jejuni* undergoes global expression changes in numerous metabolic genes during cell infection (E. Gaynor, unpublished), and real-time quantitative PCR data suggest that expression of *dcuA*, encoding a likely C4-dicarboxylate transporter, is reduced 10-fold in Δ*cprS* (S. Svensson and E. Gaynor, unpublished). Finally, distinct metabolic pathways are initiated within the avian gastrointestinal tract (Woodall *et al*., 2005); thus the metabolic changes in Δ*cprS* may also account for its decreased chick colonization.

Importantly, the proteomics analysis also led us to perform additional experiments which provided further insight not only into the colonization and biofilm phenotypes of Δ*cprS* but also into factors affecting biofilm formation of WT *C. jejuni*. For instance, elevated MOMP porin expression in Δ*cprS* led us to hypothesize that outer membrane permeability may be altered, consistent with subsequent observations that Δ*cprS* exhibited a striking osmotolerance defect. Interestingly, we also observed a slight increase in sensitivity to oxidative stress agents such as t-butylhydroperoxide. This appears to contrast the observation that *C. jejuni* residing in biofilms are more resistant to oxidative stress than their planktonic counterparts (Trachoo and Frank, 2002) as well as our proteomics data, where numerous proteins involved in oxidative stress tolerance were upregulated in Δ*cprS*. However, it is possible that upregulation of oxidative stress proteins may be the result of a general stress response to disregulation of essential genes. Alternatively, increased levels of MOMP may increase outer membrane permeability to oxidative agents, resulting in increased oxidative stress sensitivity and upregulation of stress response proteins. It is nonetheless interesting to hypothesize that the increased osmotic and oxidative stress susceptibilities of Δ*cprS* may in part account for its chick colonization defect.

FlaA was also present at higher levels in the mutant strain, which was supported by our observation of a slight increase in motility in Δ*cprS* compared with WT. As noted, the flagellar filament is important for *C. jejuni* bioflm formation, and while a role for motility *per se* (i.e. via analysis of a filament-positive, motility-minus mutant) has not yet been proven for *C. jejuni*, it is not unreasonable to predict,

based on work in other bacteria, that motility will prove important for *C. jejuni* biofilm formation as well. The increased expression of FlaA in Δ*cprS* is also consistent with our observation of an increased amount and number of protein species in the media fraction of Δ*cprS* cultures. As noted above, *C. jejuni* lacks dedicated type III secretion systems; however, secretion of *C. jejuni* virulence factors called Cia (*Campylobacter* invasion antigen) proteins is dependent on a functional flagellar apparatus (Konkel *et al*., 2004). At present, we cannot rule out the possibility that the appearance of proteins in the Δ*cprS* media fraction may represent loss of membrane integrity; however, there are several striking dissimilarities between the pattern of media versus periplasmic or cytoplasmic proteins, suggesting some specificity in the proteins present in the Δ*cprS* media. Future studies to identify these proteins will allow us to explore the connection between this phenotype and those involving biofilms and host-related properties.

Interestingly, Cia protein expression and secretion is stimulated by 0.05% DOC (Malik-Kale *et al*., 2008). We found that a similar concentration of DOC stimulated Δ*cprS*-level biofilm formation in WT *C. jejuni*. DOC also promotes biofilm formation in other enteric pathogens such as *Vibrio cholerae* (Hung *et al*., 2006). Thus in addition to the DNA component mentioned above, it is possible that the synthesis and/or secretion of proteins present in the media fraction of Δ*cprS* that contribute to biofilm formation are also induced by DOC, accounting at least in part for increased biofilm formation of WT *C. jejuni* in the presence of sub-MIC levels of DOC.

Our observation that numerous metabolic genes were altered in Δ*cprS* led us to hypothesize that nutrient availability may influence *C. jejuni* biofilm formation. Assays investigating the effect of a variety of carbon sources on WT biofilms demonstrated that the bacteria favoured planktonic growth when fumarate was added to the media. This correlates with observations in other bacteria where nutrient availability can trigger biofilm dispersal (Gjermansen *et al*., 2005). For example, control of swarming motility and biofilm architecture by quorum sensing in *P. aeruginosa* is nutritionally conditional (Shrout *et al*., 2006). Likewise, in *Shewanella*, different electron acceptors (i.e. nitrate versus fumarate) result in changes in carbohydrate exopolymer production and surface-associated behaviour (Neal *et al*., 2007). In *C. jejuni*, biofilm formation also appears to be promoted by differences in nutrient availability (Reeser *et al*., 2007). It is conceivable that CprRS allows *C. jejuni* to respond to nutrient availability cues by inducing and/or repressing genes related to biofilm formation.

In summary, our genetic analysis of a TCRS in *C. jejuni*, within the confines of an essential RR, has identified complex phenotypic and protein expression changes which point to a role for CprRS in controlling changes in physiology and metabolism involved in biofilm dynamics, stress tolerance and pathogenesis-related phenotypes. Strong conservation of CprRS in only the Campylobacteraceae suggests that CprRS controls campylobacter-specific phenomena, many of which may be shared by related, and even more understudied, pathogens that cause severe enteric disease (i.e. *C. coli*, *C. upsaliensis* and *A. butzleri*) and spontaneous abortion in livestock (*C. fetus*). Absence of CprRS from *H. pylori*, essentially host-restricted, is consistent with control of phenotypes relating to passage between hosts and/or phenotypes relating specifically to adaptation to an avian host. Our data also contribute to the understanding of other pathogens that use different paradigms from model bacteria. For instance, while genes involved in processes such as stress tolerance and biofilm formation may not fit the definition of virulence factors set out in molecular Koch's postulates (Falkow, 2004), they may allow survival of an apparently fastidious pathogen such as *C. jejuni* within a commensal reservoir, between hosts during transmission, or persistence within a susceptible host long enough to cause significant inflammation. The diverse phenotypes displayed by Δ*cprS* suggest that this TCRS is pleiotropic and likely controls many phenomena in *C. jejuni*, providing intriguing hypotheses for further testing.

Experimental procedures

Bacterial strains and growth conditions

Studies were performed using the *C. jejuni* WT strain 81-176, a highly invasive isolate from a raw milk outbreak (Korlath *et al*., 1985). All strains are listed in Table S2. *C. jejuni* was routinely cultured in Mueller–Hinton broth (MH; Oxoid, Hampshire, England) or agar plates. All incubations were performed at 37°C under microaerobic conditions (6% O_2, 12% CO_2) in a Heraeus tri-gas incubator (plates and biofilms) or generated using the Oxoid CampyGen system (broth cultures). Media used to culture *C. jejuni* was supplemented with 10 μg ml^{-1} vancomycin and 5 μg ml^{-1} trimethoprim. Where appropriate, the selective antibiotics (Sigma, Oakville, ON) kanamycin and chloramphenicol were added to a final concentration of 40 μg ml^{-1} and 25 μg ml^{-1} respectively. *C. jejuni*-selective MH agar plates were used for growth of *C. jejuni* for the chick colonization studies (see below). *E. coli* strain DH5α was used for recombinant DNA manipulations and was routinely cultured in LB broth (Sigma) with antibiotics added at the following concentrations: ampicillin, 100 μg ml^{-1}, kanamycin 50 μg ml^{-1}, chloramphenicol, 25 μg ml^{-1}, and tetracycline, 20 μg ml^{-1}.

General recombinant DNA techniques

Recombinant DNA techniques were performed according to Sambrook and Russell (2001). Restriction enzymes and DNA modifying enzymes were purchased from New England

Biolabs (Mississauga, ON) or Invitrogen (Burlington, ON). Plasmids were isolated from bacteria using the Qiagen Qiaprep Spin miniprep kit (Qiagen, Mississauga, ON). Primers are listed in Table S3.

Targeted mutagenesis of C. jejuni

Targeted deletion mutagenesis of *C. jejuni* was achieved by double-cross-over homologous recombination with constructs prepared in a *C. jejuni* suicide vector containing approximately 500 base pairs of homologous DNA of the target gene region flanking either a *cat* (chloramphenicol acetyltransferase, Cm^R) or *aph-3* (aminoglycoside 3′-phosphotransferase, Km^R) cassette (Ounissi *et al*., 1990; Yao *et al*., 1993), with the majority of coding sequences removed by inverse PCR. Unless otherwise stated, experiments were performed using a *cprS*::*cat* strain (Δ*cprS*) constructed by replacing the majority of *cprS* coding sequence with a Cm^R marker, as follows. Briefly, the entire coding sequence of *cprS*, along with approximately 500 base pairs of flanking sequence on each side, was amplified by PCR using primers *cprS*-TOPO FWD/REV from genomic DNA isolated from *C. jejuni* 81-176 (DNeasy Kit, Qiagen), and this approximately 2300-base-pair product was ligated directly into the pCR2.1-Topo vector (Invitrogen). Inverse PCR using primers *cprS*-INV FWD/REV was then used to remove the majority of the *cprS* gene and engineer MfeI sites. A *cat* cassette was excised from plasmid pRY109 (Yao *et al*., 1993) using EcoRI and ligated into the MfeI-digested inverse PCR product. The resulting construct, containing approximately 86 and 137 base pairs of 5′ and 3′ coding sequence of *cprS*, was then used to naturally transform *C. jejuni* 81-176, and Cm^R recombinants were recovered and confirmed to be Δ*cprS* by PCR. A *cprS::aph-3* strain (Δ*cprS*::Km^R) was also constructed in a similar manner to the *cat* strain, using the primers *cprS*-pGEM FWD/REV and *cprS*-INV2 FWD/REV.

Complementation of ΔcprS

Insertion of copies of *cprS* for complementation was achieved using pRRK [J. Ketley, unpublished, based on pRRC integration vectors (Karlyshev and Wren, 2005)]. The coding region of *cprS* was amplified by PCR using primers *cprS*-pRRC FWD/REV, which introduced 5′-XbaI sites and 3′-MfeI sites, as well as the native ribosome binding site of *cprR* (Wosten *et al*., 1998). Following digestion with MfeI and XbaI, this product was ligated into the pRRK vector. The resulting construct was naturally transformed into *C. jejuni* Δ*cprS*::Km^R, and putative Δ*cprS*^{C} complemented clones were recovered on plates containing kanamycin. Single insertions of *cprS* in the rRNA spacer region adjacent to *cj0029* were confirmed by PCR using primers ak233, ak234, ak235 and PKanF.

Bacterial two-hybrid analysis

Protein–protein interactions were determined using the Bacteriomatch II Two-hybrid System (Stratagene, La Jolla, CA). Coding regions for CprR and DccR were amplified by PCR using primers BT-*cprR* FWD/REV or BT-*dccR* FWD/REV, digested with BamHI and XhoI, and ligated into similarly digested pBT. The C-terminal domain (amino acids 187–415) of CprS was amplified by PCR using primers TRG-*cprS*-CTD FWD/REV, digested with BamHI and SpeI, and ligated into the similarly digested plasmid pTRG. Electrocompetent *E. coli* Bacteriomatch II cells were co-transformed with 50 ng of each bait and prey plasmid, followed by recovery for 90 min in LB broth at 37°C. Cells were then washed with M9+ His dropout broth and conditioned for 2 h in this medium at 37°C prior to plating dilutions on Non-selective and Selective (3-AT) plates.

RNA extraction, cDNA synthesis and quantification

RNA was extracted from mid-log-phase broth cultures (OD_{600} 0.2–0.5) or overnight cultures on MH agar as previously described (Gaynor *et al*., 2004). Briefly, 1/10 volume of 10× Stop solution (5% buffer-saturated phenol in ethanol) was added to 0.5 OD_{600} of bacteria, and cells were collected by centrifugation at 10 000 *g* for 5 min at room temperature followed by immediate freezing at –80°C. Cells were thawed at room temperature, re-suspended in 50 μl of 0.4 mg ml^{-1} lysozyme (Sigma) in 10 mM Tris 1 mM EDTA, pH 8.0 and incubated at room temperature for 5 min. Cells were then lysed by addition of 950 μl of Tri-reagent (Sigma) and vortexing for 1 min, followed by the addition of 200 μl of chloroform. After centrifugation at 14 000 *g* for 15 min at 4°C, the top phase was transferred to a new tube. An equal volume of 70% ethanol was added dropwise, followed by application to an RNeasy column and washing according to the manufacturer's instructions. Samples were digested using the Qiagen on-column RNase-free DNase kit according to the manufacturer's instructions, and RNA was eluted in 30 μl of RNase-free water. cDNA was generated from the above preparation of total RNA using SSII enzyme (Invitrogen) and cleaned up using the Qiagen Qiaquick PCR purification kit. Concentrations of DNA and RNA were quantified using a NanoDrop ND-1000 spectrophotometer (Wilmington, DE), according to the manufacturer's instructions, and RNA quality was assessed by electrophoresis on 1% agarose TAE gels. Absence of genomic DNA within RNA samples was confirmed by PCR.

Transcript analysis

Independent transcription of *cprR* and the upstream *htrA* gene was achieved using cDNA extracted as described above. PCR reactions with *Taq* DNA polymerase using primer sets *htrA*-3′/*cprR*-BT REV and *cprR* BT FWD/REV were performed using cDNA or genomic DNA from WT *C. jejuni* 81-176, and the presence of amplicons was determined by agarose gel electrophoresis.

Growth curve assays

Campylobacter jejuni strains were grown in MH broth overnight to mid-log phase then diluted to an OD_{600} of 0.1 in MH broth. Cultures were incubated microaerobically at 37°C with shaking at 200 r.p.m. Growth and viability were assessed at various times post dilution by measuring the OD_{600} and plating serial 10-fold dilutions on MH agar.

Biofilm assays and broth culture tube staining

Biofilm formation was assessed as previously described (O'Toole and Kolter, 1998; McLennan *et al*., 2007) with modifications. Briefly, overnight broth cultures of *C. jejuni* strains were diluted to an OD_{600} of 0.002 in fresh media, and 1 ml was added to borosilicate glass tubes. Tubes were incubated without agitation in a Trigas incubator under microaerobic conditions for 1 or 2 days, followed by staining for 10 min by addition of 250 μl of 1% crystal violet (Sigma) dissolved in 100% ethanol. Tubes were then rinsed with distilled water and dried, followed by photographic documentation and/or quantification of adhered crystal violet by dissolving with 30% methanol/10% acetic acid and measuring the absorbance at 570 nm. Where indicated in figure legend 9, chemicals were included in the MH broth of suspensions introduced into borosilicate tubes prior to initiation of biofilm growth. Shaking broth culture tubes were stained with crystal violet as above.

Bright field and scanning electron microscopy of biofilms

Campylobacter jejuni biofilms were grown in borosilicate tubes as described above, with a glass coverslip standing upright in the culture. After 48 h of incubation, coverslips were either stained with crystal violet as described above, stained with PI using the LIVE/DEAD BacLight Bacterial Viability Kit according to the manufacturer's instructions, or removed and processed for SEM as follows. The coverslip was removed and gently rinsed once in 0.1 M cacodylate buffer, and the biofilms were fixed (2.5% glutaraldehyde in 0.1 M cacodylate) for 1 h. Cover glasses containing the biofilms were processed and visualized using SEM.

Carbohydrate analyses

Campylobacter jejuni strains were grown on MH agar for 48 h microaerobically and prepared based on the method of Hitchcock and Brown (1983). Briefly, bacteria were harvested with PBS and diluted to an OD_{600} of 10 in 100 μl. An equal volume of 2× lysis buffer [4% SDS, 8% β-mercaptoethanol, 20% glycerol, 0.125 M Tris (pH 6.8), 0.025% bromophenol blue] was added to the cell suspension, which was then heated at 95°C for10 min. After the samples cooled to room temperature, 10 μl of 10 mg ml^{-1} proteinase K was added and samples were incubated overnight at 37°C or 55°C. Samples were then incubated at 65°C for 1 h or 95°C for 5 min and separated by SDS-PAGE and silver-stained as previously described (Tsai and Frasch, 1982) or electroblotted to PVDF membrane for Western blotting with an α-O36 serotype antibody (a gift from Kris Rahn, Public Health Agency Canada Laboratory for Foodborne Zoonoses, Guelph, ON). Assessment of CFW reactivity has been described elsewhere (McLennan *et al*., 2007). Briefly, overnight cultures of each strain were diluted to similar OD_{600} and spotted on BHI agar containing 0.002% CFW (Sigma).

DNase I treatment of biofilms

Biofilms were grown as described above. Following 2 days of incubation, the medium was removed by pipetting, and tubes were washed with 1.5 ml of PBS, pH 7.4 (Invitrogen). DNase I buffer alone, or buffer containing DNase I (Fermentas) at 4 U ml^{-1} in PBS was added to each tube, followed by incubation for 3 h at 38°C under microaerophilic conditions and staining with crystal violet as described above. Bacterial viability under each condition was also assessed by quantifying cfu of each strain in an OD_{600} 0.02 suspension in each condition after 3 h of incubation.

Adherence, invasion and intracellular survival in INT407 cells

In vitro gentamicin protection assays were performed essentially as described previously (Gaynor *et al*., 2005). Briefly, INT407 cells in Minimal Essential Medium (MEM) with 15% fetal bovine serum (FBS) (Invitrogen) were seeded into 24-well plates 16 h before infection (~10^5 cells per well) and incubated in a humidified 5% CO_2 atmosphere. *C. jejuni* strains were harvested from healthy MH plates and inoculated into MH biphasic tubes to an OD_{600} of 0.002. After approximately 16 h of growth, bacteria were washed two times with MEM, and 1 ml of bacteria in MEM without FBS was used to infect INT407 cells, which had been washed once with MEM without FBS before infection, at a multiplicity of infection of ~100 (~10^7 bacteria per well). Infections were carried out in a 5% CO_2 incubator at 37°C. Survival in media above cells and the number of adhered and/or invaded bacteria ('adherence' time point) were assayed after 3 h; gentamicin treatment (150 μg ml^{-1}) was initiated at this point. Two hours following initiation of gentamicin treatment, invaded/intracellular bacteria were harvested by lysing the INT407 cells with sterile distilled water and a 27-gauge syringe and viability of the bacteria was assessed by plating serial dilutions on MH agar ('invasion' time point). Intracellular survival was tested by replacing the medium with MEM with 10 μg ml^{-1} gentamicin to prevent growth of bacteria released from lysing INT407 cells, followed by further incubation for 5 h and harvesting of intracellular bacteria as in above ('intracellular survival' time point). Experiments were performed in triplicate.

Chick colonization assays

The chick colonization assay was performed as previously described (Hendrixson and DiRita, 2004). Briefly, day-of-hatch chicks (white-leghorn, Charles River Laboratories) were orally inoculated with 10^2, 10^4 or 10^6 cfu *C. jejuni* diluted in PBS. The *C. jejuni* strains were grown on MH agar containing 10 μg ml^{-1} trimethoprim for 18 h in a trigas incubator at 37°C. After 6 days, chicks were euthanized, and their caeca removed. Caecal contents were weighed, diluted, and plated onto MH agar containing 10 μg ml^{-1} trimethoprim and 30 μg ml^{-1} cefoperazone. *C. jejuni* colonies were counted, cfu g^{-1} caecal matter recorded and log cfu g^{-1} reported. To quantify the average recovered bacteria from each infected group, chicks harbouring fewer cfu than the detection limit were assigned a log cfu g^{-1} value of 2 (i.e. 100 cfu g^{-1}, the limit of detection).

Proteomic comparison of WT, ΔcprS and ΔcprS^C

Proteome analysis was performed using Differential In-Gel Electrophoresis (DIGE) technology (GE Biosystems, Piscat-

away, NJ). Briefly, *C. jejuni* WT, Δ*cprS* and Δ*cprS*^{C} cells were grown to mid-log phase at 37°C in MH broth. Protein lysates were prepared by washing cells three times in wash buffer (10 mM Tris, pH 8.0, 5 mM magnesium acetate) at 4°C for 4 min at 12 000 *g*. One millilitre of lysis buffer [8 M urea, 30 mM Tris, pH 8.5, 5 mM magnesium acetate, 4% (w/v) CHAPS] was used to re-suspend the pellet, and the suspension incubated for 30 min on ice, then sonicated for six 10 s bursts (Model 100 Sonic Dismembrator, Fisher Scientific). Insoluble material was removed by centrifugation at 4°C for 10 min at 12 000 *g* and the soluble component used for further studies. Protein concentrations were determined using a BCA assay kit (Pierce, Rockford, IL). Protein samples from WT, Δ*cprS* and Δ*cprS*^{C} were labelled individually with Cy2, Cy3 and Cy5 dyes according to the protocol supplied by the manufacturer (GE Biosystems). Briefly, 25 mg of each protein sample (WT, Δ*cprS* and Δ*cprS*^{C}) were labelled at lysine residues with 1 ml Cy2, Cy3 and Cy5 dye conjugates respectively (for 10 min in the dark). The reactions were stopped by addition of 1 mM lysine. The Cy2-, Cy3- and Cy5-labelled proteins were then mixed with an equal amount of unlabelled protein, and finally all proteins mixed together (total of 150 mg of proteins in a single mixture). The protein mixture was subjected to isoelectric focusing (IEF) using IPGPhor IEF strips (range of 3–10, non-linear). After IEF, the strip was rinsed in equilibration buffer (6 M urea, 10 mM Tris, pH 6.8, 30% glycerol, 1% SDS), and then placed onto a 12% SDS-polyacrylamide gel for second dimension separation.

Following separation, the gel was scanned on a Typhoon fluorescent scanner (GE Biosystems), at the following wavelengths: Cy2, 488 nm excitation, 520 nm emission; Cy3, 532 nm excitation, 580 nm emission; Cy5, 633 nm excitation, 670 nm emission. Images were overlaid and analysed with Decyder Differential In-Gel Analysis (DIA) software (version 4.0, GE Biosystems) for pairwise identification of proteins with higher expression in pairs of the three strains. Proteins were designated as having statistically significant expression differences among strains if the abundance of an individual protein spot was more than two standard deviations greater or lesser than the mean variance of the abundance of all cellular proteins.

Differentially expressed proteins were excised, digested with trypsin (Invitrogen), and tryptic peptides were analysed using a matrix-associated laser desorption ionization – time-of-flight/time-of-flight (MALDI-ToF/ToF) spectrometer (Applied Biosystems, Foster City, CA). Protein identifications resulted from querying protein databases with both tryptic fingerprint data as well as primary amino acid sequence of peptides following collision-induced fragmentation and MS/MS.

In vitro *stress tolerance and phenotyping assays*

Growth under hyperosmotic stress was assessed by observing growth of serial 10-fold dilutions of overnight cultures on MH agar supplemented with 1% NaCl (Sigma). Oxidative stress tolerance was assessed by suspending each strain at an optical density of 0.02 in MH broth, followed by the addition of t-butylhydroperoxide (Sigma) to a final concentration of 0.1 mM. Following 30 min of incubation at 37°C microaerobically, samples of each strain were serially diluted and plated on MH plates for determination of cfu. Motility was determined by inspection of wet mounts by microscopy or measuring the halo of growth, after 24 h of microaerobic incubation, surrounding the point of inoculation of equal numbers (as determined by OD_{600}) of bacteria into MH plates containing 0.4% agar.

Subcellular fractionation and analysis of secreted proteins

Culture supernatants (Poly *et al*., 2007) and subcellular fractions (Leon-Kempis Mdel *et al*., 2006) were prepared essentially as described previously. Each strain of *C. jejuni* was grown microaerobically overnight in 100 ml of MH broth and cells were collected by centrifugation at 4800 *g* for 30 min at 4°C. The supernatant was filtered through a 0.22 μM filter (Millipore, Bellerica, MA), proteins were precipitated by the addition of TCA (Sigma) to a final concentration of 10% and incubation at 4°C overnight, and precipitated material was collected by centrifugation at 9800 *g* for 1 h. Prior to loading, the sample was washed 2× with acetone and then re-suspended in 100 μl of SDS-PAGE sample buffer. The cell pellet was re-suspended in 10 ml of ST buffer (20% sucrose, 20 mM Tris-HCl, pH 8.0) and EDTA was added to a final concentration of 1 mM. Following gentle agitation for 10 min at room temperature, cells were collected by centrifugation at 8000 *g* for 10 min and re-suspended in 5 ml of ice-cold 10 mM Tris, pH 7.5 and incubated on ice for 10 min. The supernatant, containing periplasmic proteins, was collected by centrifugation at 15 000 *g* for 15 min at 4°C and then proteins were precipitated with TCA as described above. The cell pellet was then suspended in 2.5 ml of ice-cold 10 mM Tris, pH 8.0, sonicated for 3 × 30 s, and then subjected to centrifugation for 10 min at 13 000 *g*. The pellet was discarded, and the supernatant was subjected to centrifugation on a Beckman Optima L-90K Ultracentrifuge at 100 000 *g* to collect cell membranes. The supernatant was saved as the cytoplasmic fraction, and the pellet was washed and re-suspended in 100 μl of 10 mM HEPES, pH 7.4, followed by the addition of an equal volume of 2% sarcosine (Sigma) in HEPES buffer. After incubation at room temperature for 30 min to solubilize inner membranes, the insoluble outer membranes were collected by centrifugation at 15 600 *g* for 30 min at 4°C and re-suspended in 100 μl of HEPES buffer. Equal volumes of each fraction were separated by SDS-PAGE followed by silver staining.

Growth of biofilms in the presence of carbon sources and antimicrobial agents

To test carbon sources, biofilms were grown as in above in MH broth or MH broth supplemented with 50 mM fumarate, glycine or glutamine. After 2 days of microaerobic incubation, samples were carefully removed from the media (planktonic) fraction to measure OD_{600}, and biofilms were then quantified by crystal violet staining. Shown is an average of the planktonic (OD_{600}) to biofilm (A_{570}) ratio for each strain. To test the effect of antimicrobial compounds, biofilms were again grown as in above in MH broth or MH broth supplemented with the following concentrations of each compound: DOC, 0.05%; Triton X-100, 0.0005%; Tween-20, 0.002%; and SDS, 0.00025%.

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