MOLECULAR MECHANISMS OF *CAMPYLOBACTER JEJUNI* SURVIVAL: CHARACTERIZATION OF THE CprRS TWO-COMPONENT REGULATORY SYSTEM AND BIOFILM FORMATION

by

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ABSTRACT

Campylobacter jejuni is the leading cause of foodborne bacterial gastroenteritis in the developed world. Although illness is usually self-limiting, immunocompromised individuals are at risk for infections recalcitrant to antibiotic treatment. Prior infection with *C. jejuni* also correlates with serious sequelae such as Guillain-Barré syndrome. The success of *C. jejuni* as a zoonotic pathogen indicates it can adapt to varied conditions encountered during pathogenesis, despite apparent fastidiousness in the lab. Understanding how *C. jejuni* survives in common reservoirs may allow development of strategies to limit survival in infection reservoirs or during pathogenesis, and greatly reduce the impact of *C. jejuni*-mediated disease. A two-component regulatory system, (CprRS; *Campylobacter* planktonic growth regulation) was previously identified in a screen for genes that may be required for adaptation to the host. Subsequent characterization of CprRS has contributed to understanding of two themes related to *C. jejuni* survival: environmental gene regulation and biofilm formation. The CprR response regulator was essential for viability, and while the CprS sensor kinase was dispensable, a Δ*cprS* mutant showed significant phenotypic differences from WT. Initial characterization of Δ*cprS* using phenotypic and proteomic means provided evidence that CprRS affects phenomena related to biofilm formation. Further characterization of CprRS was undertaken through transcriptomics of Δ*cprS,* molecular analysis of CprR, and promoter analysis. The CprRS regulon suggests that the system may control aspects of the cell envelope, including expression of the HtrA periplasmic protease. Finally, subsequent analysis of the biofilm-enhanced Δ*cprS* mutant, together with epistatic analyses and analysis of WT *C. jejuni* under stress conditions, has provided insight into *C. jejuni* biofilm initiation, maturation, and physiology. A specific role for flagella in biofilm initiation was demonstrated, and lysis and extracellular DNA release during biofilm maturation was also observed. Furthermore, evidence that the *C. jejuni* biofilm lifestyle confers stress tolerance that is not present in planktonic counterparts was obtained. Characterization of CprRS has thus contributed to knowledge of both physiological and regulatory themes that provide *C. jejuni*, a pathogen which diverges from paradigms set out in model bacteria, with its surprising resilience during zoonosis, and has also identified novel targets for infection control.

PREFACE

Portions of the **Introduction** have been published [Svensson *et al.* 2008. Survival Strategies of *Campylobacter jejuni*: Stress Responses, the Viable but Non-culturable State, and Biofilms. *in* I. Nachamkin, C. M. Szymanski, and M. J. Blaser (eds.), *Campylobacter*, 3rd edition][1].

All Chapters are based on research designed by Sarah Svensson and Associate Professor Dr. Erin Gaynor. Experiments were performed by Sarah Svensson in the laboratory of Dr. Gaynor (Department of Microbiology and Immunology, UBC, Vancouver, BC), unless otherwise stated. Research was performed under the UBC Research Ethics Board Biosafety Committee Certificate of Approval #B10-0061.

A version of **CHAPTER 2** has been published [Svensson *et al.* 2009. The CprS sensor kinase of the zoonotic pathogen *Campylobacter jejuni* influences biofilm formation and is required for optimal chick colonization. Mol. Microbiol. **71**:253-72][2]. Proteomics work was performed by Mohanasundari Pajaniappan in the laboratory of Dr. Stu Thompson (Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, GA), and chick infections were undertaken by Dr. Gaynor in the laboratory of Dr. Vic DiRita (Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI) under University of Michigan Committee on Use and Care of Animals (UCUCA) approval number 10462 and NIH OLAW file A3114-01. Cm^R deletion constructs for *cprR* and *cprS* were constructed by Dr. Joanna MacKichan at Stanford University.

Portions of **CHAPTER 3** are in preparation for publication [The CprRS two-component regulatory system of *Campylobacter jejuni* regulates essential aspects of the cell envelope]. Microarray analysis was performed by Sarah Svensson in the laboratory of Dr. Craig Parker the USDA Western Research Center in Albany, CA, with the help of Steven Huynh. TEM samples were prepared by Sarah Svensson and visualized by Jenny Vermeulen at the UBC Bioimaging Facility (Vancouver BC), and RacR one-hybrid analysis was performed by Dmitry Apel. The *cprR*^{KD} knockdown and *cprR*^{OE} overexpression strains were constructed by Jenny Vermeulen and Andrew Cameron. Samples for peptidoglycan analysis were prepared by Jenny Vermeulen, and analyzed by Dr. Jacob Biboy in the laboratory of Dr. Waldemar Vollmer (The Centre for Bacterial Cell Biology, Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne, UK).

A version of **CHAPTER 4** is in preparation for publication [Flagella-mediated adhesion and release of extracellular DNA contribute to biofilm formation and stress tolerance of *Campylobacter jejuni*]. Samples for confocal microscopy were prepared by Sarah Svensson, and visualized by Mark Pryjma in the laboratory of Dr. Robert Nabi (Department of Cellular and Physiological Sciences, UBC, Vancouver, BC).

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A	adenosine
A_{570}	absorbance at 570 nm
3-AT	3-aminotriazole
ABC	ATP-binding cassette
AcP	acetyl phosphate
AI-2	autoinducer-2
AIDS	acquired immune deficiency syndrome
Ala	alanine
Amp	ampicillin
Amp^R	ampicillin resistant
AP-1	activator protein 1
aph-3	aminoglycoside 3′-phosphotransferase
Asn	asparagine
Asp	aspartate
ATP	adenosine triphosphate
BHI	brain-heart infusion
bp	base pairs
C	cytosine
^oC	degrees Celsius
Caco-2	human epithelial colorectal adenocarcinoma cell line
cAMP	cyclic adenosine monophosphate
CAT	chloramphenicol acetyltransferase
CCV	*Campylobacter*-containing vacuole
cDNA	complementary DNA
c-di-GMP	cyclic diguanylate
CFU	colony-forming units
CFW	Calcofluor White
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
chrDNA	chromosomal DNA
Cia	*Campylobacter* invasion antigen
Cm	chloramphenicol
Cm^R	chloramphenicol resistant
CoA	coenzyme A
CPS	capsular polysaccharide
CTD	C-terminal domain
CV	crystal violet
Cy2	cyanine dye, 520 nm emission
Cy3	cyanine dye, 580 nm emission
Cy5	cyanine dye, 670 nm emission
DAPI	4',6-diamidino-2-phenylindole
DIA	Differential In-gel Analysis
DIGE	Differential In-Gel Electrophoresis
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide
DOC	sodium deoxycholate
eDNA	extracellular DNA
EDTA	ethylenediaminetetraacetic acid
ε-proteobacteria	epsilon proteobacteria
EPS	exopolysaccharide
FBS	fetal bovine serum
F_cR	crystallizable fragment receptor

FWD	forward
g	gram
G	guanosine
x *g*	relative centrifugal force
GBS	Guillain–Barré syndrome
GC	guanosine + cytosine
gDNA	genomic DNA
GFP	green fluorescent protein
GGDEF	glycine-glycine-aspartate-glutamate-phenylalanine
GGT	gamma-glutamyltranspeptidase
Glc	glucose
Glc*N*Ac	*N*-acetylglucosamine
Glu	glutamine
GTP	guanosine triphosphate
h	hour
HAMP	histidine kinase-adenylyl cyclase-methyl-accepting protein-phosphatase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	histidine
HIV	human immunodeficiency virus
IBD	inflammatory bowel disease
IEF	isoelectric focusing
IFN-γ	interferon-gamma
IL-8	interleukin-8
INT407	human intestinal epithelial cell line
INV	inverse
Kan	kanamycin
Kan^R	kanamycin resistant
KD	knockdown
kDa	kilodalton
KO	knockout
L	litres
LB	Luria-Bertani
log	logarithm
LOS	lipooligosaccharide
LPS	lipopolysaccharide
M	molar
M9	minimal media
MALDI-ToF	matrix-assisted laser desorption/ionization-time-of-flight
MAP	mitogen-activated protein
Mb	megabases
MCP	methyl-accepting chemotaxis protein
MDE	multi-drug exporter
mg	milligram
mg	microgram
min.	minute
MEM	Minimum Essential Medium
MH	Mueller-Hinton
MIC	minimum inhibitory concentration
mJ	millijoule
mM	millimolar
μm	micrometre
μM	micromolar
MOI	multiplicity of infection
MOMP	major outer membrane protein

mOsm	milliosmole
MS/MS	tandem mass spectrometry
Mur*N*Ac	*N*-acetylmuramic acid
N	any nucleotide base
N-linked	asparagine or arginine nitrogen-linked
ND	not determined
NF-κB	nuclear factor-kappa B
ng	nanogram
nm	nanometre
NOD1	nucleotide-binding oligomerization domain-containing protein 1
NPN	1-*N*-phenylnaphthylamine
NS	not significant
NTD	N-terminal domain
O-linked	serine, threonine, or tyrosine oxygen-linked
OD_{600}	optical density measured at 600 nm
ORF	open reading frame
Osm	osmole
p	*p*-value
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PG	peptidoglycan
pH	acidity
PI	propidium iodide
PolyP	polyphosphate
ppGpp	guanosine pentaphosphate
PVDF	polyvinylidene fluoride
PxB	polymyxin B
QPCR	quantitative real time polymerase chain reaction
R	adenine or guanine
RACE	rapid amplification of cDNA ends
REV	reverse
RNA	ribonucleic acid
RNAP	RNA polymerase
RNAPα	RNA polymerase alpha subunit
RNase	ribonuclease
RNA-seq	whole transcriptome shotgun sequencing
rpm	revolutions per minute
rRNA	ribosomal RNA
RT-PCR	reverse transcription-polymerase chain reaction
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
s	second
σ	sigma
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
Ser	serine
siRNA	small interfering RNA
ssDNA	salmon sperm DNA
Str	streptomycin
Str^R	streptomycin resistant
T	thymine
T84	human colonic adenocarcinoma cell line
TAE	Tris-acetate-EDTA
TAT	twin-arginine transporter

TCA	trichloroacetic acid
TCRS	two-component regulatory system
TEM	transmission electron microscopy
Tet	tetracycline
Tet^R	tetracycline resistant
Th1	Type 1 T helper
Thr	threonine
TNF-α	tumour necrosis factor-alpha
tRNA	transfer RNA
TLR	Toll-like receptor
Tris	tris(hydroxymethyl)aminomethane
Tw20	Tween-20
Tx100	Triton X-100
U	enzyme unit
UBC	University of British Columbia
UDP	uridine diphosphate
USDA	United States Department of Agriculture
UV	ultraviolet
VBNC	viable but non-culturable
W	adenine or thymine
WT	wild type
w/v	percent weight/volume
×	magnification
X	solution strength
Xaa	any amino acid
mL	millilitre
μL	microlitre

1 GENERAL INTRODUCTION

1.1 Characteristics, prevalence, and treatment of *Campylobacter*-mediated disease

Campylobacter jejuni is a motile, helical Gram-negative bacterium belonging to the epsilon (ε)-proteobacteria subdivision [3]. While first described by Escherich in 1886, it was not until the 1970s that campylobacters were recognized as significant human pathogens [4]. Common pathogenic *Campylobacter* species include *C. coli*, which causes gastroenteritis in humans, *C. concisus*, which is associated with periodontal disease, and *C. jejuni*, which is isolated from approximately 90% of *Campylobacter*-mediated infections [5]. Members of the genus *Campylobacter* are also important animal pathogens, such as the type species, *C. fetus*, which causes abortion in sheep and cattle [6].

1.1.1 *C. jejuni*-mediated infections.

C. jejuni causes a spectrum of diseases in humans. Manifestations of *C. jejuni* infection range from acute gastroenteritis to septicaemia and in some cases, neurological conditions. However, *C. jejuni* is most often associated with foodbourne enteric infection. *C. jejuni*-associated gastroenteritis commonly presents as an inflammatory, dysenteric syndrome, marked by symptoms such as intense (often bloody) diarrhea, vomiting, fever, and stomach cramps. While acute infection can be life-threatening in young, elderly, and immunocompromised individuals [7], infection is usually self-limiting and resolves after one or two weeks in healthy patients. Invasive cases of *C. jejuni* gastroenteritis are becoming more noteworthy with the increased prevalence of cases in patients with underlying conditions affecting immune competence, such as HIV infection [8]. Reactivation has also been reported [9]. Finally, even though campylobacteriosis is self-limiting, in some cases *C. jejuni* infection is an antecedent to more threatening conditions, including reactive arthritis, inflammatory bowel disease (IBD), and importantly, Guillain-Barré syndrome (GBS), a significant cause of ascending bilateral paralysis [10, 11].

1.1.2 Epidemiology and impact of *C. jejuni*-mediated infections.

C. jejuni is considered the leading cause of bacterial gastroenteritis worldwide [3]. It is estimated that 1% of the US and Canadian populations are infected annually, which makes cases of *C. jejuni*-mediated gastroenteritis more prevalent than those caused by prototypical enteric pathogens such as *Salmonella*, *Shigella*, and *Escherichia coli* combined [12]. In Canada, 9345 cases were reported in 2004 (a rate of 30.2 per 100,000) [13]. In comparison, the rate of salmonellosis in the same time period was 16 per 100,000. Rates are thought to be even higher in developing countries, where infection may go unreported [14]. *Campylobacter* spp. are also one of the most common causes of diarrhea in Canadians traveling to developing countries [15]. Infection with *C. jejuni* is age-related, with peaks of incidence occurring under 4 years of age and between 20-29 years of age [16]. Immune system compromisation is also both a risk factor for infection, and may predispose patients to serious complications. For example, patients with AIDS have an increased incidence of campylobacteriosis, and patients with bacteraemia are often older or have comorbid conditions [17, 18]. Due to prevalence of

infection, the cost of campylobacteriosis cases in the US is estimated to be in the range of $1.3-$6.2 billion [19]. In addition, while only 1% of cases go on to manifest as serious medical sequelae, the cost of these is estimated to be $0.2-$1.8 billion. Furthermore, sequelae such as GBS appear following resolution of gastroenteritis in healthy individuals, and antibiotic treatment has no effect on its manifestation [20]. Thus, the economic impact of *C. jejuni* stems from both the high prevalence of food-bourne illness and the seriousness of the few cases that progress to autoimmune conditions, even upon resolution of acute infection, and it follows that prevention of infection from occurring in the first place is essential to limiting the impact of this pathogen.

1.1.3 Sources of infection.

Some studies report that a dose of as few as 800 CFU can cause significant infection in healthy individuals [21]. *C. jejuni* is a naturally zoonotic pathogen and resides asymptomatically as a commensal in the intestinal mucosa of a wide range of animal species. *C. jejuni* has been detected in migrating birds, farm animals (cattle, sheep, poultry, pigs), and pets [22, 23]. *C. jejuni* thrives in mammalian and avian guts, but it also survives extended periods outside of animals in cold, dark, moist environments [24]. Transmission from commensal hosts either directly to humans, or indirectly through food and water sources, underlies both outbreaks and sporadic infection (**FIG. 1.1**). Campylobacteriosis in developed countries is highly seasonal, which may result from outbreaks related to environmental reservoirs. *Campylobacter* strains are often isolated from sewage and marine environments [16, 25], and outbreaks of campylobacteriosis have been associated with *C. jejuni* present in the environment following heavy rains [26]. Highly significant are outbreaks related to contaminated drinking water, such as in the Walkerton, ON outbreak where municipal water was contaminated with livestock runoff and improperly treated [27]. Improperly pasteurized or raw milk is also a source of outbreaks, as is contaminated produce [28, 29]. Close contact with farm animals or pets is also a risk factor for infection [30, 31]. Most epidemiological studies point to the food chain as the primary transmission route. For example, a recent analysis suggested that 97% of cases had chicken, cattle, or sheep as the source of infection, while only 3% were attributed to environmental sources [24]. Sporadic cases are associated with consumption of contaminated poultry that has been improperly prepared [32], as up to 90% of commercial poultry products harbour campylobacters [33]. Thus, prevalence of *C. jejuni* in animal hosts serves as a major reservoir for both sporadic infection and outbreaks, and leads to both direct exposure and indirect routes of infection, via the environment.

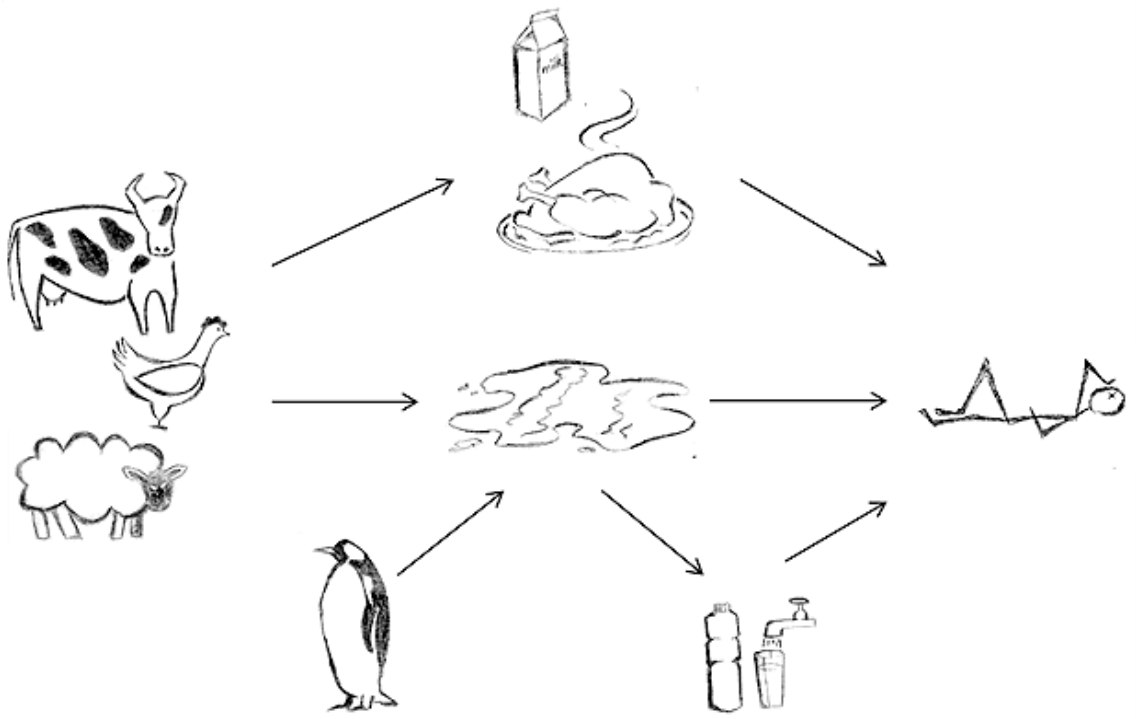


FIG. 1.1. Transmission and pathogenesis of *C. jejuni.* Animals (both agricultural and wild) harbour *C. jejuni* as a commensal in their gastrointestinal tracts. The major source of sporadic infection is contaminated poultry products, but improperly purified water contributes to outbreaks. Minor reservoirs of infection are pets, and environmental sources, and raw milk.

1.1.4 Current strategies for treatment and prevention of *C. jejuni* infection.

Naïve, immunocompetent adults who are exposed to *C. jejuni* normally resolve infection before an acquired immune response is mounted, suggesting the innate immune system plays an important role in the response to *C. jejuni* [34]. Innate defenses such as low stomach pH, bile acids, iron limitation, defensins, and complement are all important [34]. Exposure can confer acquired resistance leading to attenuated disease upon reinfection, although immunity wanes after one year post-exposure [35]. Antibodies to several *C. jejuni* structures have been observed in human sera, including flagella, the major outer-membrane protein (MOMP), and surface carbohydrates [36-38]. Resolution of *C. jejuni* infection is associated with production of IFN-γ (interferon-gamma), suggesting that Th1 polarization is central to immunity [35]. However, polarization may also affect development of GBS [39, 40]. Furthermore, it is unclear whether the strong inflammatory response that follows *C. jejuni* infection is solely protective, or whether it also contributes to disease.

In immunocompetent patients, treatment usually involves only fluid and electrolyte replacement therapy, as in most cases *C. jejuni* infection is self-limiting. Antimicrobial therapy is usually reserved for severe, complicated, or systemic infections presenting in immunocompromised individuals. However, the emergence of drug-resistant strains is drastically limiting utility of antimicrobials [41]. Furthermore, some cases of bacteremia are recalcitrant to antibiotic treatment, despite blood cultures showing susceptibility [42]. The first choice of treatment is erythromycin, because of the rates of fluoroquinolone resistance that have resulted from its

widespread use in animal husbandry [43]. Development of resistance during treatment has also been observed [44]. At present, a vaccine against *C. jejuni* is not available for widespread use, and vaccine production is hampered by the correlation between *C. jejuni* exposure and development of GBS; thus, attempts have been made to develop a live vaccine strain that does not express ganglioside mimics that may lead to autoimmune reactions [45]. Subunit vaccines have also been developed, including those composed of PorA, CmeC, Peb1a, FlaC, FspA1/2, and polysaccharide [46-50].

1.1.5 Novel strategies for limiting impact of *C. jejuni*.

It has been demonstrated that a 2 log reduction in the number of campylobacters present on carcasses leaving the slaughterhouse results in a 30-fold reduction in the incidence of campylobacteriosis [51], suggesting that reducing bacterial load in reservoirs such as chickens may significantly reduce incidence of infection. Thus, the current approach involves analysis of all potential points of hazard and control points, in a 'farm-to-fork' strategy [20], and specific strategies for limiting *C. jejuni* prevalence between producer and consumer have mainly focused on reducing levels of the organism in poultry [52]. These include use of bacteriocins, phage therapy, vaccination, and selection of colonization-resistant chickens. Such measures have led to significant decreases in countries with smaller poultry industries, such as Iceland and New Zealand [53, 54]. However, as such strategies may not be feasible in larger scale industries, new ones are needed [20]. Due to the zoonotic nature of *C. jejuni*, mechanisms must exist to aid its survival and infectivity in numerous environments encountered during transmission and colonization. Moreover, identifying these mechanisms may aid development of novel strategies that can be applied to large-scale industries. Current understanding of adaptation of *C. jejuni* to environments encountered during transmission and pathogenesis include central biological processes (**Section 1.2**), interactions with human and animal hosts (**Section 1.3**), stress tolerance and biofilm formation (**Sections 1.4**-**1.5**), and gene regulation (**Sections 1.6-1.7**). Finally, understanding molecular factors that intersect with all of these phenomena, such as the CprRS two-component regulatory system (TCRS) (**Section 1.8**), may provide greater understanding of how *C. jejuni* is such a prevalent zoonotic pathogen.

1.2 Unique aspects of *C. jejuni* biology that contribute to survival and pathogenesis

C. jejuni belongs to the ε-proteobacteria, a group of Gram-negative bacteria that inhabit a diversity of niches, ranging from the gastrointestinal tract of animals to deep sea vents. More specifically, *C. jejuni* is part of the family Campylobacteraceae, which includes both commensals or parasites of animal species (*Campylobacter* and *Arcobacter*), as well as free-living environmental genera (*Sulfurospirillum*)[6]. The related Family Helicobacteraceae contains the important human gastric pathogen *Helicobacter pylori*, with which *C. jejuni* shares unique biological characteristics, but also displays contrasting virulence mechanisms. Campylobacteraceae have microaerobic growth requirements, respiratory and chemoorganotrophic metabolisms, and are (generally) asaccharolytic and non-fermentative. They often exhibit helical morphology and have a relatively

small size: 0.2-0.8 μM x 0.5-5 μM. Species are non-spore-forming, but can progress to coccoid forms. *C. jejuni* cells bear either one or two polar unsheathed flagella that confer characteristic corkscrew-like motility.

The first sequenced genome *C. jejuni* strain was that of strain 11168 [55]. Genomes of *C. jejuni* strains range between 1.64-1.85 Mb in size, with a GC content of approximately 30%, and encode 1600-1800 open reading frames (ORFs). *C. jejuni* genomes are relatively devoid of insertion sequences and genomic islands; however, strain 81-176 harbours two plasmids [56]. Poly-GC tracts, important for phase variation, are also present. Extensive hypervariable regions are associated with genes encoding cell-surface components, such as carbohydrates and flagella [55]. Notable absences are genomic islands dedicated to virulence factors, as well as many stress response and regulatory proteins. However, *C. jejuni* genome sequences have provided an important framework for molecular studies, which have highlighted the importance of essential biological processes in pathogenesis, including metabolism, cell surface carbohydrates, motility, and genetic variation.

1.2.1 Metabolism.

C. jejuni has relatively fastidious requirements for growth under laboratory conditions, and this reflects, at least in part, aspects of its metabolism. *C. jejuni* strains are generally asaccharolytic, as they lack 6-phosphofructokinase [57], although some strains can utilize L-fucose [58, 59]. *C. jejuni* is microaerophilic and cannot grow under strict anaerobic conditions due to the requirement of O_2 by its ribonucleotide reductase homologue [60]. Mechanisms underlying microaerophily are poorly understood, but may include increased sensitivity to reactive O_2 species, strong inhibition of certain respiratory enzymes by O_2, and/or significant metabolic generation of reactive O_2 species [61]. *C. jejuni* is also capnophilic, in that it prefers a higher concentration of CO_2 (1.0% to 10%) than is available in the atmosphere [62]. This has been proposed to be due to reliance on carbon fixation from CO_2 to pyruvate, in the absence of a complete glycolysis pathway [63]. *C. jejuni* does encode a complete citric acid cycle. Many genes that are essential for viability (at least under laboratory conditions) are related to central metabolism [64, 65].

Despite being microaerophilic and capnophilic, *C. jejuni* retains some of the metabolic flexibility of its ε-proteobacteria cousins [66]. A hallmark of *C. jejuni* metabolism is its highly branched electron transport chain [67]. *C. jejuni* has two terminal oxidases, but can also utilize alternative electron acceptors, such as fumarate, nitrate, nitrite, and dimethyl sulfoxide. *C. jejuni* can also use a variety of molecules as energy sources, such as formate, H_2, organic acids, and gluconate. As catabolysis of glucose is not possible, it relies on amino acids as a primary carbon source [67, 68]. Amino acids also serve as an important source of nitrogen. *C. jejuni* appears to be partial to serine, but also utilizes glutamate, glutamine, aspartate, asparagine, and proline. Although *C. jejuni* can synthesize all amino acids it requires (and in contrast to *H. pylori*) [64], transporters for many amino acids are also encoded in the genome [55]. Some appear to have bifunctional roles in both metabolism and pathogenesis. For example, the Peb1a ABC transporter imports aspartate and glutamate, but also mediates interactions with host cells [69, 70]. Also, while the LIV branched-chain amino acid uptake system is required

for colonization, this appears to be independent of transport functions and may involve binding to caecal components [71].

C. jejuni metabolism is well-adapted to conditions present in the avian gastrointestinal tract [71], and microarray analysis of *C. jejuni* in the chick caecum suggests that colonization depends on adaptation to a low-O_2 environment [72]. The avian gut contains dense populations of anaerobes that excrete organic acids such as lactate [73]. Two respiratory oxidases confer growth of *C. jejuni* on lactate, encoded by the Cj0075c-73c and Cj1585c loci [74]. Uptake of C4-dicarboxylates, via transporters such as DcuA and DcuB, also appears to be important [72, 75]. An acetate switch mechanism, whereby *C. jejuni* excretes acetate when favourable carbon sources are available, followed by scavenging of acetate upon entry into stationary phase, has been identified [76]. Strain specific metabolic capacities that contribute to host interactions also exist. Strain 81-176 harbours a gamma-glutamyltranspeptidase (GGT) which enables it to use glutamine and glutathione [77]. This strain has also acquired the ability to secrete an L-asparaginase, allowing it to use asparagine as a nutrient source. While inactivation of *ggt* affects intestinal colonization of mice, the asparaginase is required for liver colonization [78], suggesting that metabolic flexibility contributes to tissue tropism [79].

1.2.2 Surface carbohydrates.

C. jejuni has an extensive repertoire of glycosylated molecules decorating its surface. As much as 10% of the genome may be dedicated to carbohydrate biosynthesis [80], and much of the genetic variation harboured by strains is localized to carbohydrate biosynthesis loci [55]. The genome contains genes responsible for biosynthesis of four distinct classes of surface carbohydrates: capsular polysaccharide (CPS), lipooligosaccharide (LOS), *O*-linked protein glycosylation, and *N*-linked protein glycosylation [80]. *C. jejuni* produces a high molecular weight CPS, which is responsible for the Penner serotyping scheme [81]. Expression of CPS is dependent on Type II and Type III capsule transport genes encoded in the *kpsCS* and *kpsMTEDF* loci [55]. CPS expression is phase variable (both on-off, and variation of modifications), and is required for adherence to and invasion of epithelial cells, antimicrobial peptide resistance, serum resistance, and mouse colonization [82-84]. Like mucosal pathogens, *C. jejuni* expresses a rough lipopolysaccharide (LPS), termed LOS. Several genes of the LOS biosynthetic cluster are also phase variable [85]. Interestingly, LOS is not essential for viability, as a mutant harbouring a large deletion causing absence of the LOS core is viable [86]. However, this strain is markedly affected for growth; sensitive to polymyxin B (PxB), sodium dodecyl sulphate (SDS), and novobiocin; unable to invade cells; and defective for natural transformation. More subtle truncations of LOS, such as those caused by deletion of *waaF,* also increase sensitivity to PxB and cause defects in intracellular survival and chick colonization [87]. *C. jejuni* also extensively modifies surface proteins by both *O*- and *N*-linked glycosylation. Flagella in strain 81-176 are modified by *O*-linked addition of pseudaminic acid [88, 89]; however, the flagellar modification locus is one of the most variable in the genome, and many other modifications exist [90]. Mutations that affect glycosylation of flagella reduce adherence and invasion of INT407 cells, and limit ability to cause disease in ferrets [91]. Bacterial *N*-linked protein

glycosylation was first identified in *C. jejuni* [92]. An *N*-linked glycan is attached to at least 45 proteins at an Asn residue in the motif Asn-Xaa-Ser/Thr [85], and loss of *N*-linked glycosylation has pleiotropic effects, as *pgl* mutants show defective competence, host cell interactions, and mouse colonization [93, 94].

1.2.3 Flagella and motility.

C. jejuni cells have one or two polar flagella that are thought to facilitate motility through viscous environments within animal hosts [95, 96]. The structure of *C. jejuni* flagella is similar of those expressed by other Gram-negative bacteria and has been reviewed recently [97]. Briefly, it contains a hook-basal body complex and a filament. The basal body harbours the flagellar Type III secretion system. The flagellar filament is composed of two different flagellins: the FlaA flagellin comprises the majority of the filament and is required for full motility, whereas FlaB is a minor component [98]. As mentioned above, *C. jejuni* flagella are decorated with *O*-linked sugars, and must be glycosylated for filament biosynthesis [91, 99]. Flagellar rotation is controlled by chemotaxis machinery, including ten methyl-accepting chemotaxis proteins (MCPs) for detection of specific signals [97], to allow directional movement along favourable chemical gradients [100]. Methodology used for testing of chemotaxis of *C. jejuni* was recently brought into question [101]; however, *C. jejuni* does display a chemotactic response towards fucose, pyruvate, fumarate, aspartate, and formate [102-106]. Bile is a chemoattractant, although this has been attributed to the mucin component [104]. *C. jejuni* also exhibits energy taxis, mediated by a novel bipartite system (CetAB), that is loosely homologous to *E. coli* Aer [107]. In addition to reduced motility, flagellar mutants are also defect for phenotypes such as autoagglutination, secretion, and biofilm formation (summarized later in **TABLE 5.1**). In the absence of pili [108], flagella are also thought to mediate adhesion [109]. Furthermore, motility and chemotaxis are closely intertwined with pathogenesis, as mutation of genes required for these processes often results in defects in host cell interactions and colonization of animals [88, 109-113].

1.2.4 Protein secretion.

C. jejuni encodes components of the general secretion pathway for protein export [114]. A twin-arginine transporter (TAT) is required for secretion of proteins involved in survival and pathogenesis. For example, mutants defective for TAT secretion display defective biofilm formation, flagellar expression, and antimicrobial resistance [115]. Many components of the electron transport chain, as well as the PhoX alkaline phosphatase, are also secreted by the TAT system [116-118]. A putative Type IV secretion system is also encoded by the pVIR plasmid in certain hyperinvasive strains [119, 120]. However, a specific role for this secretion system has not been identified. Unlike other Gram-negative enteric pathogens, *C. jejuni* does not harbour a Type III secretion system dedicated to secretion of virulence factors. Instead, secretion of virulence-associated Cia (*Campylobacter* invasion antigen) proteins, such as CiaB, depends on a functional flagellar export apparatus [121]. Synthesis and secretion of Cia proteins is uncoupled, as Cia protein expression is induced in deoxycholate (DOC), but secretion requires a stimulatory signal [122-124]. Numerous other Cia proteins, some of which affect host cell interactions, have been identified that are

secreted in a flagellar export apparatus-dependent fashion [122, 125-128].

1.2.5 Natural transformation, recombination, and genetic diversity.

Many strains are naturally transformable, with frequencies of 10^{-4} reported for genomic DNA (gDNA) [129]. Genetic exchange of resistance markers in chickens has been observed [130], suggesting that natural transformation may be relevant *in vivo*. Following uptake, recombination can occur with as little as 200 base pairs (bp) of homologous DNA [131], although integration of self DNA is much more efficient than that from other species, likely due to the presence of restriction-modification systems [132]. Transformation is also inhibited in strains harbouring integrated Mu prophage-like-encoded DNases (deoxyribonucleases) [133, 134]. The rate of transformation is variable between strains, with some isolates being non-transformable, even by self DNA [131]. However, DNA uptake is mediated by a Type II secretion system [135], which appears to be conserved across *C. jejuni* isolates. WT (wild type) levels of transformation also require a periplasmic single- and double-stranded DNA-binding protein encoded by the Cj0011c locus [136], as well as *recA* [137]. Natural transformation is also abolished by mutation of genes required for LOS core biosynthesis [86]. In *H. pylori*, a Type IV secretion system is required for natural transformation [138]. However, mutation of *virB11*, encoding a component of the pVIR type IV secretion system of some *C. jejuni* strains, has no effect on DNA uptake [56], and strains that do not harbour pVIR are naturally transformable. The *C. jejuni* genome contains regions of hypervariability, which are thought to contribute to pathogenesis [55]. Some of this variability is due to phase variation which occurs via slipped-strand mispairing at homopolymeric tracts, resulting in variation in tract length and altered expression [139]. Phase variation and can occur at a relatively significant rate – approximately 10^{-3} per cell per generation [140], and is is mainly restricted to surface structures, such as flagella, CPS, and LOS [82, 141-144]. *C. jejuni* encodes genes involved in methyl-directed mismatch repair, nucleotide excision repair, base excision repair, and recombinational repair [137]. However, the mismatch repair pathway appears to be non-functional, which may contribute to the significant genetic heterogeneity observed in *C. jejuni* populations.

1.2.6 Morphology, viable but non-culturable, and autolysis.

Bacterial cells often exhibit a characteristic morphology during different growth phases or during dormant persistence [145]. The characteristic helical morphology of *C. jejuni* exhibited during balanced growth has been hypothesized to aid colonization and host interactions by enhancing motility in the viscous mucus of the intestinal tract [146]. Like *H. pylori*, helical morphology of *C. jejuni* is thought to stem, at least partially, from peptidoglycan (PG) structure [147] (E. Frirdich and E. Gaynor, in press). PG is a polymer of repeating β-(1,4)-linked Glc*N*Ac (*N*-acetylglucosamine) and Mur*N*Ac (*N*-acetylmuramic acid) sugars, cross-linked with peptide chains into a covalently closed mesh-like layer that resides in the periplasm of Gram-negative bacteria to form the mechanical strength-providing cell wall. Biogenesis of the PG layer is highly regulated, both spatially and temporally [148], and targeted by antimicrobial agents such as β-lactams and vancomycin. While

cells growing in log phase in rich media exhibit helical morphology, upon entry into stationary phase or encountering environmental stresses, *C. jejuni* can progress to a coccoid form, which is thought to represent a VBNC (viable but non-culturable) state [149]. As the name suggests, VBNC cells cannot be cultured on routine microbiological media, and while they often exhibit decreased metabolic activity, macromolecule synthesis, and respiration rates, they also appear to maintain ATP levels, continue gene expression, and alter their cell envelope [150]. VBNC cells can also remain infectious, harbour antibiotic resistance, and retain attachment to surfaces. In addition to the VBNC transition, under certain conditions, transitions marked by regulated cell death and lysis also appear to confer fitness to some populations of bacteria [151]. Pathways of programmed cell death in bacteria include autolysis, which can contribute to development and dispersal of biofilms [152, 153]. Both VBNC-related coccoid morphology and autolysis involve changes in the PG layer. The helical to coccoid transition exhibited by *C. jejuni* and *H. pylori* is marked by specific changes in PG structure or amount [1, 154]. Autolysis can be mediated by PG hydrolases or homologues of phage holin proteins, which exhibit PG hydrolase activity [151]. To date, autolysis has not been described in *C. jejuni*, although numerous PG modification enzymes are present in the genome. In *H. pylori*, a dramatic reduction in culture turbidity, release of cytoplasmic proteins, and supernatant lytic activity is seen after log phase, which may represent a novel mechanism of autolysis [155] that may be shared by *C. jejuni*.

1.3 *C. jejuni* host interactions and pathogenesis

1.3.1 General characteristics of acute *C. jejuni* infection and pathogenesis.

Manifestations of acute gastrointestinal infection with *C. jejuni* in humans range from a mild, watery diarrhea to an inflammatory dysenteric syndrome marked by profuse, often bloody, diarrhea [139]. *C. jejuni* is thought to be a primarily extracellular pathogen; however colonic biopsies of patients with *C. jejuni* gastroenteritis suggest bacteria can invade into cells of the mucosa [156]. Furthermore, the ability of strains to adhere to or invade host cells or translocate across polarized epithelial cell monolayers *in vitro* correlates strongly with virulence [157, 158]. It has thus been proposed that invasion, resulting in translocation of bacteria across the intestinal epithelium to underlying regions where they can interact with cells of the innate immune system, causes cytokine release and a significant inflammatory response that leads to physical manifestations of infection [8].

Despite prevalence of *C. jejuni*-mediated disease, understanding of both host and bacterial factors that contribute to pathogenesis remains relatively limited. This is likely due to recalcitrance of *C. jejuni* to molecular genetics, its fastidious growth requirements in the laboratory, and its unique genomic characteristics compared to other enteric pathogens. Annotation of the first *C. jejuni* genome sequence has contributed a great deal to understanding of *Campylobacter* biology and pathogenesis [55]. However, it provided few clues as to how this organism causes disease. The chromosome appears to be devoid of the pathogenicity islands and secretion systems that are the hallmarks of other enteric pathogens [55]. Furthermore, some identified virulence factors are not conserved in virulent strains [20]. In fact, more appears to be understood about the

contribution of basic biological characteristics (such as surface carbohydrates, flagella, and stress tolerance) to pathogenesis, than about specific, dedicated virulence factors. Nonetheless, improved molecular techniques, such as transposon mutagenesis and whole-genome microarrays, as well as improved cellular biology protocols and animal models, have expanded understanding of how infection with *C. jejuni* leads to pathology of campylobacteriosis and autoimmune sequelae (**FIG. 1.2**).

Conflicting results from early *in vitro* work, due to strain variation and differences in experimental protocols, taken together with absence of a good animal disease model, have also hindered attempts to understand pathogenesis of *C. jejuni* [159]. The gut of many animals is highly colonized by *C. jejuni* with little or no inflammation [139]. Conventional inbred mice strains are also resistant to colonization [160]. A widely used measure of host interactions, which may or may not translate to pathogenic capacity of *C. jejuni* strains in humans, is colonization of one-day-old chicks [161]. In addition, mouse strains with altered immune complements, as well as limited or humanized microflora, have been also been developed that allow colonization [110, 162-164] or that approximate some outcomes of human disease [110, 165-168]. Strains also adhere to and invade epithelial cells *in vitro*, causing responses consistent with pathology of campylobacteriosis. *In vitro* infection models commonly used include INT407 (HeLa) human epithelial cells, as well as Caco-2 (human epithelial colorectal adenocarcinoma) or T84 (human colonic adenocarcinoma) polarized monolayers [169-171].

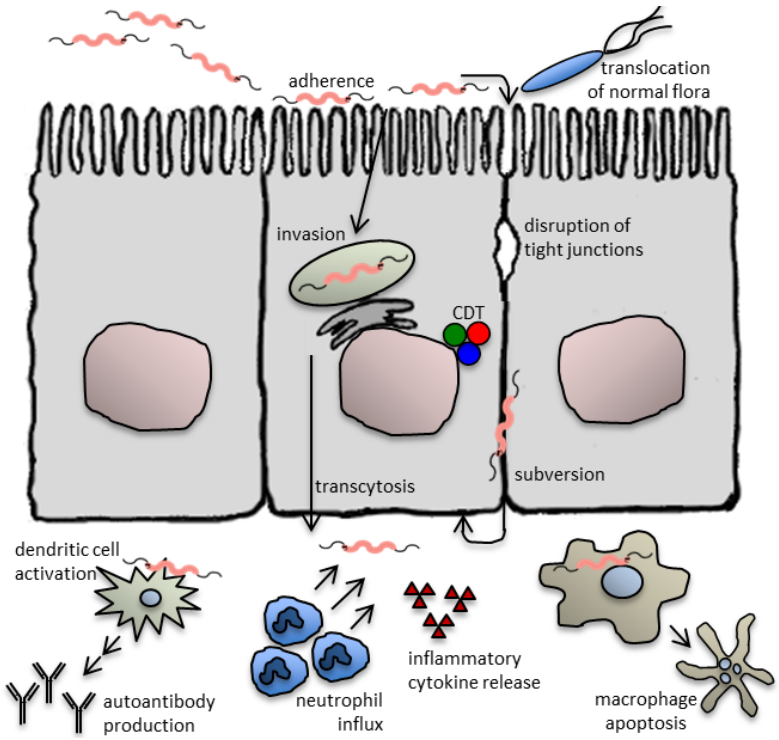


FIG. 1.2. *C. jejuni*-host-cell interactions in the intestinal epithelium of humans that may contribute to pathogenesis of campylobacteriosis and autoimmune sequelae.

1.3.2 Host and bacterial mechanisms involved in colonization and disease.

While *C. jejuni* is pathogenic to humans, it colonizes the avian gut as a commensal. Commercial broilers carry high bacterial loads (10^6-10^8 CFU g^{-1}) in their caeca, often with no overt pathology [95, 172]. A major difference between avian and human physiology is body temperature: chickens have an internal temperature of ~42°C, in contrast to the human temperature of 37°C. Thus, temperature-dependent expression of virulence-related proteins may contribute to host pathology differences. Differences in the host immune response may also underlie the tendency of each host to exhibit symptoms following colonization. An inefficient or tolerogenic inflammatory response may allow long-term colonization of chickens at high bacterial loads [172]. A variety of *C. jejuni* factors contribute to chick colonization, ranging from those involved in basic biology of the organism to some that are also required for pathogenesis in humans, including those involved in motility, protein glycosylation, and metabolism [111, 173].

Adherence to host cells allows intimate bacteria-host cell interactions that may lead to pathogenesis. *C. jejuni* does not express pili [55, 108], and instead appears to adheres to ileal tissues and monolayers via flagella [174, 175]. However, mutational studies have provided conflicting evidence for the role of flagella in host cell interactions, and it is unclear whether flagella provide motility toward cells, facilitate adhesion, or promote subsequent steps of uptake into host cells [121, 176-178]. Proteins that directly mediate adherence include lipoproteins, JlpA, and the CapA autotransporter [179-182]. The CadF and FlpA proteins also mediate binding through interaction with fibronectin [183-185], and the Peb1a and Peb3 ABC transporter proteins have been published to moonlight as adhesins [70, 186]. Possibly because of their effect on envelope protein expression, HtrA, Peb4, and disulphide bond-forming proteins are also required for adherence [187-189]. Finally, CPS, *N*-linked glycosylation, and LOS are also required for adherence to host cells [87, 190-192].

Adherence to cells of the intestinal epithelium may allow interactions leading to both non-inflammatory and inflammatory symptoms of campylobacteriosis [193]. Non-inflammatory diarrhea can be associated with production of enterotoxins by bacterial pathogens that modulate levels of intracellular signalling messengers such as cAMP or Ca^{2+}, leading to electrolyte or fluid imbalance. *C. jejuni* has been observed to stimulate Na^+ and Cl^- secretion and an increase in intracellular Ca^{2+} [194]. *C. jejuni*-infected cells upregulate genes involved in water and solute transport and show elevated cAMP levels [195-197]. However, *C. jejuni* does not encode an enterotoxin such as cholera toxin [55]. Redistribution of tight junction proteins can also cause loss of barrier integrity, leading to diarrhea, and infection of monolayers with *C. jejuni* causes loss of transepithelial electrical resistance and altered distribution of the tight junction protein occludin [198-200].

In the absence of enterotoxins, exaggerated host inflammatory mechanisms may make a significant contribution to both fluid imbalance and severe dysenteric symptoms of campylobacteriosis, such as bloody diarrhea [20]. Consistent with this, many of the 'virulence factors' reported for *C. jejuni* are actually factors that allow survival within the host, possibly leading to a strong host immune response. Thus, responsibility for

outcomes of infection may lay in the hands of both the bacterium and the host. *C. jejuni* can also induce proinflammatory responses and apoptosis in macrophage-like cell lines [201, 202]. Infection of epithelial cell monolayers with *C. jejuni* upregulates genes involved in inflammation, resulting in release of proinflammatory cytokines such as IL-8 and TNF-α [195, 203]. Cytokine release coincides with activation of NF-κB and AP-1, as well as ERK, p38, and JNK MAP kinases [199, 204-207]. Activation of NF-κB by bacterial components often occurs through Toll-like receptors (TLRs) and proteins that recognize conserved molecular motifs on pathogens. However, human TLR5 is not activated by *C. jejuni* flagella [208]. Knockdown of NOD1 expression in epithelial cells reduces IL-8 gene expression upon *C. jejuni* infection and leads to an increase in the number of intracellular bacteria [209]. *C. jejuni*-mediated intestinal inflammation is marked by release of cytokines such as IL-8 (interleukin-8) and the subsequent influx of neutrophils and macrophages [156]. Finally, *C. jejuni* can also activate anti-inflammatory cytokine production [204], thus the outcome of infection may be decided by the sum of both pro- and anti-inflammatory responses.

Two bacterial phenomena have been proposed to contribute to inflammation. Cytotoxins often underlie inflammatory diarrhea, and a cytolethal distending toxin (CDT) has also been identified in *C. jejuni*. CDT is an AB-type toxin composed of three subunits – CdtA, CdtB, and CdtC – of which CdtB is the active subunit [210]. Following entry, CDT arrests host cells in the G/M transition of the cell cycle via DNase I-like activity of CdtB [210, 211]. CDT can cause release of IL-8 from epithelial cells and apoptosis in monocytes [203, 212, 213]. While CDT has been proposed to contribute to diarrhea, inflammation, and systemic spread [8], presence of CDT genes does not solely determine clinical outcome [214]. Furthermore, clinical isolates with null mutations in genes encoding CDT have been isolated [215], and a Δ*cdt* mutant shows similar cytotoxicity to WT *in vitro* [216]. Thus, the contribution of CDT to campylobacteriosis is unclear. However, the ability of a strain to elicit IL-8 release does correlate well with its ability to translocate monolayers *in vitro* [198]. Translocation involves mechanisms by which pathogens cross the epithelium and gain access to the lamina propria, where they can cause inflammation or disseminate to other areas of the host [217]. Both transcellular (through cells) and paracellular (between cells) pathways have been reported for *C. jejuni*. Following uptake, bacteria have been observed moving intraendosomally from the apical to the basolateral surface, followed by exocytosis [169, 175]. A novel paracellular pathway ('subvasion') has also been noted where *C. jejuni* transits between cells before being internalized at the basolateral surface [112]; However, as monolayers in these experiments were grown under nutrient limitation, relevance of this mechanism has been questioned [217].

While it has been clearly shown that *C. jejuni* can enter epithelial cells, the mechanism of uptake is unclear and complicated by strain and experimental differences [217, 218]. It has been suggested that uptake requires actin filaments alone, microtubules alone, or both actin filaments and microtubules, depending on strains or methods used [219]. While multiple mechanisms of entry may exist, infection of healthy monolayers occurs at the apical surface and is microtubule-dependent [217]. In contrast, some studies indicate that the primary mechanism of uptake occurs at the basolateral surface and is microfilament-dependent [170, 185]. However,

this may depend on exposure of the basolateral surface under low serum conditions [217]. Despite some disagreement on route and mechanism of entry, *C. jejuni* does appear to modulate host signalling pathways that can lead to bacterial uptake, such as those involving receptor-mediated tyrosine kinase signalling, release of intracellular Ca^{2+}, calmodulin, and protein kinase C [218, 220, 221]. Invasion requires also caveolae [221, 222]. Bacterial factors that contribute this are unclear, but *de novo* protein synthesis by bacteria is required for uptake and activation of inflammatory signalling cascades [207, 223]. Motility may be required for invasion [224], and the flagellar export apparatus is also required for secretion of Cia proteins [121], many of which are required for uptake [122, 126]. The microfilament-dependent route of entry is mediated by the CadF and FlpA fibronectin-binding proteins, which are thought to initiate signalling events leading to membrane ruffling and uptake [225].

The ability of *C. jejuni* to elicit an inflammatory response is thought to be at least in part to its ability to survive within epithelial cells following uptake. While *C. jejuni* does not replicate within epithelial cells, it can remain viable for at least 24h [226]. Initial observations suggested *C. jejuni* resides in a membrane-bound vacuole after invasion of epithelial cells or uptake by professional phagocytes [171, 227], and it is now believed that *C. jejuni* survives by affecting endosome trafficking. The *Campylobacter*-containing vacuole (CCV) appears to be distinct from endosomes, although it transiently interacts with the early endocytic pathway and acquires Lamp-1 [226], and localizes near the Golgi apparatus. Separation of the CCV from the canonical endocytic pathway appears to allow survival within host cells, as internalization through F_cR delivers *C. jejuni* to lysosomes, where it loses viability [226]. Furthermore, survival of *C. jejuni* within epithelial cells does not requires catalase [228], which is consistent with avoidance of lysosome fusion. In contrast, *C. jejuni* survives poorly in primary macrophages, and CCVs in these cells colocalize with endocytic markers. Finally, the CiaI protein is required for intracellular survival, and CCVs harbouring a Δ*ciaI* mutant colocalize more frequently with lysosomal markers than those harbouring WT [125]. *C. jejuni* also undergoes physiological changes within cells that may contribute to survival [226]. Many stress tolerance genes contribute to survival within host cells, including *ppk1*, *ppk2*, *spoT, dps*, *htrA*, and *sodB* [229-233]. VirK, a homologue of proteins that contribute to virulence of intracellular bacterial pathogens, is also required [232]. Mutants in the glutamine transporter PaqPQ show increased intracellular survival; however, this is possibly due to decreased activation of MAP kinases and increased survival of host cells, rather than increased mutant fitness [234].

Although the majority of cases of campylobacteriosis resolve completely, a significant number of cases are followed by serious post-infectious sequelae. Perhaps the best characterized of these is GBS, an inflammatory demyelinating polyneuropathy that manifests as ascending paralysis. GBS is well correlated with prior *Campylobacter* infection [235], and pathogenesis of *C. jejuni*-mediated GBS is thought to be mediated by molecular mimicry of bacterial carbohydrate structures, such as sialic acid-containing LOS, with similar ganglioside structures on host neuronal tissues [236]. Previous *C. jejuni* infection also predisposes patients to reactive arthritis and IBD [237, 238]. Most pathogens that cause reactive arthritis are invasive enteric Gram-

negative bacteria [238]. Pathogenesis of *C. jejuni*-mediated reactive arthritis is unclear, but appears to involve both host and bacterial factors. IBD may be a consequence of disruption of homeostasis between the intestine and host microbiota [239], and *C. jejuni* can promote translocation of non-invasive *E. coli* across epithelial monolayers [240, 241].

1.4 Tolerance of specific transmission and pathogenesis-related stresses by *C. jejuni*.

During transmission and pathogenesis (**FIG. 1.1**), *C. jejuni* encounters a variety of environmental conditions that pose significant challenges to survival and replication. Prior to colonization of a commensal or susceptible host, *C. jejuni* can exist within aquatic environments, where it encounters low temperature, nutrient availability, and osmotic support. During transmission to a human host through the food chain, *C. jejuni* may encounter large fluctuations in temperature and high osmolarity. Following colonization, *C. jejuni* may encounter increases in nutrient availability and optimal growth temperatures; however, it must also contend with low pH in the stomach, compounds such as bile and mucin, competition from resident microbiota, low O_2 tensions, challenges from the immune system, antimicrobial peptides and possibly antibiotics, and oxidative burst in phagocytes. The relatively small genome of *C. jejuni*, taken together with a paucity of regulators, raises the question of how this pathogen tolerates such a wide range of conditions during zoonosis.

1.4.1 Starvation stress and stationary phase survival.

C. jejuni lacks a homologue of the RpoS σ (sigma) factor, and some evidence suggests that it lacks a stationary phase [242]. However, physiological and expression changes following logarithmic growth have been observed. Moreover, starvation induces stress tolerance in *C. jejuni*, suggesting a (possibly unorthodox) stationary phase response does exist [76, 243, 244]. *C. jejuni* harbours a *spoT* homologue that is solely responsible for production of the ppGpp (guanosine pentaphosphate) alarmone which mediates the stringent response [231]. Like in other bacteria, the *C. jejuni* stringent response is required for stationary phase and stress survival, as well as host-cell interactions. *C. jejuni* encodes a DksA ppGpp cofactor, which contributes to regulation of stable RNA synthesis, genes involved in amino acid-related metabolism, and host-related phenotypes [245], and also harbours enzymes for metabolism of polyphosphate (PolyP), a molecule central to stationary phase and stress survival, including two PolyP kinases (Ppk1 and Ppk2), an alkaline phosphatase (PhoX), and an exopolyphosphatase. Mutations affecting PolyP levels affect tolerance of *in vitro* stresses (low nutrient, osmotic, aerobic, and antibiotic), natural transformation, intracellular survival, and chick colonization [229, 230, 246].

1.4.2 Aerobic (O_2) stress and tolerance of suboptimal atmospheres.

C. jejuni is microaerophilic and capnophilic, and growth and survival under atmospheric O_2 concentrations is normally poor. Nonetheless, *C. jejuni* strains can be adapted to grow under these conditions [247, 248], and laboratory passage of *C. jejuni* (and presumably exposure to atmospheric levels of O_2) can have marked effects

on pathogenesis-related gene expression and phenotypes [75]. Incubation under atmospheric conditions can cause transition to a VBNC form [249], and aerobic adaptation also protects *C. jejuni* from acid challenge [250]. Thus, exposure to O_2 represents both an environmental challenge that *C. jejuni* tolerates, and also a trigger that may confer cross-protection to other stresses. Mutational and transcriptomic studies have identified proteins required for tolerance of atmospheric O_2 tensions by *C. jejuni*. These include the disulphide bond oxidoreductases DsbA and DsbB, AhpC, and the FdxA ferridoxin [251-254]. The chaperone activity of HtrA is also required for aerotolerance [255, 256]. Atmospheric differences beyond O_2 tension also have marked effect on *C. jejuni* gene expression and physiology [257]. Transcriptomes of cultures grown either in a Trigas incubator or a jar containing a gas pack-equilibrated atmosphere are significantly different, and include expression changes indicative of oxidative stress [257]. Mutation of *spoT* causes impaired growth under high O_2/low CO_2 conditions [231].

1.4.3 Oxidative (reactive oxygen species) stress.

O_2-dependent metabolism produces reactive oxygen species that react with cellular components. Bacteria may also encounter such molecules within macrophages or during freeze-thaw cycles [258, 259]. Expression and mutant analyses performed using stressors such as paraquat and menadione, which generate superoxide, peroxide generators such as hydrogen peroxide (H_2O_2) and cumene hydroperoxide suggest that tolerance of oxidative stress in this organism involves both canonical detoxification enzymes and novel regulators (see **Section 1.6**), and is intertwined with iron metabolism. Three prototypical proteins contribute to oxidative stress tolerance by *C. jejuni*: AhpC (akylhydroperoxide reductase), SodB (superoxide dismutase), and KatA (catalase). Expression of all three genes is upregulated under oxidative stress [260], and each contributes to tolerance of both *in vitro* and *in vivo* oxidative challenges. A Δ*sodB* mutant is most sensitive to the superoxide generator menadione, whereas Δ*katA* is most sensitive to H_2O_2, and Δ*ahpC* is most sensitive to cumene hydroperoxide [260]. The *sodB* gene is required for both entry into and/or survival within epithelial cells [224, 261], as well as for recovery after freeze-thaw treatment [262]. Catalase is required for H_2O_2 tolerance [228, 263-265]. While *katA* is dispensable for survival in epithelial cell monolayers, it is necessary for survival in macrophages [228]. All three proteins are required for chick colonization [260]. The oxidative stress response of *C. jejuni* extends beyond KatA, SodB, and AhpC [260]. Exposure to paraquat causes upregulation of the FldA flavodoxin and a pyruvate-flavodoxin oxidoreductase [266]. *C. jejuni* strains also harbour three or four Dsb homologues required for maintenance of disulphide bonds under oxidizing conditions [267], and the Rrc iron-containing rubrerythrin is also required for H_2O_2 tolerance [268]. When combined with O_2, iron can generate reactive O_2 species such as peroxides and hydroxyl radicals via Fenton and Haber–Weiss reactions [269]. Thus, it is not surprising that regulation of iron storage and metabolism intersects the *C. jejuni* oxidative stress response. Treatment of *C. jejuni* with both H_2O_2 and Fe^{2+}, compared to H_2O_2 alone, significantly increases expression of *katA*, *sodB*, and *ahpC* [260], and proteins related to iron homeostasis are also

upregulated in paraquat [266, 270]. The ferritin homologues Cft and Dps contribute to tolerance of iron-mediated oxidative stress [271, 272].

1.4.4 Nitrosative stress.

C. jejuni may also encounter nitrosative stress within animal hosts, macrophages, or as a consequence of reductive metabolism of nitrate [273-275]. Many of the proteins required for tolerance of nitrosative stress are positively regulated by the Crp-Fnr superfamily regulator NssR [276] and can be induced by agents such as *S*-nitrosoglutathione. The nitrosative stress regulon contains at least four proteins, including Cgb, a single-domain globin, and Ctb, a truncated globin [277-279]. Exposure to nitrosative stress also induces expression of heat shock genes, oxidative stress genes, and iron-related genes [280]. A TAT-translocated YedY homologue and the NrfA nitrate reductase may contribute to periplasmic nitrosative stress tolerance [116, 281]. Mutation of both *tpx* and *bcp*, as well as two methionine sulfoxide reductases, also increase sensitivity to nitrosative stress [282, 283].

1.4.5 Osmotic shock.

Variations in osmolarity are often encountered during transmission and pathogenesis. However, *C. jejuni* is more sensitive to high osmolarity than most gastrointestinal pathogens, and cannot tolerate osmolarities higher than 0.99 Osm L^{-1} *in vitro* [284]. The lower threshold for growth is 0.130-0.175 mOsm L^{-1} [285]. For comparison, the human intestine and chicken caecum are approximately 0.3 Osm L^{-1} and 0.7 Osm L^{-1}, respectively [286, 287], and MH (Mueller-Hinton) broth used for routine culture of *C. jejuni* is ~0.30 Osm L^{-1} (A. Cameron, personal communication). Addition of solutes such as 1% sodium chloride (0.34 Osm L^{-1}) to MH broth is used to approximate hyperosmotic conditions *C. jejuni* may encounter during colonization or transmission outside the host [229]. The osmotic stress response of C. jejuni is poorly characterized. The LOS biosynthesis gene *htrB* is upregulated under osmotic stress [288], and targeted mutagenesis of *ppk1*, *ppk2*, and *rpoN* affects salt tolerance [229, 230, 289]. Finally, *C. jejuni* has been reported to release free oligosaccharides, derived from the *N*-glycan pathway and analogous to free glucans of other bacteria, that may contribute to osmotolerance [290].

1.4.6 Acid and alkaline pH.

Before colonization, *C. jejuni* must transit through the low pH environment of the stomach. The relatively low infectious dose that has been observed for some strains - as low as 800 CFU can cause infection [291] - suggests it must harbour mechanisms for tolerating such conditions. The *C. jejuni* genome does not harbour a homologue of the urease gene of its gastric-adapted cousin *H. pylori*. Instead, both surface characteristics and stress response genes appear to mediate tolerance of low pH. The immediate response of *C. jejuni* to acid shock involves downregulation of metabolism and upregulation of genes involved in stress tolerance, such as the oxidative, nitrosative, and heat shock responses [292]. Longer-term adaptation and growth under acidic conditions involves upregulation of respiratory pathways and phosphate transport, but downregulation of

energy generation and intermediary metabolism genes [293]. Mutants unable to adapt to acidic conditions harbour insertions in genes involved in flagella, the outer membrane, CPS, and LOS. Mutation of *rpoN*, encoding the flagella-related RpoN σ factor, also results in acid sensitivity in standing culture [289]. While *C. jejuni* may also encounter high pH, adaptation to alkaline conditions has not been studied extensively in this pathogen. Exposure of *C. jejuni* to intestinal lavage fluid is followed by induction of the heat shock chaperones GroES and GroEL [294], and genes upregulated following exposure to the alkaline compound trisodium phosphate include those encoding Na^+/H^+ antiporters [295].

1.4.7 Bile acids.

In addition to exposure to acidic pH during stomach transit, *C. jejuni* must also tolerate bile acids in the gastrointestinal tract of host animals. Bile salts are bactericidal agents which interact with membranes and/or DNA [296]. Bile salt tolerance is more pronounced among *C. jejuni* human isolates than those isolated from poultry [297]. Compounds such as DOC, a secondary bile acid produced by gut microbes rather than the host [298], are commonly used to assess bile salt stress *in vitro*, and not surprisingly, *C. jejuni* has evolved robust mechanisms to respond to such compounds. Bile salts are negatively chemotactic for *C. jejuni*, and also stimulate significant transcriptional changes. *C. jejuni* uses both prototypical and novel mechanisms for adapting to bile stress [299, 300]. Multi-drug exporter (MDE) pump inhibitors reduce bile resistance, and the CmeABC MDE pump is regulated in response to bile and is required for bile salt tolerance and chick colonization [301-303]. Flagellar filament proteins (FlaA, FlaB), envelope proteins, a porin, and stress response proteins (GroEL) are also upregulated in bile or DOC [299, 304, 305]. Expression (but not secretion) of virulence-associated Cia proteins is induced in DOC [123, 299]. Bile salts may thus act as a key environmental signal for induction of survival and virulence gene expression in this pathogen.

1.4.8 Heat and cold shock.

C. jejuni is mildly thermophilic, with an optimum growth temperature of 37-42°C (depending on the strain), reflecting its adaptation to the intestinal tract of birds. Nonetheless, in food-processing environments, it encounters temperatures that vary widely from that of the avian gut. In general, elevated temperatures cause downregulation of metabolic genes and upregulation of genes encoding chaperones and heat shock proteins. *C. jejuni* harbours some classical heat shock proteins, many of which are upregulated or required *in vivo* [55, 306]. These include the Lon and ClpB proteases, GroES and GroEL chaperones, ClpB protease, as well as DnaK, DnaJ, GrpE, and HslU [307-311]. Mutation of *dnaJ*, *clpP*, and *lon* each causes heat sensitivity [312, 313]. Similarly, the *htrA* chaperone/protease gene is also required for thermal stress [255, 256]. The *htrB* gene, encoding a Lipid A biosynthesis protein, is also upregulated upon moderate temperature upshift, suggesting envelope modification may be required to tolerate increased temperatures. Reduced temperatures are also encountered in both food processing and aquatic transmission environments. *C. jejuni* strains maintain viability after extended periods at 4°C [314]. However, unlike *E. coli*, *C. jejuni* does not appear to alter its

membrane in response to cold [315]. Cold tolerance requires polynucleotide phosphorylase and a VacJ homologue [262, 316].

1.4.9 Antimicrobial agents and toxic metals.

C. jejuni may encounter antimicrobial agents both in the natural environment and in clinical settings. Intrinsic resistance to numerous antibiotics is provided by physiological characteristics such as DNA repair, efflux pumps (MDEs), and surface carbohydrates. The Mfd protein, which mediates strand specific DNA repair, is important for ciprofloxacin resistance [317]. MDEs play a central role in antibiotic tolerance. Mutation of the gene encoding the CmeG MDE results in increased susceptibility to ciprofloxacin, erythromycin, gentamicin, tetracycline, rifampicin, and ethidium bromide, and cholic acid [318]. The CmeABC MDE is required for resistance to bacteriocins fluoroquinolones, and macrolides [319, 320]. The CmeDEF MDE appears to play a secondary role in resistance to antimicrobials such as PxB, Amp, and ethidium bromide [321]. LOS, including modification of Lipid A, is central to tolerance of antimicrobial peptides such as PxB, α-defensins, and cathelicidins [84, 87, 322, 323]. *C. jejuni* must also tolerate heavy metals in the natural environment, in poultry houses, and during treatment of infections in humans. Arsenates are added to chicken feed, and *C. jejuni* isolated from poultry is often resistant to arsenic [324]. A four-gene operon required for resistance to arsenate compounds has been identified, and encodes a membrane permease (*arsP*), an arsenate reductase (*arsC*), an efflux protein (*acr3*), and a transcriptional repressor (*arcR*) [325]. Nanoparticles of silver and zinc oxide are also being trialed as novel antimicrobial agents against *C. jejuni* [326, 327]. Two proteins mediate copper tolerance in *C. jejuni* [328]. First, a multicopper oxidase is upregulated in the presence of copper and is required for tolerance of these conditions. Second, a CopA-like copper-transporter is also required for copper tolerance. Finally, *C. jejuni* responds to cadmium stress by upregulating disulphide reductases [329].

1.5 Global stress tolerance strategies and biofilm formation

The paradox of why *C. jejuni* is such a successful zoonotic pathogen despite its fastidious growth requirements in the laboratory may be explained by its tendency to persist in distinct lifestyles in the natural environment. Phenotypes exhibited by broth-grown bacteria in the laboratory are often different from those existing under natural conditions in the environment. For example, expression of factors required for taking advantage of high nutrient conditions – such as broth culture – are often at the expense of expression of stress tolerance [330]. Furthermore, bacteria in the natural environment can exhibit stress-tolerant physiologies, such as those of VBNC, persistor, or biofilm cells [331-333].

1.5.1 Biofilm structure and function.

Upwards of 99% of bacterial species exist outside of the laboratory not as free-swimming, solitary cells, but as part of communities called biofilms [334]. Biofilms are surface-associated consortia of microorganisms, encased in a protective polymeric matrix, whose residents possess distinct phenotypic differences from their planktonic counterparts. This includes metabolic differences, changes in cell physiology, as well as increased

antibiotic resistance, virulence, and stress tolerance [335]. Activation of mechanisms such as the stringent response in biofilm bacteria may contribute to antibiotic resistance, and for microaerophilic bacteria such as *C. jejuni*, residing within a biofilm may allow tolerance of higher O_2 tensions [336, 337]. Heterogeneity of both phenotypes and genotypes can also exist in biofilms, due to their three-dimensional structure. Natural biofilms are often composed of many species, which further contributes to their unique characteristics [333].

Biofilm formation proceeds in a set of distinct steps that has been proposed to represent 'microbial development' [335]. These steps include initial interactions with a surface, microcolony formation, secretion of an extracellular matrix and maturation into a biofilm with three dimensional structure, and finally shedding or dispersal to return bacteria to a planktonic mode of growth (**FIG. 1.3**). The mechanisms and specific factors that underlie each step are distinct for each bacterial species; however, some themes have arisen from analysis of model organisms such as *Pseudomonas aeruginosa*, *Bacillus subtilis*, and Gram-positive cocci. Following attachment, accumulation of the biofilm begins and a matrix is secreted. The biofilm matrix is a mixture of hydrated extracellular polymeric substances, such as carbohydrates, proteins, lipids, and extracellular DNA (eDNA) [338]. The components of the matrix of bacteria show extensive diversity, even within a species. For example, strains of *B. subtilis* produce either an exopolysaccharide (EPS) or poly-γ-(D,L)-glutamate, whereas *Staphylococcus aureu*s produces poly-Glc*N*Ac [339]. The *P. aeruginosa* matrix contains three polysaccharides: Pel, Psl, and alginate [338].

Proteins are also common: the *B. subtilis* matrix contains amyloid fibers of the protein TasA [339]. In addition to carbohydrates, and protein, eDNA also contributes to aspects of biofilm structure and function, including structural integrity, recombination, and antibiotic resistance [340, 341]. Autolytic mechanisms often underlie either biofilm formation or dispersal, and are thought release eDNA [152, 340, 342]. Finally, specific signals that trigger biofilm dispersal have also been identified [343]. For example, in *B. subtilis*, release of TasA amyloid fibres is stimulated by D-amino acids [344].

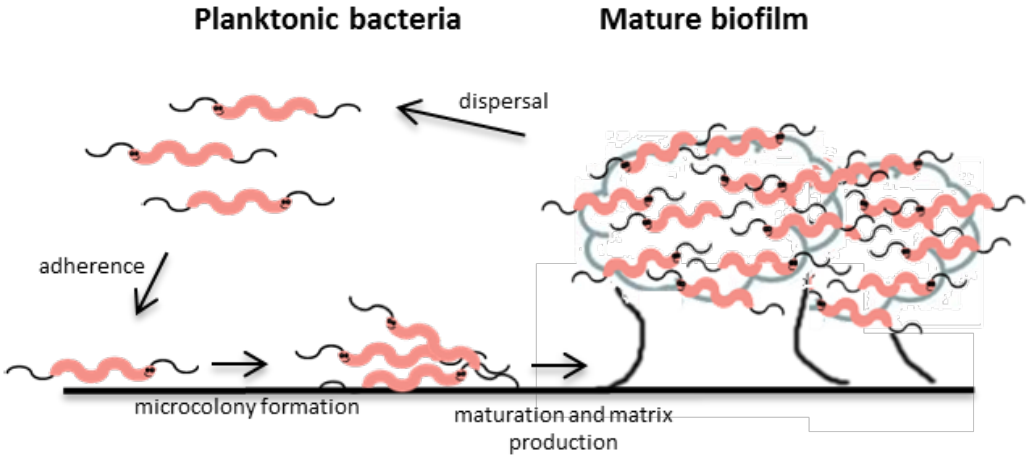


FIG. 1.3. General steps of biofilm formation by bacteria. Biofilm formation is initiated by adhesion to a surface, followed by microcolony formation, maturation and matrix production, and finally, dispersal.

Biofilms likely contribute to survival of *C. jejuni* in the food chain, from farm to fork [336]. *C. jejuni* forms biofilms under conditions in the laboratory that may be similar to environments encountered during pathogenesis. Furthermore, some evidence suggests that such a lifestyle does in fact confer it with stress tolerance in the absence of an extensive repertoire of stress response proteins. However, understanding of this process in *C. jejuni*, in comparison to other pathogens, is in its infancy. Molecular factors mediating adhesion, maturation, and matrix production, as well as specific phenotypic characteristics that define cells within the biofilm, are basically unknown.

1.5.2 Survival of *C. jejuni* in aquatic environments and mixed species biofilms.

The majority of research on *C. jejuni* biofilm formation has been in the context of survival in aqueous microcosms. Dissemination through two major reservoirs – poultry products and drinking water – both require *C. jejuni* to endure periods in water, which provides challenges of low nutrients, poor osmotic support, and ambient O_2 concentrations. Upon hatching, chicks do not yet normally contain *C. jejuni* as part of their resident microflora [33], and mixed biofilm communities in drinking water in poultry facilities is a reservoir for colonization of newly hatched birds with *C. jejuni* [345, 346]. Despite the relative fragility of *C. jejuni*, it tolerates challenges posed by aquatic environments sufficiently to be a significant public health concern [347], and biofilms may contribute to survival under such conditions. In general, *C. jejuni* cells residing within biofilms in aquatic environments survive better than their planktonic counterparts. Increased stress tolerance and maintenance of culturability has been demonstrated on stainless steel and polyvinyl chloride [149, 348-351]. One study found that biofilm-grown *C. jejuni* were less stress-tolerant [352]; however, VBNC organisms within biofilms may contribute to significant underestimation of the number of surviving bacteria in some studies, as non-culture-based methods, such as fluorescence *in situ* hybridization, detect viable cells in biofilms for extended periods [346, 349, 353, 354]. Natural biofilms are composed of numerous microbial taxa, and bacterial species isolated from poultry facilities enhance both biofilm formation and survival. Mixed-species biofilms isolated from chicken houses, meat processing facilities, or aquatic environments, as well as single species such as *P. aeruginosa, E. faecalis* or *Staphylococcus simulans* enhance the formation, structure, culturability, and survival of *C. jejuni* biofilms [348-351, 355, 356]. The mechanism by which biofilm formation is enhanced by other bacteria is unknown, although kinetics of biofilm formation may be increased by polymeric substances provided by neighbour bacteria [348]. Also, commensalism with bacteria such as *P. aeruginosa* has been reported to increase tolerance of ambient O_2 tensions by *C. jejuni* [357]. Protozoan species found in aquatic biofilms may also contribute to survival [358, 359].

1.5.3 Antimicrobial resistance and *in vivo* relevance of *C. jejuni* biofilms.

Bacteria growing within biofilms are often more resistant to chemical disinfectants and antibiotics than those grown in broth culture in the laboratory [360-362]. Reasons for this are multifactorial, but include decreased efficacy due to metabolic downshift, as well as decreased penetration due to encasement in matrix material such as EPS [361, 362]. *C. jejuni* biofilms are also more resistant than their planktonic counterparts to

commonly used sanitizers [350, 363]. Uptake by protozoa present within biofilms may contribute to resistance to chlorine disinfection [358, 364]. *C. jejuni* also encounters numerous stresses within animal hosts; however, the role of biofilms *in vivo* is unclear. *C. jejuni* has been observed forming microcolonies on intestinal epithelial tissue *in vitro,* and species of *Campylobacter* have been identified within biofilms in the upper gastrointestinal tract of patients with Barrett's esophagus [174, 365]. *H. pylori* also forms biofilm-like structures in the gastric mucosa [366, 367]. A Δ*spoT* stringent response mutant forms enhanced biofilms and retains its capacity to colonize animals, even though it displays specific *in vitro* stress-related defects [231, 368] (E.C. Gaynor, unpublished observations). In addition, Δ*ppk1* (which also exhibits stress tolerance defects *in vitro*) demonstrates a dose-dependent trend for both *in vitro* biofilm formation and chick colonization [229]. Together, this suggests that biofilms may confer stress-sensitive mutants with *in vivo* resilience.

1.5.4 Molecular themes underlying physiology and development of *C. jejuni* biofilms.

C. jejuni can form three distinct forms of biofilms: cell-cell aggregates, pellicles at the air-liquid interface, and glass-attached flocs [369]. Numerous groups have demonstrated genome-wide changes in gene expression dependent on present lifestyle (i.e., sessile/biofilm vs. planktonic). Furthermore, both global and targeted molecular genetics approaches have identified genes highly expressed in cells growing in biofilms and those required for biofilm formation (**TABLE 1.1**).

Autoagglutination. There appears to be a strong relationship between autoagglutination and host-related phenotypes in *C. jejuni* [88, 176, 370]; however, it is not yet understood how this behaviour relates to biofilm formation [371]. Nonetheless, strain 81-176, which is a strong biofilm former, has strong autoagglutination activity, and autoagglutination in this strain is dependent on a heat-labile, protease-sensitive, and acid-extractable factor [370]. In general, autoagglutination in non-motile, but flagellated, mutants (such as Δ*pflA*) resemble the parental strain, whereas non-motile aflagellate mutants (Δ*flhA* or Δ*flaA* Δ*flaB*) show reduced autoagglutination [88, 370]. Mutation of genes required to activate flagellar expression (*rpo*N), as well as those involved in chemotaxis (*cheA*, *cheY*) and energy taxis (*cetB*), also reduce autoagglutination [176], as do mutations that affect flagellin glycosylation [88, 372, 373]. Mutation of CPS export (*kpsM*) or LOS (*neuC1*) genes has intermediate effects on autoagglutination [88], whereas mutation of the *peb4* chaperone gene alters envelope protein expression and enhances this phenotype [374, 375].

flagellar expression following attachment [376], and *B. subtilis* co-expresses a clutch protein, which inhibits motility, along with EPS [377]. Targeted mutagenesis of genes encoding various flagellar proteins has clearly demonstrated that flagella are required for WT levels of biofilm formation in *C. jejuni* (**TABLE 1.1**). Mutation of the flagellin genes *flaA* and *flaB*, as well as genes encoding the flagellin-like proteins *flaG* and *flaC*, causes defective biofilm formation, as does mutation of flagellar biogenesis genes such as *flhA* or *fliS* [369, 378]. Flagellar glycosylation is also required for biofilm formation [379]. Flagellate, non-motile mutants, such as Δ*pflA*, have not previously been assessed for biofilm formation. However, despite the clear requirement of flagella for biofilm formation, it is presently unclear whether the flagellum contributes motility, adhesion, or

TABLE 1.1. Biofilm and motility phenotypes of *C. jejuni* mutants.

	Locus	Gene name	Protein product	Biofilm phenotype	Motility phenotype	Reference
Flagella	Cj1338	*flaA*	major flagellin	-	-	Kalmokoff *et al.* 2006
	Cj1339	*flaB*	flagellin	-	WT	Kalmokoff *et al.* 2006
	Cj0720c	*flaC*	flagellin homologue, adhesin	-	WT	Kalmokoff *et al.* 2006
	Cj0061c	*fliA*	flagellar sigma factor (σ^{28})	-	WT	Kalmokoff *et al.* 2006
	Cj0882c	*flhA*	flagellar secretory apparatus protein	-	-	Kalmokoff *et al.* 2006
	Cj0547	*flaG*	flagellin homologue	-	WT	Kalmokoff *et al.* 2006
	Cj0549	*fliS*	flagellar chaperone	-	-	Joshua *et al.* 2006
Metabolism	Cj0688	*pta*	phosphate acetyltransferase	-	WT	Joshua *et al.* 2006
	Cj0689	*ackA*	acetate kinase	WT	WT	Joshua *et al.* 2006
	Cj0188c	*phoX*	alkaline phosphatase	+	WT	Drozd *et al.* 2011
Carbo-	Cj1448c	*kpsM*	CPS export protein	WT/+	WT	Joshua *et al.* 2006; McLennan *et al.* 2008
hydrates	Cj1129c	*pglH*	*N*-linked protein glycosylation	WT	WT	Joshua *et al.* 2006
	Cj1441	*neuB1*	LOS sialic acid synthase	WT	WT	Joshua *et al.* 2006
	Cj1131c	*gne*	UDP-GlcNAc/Glc 4-epimerase	WT	N/A	McLennan *et al.* 2008
	Cj0279	*carB**	carbamoylphosphate synthase	-	-	McLennan *et al.* 2008
	Cj1148	*waaF*	LOS heptosyltransferase	+	WT	Naito *et al.* 2010; Kanipes *et al.* 2004
	Cj1135	*lgtF*	LOS glucosyltransferase	+	WT	Naito *et al.* 2010; Kanipes *et al.* 2008
	Cj1324	Cj1324	flagellin glycosylation	-	WT	Howard *et al.* 2009
	Cj1413c	*kpsS**	CPS modification protein	+	N/A	M. Pryjma and E. Gaynor, unpublished
Regulatory/	Cj1103	*csrA*	RNA-binding regulatory protein	-	-	Fields and Thompson 2008
stress	Cj1272	*spoT*	bifunctional ppGpp synthetase II	+	WT	McLennan *et al.* 2008
response	Cj1359	*ppk1*	PolyP kinase 1	+	WT	Candon *et al.* 2007; Gangaiah *et al.* 2009
	Cj0604	*ppk2*	PolyP kinase 2	+	WT	Gangaiah *et al.* 2010
	Cj1556	Cj1556	MarR family transcriptional regulator	-	WT	Gundogdu *et al.* 2011
	Cj1198	*luxS*	autoinducer-2 synthase	-	-	Reeser *et al.* 2007
Envelope	Cj1345c	*pgp1*	PG-directed carboxypeptidase	-	-	E. Frirdich and E. Gaynor, in press
proteins/	Cj0596	*peb4*	Peptidyl-prolyl *cis/trans* isomerase	+/-	-	Rathbun *et al.* 2009; Asakura *et al.* 2007
secretion	Cj0578c	*tatC*	twin-arginine transporter	-	-	Rajashekara *et al.* 2009

(+) : enhanced biofilm formation
(-) : defective biofilm formation
*transposon insertion
N/A: data not available

secretion to the biofilm. *C. jejuni* motility decreases in stationary phase, but flagellar gene expression is maintained [76]. Moreover, flagellar proteins, such as the FlaA and FlaB flagellins, are overexpressed in biofilm cells compared to those grown planktonically [378]. This suggests that the flagellum may be required for steps following attachment and initiation.

Biofilm matrix. The matrix of microbial biofilms is often composed of EPS, proteins, and eDNA [380]. However, the nature of the polymeric matrix encapsulating the *C. jejuni* biofilm remains ill-defined. The related pathogen *H. pylori* secretes a hydrophilic EPS during biofilm growth, and production of proteomannans has been observed during biofilm formation [381, 382]. Serendipitously, a Δ*spoT* stringent response mutant, which forms enhanced biofilms, was found to overproduce a novel CFW-reactive polysaccharide, distinct from both CPS and LOS [368]. CFW hyporeactive mutants are correspondingly poor biofilm formers [87, 368]. It is now being appreciated that eDNA is a significant component of the biofilm

matrix [340], and eDNA has been observed as part of the *C. jejuni* biofilm matrix [383]. However, both the contribution to physiology and mechanism of release of *C. jejuni* eDNA are presently uncharacterized.

Cell envelope. Mutations that affect *C. jejuni* biofilm formation (either positively or negatively), other than those affecting flagella, are in genes relating to the cell surface. A correlation between increased CFW reactivity and enhanced biofilm formation may reflect envelope changes [87]. LOS mutants that are defective for addition of inner core sugars (such as Δ*waaF* and Δ*lgtF*) exhibit CFW hyper-reactivity and enhanced biofilm formation, whereas mutants defective for addition of outer core sugars (such as Δ*galT* and Δ*cstII*) are modestly defective for biofilm formation. LOS sialylation and *N*-linked protein glycosylation do appear to affect biofilm formation [369]. Unexpectedly, impairment of CPS export has been reported to enhance biofilm formation [368]. Mutations that affect surface protein expression and secretion also affect biofilm formation. Loss of the Peb4 chaperone affects the outer membrane profile and causes decreased expression of envelope-localized proteins such as HtrA, PorA, and FlaA [187, 375, 384]. Mutation of *peb4* causes either defective or enhanced biofilm formation [187, 375]. Mutation of *tatC*, encoding a portion of the TAT secretion system, causes defective biofilm formation [115].

Stress response, metabolism, and global expression changes. Both increased stress response protein expression and decreased metabolic activity are thought to contribute to stress tolerance exhibited by biofilm bacteria. *C. jejuni* growing on solid media to mimic biofilm growth undergo a shift towards stress tolerance and nutrient uptake [385]. However, the validity of using plate-grown *C. jejuni* as a representation of biofilm cells is unknown. Iron uptake and stress response proteins such as AhpC, GroEL, GroES, thiol peroxidase, and Peb4 have also been reported to be more highly expressed in biofilms [187, 378]. Metabolic proteins are both down- and up-regulated in biofilms, suggesting that distinct changes in metabolism mark the transition between planktonic and biofilm modes of growth.

1.6 Regulation of gene expression by *C. jejuni*

As a successful zoonotic pathogen that survives in different environments, *C. jejuni* must harbour mechanisms to adapt gene expression to each niche. However, the annotated genome contains a relatively small number of regulatory proteins [55], which is often characteristic of organisms adapted to a limited number of environments. *C. jejuni* dedicates 1.8% of its genome to regulation, which is more similar to that of *H. pylori* (restricted to the human stomach, 0.9%) than to *W. succinogenes* (environmental, 5.2%)[386]. For comparison, the *E. coli* genome contains approximately 2.8% regulatory genes. Nonetheless, *C. jejuni* adapts to numerous environmental challenges, and responds transcriptionally to *in vitro* conditions representing those that may be encountered during pathogenesis, such as mucin, DOC, ciprofloxacin, acid, as well changes in temperature and O_2 tension [292, 293, 299, 317, 387, 388]. *C. jejuni* also alters gene expression upon interaction with host cells and during colonization [72, 231, 306, 389, 390]. While many identified regulators reflect challenges encountered by *C. jejuni* (**TABLE 1.2**), absence of many classical regulators raises the question of how *C. jejuni* adapts expression to such *in vitro* conditions and those encountered during zoonosis.

TABLE 1.2. Environmental and stress-response-related regulators characterized to date in *C. jejuni*.

Regulator	Type	Phenotypes regulated	Reference
RpoD	sigma factor (σ^{70})	housekeeping genes; early flagellar gene expression	Wösten *et al.* 1998
			Wright *et al.* 2009
RpoN	sigma factor (σ^{54})	motility; osmotic, peroxide, and acid stress tolerance	Jafannathan *et al.* 2001;
			Hwang *et al.* 2011
FliA	sigma factor (σ^{28})	motility	Jafannathan *et al.* 2001
SpoT	ppGpp synthetase II	intracellular and stationary phase survival;	Gaynor *et al.* 2005
		rifampicin resistance	
DksA	ppGpp co-regulator	metabolism	Yun *et al.* 2008
Ppk1/ Ppk2	PolyP kinases	osmotic, nutrient, and antimicrobial stress tolerance;	Candon *et al.* 2007;
		biofilms; chick colonization; intracellular survival	Gangaiah *et al.* 2009;
			Gangaiah *et al.* 2010
Fur	ferric uptake regulator	iron uptake	van Viet *et al.* 1998
PerR	Fur-like	oxidative (peroxide) stress tolerance	van Vliet *et al.* 1999
NssR	Crp-Fnr family	nitrosative stress tolerance	Elvers *et al.* 2005
RacRS	TCRS	temperature-dependent growth; chick colonization	Bras *et al.* 1999
DccRS	TCRS	mouse and chick colonization; adaptation to	MacKichan et al. 2004;
		stationary phase	Wösten et al. 2010
PhosSR	TCRS	phosphate uptake	Wösten *et al.* 2006
FlgRS	TCRS	motility; chick colonization	Wösten *et al.* 2004
CbrR	TCRS regulator	bile tolerance; chick colonization	Raphael *et al.* 2005
CosR	TCRS regulator	oxidative stress tolerance	Hwang *et al.* 2011
HrcA	heat shock repressor	heat shock tolerance	Holmes *et al.* 2010
HspR	heat shock repressor	heat shock tolerance	Anderson *et al.* 2005
CmeR	TetR family repressor	antimicrobial resistance; chick colonization	Guo *et al.* 2008
CsrA	RNA-binding regulator	oxidative stress tolerance; biofilm formation	Fields and Thompson 2008

1.6.1 Alternative sigma factors.

C. jejuni possesses only three RNA polymerase (RNAP) sigma factors [391], two of which appear to be almost solely devoted to regulating flagellar biosynthesis. In addition to the RpoD (σ^{70}) housekeeping sigma factor, *C. jejuni* encodes the alternative sigma factors RpoN and FliA [55]. Most notably absent are the RpoS (σ^{38}) stationary phase sigma factor, and the RpoH (σ^{32}) sigma factor that coordinates the heat shock response. Homologues of extracytoplasmic s factors, such as the RpoE envelope stress sigma factor, do not appear to be present in the *C. jejuni* genome. RpoN (σ^{54}) homologues often regulate aspects of nitrogen metabolism, and were recently proposed to control expression of aspects of the bacterial exterior, such as EPS, flagella, lipids, LPS, lipoproteins, and PG, as well as biofilm formation [392]. Activation of RpoN is dependent on the TCRS FlgSR, which includes an NtrC-type response regulator, and is tied to completion of early flagellar structures [393, 394]. The RpoN consensus in *C. jejuni* is represented by 5'-TTGGAACRN$_4$TTGCTT [395]. RpoN affects virulence via activation of flagellar expression [103]. Inactivation of *rpoN* affects chick colonization and results in hyperosmotic and acid tolerance defects [111, 289, 396]. The FliA sigma factor (σ^{28}) appears to be solely dedicated to expression of flagellar proteins in *C. jejuni*. FliA is required for expression of FlaA. Both Δ*flaA* and Δ*fliA* mutants have stubby flagella and are non-motile [378, 397]. FliA activity is negatively regulated by binding of the anti-s factor FlgM in a temperature-dependent fashion [398]. The FliA consensus differs slightly from that of *E. coli* (5'-TWWWN$_{13\text{-}18}$CGAT), in that it is more AT-rich and contains a unique conserved T residue [395].

1.6.2 Prototypical regulators of the enterobacteria and novel *C. jejuni* regulators.

C. jejuni encodes some homologues of key regulators well-characterized in other bacterial species (**TABLE 1.2**) Other than the alternative sigma factors mentioned above, most notably absent are cornerstones of stress response signalling in *E. coli*, including the oxidative stress regulators OxyR and SoxRS, the LexA DNA-damage regulator, and the envelope stress response TCRS CpxAR. The *E. coli* paradigm of catabolite repression also appears to not operate in *C. jejuni* [399]. In the absence of RpoH, regulation of the heat shock response is achieved by homologues of other classical proteobacterial regulators, such as HspR and HrcA [400]. HspR and HrcA have antagonistic activities on overlapping regulons, with Δ*hspR* showing enhanced thermal stress, and Δ*hrcA* temperature sensitivity [401]. Iron homeostasis and oxidative stress phenotypes appear to be intertwined and are controlled by two apparently paralogous regulators, Fur (ferric uptake regulator) and PerR. PerR is a functional, but nonhomologous, substitution for the OxyR protein in *C. jejuni* [270]. The PerR regulon is composed of 104 genes [260], and mutation of *perR* de-represses expression of oxidative stress proteins and causes hyper-resistance to oxidative stress [270]. Aerotolerance is affected by a MarR-type regulator (Cj1556), HspR, and SpoT [231, 400, 402], whereas the nitrosative stress response is controlled by NssR [276]. One of the most extensively characterized regulators in *C. jejuni* is CmeR, a TetR family regulator. CmeR has pleiotropic effects on physiology and controls expression of membrane, CPS-related, metabolic proteins, and the adjacent CmeABC MDE [301, 305, 403, 404]. Bile salts interact with CmeR and inhibit binding to regulated promoters [405-407].

1.6.3 Regulation of flagellar expression.

Like other Gram-negative bacteria, flagellar expression is tightly regulated, allowing construction of the flagellum in a hierarchical fashion, starting with the export apparatus and finishing with the filament. *C. jejuni* flagellar genes are divided into three classes, based on their temporal expression: early, middle, and late [98]. Early, or Class I, genes consist of the export machinery (Flagellar Type III secretion system), the motor/switch and stator, the RpoN sigma factor, FlhF, and the FlgSR TCRS. Middle (Class II) genes are RpoN-dependent and include those for construction of the hook, rod, P-, and L-ring, the minor flagellin FlaB, as well as the FliA sigma factor and its cognate anti-sigma factor FlgM [98]. Finally, late (Class III) genes are FliA-dependent, and include genes encoding the flagellin FlaA and minor filament proteins. A master regulator of flagellar expression (such as FlhDC) has been not been identified in *C. jejuni* [397]. The top of the flagellar cascade may be regulated by a novel paradigm, or early genes may be constitutively expressed [408]. Flagellar gene classes can be divided into two subsets according to their temporal expression pattern [76]. The first subset, including Class I genes, coincides with levels of motility and decreases as bacteria enter stationary phase consistent with regulation by σ^{70}. Second, RpoN- and FliA-dependent (Class II and Class III) promoters show sustained or increasing expression through stationary phase. The two checkpoints in *C. jejuni* flagellar gene expression are related to stages in completion of the flagellar export apparatus. First, a signal relating to physical completion of at least a portion of the export apparatus activates the FlgSR TCRS,

resulting in activation of RpoN activity and transcription of Class II genes [394, 408]. The second checkpoint requires a secretion-competent export apparatus, and involves secretion of the FlgM anti-sigma factor by the completed secretory apparatus thereby releasing inhibition of FliA and allowing transcription of Class III genes. Finally, additional layers of regulation ensure the proper morphological and spatial expression of *C. jejuni* flagella. The *fliK* gene encodes a hook-length control protein [409], and the FlhF and FlhG proteins are required for proper number and/or placement of flagella [410, 411].

1.6.4 Regulation of biofilm development.

Regulation of the sequential steps of biofilm formation has been extensively characterized in other pathogens, and is under control of a variety of regulatory schemes, including global regulators, the second messenger cyclic diguanylate (c-di-GMP), quorum sensing, the stringent response, and importantly, TCRSs [412-416]. In *P. aeruginosa*, a series of TCRSs appear to pace the progression of each stage of development [417]. TCRS also control biofilm-related phenotypes in other Gram-negative and Gram-positive bacteria [418, 419]. In *Bacillus* spp., developmental programs for motility, sporulation, and biofilm formation are connected and share global regulators [339]. It is currently unclear if such regulatory schemes control *C. jejuni* biofilm formation. However, this process does appear to be regulated, as *de novo* protein synthesis is required, and specific conditions, such as nutrient availability, O_2 tension and osmolytes (sucrose or NaCl) affect biofilm formation [337, 420]. Few mutants in *C. jejuni* regulatory proteins have been assessed for biofilm formation. In contrast to what is usually observed in other bacteria, mutation of *spoT* in *C. jejuni* enhances biofilm formation [368]. Deletion of genes required for PolyP synthesis also increase biofilm formation [117, 229, 230]. It is unknown whether lower ppGpp and/or PolyP levels in these strains act as a specific signal that initiates biofilm formation, or if such mutants are triggering compensatory responses that promote differentiation into the more resilient biofilm state. In contrast to the stringent response mutants, biofilm formation is reduced in the absence of CsrA [421], a post-transcriptional regulator that controls carbon metabolism and biofilms [422]. Bacterial populations coordinate gene expression in response to cell density using quorum sensing, which often also regulates biofilm phenotypes [423]. Of the best-characterized quorum sensing systems of Gram-negative species, *C. jejuni* harbours part of the AI-2-mediated system, which is thought to be a byproduct of the activated methyl cycle and is produced by the LuxS protein [424]. *C. jejuni* encodes a homologue of LuxS, and *C. jejuni* culture supernatants contain AI-2 [425]; however, genes encoding proteins for detecting AI-2 appear to be absent from the genome. While *H. pylori* uses the MCP TlpB to detect AI-2 as a chemorepellent [426], it is unknown whether *C. jejuni* uses a similar strategy. Not surprisingly (because of the apparent absence of an AI-2 sensing mechanism), conflicting reports on the role of AI-2 in *C. jejuni* physiology and biofilm formation exist. AI-2 does function in quorum sensing during growth in broth culture [427], and analysis of transcription in a Δ*luxS* mutant identified changes consistent only with alteration of the activated methyl cycle. Furthermore, addition of exogenous AI-2 to both WT and Δ*luxS* cultures has no effect on transcription. Mutation of *luxS* negatively affects transcription of the *flaA* flagellin gene [428], which may

underlie decreased autoagglutination in this strain [88]. Biofilm formation is also defective in a Δ*luxS* mutant [420]. However, until an AI-2 detection mechanism is identified, it is unclear whether quorum sensing plays a regulatory role in any phenotypes of *C. jejuni* [427].

1.7 Two-component signal transduction

A common strategy for regulating gene expression in prokaryotes in according to environmental conditions is the TCRS [429]. The prototypical system consists of a transmembrane sensor histidine kinase, and a cytoplasmic response regulator (**FIG. 1.2A**). The extracellular domain of the sensor kinase detects a specific environmental signal, which results in ATP-dependent autophosphorylation of its intracellular domain on a conserved His residue [429]. This phospho-His serves as a substrate for a cognate response regulator, which itself becomes phosphorylated on an Asp residue on its N-terminal receiver domain, causing a conformational change that allows its C-terminal output domain to elicit a response that is appropriate for the particular environmental stimulus [430]. Variations from the prototypical organization of EnvZ-OmpR also exist. This includes multi-step relay systems [431] and linker proteins that shuttle phosphate between TCRSs [432]. However, no such proteins have been identified in *C. jejuni*.

1.7.1 Structure and function of TCRS components.

Both components of the prototypical TCRS are modular in nature and harbour both highly conserved and variable domains in a relatively consistent configuration [429]. Prototypical sensor kinases (**FIG. 1.4A**) are composed of an N-terminal domain (NTD) containing two transmembrane domains flanking a variable periplasmic signal detection loop, separated by a HAMP linker from a highly conserved C-terminal domain (CTD). The CTD contains the histidine kinase core, including an ATP-binding region and the phosphorylatable His residue (H221 in CprS). Sensor kinases homodimerize to mediate trans-phosphorylation of this domain [431], which then interacts with the receiver domain of the response regulator. Response regulators are comprised of an N-terminal receiver domain and a C-terminal output domain (**FIG. 1.4B**). The NTD receiver interacts with the sensor, and contains the phosphorylatable Asp residue (Asp52 in CprR). The receiver domain catalyzes phosphotransfer from the sensor, but can also be phosphorylated by molecules such as acetyl phosphate (AcP) [433]. Phosphorylation often triggers homodimerization of the regulator, which is also mediated by the NTD. The receiver is fused to a variable C-terminal output domain that determines the outcome of signalling.

The variable domains of the sensor kinase and response regulator proteins are specific to the particular stimulus or output of each TCRS. The variable N-terminal periplasmic signal detection loop of sensor kinases determines the condition a TCRS responds to, such as specific nutrients, metal ions, changes in osmolarity, and changes in pH. Signals detected by sensor kinases are often difficult to identify; nonetheless, sensor kinases with identified signals include LuxQ (AI-2), DcuS (C4-dicarboxylates), VanS (vancomycin), and PhoQ (divalent cations)[434]. The C-terminal output domain of the response regulator is also variable and

determines the physiological response triggered by a particular TCRS. A variety of output domains can be fused to the receiver, ranging from those mediating intermolecular interactions (RNA-binding, ligand binding, protein binding), those with enzymatic activity (GGDEF or c-di-GMP phosphodiesterases, methylesterases, histidine kinases, or protein kinases/phosphatases), and DNA-binding domains [435]. Approximately 60% of response regulators in annotated genomes are OmpR-, NarL-, or NtrC-type DNA-binding response regulators [436]. The CTD of OmpR regulators, such as CprR, harbour a winged-helix DNA binding domain. In general, phosphorylation of OmpR-type response regulators promotes oligomerization, followed by binding to specific DNA sequences in promoters of regulated genes to elicit gene expression changes [437]. Consensus sequences are often weakly conserved, and thus require both experimental and bioinformatic identification [431], but are usually represented by direct or inverted repeat elements separated by a 2-11 bp spacer [438]. Response regulators can act as activators and/or repressors of gene expression, and the regulon of a TCRS can range from a single operon to hundreds of genes [431].

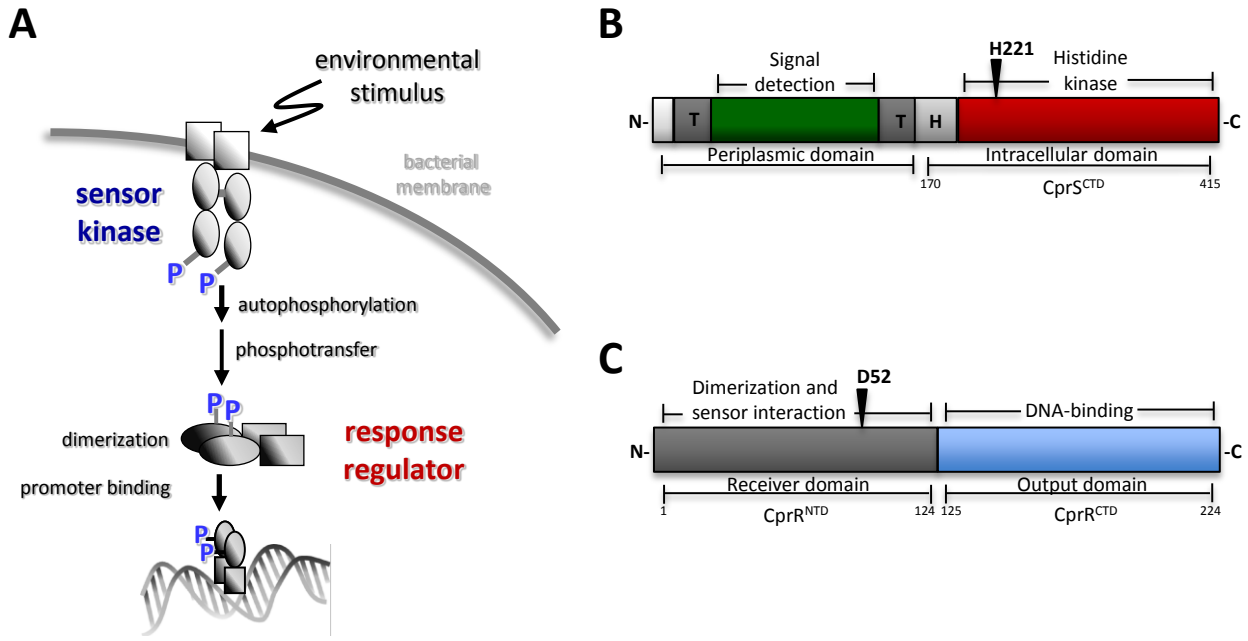


FIG. 1.4. Two-component signal transduction. **A)** Prototypical TCRSs are composed of a membrane-bound sensor kinase and its cognate cytoplasmic response regulator. Detection of a signal by the sensor kinase results in autophosphorylation on a conserved His residue on its intracellular domain. This phospho-His is a substrate for phosphorylation of the response regulator on a conserved Asp residue. The phosphorylated response regulator dimerizes and binds a specific DNA sequence in the promoter of target genes to affects their transcription. **B)** Structure of an EnvZ-type histidine kinase. Green: periplasmic signal detection domain; red: intracellular histidine kinase domain; T: transmembrane domains; H: HAMP domain. **C)** Structure of an OmpR-family response regulator. Grey: sensor kinase interaction, phosphorylation, and dimerization domain; blue: winged helix DNA-binding domain. The $CprS^{CTD}$, $CprR^{NTD}$, and $CprR^{CTD}$ domains used in **CHAPTER 3** are shown.

1.7.2 Regulation of TCRS activity, expression, and specificity.

Activity of a TCRS is controlled both post-translationally and transcriptionally. In general, activity of an TCRS is ultimately determined by the phosphorylation state of the response regulator, and this depends on the sum of the rates of its phosphorylation and dephosphorylation [439]. Both of these rates can be affected by activity of the sensor kinase, as they often exhibit two enzymatic activities: response regulator phosphorylation, and regulator-specific phosphatase activity [440]. Either activity can be modulated by presence of the environmental signal [431]. Moreover, the response regulator can also exhibit autophosphatase activity [431]. Phosphorylation-dependent dimerization of the regulator often facilitates DNA binding [431], in some cases by releasing inhibition of the C-terminal output domain [441]. TCRS activity is also controlled transcriptionally, often by autoregulation. In many cases, the components are encoded in an operon expressed from two promoters: one constitutive, and one that is autoregulated [442]. For example, in *Salmonella*, basal levels of PhoPQ are expressed constitutively, but positive feedback and autoregulation increases PhoPQ expression when the PhoQ-activating signal is present [443]. One-third of the TCRSs in *B. cereus* harbour their response regulator consensus sequence upstream of their own operons [438]. In addition to autoregulation, expression of TCRSs can also be controlled by other regulatory proteins, thereby linking signalling pathways into networks. Finally, while the modular nature of TCRSs may provide the opportunity for cross-talk and signal integration, it also suggests mechanisms for signal insulation must also exist. Similarities in component structure between TCRS proteins may underlie the observation of response regulator phosphorylation by non-cognate sensors, although it is unclear whether these observations are relevant *in vivo* [444]. Response regulators can also be phosphorylated by molecules such as AcP, an intermediate in the *pta-ackA* pathway [433], although domain interactions that restrict conformational changes in response regulators are thought to prevent phosphorylation by AcP [445]. Nonetheless, AcP has been proposed to be a global signalling molecule because of its effect on phosphorylation of response regulators, and AcP levels affect expression and biofilm formation in some bacteria [446, 447].

1.7.3 TCRS as targets for novel antimicrobials.

Many TCRSs are essential for viability. While some are master regulators that control the cell cycle, such as CtrA of *Caulobacter* spp. [448], others control essential processes such as maintenance of the cell envelope. For example, WalKR controls envelope metabolism and is essential for viability in many low-GC Gram-positive bacteria [449]. TCRSs also control survival- and virulence-related phenotypes, including quorum sensing, antimicrobial peptide resistance, toxin production, intramacrophage survival, and importantly, biofilm formation [450-453]. In addition to being essential for viability and expression of pathogenesis-related phenotypes, TCRSs are generally restricted to prokaryotes [454, 455], and conservation of enzymatic domains makes TCRS components attractive for broad-spectrum drug design [454]. These systems have thus been proposed as targets for novel antimicrobials [456]. Both sensors and regulators have already been targeted, such as PhoQ of *S. flexneri* and WalK of Gram-positives, as well as WalR [457-460].

1.7.4 TCRSs in *C. jejuni* and the ε-proteobacteria.

C. jejuni has a relatively limited repertoire of TCRSs (7 sensor kinases and 12 response regulators), as does its cousin *H. pylori* (4 sensors and 6 regulators)[386]. In contrast, the ε-proteobacterium *W. succinogenes* encodes 39 sensors and 52 regulators. Many TCRSs in *H. pylori* and *C. jejuni* are essential for viability or colonization, and display some novel mechanisms of signal transduction. In *H. pylori*, ArsRS controls acid responsive gene expression [461-465], whereas CrdRS regulates copper tolerance [466]. Three *H. pylori* response regulators are essential: ArsR, HP1043, and HP1021 [467]. One non-essential response regulator (CrdR) and three non-essential sensor kinases (HP0244, ArsS, and CrdS) are required for colonization of mice [468]. HP1021 and HP1043 do not require phosphorylation, as mutant forms that cannot be phosphorylated are sufficient for viability [469].

Other than CprRS, five TCRS pairs have been characterized in *C. jejuni*. Four of these contain a membrane-bound sensor kinase and an OmpR-family response regulator. PhosSR is a functional homologue of PhoBR phosphate sensing systems, and directly regulates the *pstSCAB* phosphate uptake operon and *phoX* [118, 470]. PhosR binds a unique Pho box (represented by 5'-GTTTCNA$_4$NGTTTC). The DccRS system is required for optimal colonization, but is dispensable for *in vitro* phenotypes, and is activated in late stationary phase, possibly in response to metabolic products [471, 472]. The DccR response regulator binds the direct repeat sequence 5'-WTTCACN$_6$TTCACW, found in front of genes encoding envelope proteins and a Type I secretion system. RacRS is thought to regulate genes for temperature adaptation, as a Δ*rac*R mutant is temperature sensitive and constitutively expresses temperature-dependent proteins [473]. A Δ*rac*R mutant is also defective for chick colonization (internal temperature ~42^{o}C). FlgSR has been extensively characterized. FlgSR activates RpoN-dependent flagellar genes, and a Δ*flg*R mutant is aflagellate [397, 409, 474]. Unlike canonical NtrC-like regulators, the DNA-binding domain of FlgR is absent, and the CTD may ensure specificity of phosphorylation [393, 394, 475]. Two orphan response regulators have been characterized in *C. jejuni*. CosR, which can complement the *H. pylori* response regulator HP1043, is essential for *C. jejuni* viability and plays a role In oxidative stress tolerance [266, 476, 477]. CosR binds the consensus sequence 5'-tttaAanAaAAaTtAtgaTTt in promoters of both positively and negatively regulated genes. The CbrR response regulator has also been characterized. CbrR harbours the only GGDEF domain of *C. jejuni*, and may thus be solely responsible for c-di-GMP production and related phenomena, such as biofilm formation [478]. A Δ*cbr*R mutant cannot grow on plates containing 1% DOC, suggesting CbrR mediates bile resistance [479]. CbrR harbours two N-terminal receiver domains and thus putatively interacts with two (unidentified) sensor kinases. CbrR is essential in strain 81-176 (S. Svensson and E. Gaynor, unpublished observations).

1.8 A novel TCRS may control stress- and virulence-related phenotypes in *C. jejuni*

1.8.1 Identification of CprRS, a TCRS upregulated in response to human host cells.

As it was relatively unclear how *C. jejuni* adapts to *in vivo* environments, a microarray-based screen was employed to identify genes important for adapting to the human host environment [231]. Numerous genes were observed to be upregulated in the presence of live, but not fixed, host cells, including *spoT* and genes comprising part of a putative amino acid ABC transporter. These genes were subsequently shown to contribute to survival and virulence-related phenotypes [231, 234]. Clones from the pVIR plasmid, which encodes a putative Type IV secretion system and is harboured by the invasive 81-176 strain [56] were also upregulated. In the presence of live host cells, *C. jejuni* also upregulated an operon encoding a putative TCRS (Cj1227c-Cj1226c). This TCRS was uncharacterized; however, previous work suggested it was essential for viability of *C. jejuni* [J. MacKichan and E. Gaynor, unpublished observations, [479]].

1.8.2 Hypothesis.

Because Cj1227c-Cj1226c was upregulated in the presence of live host cells, it was hypothesized that this TCRS may control phenotypes central to adaptation of *C. jejuni* to pathogenesis-related environments. Furthermore, initial characterization of a sensor kinase mutant lead to the proposal that this TCRS (later renamed CprRS for *Campylobacter* planktonic growth regulation, sensor and regulator) controls phenomena central to biofilm formation in *C. jejuni*.

1.8.3 Specific aims.

To identify the specific role of this TCRS in *C. jejuni* gene regulation, biofilm formation, survival, and pathogenesis, we sought to:

I. Generate and characterize phenotypes of loss- and/or gain-of-function *cprR* and *cprS* mutants;

II. Identify and characterize genes controlled by CprRS that comprise the CprRS regulon;

III. Elucidate the nature of CprRS phosphorelay and identify other regulatory networks that may interact with CprRS;

IV. Use expression changes and phenotypes of *cprRS* mutants to characterize global changes in physiology, such as biofilm formation, that may contribute to stress tolerance in *C. jejuni*.

2 THE CPRS SENSOR KINASE INFLUENCES BIOFILM FORMATION AND IS REQUIRED FOR OPTIMAL CHICK COLONIZATION

2.1 Introduction and synopsis

In this study, genetic, molecular, and proteomic evidence are provided to support the hypothesis that the TCRS encoded by Cj1226c and Cj1227c affects survival- and pathogenesis-related phenotypes of *C. jejuni* through control of essential biological processes, stress tolerance and biofilm formation, thereby contributing to the success of this apparently fragile zoonotic pathogen. Analyses described herein led to designation of the system as CprRS (*Campylobacter* planktonic growth regulation). While the CprR response regulator was essential, a Δ*cprS* sensor kinase mutant was viable, although this strain displayed an apparent growth defect in broth culture. Furthermore, the mutant formed dramatically enhanced biofilms in a mechanism that did not involve upregulation of previously characterized surface polysaccharides, suggesting that analysis of Δ*cprS* may provide insight into *C. jejuni* biofilm formation. Proteomics analysis of Δ*cprS* showed expression changes essential metabolic genes, upregulation of stress tolerance proteins, and increased expression of MOMP and FlaA. Consistent with expression profiling, enhanced motility, and possibly secretion, was observed in Δ*cprS*, as well as decreased osmotic and oxidative stress tolerance. WT *C. jejuni* biofilms were also observed to contain a DNase I-sensitive component, and biofilm formation was influenced by DOC and fumarate. Finally, consistent with identification of CprRS as upregulated in the presence of host cells, Δ*cprS* displayed a dose-dependent defect for colonization of chicks. These results suggest that CprRS may influence expression of factors important for colonization, stress tolerance, and biofilm formation, and may thus contribute to survival of this zoonotic pathogen. Furthermore, characterization of the Δ*cprS* mutant in this work has also provided a framework for subsequent analysis of *C. jejuni* biofilm physiology.

2.1.1 Information about collaborators. Proteomics was performed in the laboratory of Dr. Stu Thompson (Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, GA). Chick infections were performed in the laboratory of Dr. Vic DiRita (Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI).

2.2 Materials and Methods

2.2.1 Routine and experimental bacterial culture.

Bacterial strains and growth conditions. Studies were performed using *C. jejuni* WT strain 81-176, a highly invasive isolate from a raw milk outbreak [28]. All strains are listed in **TABLE A.1**. *C. jejuni* was routinely cultured in MH broth (Oxoid, Hampshire, England) or agar (1.7%) plates. All incubations were performed at 37°C under microaerobic conditions (6% O_2, 12% CO_2) in a Sanyo 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 (Sigma, Oakville, ON). Where appropriate, the selective antibiotics Kan and Cm 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 (Luria-Bertani) broth (Sigma) with antibiotics added at the following concentrations: ampicillin (Amp), 100 μg mL^{-1}; kanamycin (Kan), 50 μg mL^{-1}; chloramphenicol (Cm), 25 μg mL^{-1}; tetracycline (Tet), 20 μg mL^{-1}.

Growth curve assays. *C. jejuni* strains were grown in MH broth overnight to mid-log phase then diluted to an optical density at 600 nm (OD_{600}) of 0.1 in MH broth. Cultures were incubated microaerobically at 37°C with shaking at 200 rpm (revolutions per minute). 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 [335, 368] with modifications. Briefly, overnight broth cultures 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 tri-gas incubator under microaerobic conditions (6% O_2, 12% CO_2) for one or two days, followed by staining for 10 min. by addition of 250 μL of 1% crystal violet (CV; Sigma) dissolved in 100% ethanol. Tubes were then rinsed with distilled water and dried, followed by photographic documentation and/or quantification of adhered CV by dissolving with 30% methanol/10% acetic acid and measuring absorbance at 570 nm (A_{570}). Where indicated in figure legends, chemicals were included in the MH broth of suspensions introduced into tubes prior to initiation of biofilm growth. Shaking broth culture tubes were stained with CV as above. To test carbon sources, biofilms were grown as in above in MH broth or MH broth supplemented with 50 mM fumarate, glycine or glutamine (Sigma). 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 CV staining. Either the average of the planktonic to biofilm ratio (OD_{600}/A_{570}), or biofilm measurements only (A_{570}), are reported, depending on the experiment. Unless otherwise stated, for all experiments means are reported along with error bars representing standard deviation. To test the effect of antimicrobial compounds, biofilms were again grown as in above in MH broth or MH broth supplemented with the

following: DOC, 0.05%; Triton X-100 (Tx100), 0.0005%; Tween-20 (Tw20), 0.002%; SDS, 0.00025%. For DNase I treatment of biofilms, biofilms were grown as described above, and following 2 days of incubation, the medium was removed by pipetting, and tubes were washed with 1.5 mL of PBS (phosphate-buffered saline), pH 7.4 (Invitrogen). Buffer alone, or buffer containing DNase I (Fermentas) at 4 U mL^{-1}, was added to each tube, followed by incubation for 3h at 37°C under microaerophilic conditions, followed by staining with CV. Bacterial viability under each condition was also assessed by quantifying colony-forming units (CFU) of each strain in an OD_{600} 0.02 suspension in each condition after 3h of incubation.

***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 OD_{600} 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 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 24h of incubation, surrounding the point of inoculation of equal numbers (as determined by OD_{600}) of bacteria into MH plates with 0.4% agar.

2.2.2 Recombinant DNA techniques.

General recombinant DNA techniques. Recombinant DNA manipulations were performed according to Sambrook and Russell [480]. Restriction 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 B.1**.

Targeted mutagenesis of *C. jejuni*. Targeted deletion mutagenesis of *C. jejuni* was achieved by double-cross-over homologous recombination with deletion constructs prepared in a suicide vector containing approximately 500 bp of homologous DNA of the target gene region flanking either a *cat* (chloramphenicol acetyltransferase, Cm^R) or *aph-3* (aminoglycoside 3′-phosphotransferase, Kan^R) cassette [481, 482]. The majority of coding sequences removed by inverse polymerase chain reaction (PCR). Attempts were made to delete either *cprS* or *cprR* using the Cm^R cassette; however, only Δ*cprS* mutants were recovered. The *cprS* gene was also deleted with a Kan^R cassette. Unless otherwise stated, experiments were performed using a Δ*cprS*::Cm^R strain (Δ*cprS*). Cm^R deletion of *cprS* was performed as follows. Briefly, the entire coding sequence of *cprS*, along with approximately 500 bp of flanking sequence on each side, was amplified by PCR using primers *cprS*-TOPO-FWD/REV from gDNA isolated from *C. jejuni* 81-176 (DNeasy Kit, Qiagen), and ligated directly into the pCR-XL-TOPO® vector (Invitrogen). Inverse PCR using primers *cprS*-INV-FWD/REV was then used to remove the majority of the *cprS* coding regions and engineer *Mfe*I sites. A *cat* cassette was excised from plasmid pRY109 [482] using *Eco*RI and ligated into the *Mfe*I-digested inverse PCR product. The resulting construct (pJM1), containing 86 and 137 bp of 5′ and 3′ coding sequence of *cprS*, was

then used to naturally transform WT *C. jejuni*, and Cm^R recombinants were recovered and confirmed to be Δ*cprS* by PCR. A similar procedure was used to attempt deletion of *cprR*, with the resulting pJM2 deletion construct; however, legitimate recombinants were not recovered. A Δ*cprS*::*aph-3* strain (Δ*cprS*::Kan^R) was also constructed in a similar manner to the Cm^R strain, using primers *cprS*-pGEM-FWD/REV and *cprS*-INV2-FWD/REV to create knockout plasmid pSS3 in pGEM-T. The Δ*spoT* mutant has been described previously [231].

Complementation of Δ*cprS*. Insertion of copies of *cprS* for complementation was achieved using pRRK [J. Ketley, unpublished, based on pRRC integration vectors [483]]. The coding region of *cprS* was amplified by PCR using primers *cprS*-pRRC-FWD/REV, which introduced 5′-*Xba*I sites and 3′-*Mfe*I sites, as well as the native ribosome binding site of *cprR* [484]. Following digestion with *Mfe*I and *Xba*I, this product was ligated into the pRRK vector to create plasmid pSS50. The resulting construct was naturally transformed into *C. jejuni* Δ*cprS*::Kan^R, and putative Δ$cprS^C$ complemented clones were recovered on plates containing Kan and Cm. Single insertions of *cprS* in the rRNA (ribosomal RNA) spacer region adjacent to Cj0029 were confirmed by PCR using primers ak233 and PKanF.

2.2.3 Microscopy.

Bright field and scanning electron microscopy of biofilms. *C. jejuni* biofilms were grown in borosilicate tubes as described above, with a glass coverslip standing upright in the culture. After 48h of incubation, coverslips were either stained with CV, stained with PI using the LIVE/DEAD BacLight Bacterial Viability Kit according to the manufacturer's instructions, or removed and processed for scanning electron microscopy (SEM) as follows. The coverslip was removed and gently rinsed once in 0.1 M cacodylate buffer. Biofilms were then fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1h. Coverslips containing the biofilms were processed and visualized at the UBC Bioimaging facility.

2.2.4 Molecular and biochemical analyses.

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 *cprR*-BT-FWD/REV or *dccR*-BT-FWD/REV, digested with *Bam*HI and *Xho*I, and ligated into similarly digested pBT to create plasmids pSS41 and pSS39, respectively. The CTD (amino acids 187–415) of CprS was amplified by PCR using primers *cprS*^{CTD}-TRG-FWD/REV, digested with *Bam*HI and *Spe*I, and ligated into the similarly digested plasmid pTRG to create plasmid pSS38. Electrocompetent *E. coli* Bacteriomatch II cells were co-transformed with 50 ng each of 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, conditioned for 2h in this medium at 37°C, and cells were then plated on Non-selective and Selective plates, lacking or containing 3-aminotriazole (3-AT), respectively.

RNA extraction, cDNA synthesis and quantification. RNA was extracted from mid-log-phase broth cultures at an OD_{600} of 0.2–0.5 or overnight cultures on MH agar as previously described [75]. 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 x *g* for 5 min. at room temperature followed by immediate freezing and storage at −80°C. Cells were thawed at room temperature, resuspended in 50 μL of 0.4 mg mL^{-1} lysozyme (Sigma) in 10 mM Tris pH 8.0, 1 mM EDTA, 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 addition of 200 μL of chloroform. After centrifugation at 14,000 x *g* for 15 min. at 4°C, the top phase was transferred to a new tube. An equal volume of 70% ethanol was added drop-wise, followed by application to an RNeasy column and washing according to the manufacturer's instructions. Samples were digested using the Qiagen on-column RNase (ribonuclease)-free DNase kit according to the manufacturer's instructions, and RNA was eluted in 30 μL of RNase-free water. Complementary DNA (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), and RNA quality was assessed by electrophoresis on 1% agarose TAE (Tris-acetate-EDTA) gels. Absence of gDNA within RNA samples was confirmed by PCR.

Transcript analysis. RT-PCR (reverse transcription-PCR) was used to demonstrate separate transcription of *cprR* and the upstream *htrA* gene. PCR reactions with *Taq* DNA polymerase and primer sets *htrA*-3′/*cprR*-BT-REV and *cprR*-BT-FWD/REV were performed using cDNA or gDNA from WT *C. jejuni* 81-176, and amplification was determined by agarose gel electrophoresis.

Proteomic comparison of WT, Δ*cprS* and Δ*cprS*^{C}. Proteome analysis was performed using Differential In-Gel Electrophoresis (DIGE) technology (GE Biosystems, Piscataway, NJ). *C. jejuni* WT, Δ*cprS* and Δ*cprS*^{C} cells were grown to mid-log phase at 37°C in MH broth. Cells were rinsed three times by suspending in wash buffer (10 mM Tris, pH 8.0, 5 mM magnesium acetate), followed by centrifugation for 4 min. at 12,000 x *g* at 4°C. Cells were lysed by suspension of the pellet of 1 mL of lysis buffer (8 M urea, 30 mM Tris, pH 8.5, 5 mM magnesium acetate, 4% (w/v) CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate). The lysate was incubated for 30 min. on ice, and 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 x *g*. Protein concentrations of the soluble component, used for subsequent proteomic analyses, were determined using a BCA assay kit (Pierce, Rockford, IL). Samples from WT, Δ*cprS* and Δ*cprS*^{C} were labeled 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 labeled at lysine residues with 1 μL 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 unlabeled 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-PAGE (SDS-polyacrylamide gel electrophoresis) 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). Proteins were identified by querying protein databases with both tryptic fingerprint data as well as primary amino acid sequence of peptides following collision-induced fragmentation and MS/MS.

Subcellular fractionation and analysis of secreted proteins. Culture supernatants [127] and subcellular fractions [69] were prepared essentially as described previously. Each strain was grown overnight in 100 mL of MH broth and cells were collected by centrifugation at 4,800 x *g* for 30 min. at 4°C. The supernatant was filtered through a 0.22 μM filter (Millipore, Bellerica, MA), and proteins were precipitated by the addition of trichloroacetic acid (TCA) (Sigma) to a final concentration of 10% and incubation at 4°C overnight. Precipitated material was then collected by centrifugation at 9,800 x *g* for 1h. The pellet was washed 2X with acetone and resuspended in 100 μL of SDS-PAGE sample buffer. The cell pellet was resuspended in 10 mL of sucrose-Tris 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 8,000 x *g* for 10 min. and resuspended 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 x *g* for 15 min. at 4°C and then proteins were precipitated with TCA as described above. The cell pellet was suspended in 2.5 mL of ice-cold 10 mM Tris, pH 8.0, sonicated for 3 × 30 s, and centrifuged for 10 min. at 13,000 x *g*. The pellet was discarded, and the supernatant was centrifuged on a Beckman Optima L-90K Ultracentrifuge at 100,000 x *g* to collect cell membranes. The supernatant was saved as the cytoplasmic fraction, and the pellet was washed and resuspended in 100 μL of 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4, followed by the addition of an equal volume of 2% sarcosine (Sigma) in HEPES. After incubation at room temperature for 30 min. to solubilize inner membranes, insoluble outer membranes were

collected by centrifugation at 15,600 x *g* for 30 min. at 4°C and resuspended in 100 µL HEPES. Equal volumes of each fraction were separated by SDS-PAGE followed by silver staining [485].

Carbohydrate analyses. Strains were grown on MH agar for 48h microaerobically and carbohydrates were prepared as described previously [486]. Briefly, bacteria were harvested with PBS and diluted to an OD_{600} of 10.0 in 100 µL. An equal volume of 2X 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 for 10 min. After samples cooled to room temperature, 10 µL of 10 mg mL^{-1} proteinase K was added and samples were incubated overnight at 55°C. Samples were then incubated at 95°C for 5 min. and separated by SDS-PAGE and silver-stained or electroblotted to PVDF (polyvinylidene difluoride) membrane for Western blotting with an anti-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 [368]. Briefly, overnight cultures were diluted to a similar OD_{600} and spotted on Brain-Heart Infusion (BHI) agar containing 0.002% CFW (Sigma), followed by incubation microaerobically for 24-48h..

2.2.5 Host interactions.

Adherence, invasion and intracellular survival in INT407 cells. *In vitro* gentamicin protection assays were performed essentially as described previously [231]. INT407 cells in Minimal Essential Medium (MEM) with 15% fetal bovine serum (FBS) (Invitrogen) were seeded into 24-well plates 16h before infection (~10^5 cells per well) and incubated in a humidified 5% CO_2 atmosphere. *C. jejuni* strains were harvested from MH plates and inoculated into MH biphasic tubes to an OD_{600} of 0.002. After approximately 16h 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 (MOI) 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 3h; 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 8h 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 [111]. Day-of-hatch chicks (white-leghorn, Charles River Laboratories) were orally inoculated with 10^2, 10^4 or 10^6 CFU of *C. jejuni* diluted in PBS. The *C. jejuni* strains were grown on MH agar containing 10 µg mL^{-1} trimethoprim for 18h in a tri-gas 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).

2.3 Results

The *C. jejuni* genes Cj1226c and Cj1227c encode a TCRS pair (CprRS); the CprR response regulator is essential for viability. The annotated *C. jejuni* NCTC 11168 genome contains 7 sensor kinases and 12 response regulators [55], and among these are five TCRS pairs in which the sensor kinase appears to be encoded immediately downstream of the response regulator. One such pair, encoded by Cj1226c and Cj1227c, was previously identified as upregulated in the presence of live epithelial cells *in vitro* [231]. Bioinformatic analysis of each component suggested that Cj1226c encodes an EnvZ-family sensor kinase, and the protein encoded by Cj1227c contains domains characteristic of the OmpR family of DNA-binding response regulators [487]. The organization of the region surrounding Cj1226c and Cj1227c in various *Campylobacter* spp. is shown in **FIG. 2.1**.

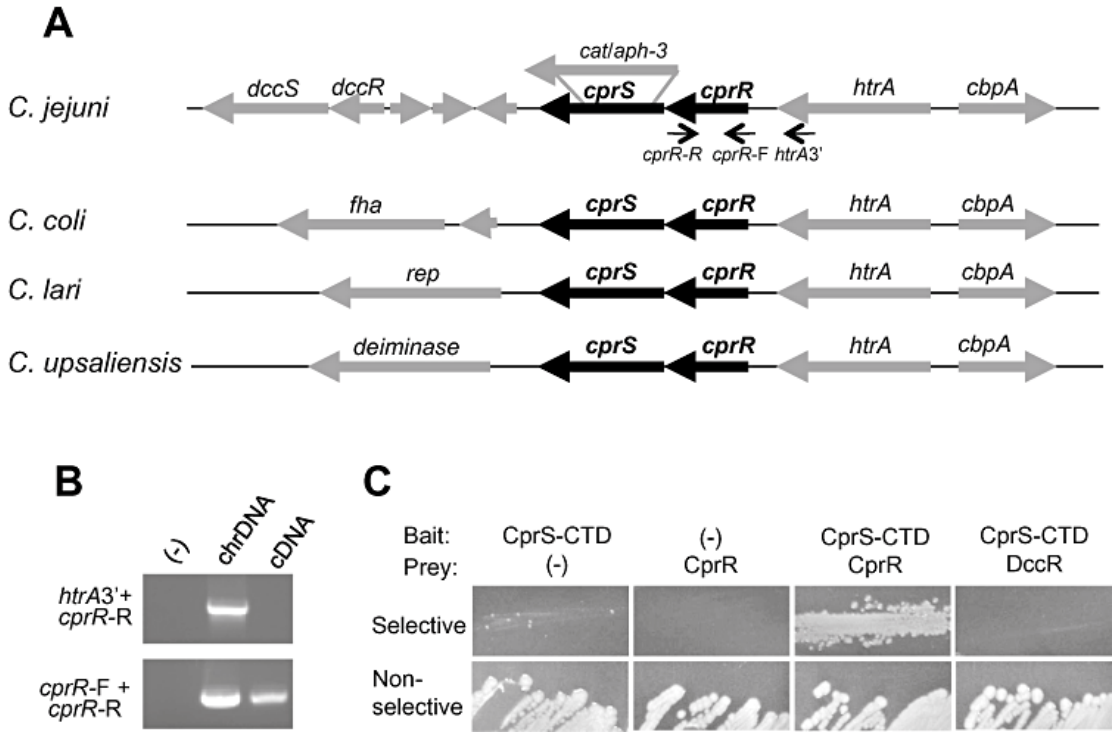


FIG. 2.1. Identification and genetic analysis of a *Campylobacter*-specific TCRS, *cprRS*. A) Location of the *cprR* response regulator and *cprS* sensor kinase homologues (annotated as Cj1226c and Cj1227c in strain 11168) in the genomes of *Campylobacter* spp., using CampyDB (http://xbase.bham.ac.uk/campydb/). Approximate location of *cat*- and *aph-3*-marked deletions in *cprS* are shown, resulting in strains designated Δ*cprS* and Δ*cprS*::Kan^R respectively. **B)** The *cprR* and *htrA* genes are transcribed separately. 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 CTD interacts with CprR, but not a control *C. jejuni* response regulator, DccR.

The TCRS encoded by Cj1226c and Cj1227c appears to be well conserved only among *Campylobacter* spp. 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; *A. butzleri*, 71% and 52% similarity). In each case, the genes are encoded adjacent to the *htrA* gene, strongly suggesting orthology (**FIG. 2.1A**). 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; *H. 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 [66], 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), the system was renamed CprRS (*Campylobacter* planktonic growth regulation response regulator and sensor kinase).

To explore the physiological role of CprRS in *C. jejuni*, mutational analysis was performed on each gene. Numerous attempts to delete the response regulator gene were made using different mutagenesis constructs (J.K. MacKichan, E.C. Gaynor and S. Falkow, unpublished observations). In addition, it has been reported that Cj1227c could only be inactivated when a second copy was present at a heterologous location [479], and transposon mutagenesis of this region resulted in inserts in both *htrA* and Cj1226c, but not Cj1227c [488]. These results thus strongly suggested that activity of this response regulator was essential for viability of *C. jejuni* under laboratory conditions. In contrast, deletion 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 Kan^R cassette, suggesting that activity of CprS was dispensable. The resulting strains were designated Δ*cprS* and Δ*cprS*::Kan^R; as they behaved similarly, they were used interchangeably.

The non-essential nature of the sensor kinase, together with the fact that the response regulator appeared to be 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 [489] and microarray expression analysis [E. Gaynor, unpublished observations; [75]] suggest that they are encoded in a stand-alone two-gene operon. Nonetheless, because of the conservation of this genomic organization within the campylobacters, the transcriptional (and thus the possible functional) relationship between *cprRS* and *htrA* was explored. RT-PCR

was performed using primers annealing to the response regulator gene only, or the 3′ end of *htrA* and the 3′ end of the response regulator (**FIG. 2.1A**). While amplicons were observed for both primer sets when chromosomal DNA (chrDNA) was used as template, PCR products using cDNA as a template were only obtained for the *cprR* primer set (**FIG. 2.1B**), suggesting that *cprR* may be transcribed independently from *htrA*. Although the above transcriptional and operon analyses strongly suggest that these genes encode a cognate TCRS, confirmation of this was sought by demonstrating a direct physical interaction between CprR and the CTD of CprS. A bacterial two-hybrid system was used to measure physical association between the two proteins as has been previously employed [490]. 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 response regulator (DccR) (**FIG. 2.1C**), indicating that only interaction of CprS with CprR was strong enough to activate expression of the reporter genes.

Δ*cprS* displays an apparent growth defect in broth culture. The role of CprRS in *C. jejuni* biology was explored via extensive phenotypic characterization of the Δ*cprS* mutant. The majority of phenotypes tested were the same for WT and Δ*cprS* (**TABLE 2.1**). However, a growth defect in the Δ*cprS* mutant was immediately noted, as it formed much smaller colonies on rich media than the WT strain. Furthermore, in shaking broth culture, Δ*cprS* reached log phase slower than WT and also displayed a late-stage culturability defect (**FIG. 2.2**), with a 4-log decrease in recovery of culturable bacteria (CFU mL^{-1}) beyond 48h of culture. These results were not surprising, given the essential nature of the cognate response regulator. The Kan^R cassette used to make one Δ*cprS* deletion mutant is non-polar, and RT-PCR data suggested that *cprRS* is a stand-alone two-gene operon (**FIG. 2.1B**); furthermore, the downstream genes are likely essential (tRNA-Asn, *cprR*). Nonetheless, the possibility that this phenotype was not linked to the targeted mutation was addressed by inserting a WT copy of *cprS* into a heterologous location in the chromosome of Δ*cprS* to create $\Delta cprS^C$, which partially complemented growth (data not shown).

TABLE 2.1. The Δ*cprS* mutant is not defective for numerous *in vitro* phenotypes. *In vitro* stress-related phenotypes ('Phenotype') were tested by the indicated procedures ('Experiment').

	Phenotype	Experiment
Pathogenesis-related:	Adherence and invasion	*in vitro* INT407 gentamicin protection assay
	Serum sensitivity	10% human serum survival
Antimicrobial tolerance:	Antibiotic resistance	MICs: gentamicin, Amp, rifamipicin
	Antimicrobial tolerance	MICs: Tween-20, Triton X-100, ethidium bromide, EDTA
	Low pH survival	acetic acid disk diffusion assay, MH pH 5.5 survival
Atmospheric tolerance:	Aerobic/aerobic survival	Ambient/ anaerobic atmosphere broth culture survival
	Oxidative sensitivity	30% H_2O_2 disk diffusion assay
	Low CO_2 tolerance	5% CO_2 atmosphere growth
Carbohydrates:	Surface polysaccharides	CFW, Congo red, and Sudan black reactivity
	LOS	SDS-PAGE - silver stain profile
	CPS	SDS-PAGE - Alcian blue stain and Penner immunoblotting
Nutritional stress:	Low iron (Fe^{2+} and Fe^{3+})	dipyridyl and desferal sensitivity
	Nutritional downshift	survival in Minimal Essential media
Other:	Heat tolerance	growth at 45°C
	Low osmotic tolerance	survival in water

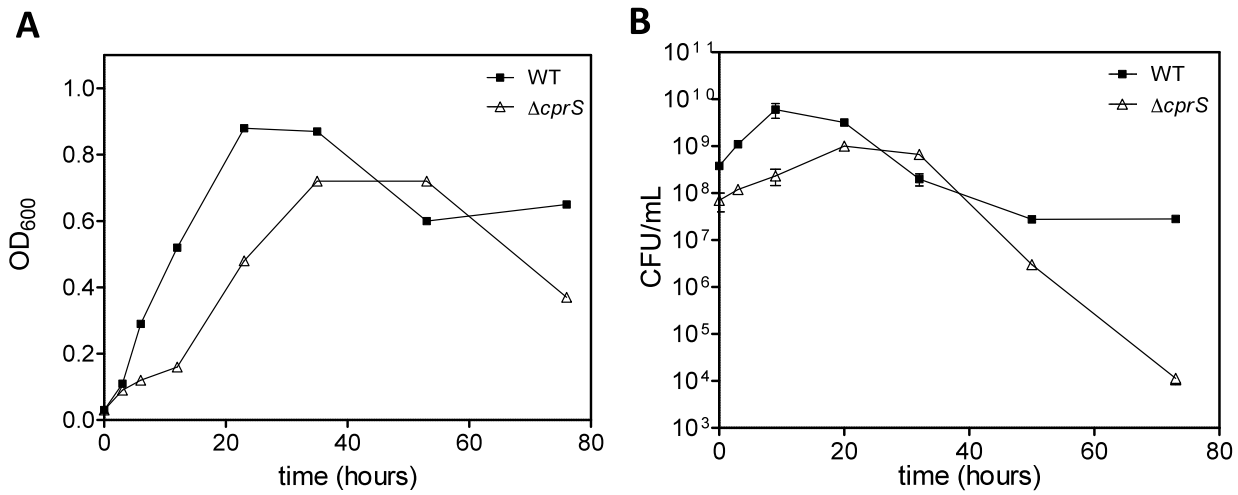


FIG. 2.2. A Δ*cprS* sensor kinase mutant displays a growth defect compared with the WT strain in rich broth culture. WT (black squares), Δ*cprS* (grey squares) and Δ*cprS*^{C} (white squares) were cultured in MH broth; at indicated time points, samples were taken to measure OD_{600} (A) or CFU (B). Error bars are present but in most cases are too small to see.

Δ*cprS* exhibits enhanced and accelerated biofilm formation. Upon closer observation of broth cultures prepared for further experiments, aggregates of bacteria were clearly visible in flasks containing Δ*cprS* cultures. A 30% increase in autoagglutination for Δ*cprS* compared with WT (data not shown), and CV staining suggested that Δ*cprS* was adhering to shaking culture tubes (**FIG. 2.3A**). This raised the possibility that the apparent growth defect shown in **FIG. 2.2** 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 was that Δ*cprS* may have favoured surface-attached biofilm growth, rather planktonic growth. Indeed, standing culture biofilm CV assays [368, 491] clearly demonstrated that Δ*cprS* exhibited a visible enhancement of biofilm formation compared with WT after only 1 day of incubation (**FIG. 2.3B**, left). Not surprisingly, when adhered CV was dissolved and quantified, a statistically significant ($p<0.005$) difference for Δ*cprS* compared with the WT strain was seen (**FIG. 2.3B**, right). The complemented Δ*cprS*^{C} strain exhibited biofilm formation at levels comparable to WT, supporting the hypothesis that deletion of *cprS* may have affected aspects of *C. jejuni* physiology related to biofilm formation and/or planktonic growth.

Microscopy was performed to confirm that the CV-stained material represented healthy *C. jejuni* biofilms present at the air–liquid interface. Bright field microscopy and CV staining (10×–100× magnifications) clearly identified microcolony structures, suggesting active biofilm development rather than non-specific aggregation and adherence to the surface or acellular material (**Fig. 2.3C**). Biofilm formation appeared to be both accelerated and enhanced for Δ*cprS* 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 VBNC state at later growth stages, which is often defined by a switch from helical to coccoid physiology. As the Δ*cprS* mutant appeared to display a late-stage

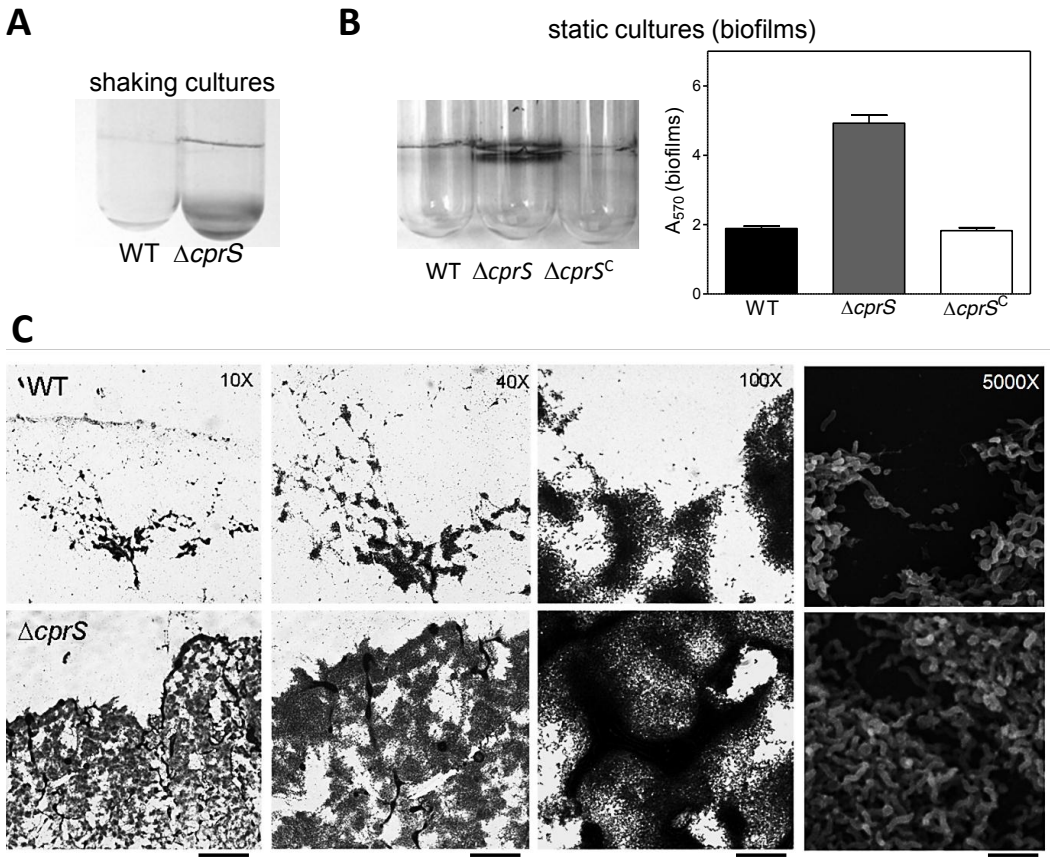


FIG. 2.3. Δ*cprS* exhibits biofilm formation compared to WT. **A)** Δ*cprS* adheres to shaking broth culture tubes. WT and Δ*cprS* were grown overnight with shaking in MH broth, and adherent bacteria were stained with 0.2% CV. **B)** Δ*cprS* shows enhanced biofilm formation. Overnight cultures of WT, Δ*cprS*, and Δ*cprS*^{C} were diluted to an OD_{600} of 0.002 in MH, added to borosilicate glass tubes and incubated without shaking for 2 days. Biofilms were stained by addition of CV 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 CV 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 compared with WT and are composed of helical-shaped bacteria. Biofilms were grown on glass coverslips in tubes prepared as in **FIG. 2.4**, and the region at the air–liquid interface was either stained with CV for bright field microscopy (10×, 40×, 100×; first three panels) or prepared for SEM (5,000×; 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.

culturability defect (**Fig. 2.2B**), the morphology of bacteria within biofilms was observed to solidify the conclusion that the growth 'defect' in liquid culture may at least in part be attributed to increased sessile growth rather than solely to loss of culturability. SEM (5000× magnification) confirmed helical morphology for both WT and Δ*cprS* (**Fig. 2.3C**, far right panels).

Accelerated and enhanced Δ*cprS* biofilms form independently of changes in surface carbohydrates; *C. jejuni* biofilms are DNase I-sensitive and contain fibres that react with DNA stains. Further analyses were performed to explore the molecular mechanisms underlying the enhanced biofilm phenotype of Δ*cprS*. As the importance of surface carbohydrates in *C. jejuni* biofilms has been demonstrated [368, 378], it

was hypothesized that changes in such polysaccharides may have been occurring in Δ*cprS*. Total carbohydrates were extracted from both WT and Δ*cprS* for separation by SDS- PAGE, followed by silver

staining to visualize LOS and Western blotting with Penner antiserum to compare levels of 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. 2.4A**). A *C. jejuni* Δ*spoT* mutant forms enhanced biofilms, commensurate with upregulation of a CFW-reactive polysaccharide [368]. However, CFW reactivity for Δ*cprS* was nearly identical to the WT strain (**FIG. 2.4B**). 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 carbohydrates.

Microscopy of CV-stained biofilms consistently suggested that the bacteria and microcolonies were connected by fibrous-like structures as exemplified in **FIG. 2.3C** (WT panel, 40× magnification). Closer examination showed the presence of fibres connecting bacteria and microcolonies (**FIG. 2.4C**, left). These fibres were seen under non-fixed conditions, and also stained with propidium iodide (PI) (**FIG. 2.4C**, right). Shown are Δ*cprS* samples; similar fibres were also observed for WT biofilms. As both CV and PI can stain DNA, it appeared 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. DNase I treatment resulted in a decrease in the amount of stained 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 evidence for the presence of DNA within the matrix surrounding *C. jejuni* biofilms.

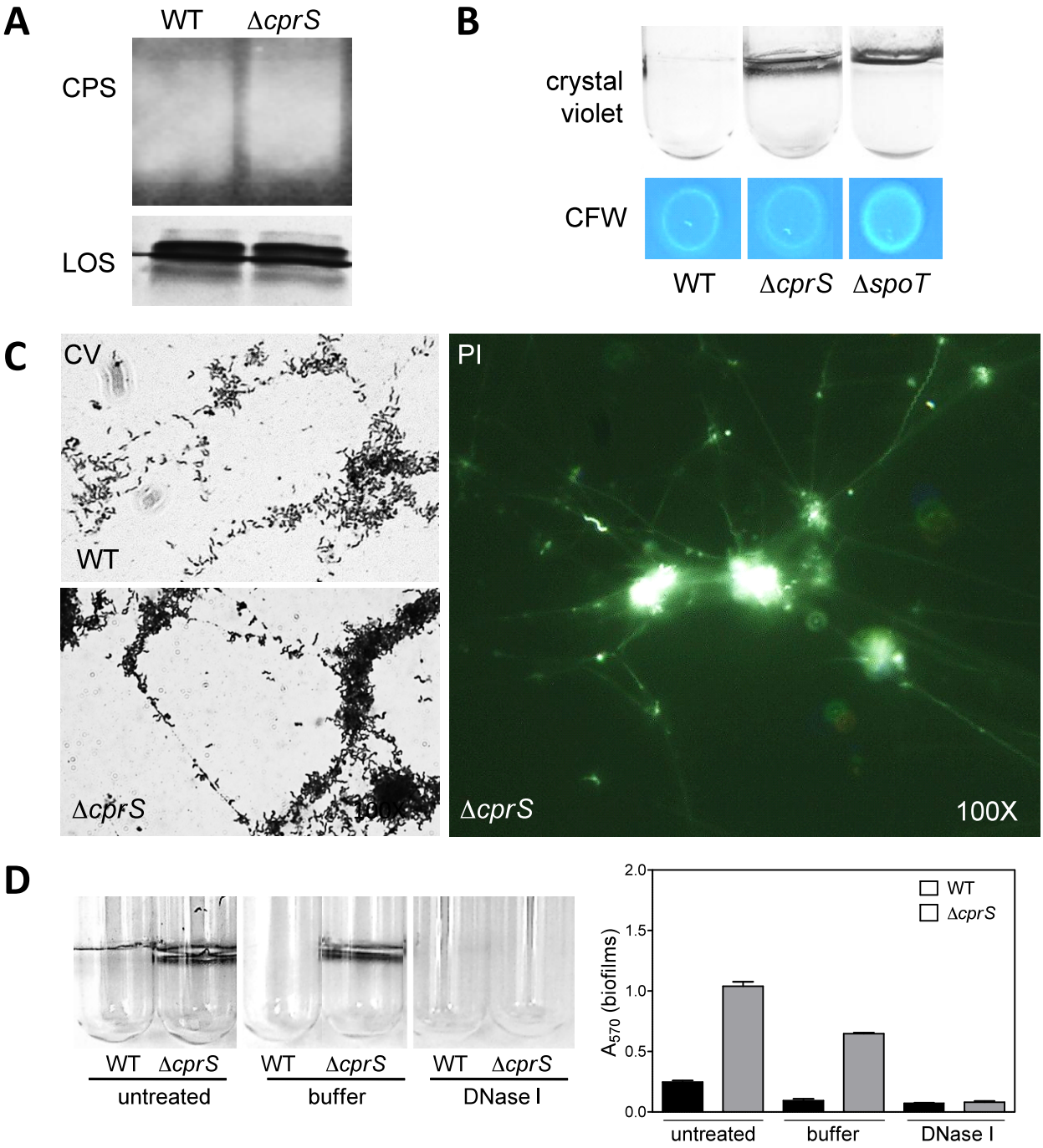


FIG. 2.4. Enhanced biofilm formation in Δ*cprS* occurs independently of changes in surface polysaccharides; the *C. jejuni* biofilm matrix contains DNA. **A)** Formation of enhanced biofilms Δ*cprS* occurs independently of changes in LOS and CPS. Total carbohydrates were extracted from plate-grown bacteria and subjected to SDS-PAGE and silver staining of LOS, or Western blot analysis of CPS with a Penner anti-O36 serotype antibody. **B)** Δ*cprS* does not overproduce a CFW-reactive surface polysaccharide. Overnight cultures of WT, Δ*cprS* and the positive control D*spoT* were spotted on BHI agar supplemented with 0.002% CFW to assess of production of carbohydrates with β(1-3) and β(1-4) linkages. CV biofilms are shown for comparison. **C)** Fibres are seen in both Δ*cprS* and WT biofilms. Biofilms were grown on glass coverslips, stained with either CV or PI and visualized by microscopy. Bars represent ~25 μm (CV) and ~20 μm (PI). **D**) *C. jejuni* biofilms can be disrupted by DNase I. WT and Δ*cprS* biofilms were either left untreated or rinsed with PBS, then incubated for 3h in buffer alone or buffer with DNase I, followed by CV staining.

Δ*cprS* shows modestly increased intracellular survival but is defective for colonization of one-day-old chicks. Because of initial identification of the genes encoding the CprRS TCRS in a cell infection screen, the role of CprS in host-related phenotypes was explored. First, invasion and intracellular survival of Δ*cprS* in human epithelial cells was assessed. INT407 cell monolayers were infected with WT, Δ*cprS* or Δ*cprS*^{C} bacteria. There was no significant difference in the number of bacteria recovered at the invasion time point following 3h of infection and 2h of gentamicin treatment (**FIG. 2.5A**). However, a surprising and reproducible two- to fivefold increase in the number of bacteria surviving an additional 5h 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 water and syringe lysis procedures; likewise, no differences were observed for bacterial survival in media above cells during the infection period, or for adherence (data not shown).

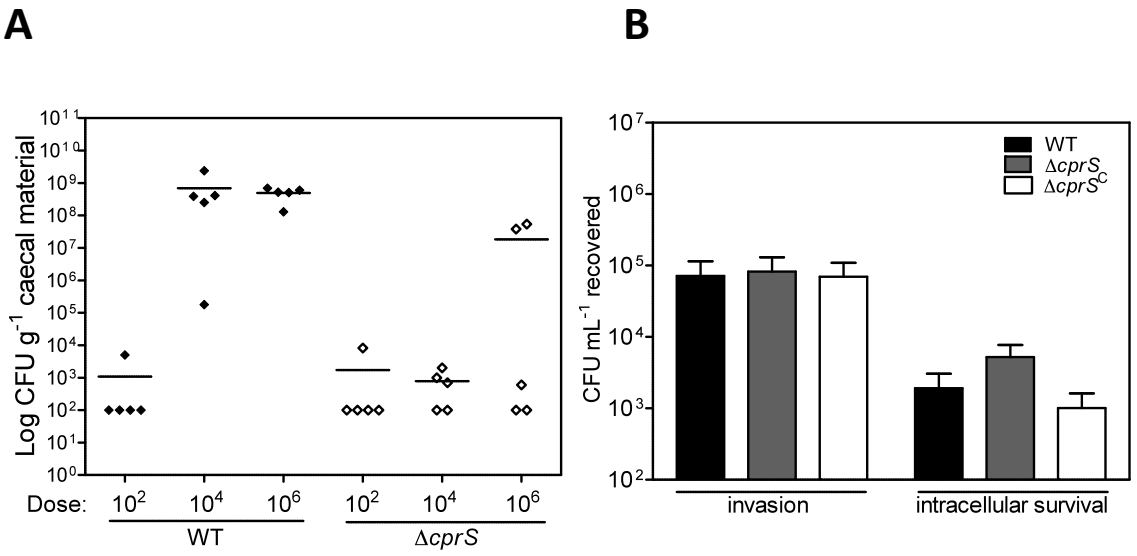
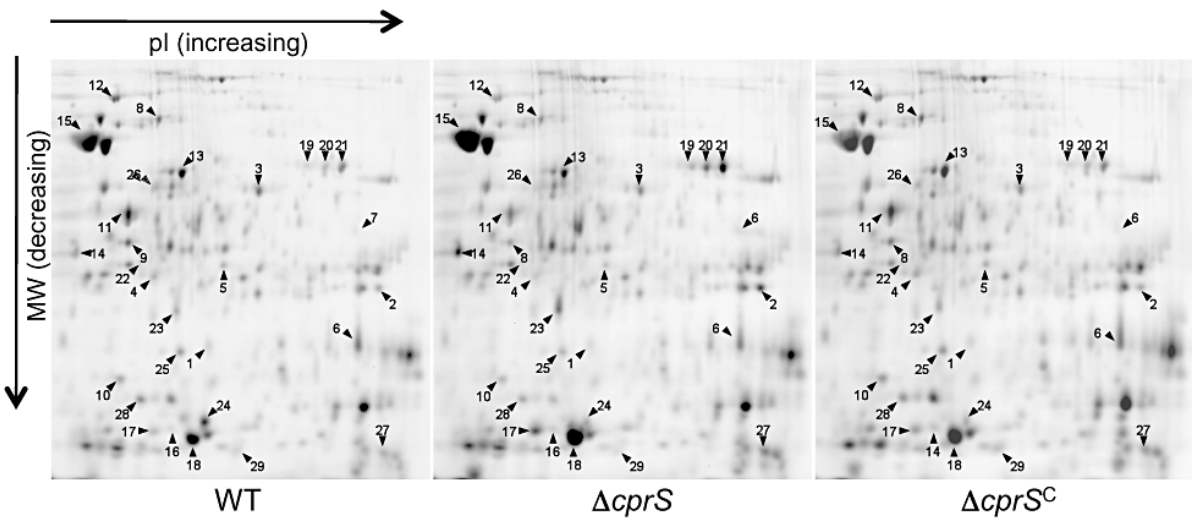


FIG. 2.5. The Δ*cprS* mutant shows differences from WT for pathogenesis-related phenotypes. **A**) The Δ*cprS* mutant invades cells at levels comparable to WT but exhibits a modest increase in intracellular survival. WT, Δ*cprS* and Δ*cprS*^{C} were grown biphasically and used to infect INT407 monolayers at an MOI of ~100. Following 3h of infection and 2h of gentamicin treatment, cells were lysed and serial dilutions were plated to determine intracellular bacteria (invasion). Intracellular survival was determined after an additional 5h of incubation. **B**) Δ*cprS* displays a dose-dependent chick colonization defect. One-day-old chicks were orally challenged with the indicated number of CFU of WT or Δ*cprS*. 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.in the number of bacteria surviving an additional 5h 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 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.

Next, the ability of Δ*cprS* to colonize one-day-old chicks was tested (**FIG. 2.5B**). 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 at high levels. Both WT and Δ*cprS* colonized equally well at doses above 10^6 CFU (data not shown). These data suggest that CprS is required for optimal colonization of chicks at lower doses, and that this defect could be partially overcome when chicks were administered higher doses of bacteria.

Proteomics identifies specific protein expression differences between WT and Δ*cprS* strains. To determine expression differences that may underlie the biofilm and chick colonization phenotypes of Δ*cprS* the global protein expression profiles of WT, Δ*cprS* and Δ*cprS*^{C} was compared. Two-dimensional gel electrophoresis and mass spectrometry revealed that numerous proteins varied significantly in expression between WT and Δ*cprS*, most of which are reported to be related to stress tolerance, cell surface structures, regulation, and metabolic pathways (**FIG. 2.6**). 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 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 MOMP (encoded by *porA*) and the flagellar filament protein FlaA were upregulated in Δ*cprS* compared to 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* 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 responseregulator, as well as the AI-2 synthase, LuxS. Finally, two unknown proteins encoded by Cj0706 and Cj0092 were also dysregulated in Δ*cprS*. The diversity of dysregulated proteins observed 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*.

A



B

Class	Spot	Protein /ORF	Putative function	Fold change Δ*cprS* vs.WT	Fold change 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	7	HisS	histidyl-tRNA synthetase	1.96	-1.91
synthesis	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	16/17/18	AhpC	antioxidant, AhpC/Tsa family	1.75/3.31/2.29	-1.07/-2.19/-1.93
response	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	CosR	two-component response regulator	-2.04	1.53
	29	LuxS	autoinducer-2 synthase	-1.65	2.13

FIG. 2.6. 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 as described in Experimental procedures. **A)** Two-dimensional SDS-PAGE analysis of WT, Δ*cprS* and Δ*cprS*^{C}. **B)** Proteins showing significant increases or decreases in expression in Δ*cprS* compared with WT.

Consistent with several proteomics observations, Δ*cprS* exhibits enhanced motility, protein secretion, and osmotic and oxidative stress susceptibilities. To explore connections between the proteomics data and Δ*cprS* phenotypes, follow-up experiments were performed. Because higher FlaA expression was seen in Δ*cprS*, this strain was assessed for motility. Microscopy suggested that Δ*cprS* was highly motile, and agar stab assays likewise showed an increase in motility in this strain (**FIG. 2.7A**). Because the flagellar apparatus has been proposed to function in protein secretion in *C. jejuni* [121], the subcellular protein localization or secreted protein profiles of Δ*cprS* was also compared to WT. Interestingly, while subcellular fractionation did not reveal notable differences in distribution of cytoplasmic proteins, an increase in several proteins in the media of Δ*cprS* compared to WT (**FIG. 2.7B**).

Initial 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 (**TABLE 2.1**). 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. 2.7C**), 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. 2.7C**). These data indicate that Δ*cprS* was 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). The Δ*cprS* mutant was also observed to be defective for forming colonies on plates containing sucrose, suggesting a general osmotolerance defect. After noting changes in expression of oxidative stress proteins, survival of Δ*cprS* in broth culture supplemented with the oxidative stress agent *t*-butylhydroperoxide was also tested. A small but reproducible increase in sensitivity ($p<0.05$) in the Δ*cprS* mutant compared with WT (**FIG. 2.7D**). A slight increase in sensitivity to both H_2O_2 and paraquat has also been seen (data not shown).

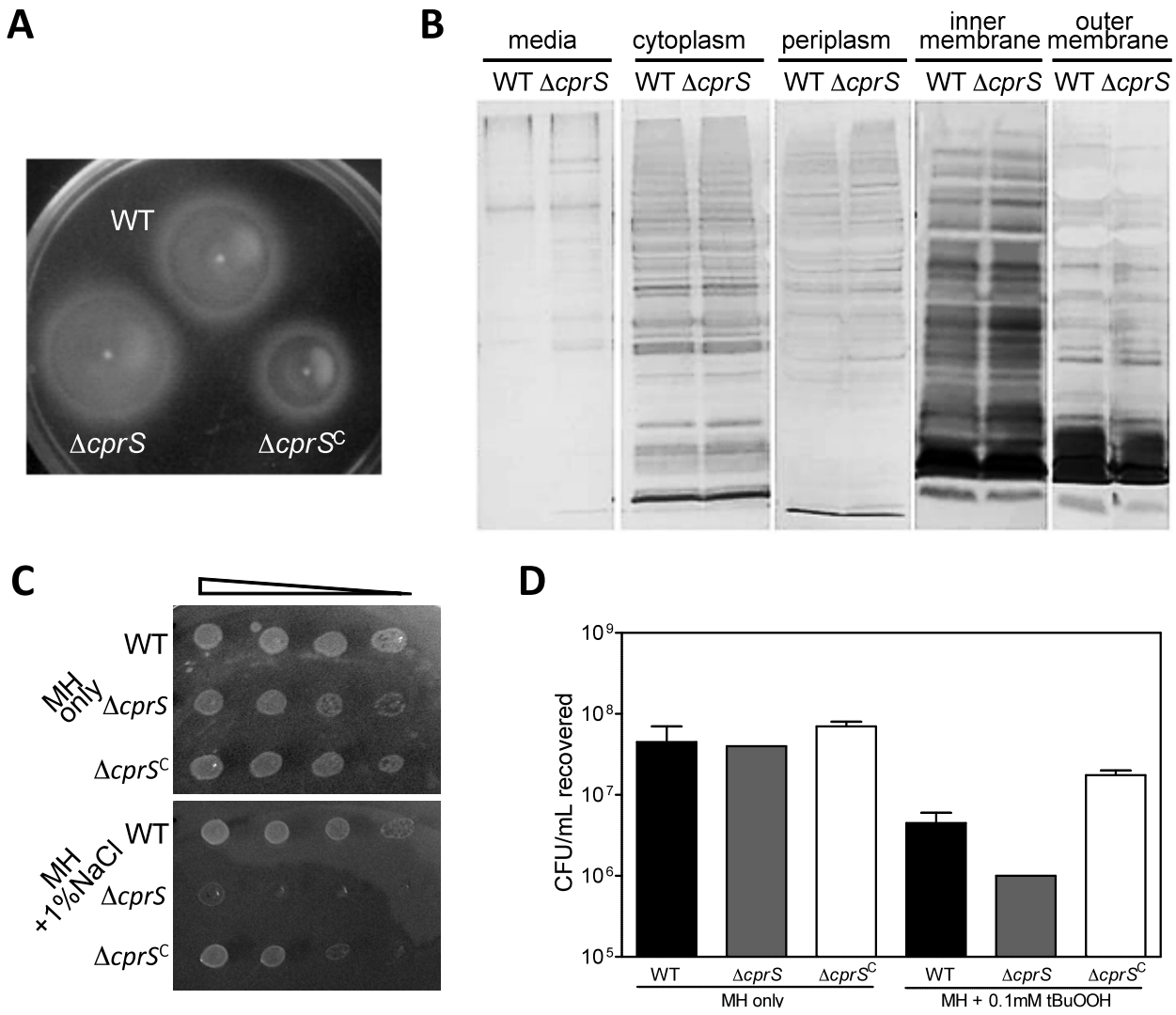


FIG. 2.7. Proteomics follow-up studies reveal enhanced motility, protein secretion, and osmotic and oxidative sensitivity differences between WT and Δ*cprS*. **A)** Δ*cprS* shows an increase in motility compared with WT. Overnight cultures of WT, Δ*cprS* and Δ*cprS*^{C} were stabbed into MH plates with 0.4% agar and incubated microaerobically for 24h. **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 OD_{600} 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 24h 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 (*t*BuOOH). Following 30 min. of incubation at 37°C microaerobically, samples were serially diluted and plated for CFU counts.

Biofilm formation in *C. jejuni* is suppressed by fumarate and promoted by deoxycholate. In *P. aeruginosa*, the availability of carbon sources such as glucose, succinate and glutamate directs the maturation of biofilms [492]. Because metabolic changes in the Δ*cprS* hyperbiofilm-forming strain were observed by proteomics, biofilm formation in media supplemented with different metabolic substrates was tested (**FIG. 2.8A**). 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 biofilms (data not shown).

Finally, because of the expression changes in surface proteins observed in Δ*cprS*, the effect of various detergents on biofilm formation was tested (**FIG. 2.8B**). Biofilms were grown in the presence of sub-MIC levels of DOC, Tx100, Tw20 and 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). PI staining of WT (**FIG. 2.8C**) 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. 2.4C**. No fibres were observed by PI 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. 2.8D**; $p<0.005$ for DNase I-treated versus buffer-treated biofilms), with A_{570} values for all samples similar to those shown in **FIG. 2.4D** 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.

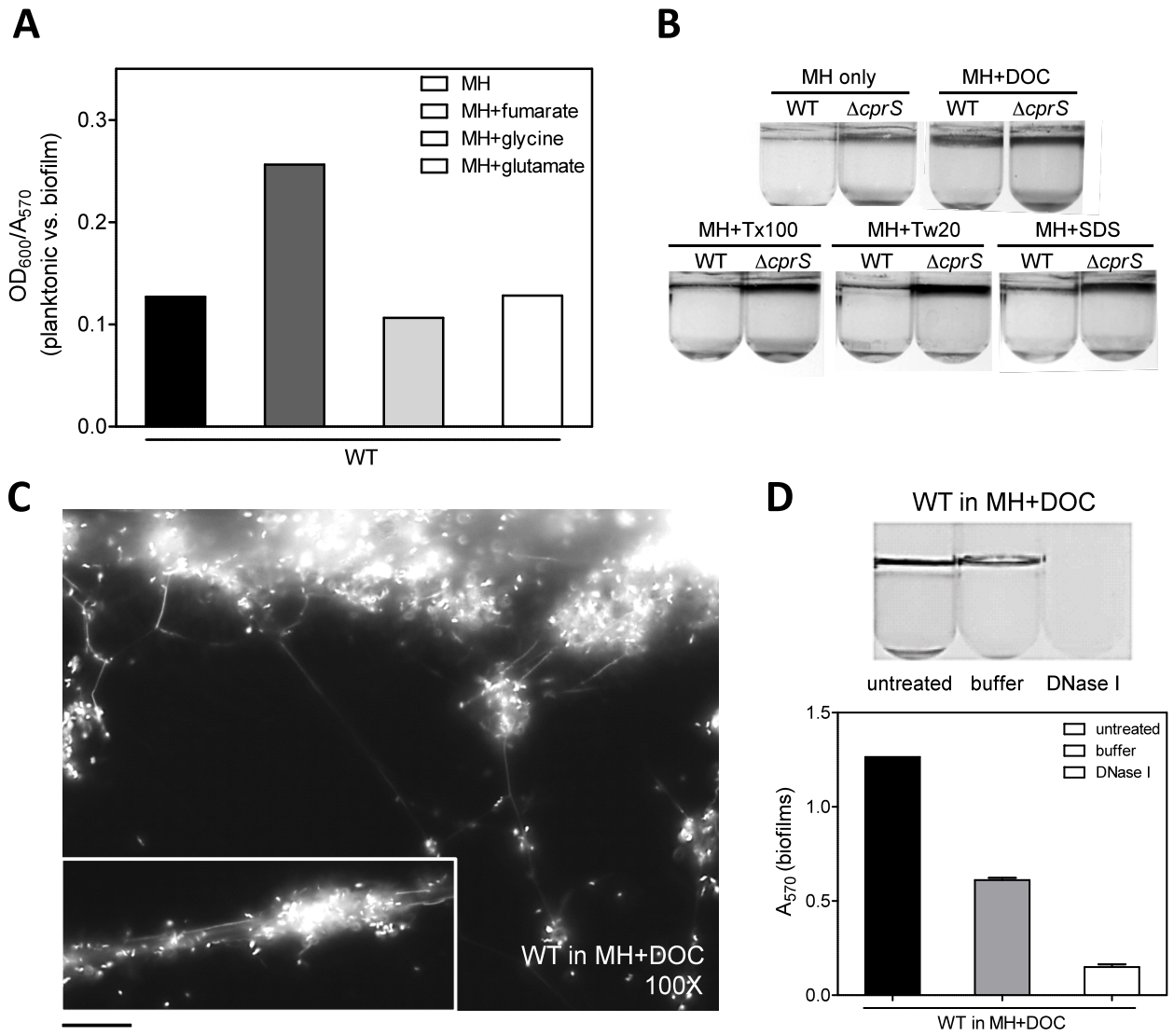


FIG. 2.8. *C. jejuni* biofilm formation is inhibited by fumarate and enhanced by deoxycholate. **A)** Fumarate promotes planktonic growth of *C. jejuni*. Biofilms were grown in MH broth or MH broth supplemented with 50 mM fumarate, glycine or glutamine. After 2 days of microaerobic incubation, samples were carefully removed from the media (planktonic) fraction to measure OD_{600}, and biofilms were then quantified by CV 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 (Tx100), 0.0005%; Tween-20 (Tw20), 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 PI, 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 3h in buffer alone or buffer with DNase I, followed by CV staining.

2.4 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. Characterization of the *C. jejuni* CprS sensor kinase has been undertaken, a component of a TCRS 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*. Preliminary genetic analyses indicated that the activity of the CprR response regulator, but not the CprS sensor kinase, was essential for viability of *C. jejuni* in the laboratory. It is possible that CprR mutants enter into a VBNC state and are not recoverable by colony isolation except under specific unknown conditions. Nonetheless, it was intriguing that only the response regulator appeared 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, unpublished observations), the CTD of CprS interacted specifically with CprR by two-hybrid analysis. Based on work in other systems [490] this interaction strongly supports a functional relationship between the two components.

The essential nature of CprR suggested that this TCRS might control aspects of physiology central to the biology of *C. jejuni*, similar to the *Caulobacter* response regulator CtrA [448] or the YycF response regulator of *B. subtilis* [493]. Consistent with control of essential processes, apparent growth defects were observed for Δ*cprS* in rich broth. Behaviour of Δ*cprS* also suggested that this TCRS may influence global changes such as the biofilm-planktonic switch. The essential nature of only the response regulator was 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 sensor kinase [444]. This may be especially significant for a TCRS (such as CprRS) that regulates genes required for viability, where non-cognate sensor kinase(s) may affect phosphotransfer to the response regulator when the native cognate sensor kinase is absent. Furthermore, AcP can also phosphorylate response regulators in *E. coli* [494], and in *C. jejuni*, this may allow basal levels of response regulator phosphorylation that permit viability. Alternatively, phosphotransfer may not be required for CprR to modulate essential genes, similar to the *H. pylori* essential response regulators HP1021 and HP1043 [477]. *H. pylori* also encodes a TCRS, ArsRS, where like CprRS, only the response regulator is essential for growth [469]. In this system, there are two types of response regulator-controlled promoters – one recognized by ArsR (which presumably regulates essential genes) and a second, lower-affinity class of promoters bound by phospho-ArsR (which activates dispensable genes) only when environmental conditions stimulate phosphorylation. In a bacterium with limited regulatory capacity such as *C. jejuni*, this scenario is especially attractive, as it would allow the response regulator to control two separate regulons.

There is debate as to whether biofilms represent the default lifestyle for bacteria, or whether planktonic growth is simply an artifact of laboratory culture. The hyperbiofilm phenotype of Δ*cprS* suggests that this

phenomenon may be a regulated process in *C. jejuni*, and this work suggests that CprS may play a role in this regulation. Numerous TCRSs have been implicated in regulation of biofilm formation in other pathogens through control of phenomena such as adhesin expression [490], EPS production [495] and quorum sensing [496]. Direct involvement of a TCRS in *C. jejuni* biofilms is supported by observations of defective biofilm formation upon inactivation of Cj0688, encoding a phosphate acetyltransferase [369] which may modulate AcP levels and response regulator phosphorylation. Furthermore, the response regulator CbrR contains a putative GGDEF domain [479] which may be and may mediate formation of c-di-GMP, a mediator of biofilm formation [497]. Finally, the CosR response regulator was also downregulated in Δ*cprS*. Like CprR, CosR is also essential [266, 479]; however, downregulation of this protein in Δ*cprS* was in apparent contrast to work which noted upregulation in biofilms [378].

Mechanistic insight into *C. jejuni* biofilm formation is relatively limited, and it was 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 [380]. While *H. pylori* secretes an EPS during biofilm growth [381] and other enteric pathogens such as *Salmonella* and *E. coli* also utilize carbohydrates extensively [498], the matrix surrounding *C. jejuni* biofilms remains ill-defined. A Δ*spoT* stringent response mutant appears to overexpress a CFW-reactive polysaccharide, production of which correlates with biofilm formation [368]. Analyses of LOS profile, CPS production, and CFW reactivity of Δ*cprS* revealed no differences from WT, suggesting that the hyperbiofilm phenotype of Δ*cprS* was independent of changes in expression of previously characterized surface carbohydrates. Furthermore, analysis of the *C. jejuni* genome did not identify homologues of biofilm-associated proteins such as Bap or Esp [499, 500].

Other than proteins and carbohydrates, the matrix of other bacteria often contains eDNA [501]. Fibres were observed extending between microcolonies in *C. jejuni* biofilms, and these structures stained with CV or PI, both of which stain DNA. The presence of DNA in the *C. jejuni* biofilm matrix was further supported by observations that biofilms formed by both WT and Δ*cprS* could be visibly disrupted by treatment with DNase I, and that enhanced WT biofilms formed in the presence of DOC likewise exhibited DNase I sensitivity. Interestingly, strain 81-176 forms more robust biofilms than strain 11168 (S. Svensson and E. Gaynor, unpublished observations) and also harbours a plasmid (pVIR) containing components of a putative Type IV secretion system [56]. It may be interesting, in future work, to explore a possible involvement of this plasmid in release of eDNA.

Because interest in CprRS was initially piqued by its identification in a cell infection screen, it was hypothesized that CprRS may control phenotypes 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. Furthermore, higher numbers of Δ*cprS* than WT were recovered from INT407 cells following several hours

of intracellular survival. It remains to be seen whether this was due to increased bacterial or epithelial cell survival, either of which could result in enhanced recovery of intracellular bacteria. In contrast to *in vitro* infection data, the Δ*cprS* mutant was significantly impaired for chick colonization, similar to mutants in several other *C. jejuni* TCRS [470, 471, 473, 474, 479]. Inactivation of *ppk1* in *C. jejuni* also results in a colonization defect that, like Δ*cprS*, is rescued by increasing the inoculating dose [229]. 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 [229], and similar mechanisms may exist in Δ*cprS*. Consistent with a role for biofilms *in vivo*, 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 [502]. 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 dysregulation of essential genes.

A global proteomics-based approach was undertaken to identify protein expression changes that may explain the diverse phenotypes observed for Δ*cprS*. This identified approximately 20 differentially expressed proteins in the Δ*cprS* mutant compared with WT, with the majority of these proteins present at WT levels in Δ*cprS*^{C}. Differences in expression of proteins involved in several distinct aspects of physiology were detected, 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 [187]. Analysis of agar-grown (sessile) *C. jejuni* also identified succinyl-CoA synthetase and trigger factor as upregulated [385], and increased expression of TrxB [187], AhpC and FlaA in Δ*cprS* was also consistent with proteomics analyses of WT *C. jejuni* biofilms [378]. MOMP and FlaA also serve as adhesins in *C. jejuni* and therefore have relevance to biofilm formation [503], and previous work suggests that biofilm-residing *C. jejuni* retain their flagella [378]. Downregulation of LuxS in Δ*cprS* was surprising. While a role for AI-2 in quorum sensing in *C. jejuni* biofilm formation has not been demonstrated, it has been reported that *C. jejuni luxS* mutants exhibit decreased motility, autoagglutination [428] and biofilm formation [420]. Expression profiling thus supports an AI-2-independent mechanism for the biofilm phenotype of Δ*cprS*.

Altered expression of metabolic proteins in Δ*cprS* was 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 response regulator, and proteins representing of a number of metabolic pathways such as glycolysis (Fba), the citric acid 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* compared to WT. RT-qPCR data suggest that expression of *dcuA*, encoding a likely C4-dicarboxylate transporter, has also been observed to be reduced 10-

fold in Δ*cprS* (S. Svensson and E. Gaynor, unpublished observations). Some of these expression differences in metabolic genes and proteins might reflect shifts that occur when *C. jejuni* enters a biofilm. Likewise, metabolic alterations may help explain the modest increase in recovery of Δ*cprS* from within INT407 cells. Metabolic adaptation of *C. jejuni* appears to occur within the nutrient-poor intracellular environment [226, 229, 231]. Consistent with this, microarray analyses suggest that *C. jejuni* undergoes global expression changes in numerous metabolic genes during cell infection (E. Gaynor, unpublished observations), Finally, distinct metabolic pathways are initiated within the avian gut [72]; thus metabolic changes in Δ*cprS* may also account for decreased chick colonization at lower doses.

Importantly, proteomics analysis also led to performance of additional experiments which provided further insight not only into the colonization and stress-related 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 to the hypothesis that outer membrane permeability of Δ*cprS* may be altered, consistent with subsequent observations that Δ*cprS* exhibited a striking osmotolerance defect. A slight increase in sensitivity to oxidative stress agents such as *t*-butylhydroperoxide was also observed. This appeared to contrast reports that *C. jejuni* residing in biofilms are more resistant to oxidative stress than their planktonic counterparts [350], as well as 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 in Δ*cprS* may have been the result of a general stress response to dysregulation of essential genes which still results in stress sensitivities. 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 expressed at higher levels in the mutant, which was supported by observation of a modest increase in motility in Δ*cprS* compared with WT. As noted, the flagellar filament is important *for C. jejuni* biofilm formation, and while a role for motility *per se* (i.e. via analysis of a filament-positive, motility-minus mutant) in biofilms has not yet been shown for *C. jejuni*, it is not unreasonable to predict, based on work in other bacteria, that motility will prove important for biofilm formation in this organism as well. The increased expression of FlaA observed in Δ*cprS* was also consistent with 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 proteins is dependent on a functional flagellar apparatus [121]. However, there were dissimilarities between the pattern of media versus periplasmic or cytoplasmic proteins, suggesting some specificity in the proteins present in the Δ*cprS* media. Identification of these proteins will allow exploration of the connection between this phenotype and those involving biofilms and host-related properties. Interestingly, Cia protein expression is stimulated by 0.05% DOC [299]. 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* [504]. Thus, in addition to the DNA component mentioned above, it is possible that the synthesis and/or secretion of

proteins that were present in the media fraction of Δ*cprS* (that contribute to biofilm formation) were also induced by DOC. This would account at least in part for the increased biofilm formation of WT *C. jejuni* that was observed in the presence of sub-MIC levels of DOC. However, in addition to induction of transcription by compounds such as DOC, Cia protein secretion also requires a stimulatory signal, such as host cell components [123]. As it is unknown whether such signals may be present during biofilm growth; thus, it also is unknown whether Cia secretion contributes to enhanced biofilm formation in DOC. At present, the possibility that the appearance of proteins in the Δ*cprS* media fraction may represent loss of membrane integrity or increased autolysis cannot be ruled out.

The observation that numerous metabolic genes were altered in Δ*cprS* led to the hypothesis 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 was similar to observations in other bacteria where nutrient availability can trigger biofilm dispersal [505]. For example, control of swarming motility and biofilm architecture by quorum sensing in *P. aeruginosa* is nutritionally conditional [492]. Likewise, in *Shewanella*, different electron acceptors (i.e. nitrate versus fumarate) result in changes in carbohydrate exopolymer production and surface-associated behaviour [506]. In *C. jejuni*, biofilm formation also appears to be promoted by differences in nutrient availability [420], and it is conceivable that CprRS may allow response to nutrient availability cues by inducing and/or repressing genes related to biofilm formation.

In summary, genetic analysis of a TCRS in *C. jejuni*, within the confines of an essential response regulator, 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 *Campylobacter* spp. suggests that CprRS controls phenomena specific to these bacteria, many of which cause significant human and animal disease (i.e. *C. coli*, *C. upsaliensis*, *C. fetus*, and *A. butzleri*). Absence of CprRS from *H. pylori*, essentially host-restricted, was consistent with control of phenotypes relating to passage between hosts and/or phenotypes relating specifically to adaptation to an avian host. Identification of factors contributing to survival of pathogens such as *C. jejuni,* that appear to use different virulence paradigms from model pathogens, may be important. 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 [507], 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 may be pleiotropic and likely controls many phenomena in *C. jejuni*, providing intriguing hypotheses for further testing.

3 THE CPRRS TWO-COMPONENT REGULATORY SYSTEM OF *CAMPYLOBACTER JEJUNI* REGULATES ASPECTS OF THE CELL ENVELOPE

3.1 Introduction and synopsis

A two-component regulatory system, CprRS, was previously identified which controls essential biological processes, stress tolerance phenotypes, and phenomena related to biofilm formation in *C. jejuni*, thereby contributing to the success of this zoonotic pathogen. While previous work focused on phenotypic characterization of a biofilm-enhanced Δ*cprS* sensor kinase mutant, this work further characterized CprRS through expansion of genetic and molecular analyses of the CprR response regulator, in-depth expression profiling, and more extensive physiological characterization of *cprRS* strains. The essential nature of CprR necessitated more elaborate genetic and molecular characterization. This included construction of strains expressing point mutant (Asp52Ala, Asp52Glu), siRNA (small interfering RNA) knockdown (KD), and dominant negative forms of *cprR*, as well as one-hybrid identification of the CprR-binding DNA consensus sequence. Analysis suggested that not only was *cprR* essential for viability of *C. jejuni*, but phosphorylation of CprR was also required. CprR appeared to share a consensus sequence with the related response regulator RacR, and binding to this sequence requires phosphorylation. The *cprRS* promoter was also identified, and was expressed most highly during log phase, and found that CprRS exhibits autoregulation. In general, genetic manipulations that would be expected to negatively affect the level and/or activity of the CprR response regulator resulted in phenotypes shown by Δ*cprS*, such as enhanced biofilm formation and osmotolerance defect. Microarray analysis of the Δ*cprS* sensor kinase mutant identified putative members of the CprRS regulon Expression changes in Δ*cprS* suggested that CprRS may directly control genes involved in biogenesis and/or maintenance of the cell envelope, including the adjacent *htrA* gene, encoding a periplasmic serine protease. Microarray analysis also identified genes that were dysregulated, possibly indirectly, in Δ*cprS* that suggested this strain may be exiting log phase before WT. Moreover, further phenotypic analysis of Δ*cprS* and various *cprR* mutants were consistent with observations of dysregulation of envelope proteins. Both Δ*cprS* and $cprR^{OE}$ strains showed altered morphology and PG differences from WT. Finally, evidence was obtained the suggested that envelope stress and biofilm formation may be related in *C. jejuni*.

3.1.1 Information about collaborators. Microarray experiments were performed by Sarah Svensson in the laboratory of Dr. Craig Parker (USDA Western Research Centre, Albany, CA). PG analysis was performed by Jacob Biboy and Dr. Waldemar Vollmer (The Centre for Bacterial Cell Biology, Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne, UK).

3.2 Materials and Methods

3.2.1 Routine and experimental bacterial culture.

Bacterial strains and routine culture conditions. As in **CHAPTER 2**, studies were performed using the *C. jejuni* WT strain 81-176 [28]. Strains are listed in **TABLE B.1**. *C. jejuni* was routinely cultured under microaerobic conditions using MH broth or agar. Where appropriate, the antibiotics (Sigma) Kan, Cm, and Str (streptomycin) were added to a final concentration of 40 μg mL^{-1}, 15 μg mL^{-1}, and 100 mg mL^{-1}, respectively. *E. coli* used for DNA manipulations was routinely cultured in LB supplemented with the following antibiotics, where appropriate: Amp, 100 μg mL^{-1}; Kan, 25 μg mL^{-1}; and Cm, 25 μg mL^{-1}.

Growth curve analysis in broth culture and growth of cultures for microarray analysis. For standard growth curve analysis, *C. jejuni* strains were grown biphasically 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 rpm. Growth and viability were assessed at various times post dilution by measuring the OD_{600} and plating serial 10-fold dilutions on MH agar. For microarray analysis, WT, Δ*cprS*, and Δ*cprS*^{C} bacteria were grown biphasically overnight in MH broth with trimethoprim and vancomycin. Biphasic cultures of the mutant and complemented strains were grown in MH broth supplemented with 20 μg mL^{-1} Cm to maintain selection of Δ*cprS* alleles. Overnight biphasic cultures were then diluted into fresh MH broth with trimethoprim and vancomycin only at an OD_{600} of 0.05, and cultures were grown microaerobically with shaking at 200 rpm. At the indicated time points, samples were removed onto ice and processed immediately.

Biofilm assay. Biofilm formation was assessed as previously described (**CHAPTER 2**). Where indicated, chloride salts of divalent cations (Mg^{2+}, Ca^{2+}) (Sigma) were added to MH broth used for biofilm assays. For broth concentration experiments, MH broth (Oxoid) was prepared at 0.5X, 1.0X, or 1.5X concentration, according to the manufacturer's instructions. For experiments comparing biofilm formation in strains harbouring pRY112-based plasmids, all bacteria harboured a plasmid (either empty vector, or containing *cprR*) and biofilms were grown in MH broth with Cm.

***In vitro* stress tolerance and phenotyping assays.** Growth under hyperosmotic stress was assessed by measuring OD_{600} of cultures in MH broth supplemented with 150 mM NaCl (Sigma) and grown with shaking for 24h under microaerobic conditions.

3.2.2 Recombinant DNA techniques.

General recombinant DNA techniques. Recombinant DNA techniques were performed as in **CHAPTER 2**. Primers are listed in **TABLE A.1**, and DNA constructs are listed in **TABLE B.1**.

Site-directed mutagenesis of *cprR*. The *cprR* gene, including approximately 200 bp of upstream DNA harbouring the *cprR* promoter, was amplified by PCR using primers *cprR*-pRY112-FWD/REV, digested with

*Mfe*I and *Xba*I, and ligated into the conjugative vector pRY112 [482] that had been digested with *Eco*RI and *Xba*I to create plasmid pSS82. To construct mutant forms of *cprR* containing either an Asp52Ala or Asp52Glu mutation, PCR-based site directed mutagenesis was performed using the primers *cprR*^{Asp52Ala}-FWD/REV and *cprR*^{Asp52Glu}-FWD/REV, respectively. Following the mutagenesis reaction, DNA was re-subjected to PCR using primers *cprR*-pRY112-FWD/REV to amplify the point mutant form of *cprR*. The resulting product was digested with *Eco*RI and *Xba*I, and ligated into pRY112 to create plasmids pSS84 and pSS85. Point mutations were confirmed by sequencing (Genewiz, South Plainfield, NJ), and plasmids were then mobilized from *E. coli* into *C. jejuni* DRH461 (Δ*astA*, Str^R) [408] by triparental mating/conjugation with *E. coli* harbouring the pRK600 helper plasmid [508]. Plasmids were isolated from Cm^R conjugants and introduced into fresh WT or Δ*cprS* cells by natural transformation.

Construction of targeted deletion strains. Construction of the Δ*cprS* sensor kinase mutant and complemented Δ*cprS*^{C} strain was described in **CHAPTER 2**. Attempts to delete (KO) *cprR* in *C. jejuni* by double crossover homologous recombination were performed using a *C. jejuni* suicide vector containing approximately 500 bp of homologous DNA flanking the Kan^R cassette. Briefly, the *cprR* region was amplified using primers *cprR*^{KO}-FWD/REV and ligated into pGEM-T. Inverse PCR with primers *cprR*-INV-FWD/REV was then used to remove the coding region of *cprR*, and the resulting PCR product was digested with *Kpn*I and *Bam*HI and ligated to a similarly digested Kan^R cassette [481] to create plasmid pSS56. The resulting KO construct was prepared from *E. coli* using the Invitrogen midiprep kit and naturally transformed into WT *C. jejuni* harbouring pRY112-based plasmids (pSS82, pSS84, pSS85) containing a WT or mutated allele of *cprR* (see above). Targeted deletion of *htrA* was achieved in a similar manner. Briefly, *htrA* and ~500 bp of flanking DNA was amplified using the primers *htrA*-FWD/REV and inserted into pGEM-T. Part of the *htrA* coding region was then removed by inverse PCR using the primers *htrA*-INV-FWD/REV. This was followed by digestion with *Eco*RI and *Bam*HI and ligation to the Kan^R cassette. Construction of double mutants was achieved by natural transformation of gDNA, isolated from single mutant strains using the Wizard Genomic DNA purification Kit (Promega), into the appropriate single mutant background strain and recovery on media containing antibiotics selective for both deletion cassettes (Kan+Cm). Single mutants obtained from other laboratories (**TABLE B.1**, Δ*flhA*, Δ*flgR*, Δ*rpoN*) were reconstructed by naturally transforming gDNA from the strain of interest into WT cells and recovering colonies on the appropriate selective media. Transposon mutants (Δ*pflA*, Δ*kpsS*) were isolated and confirmed in our laboratory as previously described [103], and contain a Mariner-based transposon insertion containing a Kan^R cassette. Construction of Δ*waaF* and Δ*spoT* has been described previously [87, 231].

Construction of overexpressing or knockdown strains. Construction of strains expressing alleles of *cprS* or *cprR* from a 16S rRNA spacer was achieved using the pRRC system [483]. For construction of the *cprR*^{NTD} strain, primers *cprR*^{NTD}-FWD/REV were used to amplify a region representing amino acids 1-124 of CprR

(**FIG. 1.2**) from WT gDNA. The resulting PCR product was digested with *Xba*I and ligated into pRRC to create plasmid pSS27, which was naturally transformed into WT or Δ*cprS*, followed by selection of recombinants on media containing either Cm (transformation into WT) or Kan+ Cm (transformation into Δ*cprS*). Genotypes were then confirmed by PCR. A similar procedure was used to construct *cprS*^{CTD}, except primers *cprS*^{CTD}-FWD/REV, representing amino acids 170 to 415 of CprS (**FIG. 1.2**), were used to create the plasmid pSS55. A *peb1a* promoter overexpression plasmid was constructed by amplifying ~200 bp of the region upstream of *peb1a* using primers P$_{peb1a}$-FWD/REV, digesting the resulting product with *Apa*I and *Xho*I, and ligating it to plasmid pRY112 to create plasmid pRY112-P$_{peb1a}$. To construct a strain expressing an extra copy of *cprR* from the *peb1a* promoter ('*cprR*^{OE}'), *cprR* was amplified using primers *cprR*^{OE}-P$_{peb1a}$-FWD/REV, digested with *Pst*I and *Sma*I, and ligated into pRY112-P$_{peb1a}$. To create a *cprR* knockdown strain (*cprR*^{KD}), an antisense product specific for *cprR* was amplified with primers *cprR*^{KD}-P$_{peb1a}$-FWD/REV, digested with *Eco*RV and *Pst*I, and ligated into pRY112-P$_{peb1a}$. Plasmids were introduced into WT by conjugation as described above.

3.2.3 Microscopy.

Transmission electron microscopy. TEM was performed on cells harvested from log (10h) or stationary (24h) broth cultures. Cells were fixed with 2.5% (w/v) glutaraldehyde overnight at 4°C and stained with 2 volumes of 0.5% uranyl acetate for 1 min. Samples were added to a formvar-carbon film on 300 mesh copper grid (Canemco, Lakefield, QC), rinsed with water 10 times, dried, and visualized on a Hitachi H7600 TEM equipped with a side mount AMT Advantage (1 mega-pixel) charge-coupled device camera (Hamamatsu ORCA) at the UBC Bioimaging facility.

3.2.4 Molecular and biochemical analyses.

Bacterial one-hybrid screen. Identification of DNA sequences putatively bound by CprR was achieved using CprR^{CTD} as bait in a bacterial one-hybrid assay [509]. The region representing the C-terminal DNA-binding domain (amino acids 125-224, **FIG. 1.2**) was amplified by PCR using primers *cprR*^{CTD}-pB1H-FWD/REV, digested with *Not*I and *Avr*II, and ligated into plasmid pB1H for fusion to RNAPα (RNA polymerase alpha subunit) to create pSS83. This plasmid was introduced into *E. coli* host USO by electroporation, which was then transformed with a library of 28-mer DNA sequences in plasmid pU3H3, previously selected against constitutively active clones on fluoro-orotic acid [509]. Following recovery in LB broth and adaptation to minimal media, cells were plated on minimal media lacking histidine and containing 3-AT [509] and incubated at 37°C until colonies, putatively harbouring sequences interacting with CprR^{CTD}, appeared. Plasmids were extracted and sequenced using primer HU100 (Genewiz, South Plainfield, NJ). Twenty-five positive clones from two rounds of transformations were sequenced. Three positive clones (harbouring GTAAAT, TTAAAC, and CTAAAC sequences) were then selected and reintroduced into *E. coli*

harbouring the empty pB1H vector to demonstrate lack of growth without $CprR^{CTD}$. Motif analysis was performed using MEME (http://meme.sdsc.edu/meme4_6_1/). For analysis of binding of full-length CprR derivatives to positive library clones, pRY112 plasmids containing WT or Asp52Ala versions of *cprR* were used as templates for PCR using primers *cprR*-pRY112-FWD/REV with *Taq* polymerase and ligated to pGEM-T. Positive clones (pSS87 and pSS88, respectively) were then cotransformed along with a library clone harbouring a representative CprR consensus (GTAAAT) and tested for reporter activation on minimal media with 3-AT.

Targeted bacterial one-hybrid. For analysis of binding of $CprR^{CTD}$ to specific promoter fragments, complementary primers representing the chosen sequences were annealed, digested with *Xma*I and *Eco*RI, and ligated to a similarly digested plasmid pU3H3. The three fragments chosen represented bases -4 to -35 (primers P_{htrA}-A-FWD/REV), -33 to -64 (primers P_{htrA}-B-FWD/REV), and -65 to -97 (primers P_{htrA}-C-FWD/REV) of the region upstream of the first nucleotide of the *htrA* start codon. The resulting plasmids (pSS95, pSS96, pSS97, respectively) were cotransformed with pSS83 into BIH host strain USO, followed by assessment of reporter activity by serial dilution and spotting on LB containing Kan+Cm, or minimal media containing 3-AT to determine plating efficiency.

RNA extraction, cDNA synthesis, and RT-qPCR. For RT-qPCR (reverse transcription-quantitative PCR), RNA was extracted from broth cultures and converted to cDNA as previously described (**CHAPTER 2**). Equal volumes of cDNA were used directly in QPCR (quantitative PCR) reactions, assuming equivalent conversion in each reaction from total RNA to cDNA. QPCR was performed IQ SYBR Green Supermix and MyIQ Real-time PCR Detection System (Biorad, Mississauga, ON). Measurement of *cprR*, *htrA*, and *gyrA* cDNA levels was performed using primer sets listed in **TABLE A.1** (*cprR*-QPCR-FWD/REV; *htrA*-QPCR-FWD/REV; *gyrA*-QPCR-FWD/REV). Expression differences were calculated using the $2^{-\Delta\Delta C_T}$ method.

Construction of the microarray. DNA from ORFs was amplified with the Sigma-Genosys (The Woodlands, TX) *C. jejuni* ORFmer primer set specific for strain NCTC 11168, as described previously [510]. Additionally, unique ORFs from strain 81-176 were included using primers from Operon Technologies (Alameda, CA) designed with ArrayDesigner 2.0 (Premier Biosoft, Palo Alto, CA). All PCR products were purified with a Qiagen 8000 robot and the QIAquick 96-well Biorobot kit (Qiagen). Purified amplicons were spotted in duplicate onto Ultra-GAPS glass slides (Corning Inc., Corning, NY) using an OmniGrid Accent (GeneMachines, Ann Arbor, MI). After printing, microarrays were immediately cross-linked at 300 mJ using a Stratalinker UV Cross-linker 1800 (Stratagene, La Jolla, CA) and stored in a desiccator. Prior to use, microarrays were blocked with Pronto! prehybridization solution (Corning Inc.), used according to the manufacturer's specifications.

Microarray hybridization and analysis. Expression profiles of Δ*cprS* and $\Delta cprS^{C}$ were compared to that of the parental strain (81-176 WT) in shaking MH broth culture during a growth curve under microaerobic

conditions as described previously. Cy5-labeled test cDNA (from WT, Δ*cprS*, or Δ*cprS*^{C}) made from RNA extracted from cells at 3h, 12h, and 24h of culture was mixed with Cy3-labeled reference gDNA from strain 81-176 and hybridized to the array. Arrays were scanned using an Axon GenePix 4000B microarray laser scanner (Axon Instruments, Union City, CA). The experiment was repeated two times (biological replicate) with two technical replicate arrays and two replicate features per array for each of the time points studied. Spot and background intensity data were processed with GenePix 4.0 software, and data normalization was performed to compensate for differences in the amount of template amount or unequal Cy3 or Cy5 dye incorporation as previously described [510]. Normalized data was analyzed with GeneSpring 7.3 software (Silicon Genetics, Palo Alto, CA). A parametric statistical *t* test was used to determine the significance of the centred data at a *p* value of <0.05, adjusting the individual *p* value with the Benjamini-Hochberg false discovery rate multiple test correction in the GeneSpring analysis package. Differentially expressed genes were reported if the difference was at least twofold in WT vs. mutant (Δ*cprS*) but less than twofold in WT vs. the complemented strain (Δ*cprS*^{C}).

5'-RACE analysis. Identification of the *cprR* transcriptional start site was achieved by performing 5'-RACE (rapid amplification of cDNA ends) on RNA extracted from WT *C. jejuni* growing in log phase. Briefly, RNA and cDNA were made as described above, followed by analysis using the 5'-RACE System, Version 2.0 (Invitrogen) according the manufacturer's instructions, with primers *cprR*-GSP1, *cprR*-GSP2, and *cprR*-GSP3. Amplified bands were purified by gel extraction and sequenced (Genewiz, South Plainfield, NJ).

Luciferase reporter fusion analysis of expression. Promoter activity was measured using a promoterless *luxCDABE* operon [511] adapted for *C. jejuni* by introduction into the conjugative vector pRY112 [512]. Approximately 220 bp of the region immediately upstream of *cprR* was fused to the *lux* operon by PCR amplification of the region with primers P_{cprR}-*lux*-FWD/REV, digestion with *Not*I, and ligation into pRY112-*lux* to create plasmid pSS81. The resulting plasmid was conjugated into WT and Δ*cprS* as described above. For expression analysis, strains were grown in shaking microaerobic culture in MH+Cm, and Lux activity (light production) was measured on a Varioskan Flash plate reader (Thermo Scientific).

Measurement of bacterial cell lysis. Bacterial lysis was assessed by Western blot analysis of culture supernatants. Following growth in broth culture (10 mL), usually after 24h, a 1 mL sample of culture was harvested for analysis of total cellular protein expression. Cells from the rest of the culture were removed by centrifugation at 10,000 x *g* for 5 min. and discarded. Any cells remaining in this clarified supernatant were removed by filtration through a 0.22 μM filter. Supernatants were then concentrated approximately 10-fold from 2.5 mL to 250 μL using 3 kDa cutoff Amicon Ultra centrifugal filter units (Millipore, Billerica, MA) by centrifugation for 60 min. at 4,000 x *g*. Samples were then analyzed by SDS-PAGE and Western blotting, using an anti-CosR antibody (a gift from Dr. Stu Thompson).

Peptidoglycan isolation and muropeptide analysis. *C. jejuni* strains were passaged once from frozen stocks and then inoculated onto 20 MH plates (without antibiotics) and grown for 20h to obtain bacteria at a final OD_{600} of 200-600. Cells were collected in cold MH broth, harvested by centrifugation at 8,000 x *g* for 15 min., and then resuspended in 6 mL ice-cold H_2O. Cells were lysed by dropwise addition to 6 mL 8% SDS boiling under reflux. PG was purified from the cell lysate, digested with the muramidase cellosyl (kindly provided by Hoechst, Frankfurt, Germany), and the resulting muropeptides were reduced with sodium borohydride and separated by HPLC as previously described [513]. Muropeptide fractions were collected, concentrated in a SpeedVac, acidified with 1% trifluoroacetic acid, and analysed by offline electrospray mass spectrometry on a Finnigan LTQ-FT mass spectrometer (ThermoElectron, Bremen, Germany) at the Newcastle University Pinnacle facility as previously described [514]. Muropeptide structures were assigned based on (A) comparison with retention times of known muropeptides from *H. pylori*, *Caulobacter crescentus* and *E. coli* and (B) the obtained MS data and MS/MS fragmentation patterns (not shown).

3.3 Results

The *cprRS* operon is expressed from an autoregulated promoter directly upstream of *cprR*. It was previously shown that *cprR* was presumably transcribed separately from the upstream *htrA* gene, as transcripts containing both *htrA* and *cprR* in WT bacteria in log phase were not observed (**CHAPTER 2**). To confirm that the region upstream of *cprR* did in fact harbour an active promoter, the transcription start site for the *cprRS* operon was identified using 5'-RACE on cDNA synthesized from RNA extracted from WT bacteria during log phase. Sequencing of RACE products suggested that all transcripts started at 22 bp upstream of the *cprR* start codon (**FIG. 3.1A**). RACE results were confirmed by RNA-seq (whole transcriptome shotgun sequencing) data, which has identified separate transcription start sites at 22 bp and 53 bp upstream of *cprR* and *htrA*, respectively (G. Dugar and C. Sharma, personal communication).

Presence of an active promoter directly upstream of *cprR* was confirmed by fusing 200 bp upstream of the *cprR* start codon to a promoterless luciferase (*luxCDABE*) reporter in plasmid pRY112-*lux* (**FIG. 3.1B**). This construct produced light when introduced into *E. coli* (data not shown), as well as following conjugation into WT *C. jejuni*, suggesting the presence of an active promoter in this region. Light production by WT bacteria harbouring P_{cprR}-pRY112-*lux* increased over the course of a growth curve in rich media, with a peak in log phase (approximately 10h of growth, at an OD_{600} of 0.250) followed by a rapid decrease in Lux activity. The peak in Lux activity appeared to occur approximately 5h before the peak in OD_{600}. When reporter activity was assessed in the Δ*cprS* mutant, very little light production was observed at all points of the growth curve. Of note, bacteria harbouring the empty pRY112-*lux* plasmid had zero light production (data not shown), whereas background levels of light production were observed from the P_{cprR}-*lux* fusion in the Δ*cprS* mutant, suggesting that transcription from this promoter occurs at basal, rather than zero, levels in the absence of activation. No difference in light production from a reporter containing the promoter of Cj1500 (a putative

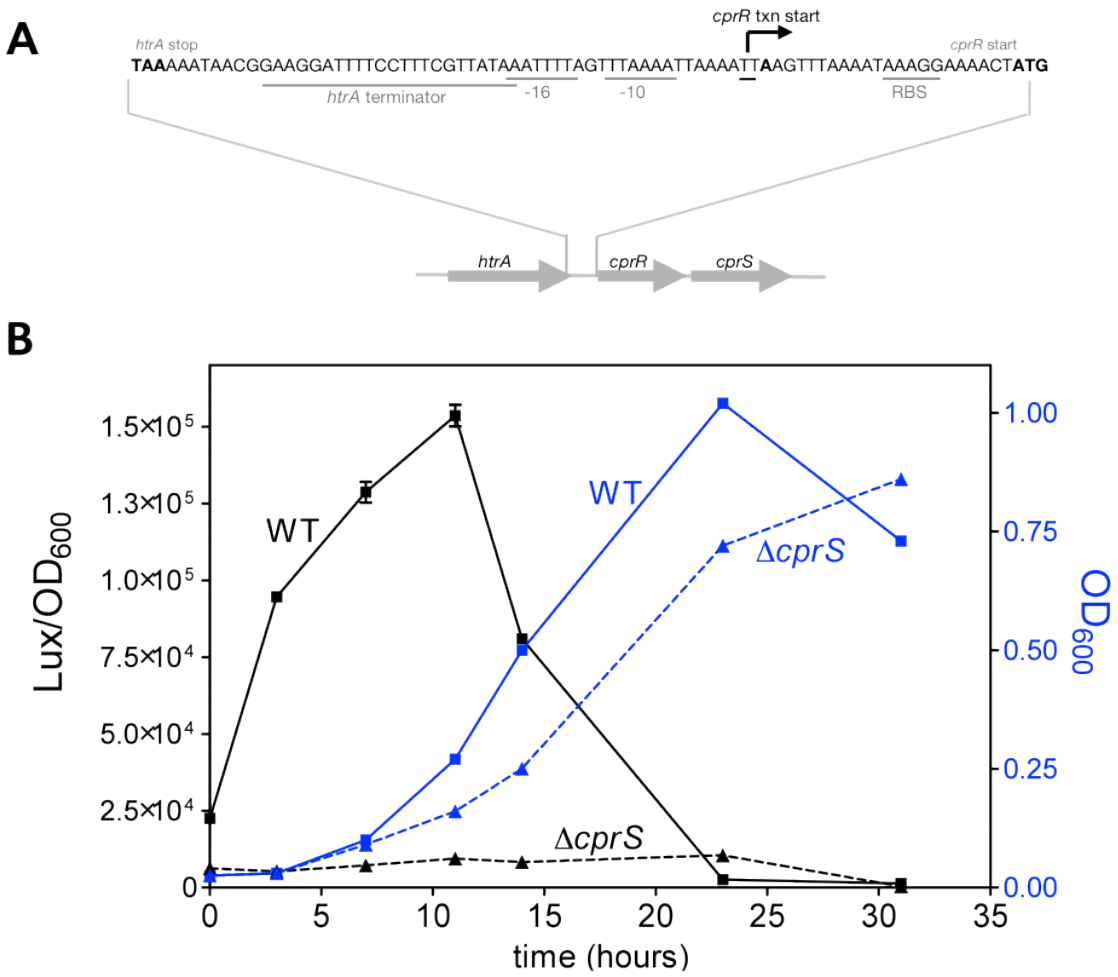


FIG. 3.1. The *cprRS* operon is expressed from an autoregulated promoter upstream of *cprR*. **A)** Transcription of *cprRS* appears to start immediately upstream of *cprR*, as 5'-RACE suggests transcripts for *cprRS* start at -22 with respect to the *cprR* start codon. The location of a putative transcriptional terminator after the *htrA* stop codon is shown [256], as well as putative *cprR* -16 and -10 sequences. **B)** Expression of the *cprRS* promoter increases through log phase in WT and is expressed at basal levels in Δ*cprS*. Expression of a region approximately 200 bp upstream of the *cprR* start codon was assessed by fusing it to a promoterless *lux* operon in plasmid pRY112-*lux*. The resulting construct (pSS81) was introduced into both WT (solid lines) and Δ*cprS* (dashed lines), and bacteria were grown in shaking MH broth culture. At each time point, samples were removed to measure OD_{600} (right axis, blue lines) and light production (left axis, black lines). Data are representative of three independent trials.

integral membrane protein) was observed between WT and Δ*cprS*, suggesting that ATP levels (which can affect Lux activity) in these strains do not differ significantly (data not shown). Together, this suggests that CprRS TCRS can be expressed separately from the adjacent *htrA* gene, from a promoter immediately upstream of *cprR*. Furthermore, the promoter appeared to be autoregulated and required activity of the CprS sensor kinase for induction of expression above basal levels. Finally, in WT, the *cprR* promoter was expressed most highly during log phase, and showed a rapid reduction of expression prior to stationary phase.

Levels of *cprR* transcript are different in Δ*cprS* and *cprR*^{OE} compared to WT. To confirm Lux reporter data, RNA was extracted from strains in log phase (~10h), and RT-qPCR was used to determine levels of the *cprR* transcript (**FIG. 3.2A**). The Δ*cprS* mutant showed an approximately 2-fold decrease in *cprR* transcript levels compared to the WT strain (p=0.006). In contrast, *cprR* transcript levels in the Δ*cprS*^{C} complemented were not significantly different from WT (p=0.88). Levels of *cprR* transcript were also measured in a strain overexpressing *cprR* from the *peb1a* promoter (*cprR*^{OE}). The *peb1a* promoter is expressed most highly in stationary phase (E. Gaynor, unpublished observations), and *cprR* was expressed from this non-native promoter to limit effect of any negative autoregulation on *cprR* transcription. In contrast to Δ*cprS*, levels of *cprR* transcript were higher in *cprR*^{OE} than in WT. Although this difference was consistently observed across experiments, it did not reach statistical significance in the experiment shown (p=0.25). Lux data indicated that the *cprR* promoter was expressed most highly during log phase, suggesting CprRS may control expression of genes that confer fitness during exponential growth in rich media. The Δ*cprS* mutant previously showed a growth defect in broth culture (**FIG. 2.2**). Comparison of RT-qPCR data (**FIG. 3.2**) and observations of growth behaviour of these strains in log phase (data not shown) suggested that in general, growth behaviour in log phase correlated with relative *cprR* transcript levels. For example, while Δ*cprS* grew more slowly than WT, the *cprR*^{OE} strain tended to enter log phase more rapidly than WT, and lower and higher levels of *cprR* expression were measured in these strains, respectively.

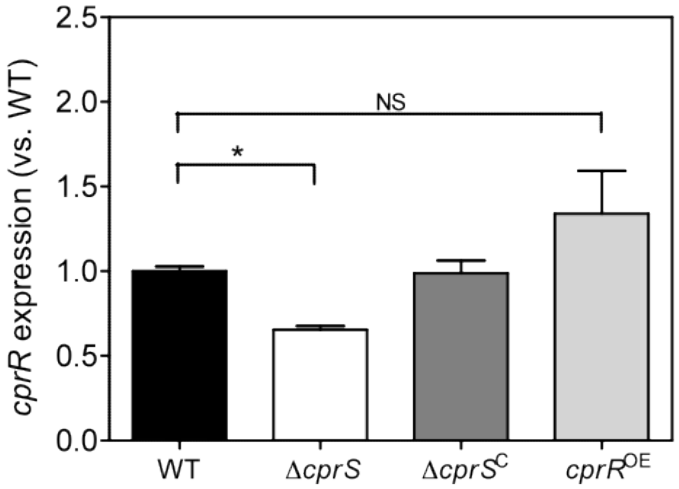
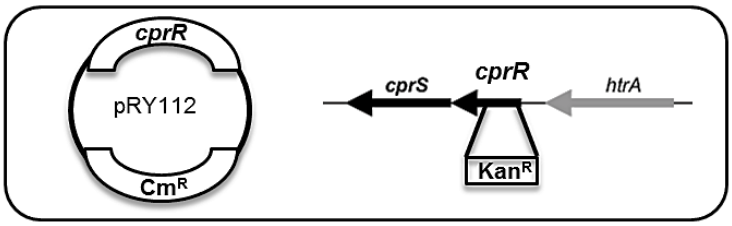


FIG. 3.2. Levels of *cprR* transcript vary between WT, Δ*cprS,* and *cprR*^{OE}. Levels of *cprR* transcript during log phase in broth culture were lowest in Δ*cprS* and highest in *cprR*^{OE}. RNA was extracted from WT, Δ*cprS*, and *cprR*^{OE} in log phase (~10h growth) and levels of *cprR* were determined by RT-qPCR with *gyrA* as a reference. Both WT and Δ*cprS* harboured the empty pRY112 vector, whereas *cprR*^{OE} contained pRY112-*cprR* (expression from the *peb1a* promoter). Strains were grown in MH broth supplemented with Cm to maintain plasmids. *p=0.0006; NS, not significant.

Phosphorylation of the CprR response regulator is essential for viability. As the CprS sensor kinase was dispensable, but CprR was not, it was unclear whether CprR phosphorylation was also essential. To determine this, construction of strains expressing solely Asp52Ala (phosphoacceptor-null) or Asp52Glu (putatively constitutively phosphorylated activity) versions of the CprR protein was attempted. This was achieved by introduction of one of three *cprR* alleles (*cprR*^{WT}, *cprR*^{Asp52Ala}, or *cprR*^{Asp52Glu}) into WT on a Cm^{R} plasmid (pRY112), followed by attempts to delete *cprR* from the chromosome with a Kan^{R} cassette (**FIG. 3.3A**). The *cprR* forms on pRY112 were expressed from its native promoter (P_{cprR}). All *cprR* alleles could be introduced into WT (**FIG. 3.3B,** third column). Furthermore, the native copy of *cprR* could be deleted in the strain carrying pRY112-*cprR*^{WT}, confirming earlier reports that the region surrounding *cprR* is significantly recalcitrant to recombination [64, 479]. While colonies were also recovered on selective plates (Kan+Cm) when deletion of *cprR* was attempted in strains harbouring pRY112- *cprR*^{Asp52Ala} or pRY112-*cprR*^{Asp52Glu}, when

A



B

Strain	Plasmid	Strain obtained (genotype confirmed)	Plasmid retention (% colonies Cm^{R})
WT	pRY112 (empty)	yes	45%
	pRY112-*cprR*^{WT}	yes	7%
	pRY112-*cprR*^{Asp52Ala}	yes	83%
	pRY112-*cprR*^{Asp52Glu}	yes	95%
Δ*cprS*	pRY112 (empty)	yes	37%
	pRY112-*cprR*^{WT}	yes	59%
	pRY112-*cprR*^{Asp52Ala}	yes	0%
	pRY112-*cprR*^{Asp52Glu}	yes	85%
Δ*cprR*	pRY112 (empty)	no	N/A
	pRY112-*cprR*^{WT}	yes	100%
	pRY112-*cprR*^{Asp52Ala}	no	N/A
	pRY112-*cprR*^{Asp52Glu}	no	N/A

FIG. 3.3. Phosphorylation of CprR affects its activity and is essential for viability. **A)** Construction of *C. jejuni strains* expressing *cprR* alleles ectopically. WT or point mutant forms of *cprR* (*cprR*^{WT}, *cprR*^{Asp52Ala}, or *cprR*^{Asp52Glu}) were introduced into WT *C. jejuni* on plasmid pRY112 to obtain Cm^{R} colonies. A Δ*cprR*::Kan^{R} (or Δ*cprS*::Kan^{R}, not shown) KO construct was then introduced into each plasmid-bearing strain and putative recombinants were selected on media containing Kan+Cm. **B)** Recovery of confirmed strains (third column) and retention of their respective pRY112 plasmid (4th column). Sequencing and Southern blotting confirmed legitimate double crossover replacement of 'native' *cprR*. Plasmid retention was assessed by streaking cells for isolated colonies on MH agar with no antibiotics, followed by transfer of individual colonies to media containing either Cm or Kan+Cm.

DNA from these colonies was analyzed by PCR, sequencing, and Southern blotting (data not shown), it was found that they were illegitimate recombinants where the deletion construct had crossed into the plasmid copy of *cprR* rather than the chromosome. In contrast, the *cprS* sensor kinase could be deleted from strains expressing all three plasmid-bourne forms of *cprR*.

Each form of *cprR* had a different effect on behaviour of WT and Δ*cprS* when expressed from pRY112. Growth behaviour, biofilm formation, and plasmid retention were assessed in WT and Δ*cprS* harbouring each allele of *cprR*. In general, growth behaviour in broth culture and biofilms was noted to be markedly different in each strain during routine culture (data not shown). It was also observed that each strain showed a different tendency to retain its *cprR*-containing plasmid without selective antibiotic pressure. Thus, the effect of version of *cprR* on behaviour was quantified by the difference in retention of each *cprR*-containing plasmid when Cm selective pressure was removed (**FIG. 3.3B**, last column). Each strain was subcultured from selective media (Cm or Kan+Cm, where appropriate) onto MH plates lacking antibiotics, and then individual colonies were patched back onto selective plates. Chromosomal markers (Δ*cprS or* Δ*cprR* marked with the Kan^R cassette, Δ*cprS* marked with the Cm^R cassette; data not shown) were retained at a rate of 100% in the absence of selection. However, the Cm^R marker on pRY112 was not retained at 100% when Cm selection was absent, suggesting that the plasmid was being lost. Furthermore, the rate of retention varied with both host genotype (WT, Δ*cprR*, or Δ*cprS*) and the *cprR* allele contained on the plasmid. For example, in a WT background, the empty pRY112 plasmid was retained by only 45% of colonies without Cm pressure, and an even lower rate of retention was found for pRY112-*cprR*^{WT} (7%). In contrast, retention of either pRY112-*cprR*^{Asp52Ala} or pRY112-*cprR*^{Asp52Glu} by WT was higher than that for empty pRY112 (83% and 95%, respectively). Like WT, retention of each plasmid by Δ*cprS* also varied. While pRY112-*cprR*^{WT} and pRY112-*cprR*^{Asp52Glu} were maintained at rates higher than the empty vector (59% and 85%, respectively, compared to 37% for pRY112), Cm^R colonies were not recovered for Δ*cprS* pRY112-*cprR*^{Asp52Ala} after one pass without Cm. Interestingly, this strain also grew very slowly in broth with Cm (data not shown). Finally, the Δ*cprR* mutant retained pRY112-*cprR*^{WT} at a rate of 100%, presumably because it requires the plasmid copy of *cprR*^{WT} to maintain viability. Thus, phosphorylation of CprR appeared to be essential, despite the fact that kinase was dispensable. Finally, mutation of the Asp52 residue of CprR affected activity of the protein, and phenotypes of the sensor kinase mutant were negatively affected by overexpression of the CprR^{Asp52Ala} phosphoacceptor-null protein.

Effect of *cprR* and *cprS* alleles on salt tolerance and biofilm formation of WT and Δ*cprS*. The modular nature of TCRS proteins means that, isolated domains, expressed separately from the regulatory activities of the full-length protein), often exhibit enzymatic and/or protein binding activities towards other TCRS components, leading to either dominant negative or constitutively active phenotypes. For example, overexpression of the NTD of response regulators (harbouring the receiver and oligomerization domain) in a WT background can cause a dominant negative phenotype, due to dimerization with the full-length protein

and subsequent interference with DNA binding, or acceptance of phosphate from the sensor kinase with no downstream effect on gene expression due to the absence of the DNA-binding domain. In contrast, overexpression of the sensor kinase CTD (containing the kinase activity) of can sometimes cause constitutive kinase activity towards the cognate response regulator. While these activities have not been confirmed for CprRS, such forms of the sensor kinase and response regulator (CprS^{CTD} or CprR^{NTD}; **FIG. 1.4B**) were expressed in either WT or Δ*cprS* genetic backgrounds. A common strategy for coping with essential proteins in genetic analyses also includes expression of a knockdown product specific for transcript of the protein of interest, and overexpression of an interfering RNA specific for *cprR* using a system developed for *C. jejuni* (A. Cameron, unpublished).

Because Δ*cprS* has reduced expression of *cprR* (**FIG. 3.1**), it was hypothesized that reduced activity of CprRS may be responsible for Δ*cprS* phenotypes, such as reduced salt tolerance and enhanced biofilm formation, we therefore used these phenotypes as a general readout of CprRS activity to provide more insight into how components of the system interact (**TABLE 3.1**). Alleles were either integrated into the chromosome ('chromosome'), or expressed from a plasmid ('pRY112'). Chromosomal genes were expressed from the non-native *cat* cassette promoter, and plasmid alleles were expressed from either the *peb1a* promoter (*cprR* knockdown) or the native *cprR* promoter (*cprR* alleles). Strains expressing different forms of either CprS or CprR were constructed. For CprS, either full-length *cprS,* or the intracellular kinase domain (*cprS*^{CTD}) were expressed. For CprR, either full-length *cprR*; the sensor interaction and dimerization domain (*cprR*^{NTD}); an antisense product specific for *cprR* (*cprR*^{KD}), or a phosphoacceptor-null version of *cprR* (*cprR*^{Asp52Ala}) were expressed.

Osmotolerance was determined by measuring growth in MH broth +150 mM NaCl (**TABLE 3.1**, fourth and fifth columns). All strains grew in MH broth alone ('+'), although Δ*cprS* pRY112-*cprR*^{Asp52Ala} showed a growth defect ('+/-'). When broth was supplemented with NaCl, WT bacteria harbouring an extra copy of either *cprS*, *cprS*^{CTD}, *cprR*^{WT}, or *cprR*^{Asp52Ala} were able to grow ('+'). In contrast, overexpression of either *cprR*^{NTD} or *cprR*^{KD} in WT caused salt sensitivity ('-'). In the Δ*cprS* background, the osmotolerance defect was complemented by expression of *cprS in trans*. In contrast, none of the *cprR* alleles rescued salt sensitivity of Δ*cprS*. The effect of each allele on biofilm formation was assessed using the CV assay (**TABLE 3.1**, last column). In the WT background, most alleles had no observable effect on biofilm formation. However, overexpression of either *cprR*^{NTD} or *cprR*^{WT} modestly enhanced biofilm formation ('++'), but not to levels shown by Δ*cprS* alone ('+++'). In the Δ*cprS* background, overexpression of *cprS* rescued biofilm formation to levels of WT, as did overexpression of *cprS*^{CTD}. Overexpression of *cprR*^{WT} also partially rescued Δ*cprS* biofilm formation ('++'), although not to levels of WT. In contrast, neither knockdown of *cprR* expression, nor overexpression of the CprR^{Asp52Ala} point mutant in *cprS* rescued biofilm formation. In fact, a modest exacerbation of the biofilm phenotype was consistently observed in these strains. Together, this suggested

TABLE 3.1. Effect of *cprS* and *cprR* alleles on salt tolerance and biofilm formation. Alleles of *cprS* or *cprR* were expressed from a heterologous location in either WT or Δ*cprS*. Overexpression from the chromosome was from the *cat* cassette promoter; overexpression from pRY112 was from the *peb1a* promoter. Salt tolerance was determined by growth in MH+150 mM NaCl (~0.88%, 300 Osm L^{-1}), and biofilms were determined by the CV assay after 2 days. Where appropriate, Cm was included to retain the pRY112 plasmids, and phenotypes were compared to WT or Δ*cprS* harbouring empty pRY112.

***cprRS* genotype**				**MH broth (growth)**	**MH+NaCl (growth)**	**Biofilms (vs. WT)**
Background genotype	**Overexpressed allele**	**Location**	**Promoter**			
WT	-	-	-	+	+	WT
	cprS	chromosome	P_{CAT}	+	+	WT
	$cprS^{CTD}$	chromosome	P_{CAT}	+	+	WT
	$cprR^{NTD}$	chromosome	P_{CAT}	+	-	++
	$cprR^{KD}$	pRY112	P_{peb1a}	+	-	WT
	$cprR^{WT}$	pRY112	P_{cprR}	+	+	++
	$cprR^{Asp52Ala}$	pRY112	P_{cprR}	+	+	++
Δ*cprS*	-	-	-	+	-	+++
	cprS	chromosome	P_{CAT}	+	+	WT
	$cprS^{CTD}$	chromosome	P_{CAT}	+	ND	WT
	$cprR^{NTD}$	chromosome	P_{CAT}	+	-	+++
	$cprR^{KD}$	pRY112	P_{peb1a}	+	-	+++
	$cprR^{WT}$	pRY112	P_{cprR}	+	-	++
	$cprR^{Asp52Ala}$	pRY112	P_{cprR}	+/-	-	++++

that in general, decreased activity of CprRS led to salt sensitivity and enhanced biofilms. It appeared that this could be achieved by either deletion of the sensor kinase, expression of putatively dominant negative *cprR* alleles ($cprR^{NTD}$; $cprR^{KD}$), or knockdown of *cprR* expression. However, it also appeared that the dosage of active CprR was critical. For example, $cprR^{WT}$ partially suppressed Δ*cprS* biofilms, but did not complement its salt defect, and knockdown of *cprR* in WT caused salt sensitivity, but did not appear to affect biofilms.

Phosphorylated CprR binds the consensus 5'-[G/C]TAAA[C/T]. A previously developed bacterial one-hybrid system [509] was used to identify the CprR-binding consensus. A library of 28-mer DNA sequences (previously selected against self-activation) was screened for sequences bound by the C-terminal DNA-binding domain of CprR ($CprR^{CTD}$). Positive sequences were used to construct a putative consensus, 5'-[G/C]TAAA[C/T] (**FIG. 3.4A**). It was subsequently confirmed that three of the sequences identified in the library screen only drove reporter expression when $CprR^{CTD}$ was present (**FIG. 3.4B**). It was also demonstrated that full-length $CprR^{WT}$, but not $CprR^{Asp52Ala}$, binds a sequence isolated in the $CprR^{CTD}$ screen (**FIG. 3.4C**). Thus, CprR appeared to bind to specific DNA sequences, and this activity appeared to be modulated by phosphorylation of the protein. The motif isolated may represent part of sequences present in promoters directly regulated by CprRS.

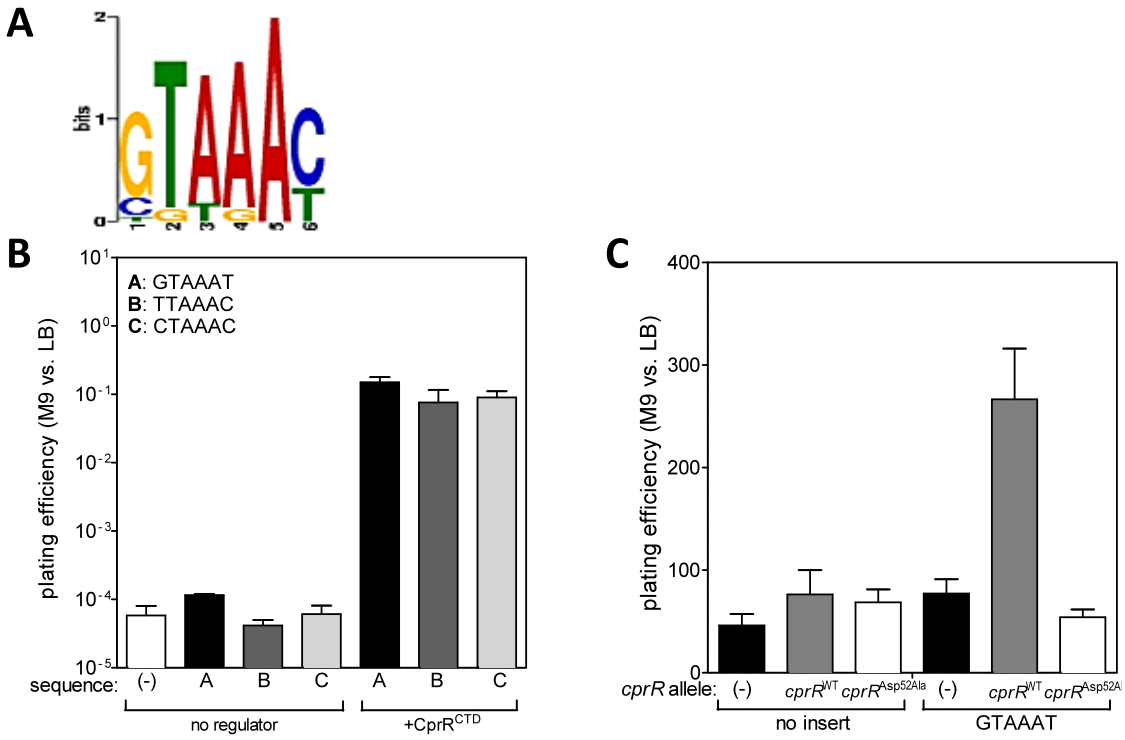


FIG. 3.4. CprR^{CTD} binds the consensus 5'-[C/G]TAAA[C/T]; binding of full-length CprR **requires phosphorylation.** A bacterial one-hybrid library of random sequences was screened with CprR^{CTD}. **A)** Sequence logo of the consensus harboured by positive, as determined by MEME. **B)** Positive sequences drove reporter expression only when CprR^{CTD} is present. Host cells were cotransformed with one of three positive sequences and either the empty bait plasmid ('no regulator') or the bait plasmid containing CprR^{CTD} ('+CprR^{CTD}). Plating efficiency was determined on media selective for reporter expression vs. LB. **C)** Binding of full-length CprR requires phosphorylation. Cells were cotransformed with plasmids harbouring CprR^{WT} or CprR^{Asp52Ala} and one of the positive sequences identified in the library screen (GTAAAT). Plating efficiency on media selective for reporter expression vs. LB was determined.

The CprR consensus may be shared by the related response regulator RacR. During a separate investigation of the RacRS TCRS of *C. jejuni* performed in our laboratory, the one-hybrid system was also used to identify the consensus bound by the RacR response regulator. Interestingly, a sequence very similar to that of CprR was obtained (**FIG. 3.5A**). Furthermore, positive sequences from the RacR screen were bound by both RacR^{CTD} and CprR^{CTD} (data not shown). In contrast, they were not bound by the *Salmonella* response regulators SsrB or PhoP (data not shown), which have been previously tested in this one-hybrid system [515]. Bioinformatics analyses suggested the CprRS and RacRS TCRSs may be related. First, alignment of the primary sequences of the response regulator proteins (**FIG. 3.5B**) showed a relatively high degree of identity (51%) and similarity (72%). The RacRS TCRS appears to be the closest homologue to CprRS – closer than to any TCRS outside the *Campylobacter* genus (**CHAPTER 2**). Although the sensor kinases of these TCRSs (CprS and RacS) showed similarity as well (39% identity, 61% similarity), it was not as high as for the response regulators. In addition to primary sequence, the genetic organization and context of the operons encoding these two systems was also similar. Both were encoded in operons beginning with the response regulator, and show a high conservation of synteny within the *Campylobacter* genus with heat shock genes (*htrA* for *cprRS*, and *dnaJ* for *racRS*) (**FIG. 3.5B**). Thus RacR and CprR may have a related evolutionary history.

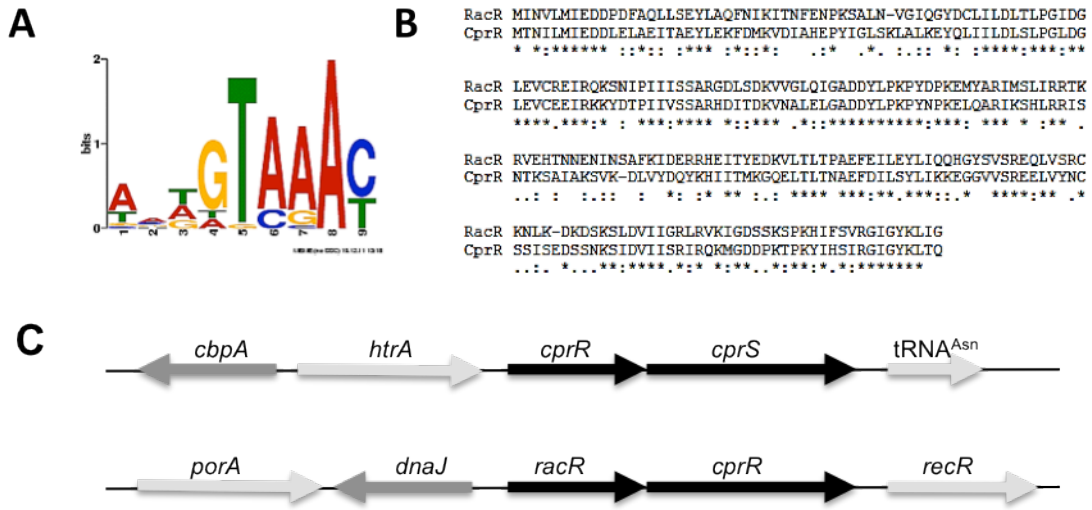


FIG. 3.5. The related response regulator RacR also binds the CprR consensus. **A)** Consensus sequence for the RacR protein determined by screening the one-hybrid library with RacR^{CTD}. **B)** CLUSTAL W alignment of CprR and RacR primary sequences shows 51% identity and 72% similarity. "*****" identical**,** "**:**"**,** conserved substitution. **C)** Genetic organization of both *cprRS* and *racRS* in *C. jejuni*, showing proximity to heat shock response genes, *htrA* and *dnaJ*, respectively. CbpA is also annotated as containing a DnaJ-like domain.

Microarray analysis of Δ*cprS* shows dysregulation of envelope-related genes and early entry into stationary phase. To elucidate the CprRS regulon, microarray analysis of WT, Δ*cprS*, and the complemented Δ*cprS*^{C} strain during shaking MH broth culture was performed. Broad expression changes between WT and Δ*cprS* (**TABLE C.1**) were observed. Furthermore, consistent with known growth differences between WT and the Δ*cprS* strain, by the 6hr time point, Δ*cprS* already showed upregulation of flagellar genes. Expression of late flagellar genes in WT bacteria usually peaks during late log phase [76]. By 24h, many flagellar genes (both RpoN- and FliA-dependent) were markedly upregulated (**TABLE 3.2**, bottom). In contrast, early flagellar genes, such as those comprising the export apparatus, were not overexpressed in Δ*cprS*. Because of the large number of expression changes observed between WT and Δ*cprS*, and also because of incomplete rescue of expression for some genes in the complemented strain, early time points (3hr) that showed some complementation were the main focus of analysis. **TABLE 3.2** (top) shows genes from the 3h time point with greater than 2-fold different expression in the Δ*cprS* mutant vs. the WT strain, but less than a 2-fold change between the complemented strain and WT. These fell into four groups, based on functional category and/or nature of dysregulation. In Group I, the *cprR* response regulator gene was upregulated in Δ*cprS* at 3h, but downregulated at 24h (not shown). The second set of genes (Group II), was also upregulated in Δ*cprS*, and included metabolism-related genes, such as *fumC* (fumarate hydratase), and the Cj0076c-Cj0073c operon (lactate dehydrogenase and permease). Group III included two envelope-located proteins (Cj1169c, putative periplasmic protein; *omp50*, porin) and showed higher expression in WT than the Δ*cprS* mutant. The final set of genes (Group IV) appear to be involved in aspects of biogenesis and maintenance of the cell envelope, including those related to PG metabolism (Cj0069, putative D-Ala ligase); expression of LOS, CPS and *N*-

TABLE 3.2. Microarray analysis of expression in WT, Δ*cprS*, and Δ*cprS*^{C}. Genes at 3h are shown if they showed a ~2-fold difference in expression in Δ*cprS* compared WT, and at least partial complementation of expression in Δ*cprS*^{C} (closer expression in WT vs. complement than WT vs. mutant). Reported differences are significant by a parametric statistical *t* test ($p<0.05$) as described in the Methods.

Time point	Group	ORF	Gene product	Fold change	
				(WT/Δ*cprS*)	(WT/Δ*cprS*^{C})
3h	I	Cj1227c	CprR response regulator	0.39	0.56
	II	Cj1364c	FumC fumarate hydratase	0.56	1.02
		Cj0073c	L-lactate dehydrogenase subunit	0.42	0.52
		Cj0074c	L-lactate dehydrogenase subunit	0.39	0.61
		Cj0075c	L-lactate dehydrogenase subunit	0.37	0.54
		Cj0076c	LctP lactate permease	0.37	0.50
	III	Cj1169c	Putative periplasmic protein	2.50	1.37
		Cj1170c	Omp50 porin	2.56	1.30
	IV	Cj1228c	HtrA serine protease/chaperone	0.33	0.82
		Cj0168c	Putative periplasmic protein	0.20	0.87
		Cj0289c	Peb3 ABC transporter solute binding protein	0.52	0.97
		Cj0420	YceI-like lipid binding protein	0.46	0.66
		Cj0596	Peb4 SurA-like chaperone	0.54	0.86
		Cj0597	Fba fructose bisphosphate aldolase	0.51	0.92
		Cj0778	Peb2 ABC transporter solute binding protein	0.47	1.02
		Cj0856	LepP signal peptidase	0.56	0.61
		Cj1130c	PglK flippase	0.61	1.01
		Cj1131c	Gne UDP-GlcNAc/Glc 4-epimerase	0.53	1.01
		Cj0069	Possible D-Ala ligase, ATP-grasp domain	0.55	1.01
		Cj1279c	FlpA fibronectin-binding protein	0.50	0.86
		Cj1380	DsbC disulfide-bond isomerase	0.61	1.08
24h		Cj0887c	FlaD Flagellin	0.12	0.16
		Cj0526c	FliE Flagellar hook-basal body protein	0.26	0.36
		Cj0527c	FlgC Flagellar basal body rod protein	0.21	0.27
		Cj0528c	FlgB Flagellar basal body rod protein	0.26	0.28
		Cj0041	FliK Hook length control protein	0.31	0.37
		Cj0042	FlgD Flagellar hook assembly protein	0.094	0.11
		Cj0043	FlgE2 Flagellar hook protein	0.13	0.16
		Cj0687c	FlgH Flagellar basal body L-ring protein	0.23	0.29
		Cj0697	FlgG2 Flagellar distal rod protein	0.20	0.27
		Cj0698	FlgG Flagellar distal rod protein	0.25	0.30
		Cj1338c	FlaA Flagellin	0.30	0.31
		Cj1293	PseB UDP-GlcNAc C6-dehydratase/C4-reductase	0.12	0.18
		Cj1294	PseC putative aminotransferase (DegT family)	0.19	0.24
		Cj1312	PseG Flagellin pseudaminic acid biosynthesis protein	0.15	0.16
		Cj1316c	PseA Flagellin pseudaminic acid biosynthesis protein	0.27	0.30
		Cj1339c	FlaB Flagellin	0.25	0.28
		Cj1462	FlgI Flagellar P-ring protein	0.051	0.061
		Cj1463	FlgJ flagellar biosynthesis-related muramidase	0.16	0.19
		Cj1464	FlgM Flagellar FliA-specific anti-s factor	0.23	0.20
		Cj1465	FlgN chaperone for hook-associated proteins	0.15	0.15
		Cj1466	FlgK Flagellar hook-associated protein	0.15	0.14

linked glycoproteins (*gne*, UDP-Glc*N*Ac/Glc 4-epimerase); lipid-binding (Cj0420, *yceI*-like); protein secretion (*lepP*, signal peptidase); envelope protein folding (*dsbC*, disulphide bond isomerase; *peb4,* SurA-like chaperone); as well as proteins that reside on the cell surface (Cj1279c, fibronectin-binding protein; *peb2,* ABC transporter substrate-binding protein). The *htrA* gene (periplasmic serine protease/chaperone), encoded immediately upstream of *cprR,* was also be included in this group.

Promoter inspection, RT-qPCR, one-hybrid, and phenotypic analyses suggest CprRS regulates the adjacent *htrA* gene. Although the specificity of CprR binding to the 5'-[C/G]TAAA[C/T] consensus remains to be elucidated in light of the RacR consensus sequence data, the upstream regions of *cprR* and *htrA* were searched for putative CprR binding sites. Two sequences reminiscent of the CprR consensus, separated by a 6 bp spacer, appeared to be present as an inverted repeat upstream of the *htrA* promoter region (**FIG. 3.6A**). The placement of these sequences was approximately 20 bp upstream from the *htrA* transcription start site (determined by RNA-seq, G. Dugar and C. Sharma, personal communication). Repeats of the putative consensus were also identified flanking the *cprR* transcription start site. Thus, promoter inspection suggested that CprR may directly regulate expression of *htrA*. To confirm microarray data suggesting that *htrA* was observed to be dysregulated in Δ*cprS*, *htrA* transcript levels in WT, Δ*cprS*, and *cprR*^{OE} bacteria during log phase in MH broth were measured by RT-qPCR (**FIG 3.6B**). Expression of *htrA* was approximately 2-fold lower in the Δ*cprS* mutant compared to WT (p=0.014) at this time point. Levels of *htrA* expression were also found to be 2-fold lower in the *cprR*^{OE} strain (p=0.016). To demonstrate that CprR may directly affect expression of *htrA*, binding of CprR^{CTD} to promoter regions using targeted one-hybrid was attempted. Approximately 40 bp of DNA representing three upstream regions of *htrA*, were cloned into the one-hybrid reporter fusion vector, and these constructs were cotransformed with either the empty RNAPα plasmid, or the plasmid containing the RNAPα-*cprR*^{CTD} fusion into the one-hybrid host strain. Unfortunately, many of the fragments selected, including two selected from the *cprR* promoter region, showed strong reporter activity in the absence of CprR^{CTD} (data not shown), suggesting they were constitutively active in *E. coli*. Thus, direct binding of CprR to its own promoter in this manner could not be assessed. However, there was a significant (p=0.023) increase in one-hybrid reporter activity when a fragment representing the (-93) to (-62) region of *htrA* was cotransformed with *cprR*^{CTD} (**FIG. 3.6C**). This region contains putative CprR consensus sequences as shown in **FIG. 3.6A**. Finally, to determine if dysregulation of *htrA* observed in Δ*cprS* was responsible for enhanced biofilm formation, a targeted *htrA* deletion mutant was constructed. The majority of the coding region was removed (both protease and chaperone domains). Biofilm formation of Δ*htrA* was then compared to WT, Δ*cprS*, and Δ*cprS*^{C} (**FIG. 3.6D**). While biofilm formation by Δ*htrA* was not as enhanced as Δ*cprS*, a modest, but significant increase in biofilm formation was observed (p=0.04).

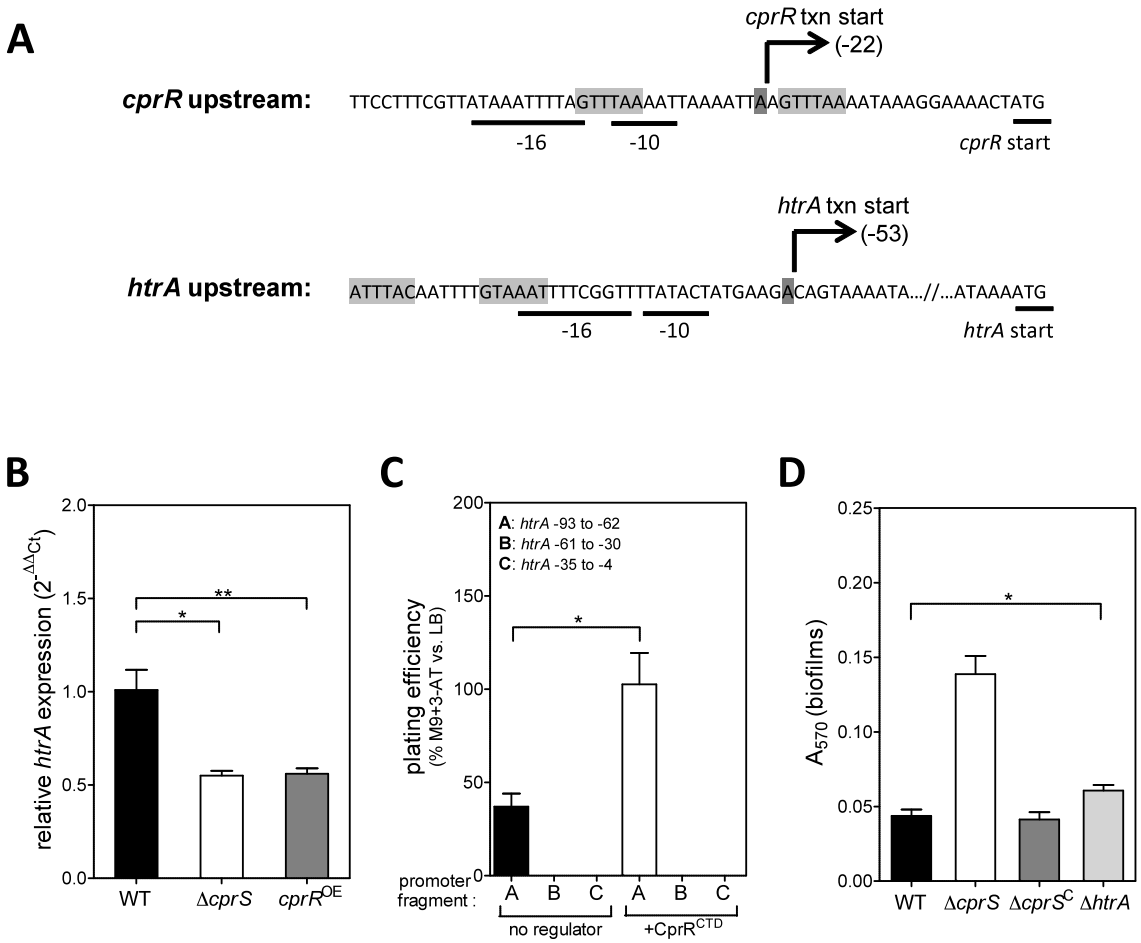


FIG. 3.6. The adjacent *htrA* gene may be directly regulated by CprRS. A) Upstream regions of *cpr*R and *htrA* may contain putative CprR binding sites. Predicted transcription start sites are shown by single highlighted A residues, and putative -10 and -16 boxes are indicated by underlines. Putative CprR binding sites are highlighted in grey. **B)** Levels of *htrA* transcript are lower in *cprRS* strains. RNA was isolated during log phase and *htrA* transcript was measured by RT-qPCR with a *gyrA* reference. **p*=0.014; ***p*=0.016. **C)** CprR^{CTD} bound a region upstream of *htrA* containing putative CprR consensus sites. One of three regions of the *htrA* promoter (A: -93 to -62; B: -61 to -30; C: -4 to -35) was inserted into the one-hybrid reporter plasmid and cotransformed into host cells with either empty bait vector ('no regulator') or the bait vector containing *cpr*R^{CTD}. **p*=0.023. **D)** Biofilm formation is modestly increased in a Δ*htrA* mutant. Biofilms were quantified for WT, Δ*cprS*, Δ*cprS*^{C}, and Δ*htrA* in MH broth using the CV biofilm assay. * *p*=0.04.

Enhanced biofilm formation by Δ*cprS* is suppressed in richer media and divalent cations. Attempts to identify suppressor mutations of the Δ*cprS* biofilm phenotype were unsuccessful due to the high rate of flagellar mutants identified in transposon suppressor screens (A. Cameron, S.L. Svensson, and E.C. Gaynor, unpublished observations). Thus, based on microarray observations, conditions that rescued Δ*cprS* biofilms to levels of the WT strain were sought. Expression analysis suggested Δ*cprS* was entering stationary phase earlier than WT, and it was hypothesized that this mutant may have been experiencing starvation stress that could be rescued by increasing broth strength, and presumably, nutrient availability. As Δ*cprS* generally grew differently from WT (**FIG. 2.2**), growth in shaking broth culture was first assessed. As expected, in 1X broth normally

used to culture *C. jejuni*, Δ*cprS* cultures reached approximately 70% of the density of WT (**FIG. 3.7A**). However, total growth of both WT and Δ*cprS* cultures appeared to be significantly affected by broth strength. Interestingly, both strains reached the highest density in 1.5X MH broth rather than 1X, with a more marked effect on growth of Δ*cprS*. Growth of the mutant was almost negligible in either 0.5X or 2X MH. In contrast, in 1.5X MH, the density of Δ*cprS* cultures was surprisingly similar to those of WT. In addition to behaviour in shaking culture, biofilm formation of Δ*cprS* was also affected by broth concentration. Like the shaking culture defect, 1.5X broth also suppressed the enhanced biofilm phenotype of Δ*cprS* (**FIG. 3.7B**). Specifically, in 1X MH, Δ*cprS* showed threefold increase in biofilm formation compared to WT. However, in 1.5X MH, there was a smaller, insignificant (p=0.08) difference in biofilm formation. Due to almost negligible growth of Δ*cprS* in 0.5X or 2X MH, biofilm data for these concentrations have not been included. Following these observations, deletion of the 'essential' *cprR* gene by recovering bacteria on plates made with 1.5X MH broth was attempted, but legitimate deletion mutants were again not recovered (data not shown).

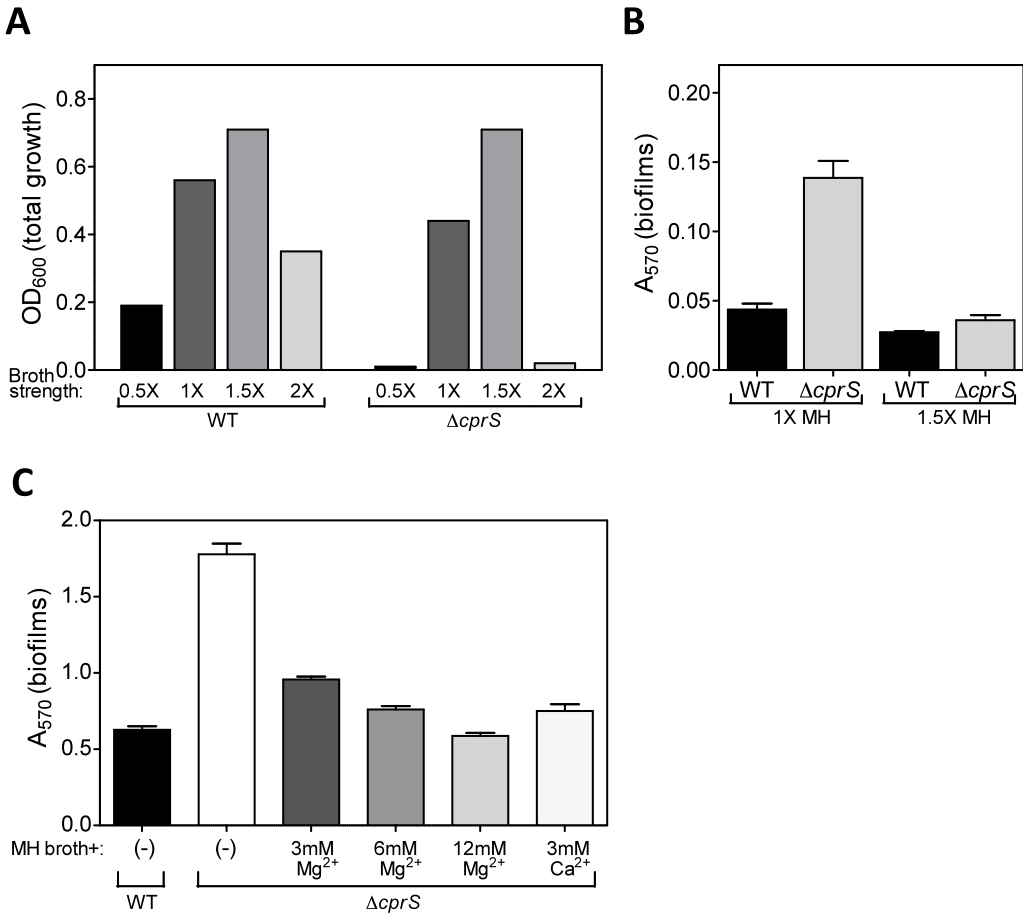


FIG. 3.7. Enhanced biofilms of Δ*cprS* are rescued by increasing broth strength or addition of divalent cations. **A)** Total growth of WT, and especially Δ*cprS*, is affected by MH broth concentration. Biofilms were grown in MH broth at various strengths (0.5X, 1X, 1.5X, and 2X) and OD_{600} was measured after vortexing to quantify total growth. **B)** Enhanced biofilm formation by Δ*cprS* was suppressed in 1.5X MH broth. Biofilms cultures were prepared as in **A)** and stained with CV. **C)** The enhanced biofilm phenotype of Δ*cprS* was suppressed by divalent cations. Biofilms of WT or Δ*cprS* were grown in MH broth supplemented with chloride salts of either Mg^{2+} or Ca^{2+}. After 2 days, biofilms were stained with CV and quantified.

While it was observed that increasing broth strength could rescue Δ*cprS* phenotypes, it was unknown whether this was due to increases in nutrient availability, or increases in other media components. Culture media contains divalent cations that can interact with various envelope structures, such as LPS, PG, and CPS [516-518], and affected their stability either positively or negatively. The Δ*cprS* mutant exhibited difficulty growing on media containing added salt (**FIG. 2.8**), as well as changes in expression of envelope-related genes (**TABLE 3.2**). Salt sensitivity can be indicative envelope changes, and it was therefore hypothesized that suppression of Δ*cprS* phenotypes observed upon increased broth strength may have represented effects on envelope stability, rather than nutrient acquisition. Growing Δ*cprS* in cation-adjusted MH II broth yielded no effect on growth or biofilm formation (data not shown). Divalent cations (Mg^{2+} and Ca^{2+}) were then added to biofilm cultures. Addition of Mg^{2+} to the media of Δ*cprS* caused a dose-dependent suppression of Δ*cprS* biofilms (**FIG. 3.7C**).

Δ*cprS* and *cprR*^{OE} show morphological differences from WT. A Δ*htrA* mutant exhibits distinct morphology from the WT strain [256], and in light of data suggesting CprRS regulates *htrA* (**FIG. 3.6**) morphology of *cprRS* strains was also examined. SEM analysis, performed previously, suggested that Δ*cprS* does not progress more rapidly to a coccoid morphology in biofilms (**FIG. 2.3**). When bacterial morphology was observed by TEM, again, no obvious differences between WT, Δ*cprS*, and *cprR*^{OE} were observed in log phase (**FIG. 3.8**). All three strains exhibited helical morphology, and appeared to be approximately similar in size. All strains also harboured flagella. However, in stationary phase, morphological differences in both Δ*cprS* and *cprR*^{OE} compared to WT were observed. While WT showed approximately 50% round cells at this time point, very few round cells were seen for either Δ*cprS* or *cprR*^{OE}. Instead, they formed apostrophe- or comma-like cells. More acellular debris was also noted surrounding Δ*cprS* and *cprR*^{OE} bacteria than WT.

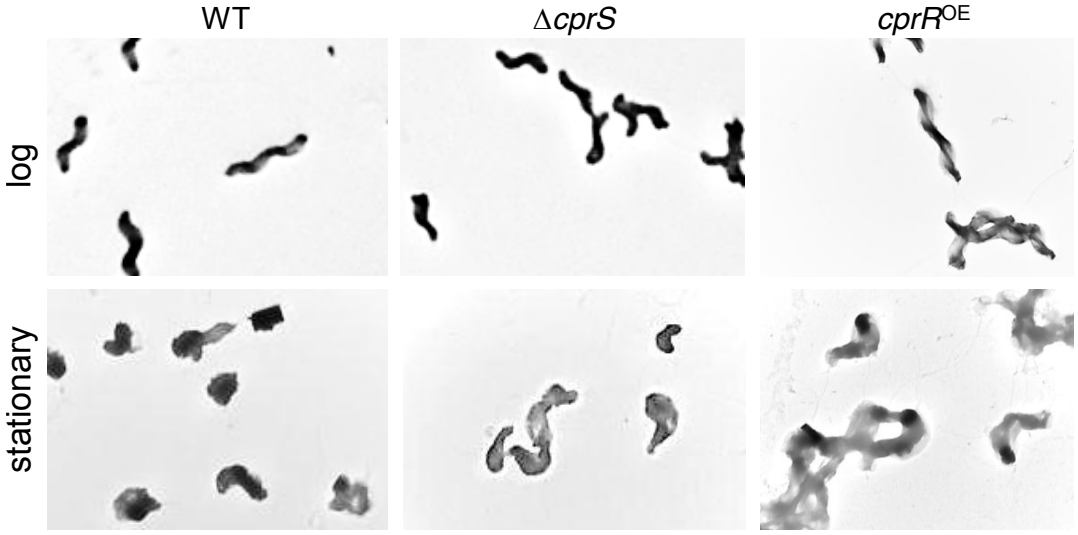


FIG. 3.8. The Δ*cprS* and *cprR*^{OE} strains show aberrant morphology in stationary phase. Morphology of Δ*cprS* or *cprR*^{OE} does not progress to coccoid like WT. Bacteria were grown to either log phase ('log') or overnight ('stationary'), fixed, stained, and visualized by TEM.

Muropeptide analysis of Δ*cprS* and *cprR*^{OE} reveal subtle differences from WT. Morphological differences were observed in Δ*cprS* and *cpr*R^{OE}, and changes to the murein layer can affect cell shape [519]. Therefore, PG was isolated from these strains on MH plates after overnight growth, digested with muramidase, and the resulting muropeptide species were analyzed by HPLC and mass spectrometry (**TABLE 3.3**). Although the majority of species did not show marked differences in amount in the mutant strains, subtle changes in PG structure were observed in both Δ*cprS* and *cpr*R^{OE} compared to WT (>10% compared to WT; in bold). For Δ*cprS*, the largest differences were observed for tetra-pentapeptide dimers (i.e., cross-linked dimer between Glc*N*Ac-Mur*N*Ac-tetrapeptide and Glc*N*Ac-Mur*N*Ac-pentapeptide) and total pentapeptides (i.e., all species containing a pentapeptide), which both were increased (212.6% compared to WT). Tetra-tripeptide dimers and total tripeptides were also modestly decreased in Δ*cprS* (90.5% and 95.1%, respectively, vs. WT). The PG of *cpr*R^{OE} showed both similarities and differences to that of Δ*cprS*, and also showed subtle differences from WT. Similar to Δ*cprS*, the *cprR*^{OE} strain had a moderate increase in pentapeptides, although not as pronounced (113.7% compared to WT). Unlike in Δ*cprS*, a small increase in dipeptide monomers and total dipeptides were observed in *cpr*R^{OE} (121.9% compared to WT), with a concurrent decrease in monomeric tripeptide and tetrapeptide species (88.0 and 88.4%, respectively, compared to WT). While differences in specific peptide species in Δ*cprS* and *cpr*R^{OE} were observed, interestingly, the overall crosslinking of PG from either of these strains, compared to WT, did not appear to be markedly different. Glycan chain length was also not significantly different from WT in either strain.

TABLE 3.3. Summary of muropeptide composition of WT, Δ*cprS*, and *cprR*^{OE}. Bacteria were harvested from plates, and PG was analyzed by HPLC and mass spectrometry. Values shown are the % peak area for each species for mutant vs. WT on plain MH plates. Bold: >10% difference from WT.

Muropeptide species	% increase compared to WT (mutant vs. WT)		
	WT	Δ*cprS*	*cprR*^{OE}
Monomers (total)	100	98.9	101.4
Di	100	100.2	**121.9**
Tri	100	99.2	**88.0**
Tetra	100	97.4	**88.4**
Dimers (total)	100	100.3	98.2
Tetra-tri	100	**90.5**	96.6
Tetra-tetra	100	102.2	98.6
Tetra-penta	100	**212.6**	**113.7**
Anhydro	100	97.3	101.1
Trimers (total)	100	103.6	103.3
Tetra-tetra-tri	100	98.2	97.9
Tetra-tetra-tetra	100	104.4	104.1
Dipeptides (total)	100	100.2	**121.9**
Tripeptides (total)	100	95.1	92.3
Tetrapeptides (total)	100	100.5	96.5
Pentapeptides (total)	100	**212.6**	**113.7**
Anhydro (total) = chain ends	100	98.2	101.4
Average chain length	100	101.8	98.6
Degree of cross-linkage	100	100.9	99.2
Peptides in cross-links (%)	100	100.8	99.0

Di, Tri, Tetra: Glc*N*Ac-Mur*N*Ac-peptide species of the indicated amino acid length (2,3, or 4).
Anhydro: 1,6-anhydro bond on Mur*N*Ac
X-X, X-X-X: cross-linked dimer or trimer of Glc*N*Ac-Mur*N*Ac-peptide species

Alterations in *cprR* or *cprS* levels cause increased lysis and enhanced biofilm formation. More protein species was noted in supernatants of Δ*cprS* cultures previously (**FIG. 2.8**), and it was hypothesized this was a result of either secretion or lysis. While increased expression of flagellar genes in Δ*cprS* was consistent with increased flagellar secretion in this strain, since dysregulation of envelope-related genes, morphological differences, and differences in PG structure were also observed in Δ*cprS*, a link between lysis and protein release was revisited. Strains were assessed for lysis by looking for a cytoplasmic protein, CosR, in culture supernatants (**FIG. 3.9A**). Consistent with lysis, significant amounts of this protein were seen in the media of Δ*cprS* and *cprR*^{OE}. In contrast, a significant difference in CosR levels in total cell extracts of each strain was not detected. CosR was present in much lower levels in WT supernatants, and only appeared after logarithmic growth. Another cytoplasmic protein, FlgR, has also been observed in culture supernatants of Δ*cprS* (data not shown), suggesting release of cytoplasmic proteins was not limited to CosR. While the amount of CosR in Δ*cprS* or *cprR*^{OE} supernatants has not been quantified, more of this protein was consistently seen in supernatants of the sensor kinase mutant than *cprR*^{OE}. Since we observed increased lysis in both Δ*cprS* and *cprR*^{OE}, finally, biofilm formation of both strains was compared to WT to determine if there was a general trend of increased lysis and increased biofilm formation (**FIG. 3.9B**). Biofilm formation was observed to be significantly increased, compared to WT, in both Δ*cprS* and *cprR*^{OE} (p=0.031 and p=0.021, respectively), although not as dramatically for *cprR*^{OE}.

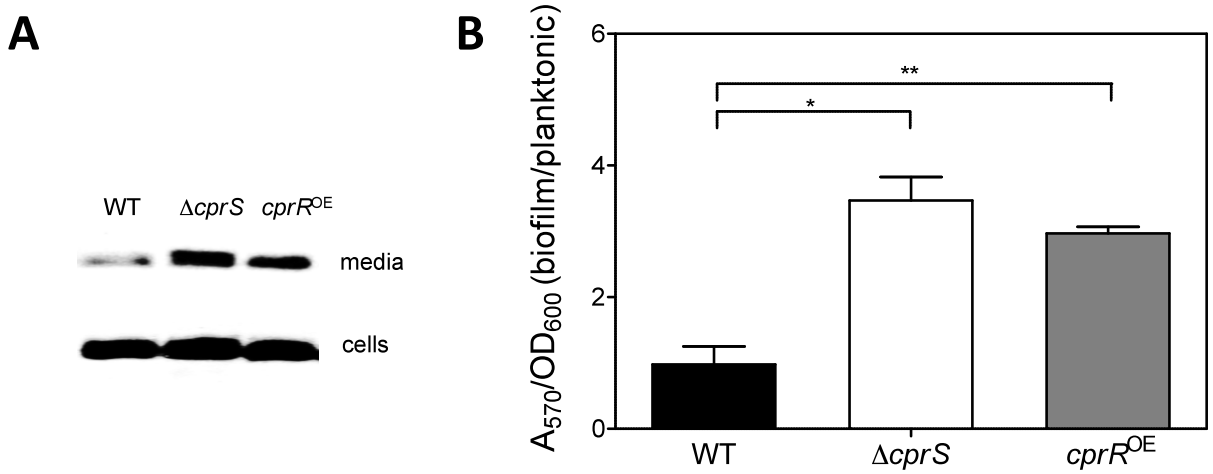


FIG. 3.9. Both Δ*cprS* and *cprR*^{OE} show increased lysis and enhanced biofilm formation. **A)** Both Δ*cprS* and *cprR*^{OE} show increased lysis. Culture supernatants from overnight MH shaking broth cultures were harvested, clarified, and concentrated. Concentrated supernatants and total cells were then analyzed for the cytoplasmic protein CosR by Western blotting. **B)** Both Δ*cprS* and *cprR*^{OE} show increased biofilm formation compared to the WT strain. Biofilms were grown for 2 days in MH broth, followed by CV staining and quantification. *p=0.031 **p=0.021.

3.4 Discussion

Characterization of the CprRS TCRS was undertaken to understand of aspects of *C. jejuni* biology relevant to survival. Following work done in **CHAPTER 2**, questions remained about the activity of the system itself, such as why only the response regulator was essential and which genes CprRS directly controls. Furthermore, it was unclear how phenotypes observed in Δ*cprS*, such as growth differences, protein release, and osmotolerance, may relate to biofilm formation. In this study, evidence is provided that phosphorylation of CprR may also be essential, that CprRS controls aspects of the cell envelope, and that the system may be most active during log phase. Furthermore, a connection between envelope stress and biofilm formation was identified through further analysis of *cprRS* strains.

Although CprR and CprS physically interact and likely comprise a cognate TCRS in *C. jejuni* (**FIG. 2.1**), *cprS* could be deleted, but *cprR* could not. The essential nature of *cprR* been confirmed by other groups [64, 479]. It was hypothesized that CprS may be dispensable because the system behaves like ArsRS of *H. pylori*, where essential functions can still be enacted with an allele that cannot be phosphorylated [469]. While a strain expressing *cprR*^{WT} from a heterologous location could be constructed, a strain expressing only *cprR*^{Asp52Ala} could not (**FIG. 3.3B**). This suggested that unlike ArsR, phosphorylation of CprR was required for its essential activities. A *C. jejuni* strain expressing only a *cprR*^{Asp52Glu} allele could also not be recovered (**FIG. 3.3B**). This could be, like the for the Asp52Ala version, due to lack of phosphorylation; however, replacement of the conserved Asp residue with Glu often results in a constitutively active conformation for many response regulators, including RcsB, OmpR, and NtrC [520-522]. Several lines of evidence suggest that the Asp52Glu version of CprR behaves differently than both the WT and Asp52Ala versions of the CprR protein. First, overexpression of CprR^{WT}, CprR^{Asp52Ala}, and CprR^{Asp52Glu} forms had different effects on *C. jejuni* behaviour. For example, Δ*cprS* bacteria harbouring pRY112-*cprR*^{Asp52Ala} could not be recovered without retaining Cm pressure, whereas Δ*cprS* maintained pRY112-*cprR*^{Asp52Glu} at a higher rate than for either the empty plasmid or pRY112-*cprR*^{WT} (**FIG. 3.3**). Different retention rates for each allele were also observed when they were overexpressed in WT, and observation of routine cultures of these strains suggested subtle but consistent differences in growth behaviour were present (data not shown). Finally, these alleles appeared to show different activity when expressed in a heterologous host. The *cprR*^{WT} gene negatively affected growth of *E. coli*, and some clones of this gene were found to acquire transposon insertions (S.L. Svensson and E.C. Gaynor, unpublished observations). Also, while performing site-directed mutagenesis, no difficulty in creating *cprR*^{Asp52Ala} was found, but numerous attempts were required to obtain *cprR*^{Asp52Glu}, with many putative clones containing primer insertions or frameshift mutations that affected expression of the protein. Thus, as mutation of Asp52 of CprR to either Ala or Glu appeared to have contrasting effects on CprR activity, taken together with the inability to create *C. jejuni* strains solely expressing either of these proteins, suggests that both phosphorylation of CprR, and the ability to switch between phosphorylated and unphosphorylated states, may be required for growth under laboratory conditions.

Observations made in this work were consistent with the phenotypes of the Δ*cprS* sensor kinase mutant being the result of reduced phosphorylation of CprR. While pRY112-*cpr*R^{WT} and pRY112-*cpr*R^{Asp52Glu} partially complemented the biofilm phenotype of Δ*cprS*, pRY112-*cpr*R^{Asp52Ala} was severely detrimental to growth of the sensor kinase mutant. Strains with (presumably) reduced activity or levels of CprR, such as *cpr*R^{NTD} or *cpr*R^{KD}, also behaved like the Δ*cprS* mutant for biofilm formation and salt tolerance (**TABLE 3.1**). Sensor kinases can exhibit both phosphatase and kinase activities directed at their cognate regulators. Like full-length *cprS*, *cprS*^{CTD} complemented biofilm formation in Δ*cprS*. As such truncated forms of sensor kinases often exhibit constitutive kinase activity [523], and given the observation of the detrimental effect of overexpression of the Asp52Ala allele on D*cprS* fitness, complementation of Δ*cprS* by *cprS*^{CTD} was likely not due to gain of phosphatase activity alone. It follows that as CprS was dispensable, but phosphorylation of CprR was essential, low levels of CprR phosphorylation must occur in Δ*cprS* via non-cognate sensor kinases or AcP. Mutation of components of the *pta-ackA* pathway do not appear to affect phenotypes of Δ*cprS* (S. Svensson, S. Thompson, and E. Gaynor, unpublished observations), providing some evidence that AcP may not significantly contribute to CprR phosphorylation in the sensor kinase mutant. The RacS sensor kinase shows the closest sequence homology to CprS, and a double Δ*racS* Δ*cprS* mutant has been observed to be markedly affected for growth (S. Svensson and E. Gaynor, unpublished observations). However, phosphorylation of CprR by RacS *in vitro* has not been reported, and interaction between these two proteins has likewise not yet been shown. Phosphorylation of CprR appeared to increase its affinity for specific DNA sequences. CprR^{WT}, but not CprR^{Asp52Ala}, bound to a putative CprR consensus sequence in the one-hybrid system (**FIG. 3.4C**). Thus, loss of Δ*cprS* presumably reduces binding of the response regulator to specific promoters. It is currently unknown whether phospho-CprR is an activator or repressor of regulated genes; however, observations of the placement of the putative consensus sequence in front of the *htrA* transcription start site, together with reduced expression of *htrA* by RT-qPCR (**FIG. 3.6**), suggest that it may be required to activate expression of at least this gene.

A major aim of this work was to identify the CprRS regulon – genes whose expression may be directly controlled by activity of the TCRS. Because of the potential for cross-talk with non-cognate sensor kinases and AcP, it was originally hoped that expression analysis could be performed on both Δ*cprS* and a response regulator point mutant, such as Δ*cprR* pRY112-*cpr*R^{Asp52Ala} and/or Δ*cprR* pRY112-*cpr*R^{Asp52Glu}. Since these strains were not viable, it was then hoped that combination of Δ*cprS* expression data with a CprR-binding consensus sequence could help to identify genes directly regulated by response regulator binding. CprR^{CTD} was found to bind to the consensus sequence 5'-[G/C]TAAA[C/T] (**FIG. 3.4**). To date, the consensus sequence for three response regulators in *C. jejuni* has been identified (see **SECTION 1.7.4**). The consensus identified in this work did not resemble any of these. Interestingly, the related response regulator RacR was also found to bind a highly similar consensus sequence to that of CprR (**FIG. 3.5**). In contrast, *Salmonella* response regulators SsrB or PhoP did not bind the CprR/RacR consensus. Thus, we believe that the putative

shared consensus identified was not a result of an artifact of the one-hybrid system, although it remains to be seen of other *C. jejuni* response regulators, for which published consensus sequences are available (such as DccR) select a similar consensus using this technology.

Three phenomena may account for identification of the same consensus for CprR and RacR. First, expression of CprR in *E. coli* consistently negatively affects growth (data not shown), and thus expression of *C. jejuni* response regulators (such as CprR and RacR) may induce expression of a regulatory protein that recognizes one-hybrid library clones harbouring the CprR/RacR consensus. The *E. coli* consensus reported in the literature that is closest to the putative CprR/RacR-binding sequence identified in this study appears to be that of CpxR: 5'-GTAAN$_{6\text{-}7}$GTAA [524]. The negative selection procedure should have removed library clones that bind CpxR from the library pool, unless CpxR levels were too low under routine growth conditions (compared to those in *E. coli* expressing CprR or RacR) to bind one-hybrid targets and elicit reporter expression. Furthermore, while CprR^{CTD} severely affected growth of *E. coli*, RacR^{CTD} did not. This suggests that CprR and RacR have distinct effects on *E. coli* physiology and thus may not both activate an *E. coli* stress response regulator that may be binding to library clones. Moreover, because binding of full-length CprR to positive clones appeared to require the phosphoacceptor Asp52 residue, reporter activation was likely dependent on specific binding interactions such as those that occur upon phosphorylation, although reduced phosphorylation of CprR^{Asp52Ala} in *E. coli* also seems to reduce the negative effect of the regulator on *E. coli* growth. Second, expression of all *C. jejuni* response regulators, which share some conservation of primary sequence, especially under non-native conditions in *E. coli* as a fusion protein, may all bind to a sequence similar to that identified for RacR and CprR. As mentioned above, screening of the one hybrid library with a third C. jejuni response regulator, such as DccR, may provide evidence to clarify this. Third, in the absence of experimental artifacts relating to activity of *C. jejuni* response regulators in *E. coli*, identification of the same consensus for CprR and RacR may represent binding activity of these proteins that may be relevant to *C. jejuni* biology. CprR shows closer homology to RacR than to any other response regulator in *C. jejuni* or outside of the genus *Campylobacter*, with 51% identity and 72% similarity at the amino acid level. Thus, these proteins may actually bind similar consensus sequences. Whether both proteins bind such sequences *in vivo,* and if this affects gene expression or behaviour, remains to be seen.

The Δ*cprS* mutant showed broad expression differences from the WT strain by microarray analysis (**TABLE 3.2**, **TABLE C.1**). Many of these became more apparent over the course of the growth curve, and it is possible that some of these may have represented secondary or compensatory changes that were a response to dysregulation of the CprRS regulon, rather than a direct result of absence of CprRS signalling. Since Δ*cprS* was a deletion mutant (rather a conditional mutant where expression was controlled via an inducible promoter), CprS levels should thus be at steady state. However, since the *cprR* promoter increases in activity through log phase, and this could represent the growth period where CprRS signalling may be required, it follows that secondary expression changes may in fact be observed if the *cprS* lesion does not have a strong

effect on bacteria outside of exponential growth phase. For example, before log phase, Δ*cprS* bacteria may behave more similarly to WT, as CprRS signalling may not normally very active at this point. However, as cultures begin to grow logarithmically, the absence of the CprS sensor kinase may be of greater consequence and secondary expression changes may thus begin to appear. Because the consensus identified for CprR in this study may be shared by RacR, and was also too short for genome-wide scans, this sequence could not be used to narrow down the directly-regulated CprRS regulon. Instead, attention was focused on genes that were significantly complemented in the Δ*cprS*^{C} strain. Furthermore, attention was focused on genes that were observed to be dysregulated at earlier time points, before putative secondary expression changes began to appear. These genes fell into four groups based on expression pattern and functional category (**TABLE 3.2**). Most notably, dysregulation of many envelope-related genes was observed, as well as both *cprR* and the adjacent *htrA* gene. Furthermore, as the growth curve progressed, increased expression of RpoN- and FliA-dependent flagellar genes was observed.

Global expression changes in the Δ*cprS* mutant was previously analyzed by proteomics (**FIG. 2.6**), and observed lower expression of translation-related proteins, increased expression of stress tolerance genes, and upregulation of the flagellin FlaA, which also suggested that the Δ*cprS* mutant was entering into stationary phase earlier than the WT strain. For a bacterium that was originally proposed to lack a stationary phase response due to the absence of RpoS [399], transition of *C. jejuni* into stationary phase is surprisingly active, and marked by a peak in motility, significant changes in membrane composition, and metabolic substrate switching, despite expression changes suggestive of metabolic downshift [76, 244]. Most of the consistencies between proteomics and microarray identified in this work lay within metabolic or flagellar pathways that may be regulated in response to growth phase. However, the increased sensitivity of microarrays allowed identification of many more stationary phase-related genes that were significantly upregulated in Δ*cprS*. Samples were taken for microarray analysis at the same time for each culture, rather than at the same culture density, and the expression differences in Δ*cprS* from WT were consistent with a marked difference in growth phase, such as accelerated expression of middle and late flagellar operons [76]. Although they did not reach the significance cutoff set out during data analysis, a general trend of decreased expression of respiratory and metabolic operons (such as the *nrf*, *nap*, *sdh*, and *frd* clusters) and protein translation-related genes (such as those encoding ribosomal proteins and translation factors), as well as increased expression of stress response genes (such as *groESL*, *sodB*, and *katA*), was observed in Δ*cprS*. Stationary phase-related genes did not appear to be as highly upregulated in the Δ*cprS*^{C} complemented strain compared to Δ*cprS*; however, complete complementation of expression in Δ*cprS*^{C} was not observed, especially at later time points. For example, *flaB* showed 4-fold upregulation in Δ*cprS* compared to WT, but 3.6-fold upregulation in Δ*cprS*^{C}. Of note, complementation was achieved by expressing *cprS* from the Kan^{R} cassette promoter using pRRK, based on the pRRC chromosomal integration system [483]. The resulting strain was often complemented for growth and biofilms, but phenotypic complementation of phenotypes between experiments was sometimes variable.

This suggests that complementation of Δ*cprS* using the pRRK system may not allow sufficient (or native) *cprS* expression under all conditions to fully complement all of the Δ*cprS* expression changes and rescue phenotypes.

Upregulation of Cj0076c-Cj0073c was observed throughout the growth curve in Δ*cprS*. This operon encodes a novel lactate dehydrogenase (LldEFG/LutABC), as well as an L-lactate transporter, that contributes to respiratory growth on lactate [74]. The Cj0076c-Cj0073c operon is expressed from a single σ^{70}-dependent promoter upstream of the permease gene, and is regulated in a growth-phase dependent fashion, with highest expression in late log and early stationary phase [74]. Thus, upregulation of this operon in Δ*cprS* may also be indicative of early entry into stationary phase. Both L- and D-lactate are good carbon sources for the microaerobic growth of *C. jejuni*, and are abundant products of fermentation produced by resident anaerobes of the gut with which *C. jejuni* shares a niche [525]. It may be interesting to determine if *C. jejuni* growing in biofilms preferentially use lactate as a carbon or energy source.

Envelope-related genes were observed to be disproportionately dysregulated in Δ*cprS* at early time points, and these may represent key members of the CprRS regulon. Some of the putatively CprRS-controlled envelope genes have been previously characterized. Omp50 is a cation-selective porin that is regulated in a temperature-dependent fashion [526, 527]. The protein encoded by Cj1279c was recently renamed FlpA: it binds fibronectin and is required for host cell adherence [183, 184]. PglK transports intermediates for *N*-linked protein glycosylation [528, 529]. A YceI homologue like that encoded by Cj0420 was recently characterized in *H. pylori*, and was proposed to sequester specific fatty acids or amides from the environment, either for its own metabolism, or as protection from detergent-like fatty acids [530]. Finally, Gne is required for biosynthesis of LOS, CPS, and *N*-linked protein glycosylation, and forms biofilms similar to the WT strain [368, 531]. Because CprR was essential for viability of *C. jejuni*, CprRS may be required for expression of essential genes. Control of envelope-related genes by CprRS was certainly consistent with the essential nature of CprR. For example, the WalKR TCRSs of Gram-positive bacteria are thought to control diverse aspects of the cell wall and are essential in most species. The WalK sensor is thought to sense Lipid II availability, and the WalR regulator binds and regulates genes such as those involved in PG metabolism [449]. Some of the putative CprRS regulon members have been reported to be essential. These include the Cj0168c periplasmic protein, Cj1169c periplasmic protein, *fumC* (fumarate hydratase), and *fba* (fructose bisphosphate aldolase) [64, 65].

The *htrA* gene was also observed to be dysregulated in the Δ*cprS* sensor kinase mutant, and is encoded adjacent to *cprRS* (**FIG. 2.1**). HtrA proteins are heat shock-induced periplasmic serine proteases that are important for maintenance of cell envelope proteins [532]. The *C. jejuni* HtrA homologue contains both chaperone and protease activities, contributes to heat and O_2 tolerance, and is required for host cell binding, likely by maintaining characteristics of the cell envelope [255, 256]. Expression of many HtrA homologues is

controlled by envelope stress-related TCRSs, such as MprAB of *M. tuberculosis*, Cpx of *Yersinia enterocolitica*, and CssRS of *B. subtilis* [533-535]. Analysis of *htrA* expression and binding of $CprR^{CTD}$ to the *htrA* promoter region suggest that expression of the *C. jejuni htrA* homologue may also controlled by a TCRS – CprRS. A putative inverted repeat containing two 6 bp CprR/RacR consensus sequences was present in the *htrA* promoter, immediately upstream of the transcription start site (**FIG. 3.7A**), and $CprR^{CTD}$ binds the region harbouring this sequence. Phenotypes of Δ*cprS* were consistent with observed dysregulation of *htrA*, including decreased osmotic and oxidative stress tolerance (**FIG. 2.7**). Morphological differences from WT were shown by Δ*cprS* and *cpr*R^{OE} (**FIG. 3.9**), as well as a Δ*htrA* mutant [256]. Modestly enhanced biofilm formation was also observed in D*htrA* (**FIG. 3.7D**). However, as biofilm formation in D*htrA* was not as enhanced as in Δ*cprS*, its biofilm phenotype could not be fully attributed to the dysregulation of *htrA* that was observed. Another *C. jejuni* mutant with a lesion in a potentially CprRS-regulated envelope biogenesis gene, D*peb4*, has been reported to have enhanced biofilm formation [375]. Peb4 encodes a SurA-like periplasmic peptidyl-prolyl *cis/trans* isomerase, and mutation of *peb4* has dramatic effects on the outer membrane profile of *C. jejuni* [375]. As mutation of *htrA* was not sufficient to replicate the enhanced biofilm phenotype of Δ*cprS*, it follows that dysregulation of numerous proteins involved in expression and maintenance of envelope proteins may contribute additively to elicit enhanced biofilm formation in the sensor kinase mutant.

The cell envelope is critically important for interaction of a bacterium with its environment, which includes nutrients, osmolytes, antimicrobial agents, metabolic byproducts, and possibly, host-related phenomena. The cell envelope may thus be central to triggering adaptation to stress conditions, such as those that require biofilm formation. Two general observations of expression changes in the Δ*cprS* mutant, together with the observation that envelope genes were overrepresented in the CprRS regulon, led to the formation of two hypotheses for the enhanced biofilm formation phenotype displayed by Δ*cprS*. The Δ*cprS* mutant enters stationary phase earlier than the WT strain, and envelope-related genes were observed to be dysregulated in Δ*cprS*. It was thus hypothesized that envelope changes in Δ*cprS* may either A) affect its ability to obtain nutrients, thus stimulating entry into stationary phase and consequently biofilm formation; or B) cause envelope stress, thus stimulating formation of stress-tolerant biofilms.

Nutrient availability is a common signal for biofilm formation and dispersal [536]. In *C. jejuni*, increased nutrient availability inhibits biofilm formation [420, 537], and it was previously noted that addition of fumarate delays biofilm formation in WT (**FIG. 2.9**). Addition of fumarate to Δ*cprS* did not suppress its enhanced biofilm phenotype (S. Svensson and E. Gaynor, unpublished observations). However the Δ*cprS* microarray suggests genes involved in uptake and metabolism of C4-dicarboxylates, such as the *dcuB* transporter genes and fumarate reductase, were downregulated in Δ*cprS* (**TABLE C.1**). Tenfold lower levels of *dcuA* transcript have also been measured in Δ*cprS* (S. Svensson and E. Gaynor, unpublished observations), suggesting that this mutant may not have been able to efficiently utilize fumarate. Nonetheless, both biofilm and growth phenotypes of Δ*cprS* were suppressed by increasing broth strength from 1X to 1.5X (**FIG. 3.7**).

This was consistent with an inability of this strain to obtain nutrients under routine culture conditions, either due to dysregulation of metabolic/transport genes, or alternatively, altered expression and maturation of envelope-localized proteins due to dysregulation of genes such as *htrA*.

While collectively, these observations suggest that *C. jejuni* biofilm formation may be a response to decreased nutrient availability and that Δ*cprS* may be forming enhanced biofilms due to a starvation response, it was also observed that Δ*cprS* cultures reached lower densities in broth concentrations both lower and higher than 1.5X (**FIG. 3.7A**). This suggested that the mutant may also be sensitive to increased concentrations of certain media components. If the mutant did in fact solely require higher nutrient concentrations, one might expect that it would show a more saturating response to changing broth concentrations, rather than a Gaussian response. Altered envelope physiology could require a higher concentration of nutrients to obtain levels that allow significant growth, but increasing broth concentration too much may increase levels of potential toxins beyond a particular threshold. Broth strength also affects levels of compounds providing osmotic support, such as divalent cations that stabilize the outer membrane, and lower broth concentrations possibly do not provide enough osmotic support for the Δ*cprS* mutant. Consistent with this, biofilm formation in D*cprS* was returned to levels of WT when 1X MH broth was supplemented with divalent cations (Mg^{2+} or Ca^{2+}) (**FIG. 3.7**). Divalent cations are normally associated with stabilization of LPS/LOS [516], thus, while this does not exclude decreased nutrient acquisition capability in Δ*cprS*, it does provide evidence that the envelope of D*cprS* may have been compromised. Gross differences in LOS species in Δ*cprS* by gel electrophoresis were not previously noted (**FIG. 2.4**), although more subtle differences in LOS structure may have been present.

Upon observation of dysregulation of envelope genes, morphology of Δ*cprS* was observed in more detail using TEM. Previously, in efforts to understand the late-stage culturability defect of Δ*cprS*, it was shown that this strain does not proceed to coccoid morphology faster than the WT strain, and exhibits helical morphology (**FIG. 2.3**). Consistent with SEM data, rather than accelerated progression to coccoid like WT, the Δ*cprS* mutant was observed forming comma- or apostrophe-like forms (**FIG. 3.8**). A *cprR*^{OE} overexpressing strain also showed morphological differences. Furthermore, in addition to stabilization of LPS, stabilization of the murein sacculus by magnesium has been noted [518]. Morphology of bacteria is closely tied to the PG layer [89], and mutation of PG modifying genes affect the shape of both *C. jejuni* (E. Frirdich and E. Gaynor, in press) and *H. pylori* [147]. Consistent with shape changes, the PG structure of both Δ*cprS* and *cprR*^{OE} was observed to be modestly different from the WT strain (**TABLE 3.3**). Most notably, the Δ*cprS* mutant appeared to have an increase in pentapeptide species. Mutations and antibiotics that affect this reaction, such as those affecting penicillin-binding proteins, can interfere with transpeptidation, which releases the terminal D-Ala residue from the donor peptide. Thus, such conditions can increase the amount of pentapeptide species. However, a decrease in the degree of crosslinking was not observed in Δ*cprS*, which would be expected if transpeptidation was inhibited.

The amount of crosslinking must be carefully controlled in order to provide the appropriate PG structure for a particular shape or for mechanical strength [148]. New PG often exhibits a higher amount of pentapeptide species, which are then trimmed by specific DD-carboxypeptidases [145]. These enzymes remove the terminal D-Ala residue from a peptide chain, and this is thought to control the number of donors for transpeptidation reactions, and therefore controls the degree of crosslinking [148]. Mutations that affect DD-carboxypeptidases can increase pentapeptide-containing muropeptide species in PG [538, 539], as was observed in Δ*cprS*. Thus, this strain may harbour lower activity of a DD-carboxypeptidase, leading to an increase in pentapeptides. To date, such an enzyme has not been characterized in *C. jejuni*. The concurrent decrease in the number of tripeptides observed in Δ*cprS* compared to WT also suggested that activity of such a DD-carboxypeptidase may be required to provide a substrate for a second carboxypeptidase required to further trim tetrapeptides to tripeptides. As new PG often exhibits higher amounts of pentapeptide species, it is also possible that Δ*cprS* did not have a defect in PG trimming, but was synthesizing PG more actively than the WT strain. Consistent with this, the increase in pentapeptides did not appear to affect the overall degree of crosslinking in Δ*cprS* PG. However, as overall muropeptide content was analyzed, there could be regions of the Δ*cprS* sacculus that do harbour reduced levels of crosslinking, which may be counterbalanced by increases elsewhere in the cell wall. Whether this is the case is currently unknown; however, a defect in PG crosslinking in some regions of the cell wall is consistent with increased osmotic sensitivity observed in Δ*cprS*. Finally, if PG differences in Δ*cprS* were a result of defects in the PG machinery (rather than an increase in nascent PG), it is unknown whether the observed PG changes were due to altered expression of components of the PG biosynthesis and modification machinery, or inappropriate localization and/or reduced activity of such components due to other changes in the envelope. Nonetheless, as biogenesis of PG must be carefully controlled both spatially and temporally an effect of CprRS on construction of the *C. jejuni* cell wall is consistent with the essential nature of the CprR response regulator.

The *cpr*R^{OE} strain also showed distinct PG changes from both WT and Δ*cprS*. Specifically, an increase in dipeptide-containing species was observed. The structure of PG often changes between growth phases, such as during the transition between log and stationary phase [150, 519], and is also thought to undergo significant changes upon transition to the VBNC state. While the structural changes that mark the *C. jejuni* transition to stationary or VBNC have not yet been elucidated, in *H. pylori*, the transition to a coccoid morphology, which may be a feature of the VBNC state, has been reported to involve an increase in dipeptide species [154]. However, while we observed an increase in dipeptides in *cpr*R^{OE}, microscopy suggested that the transition to coccoid in this strain appeared to be delayed compared to WT. Thus, such PG changes were not sufficient to allow coccoid formation in *cpr*R^{OE}. PG from *cpr*R^{OE} also showed some similarities to Δ*cprS*, in that it contained a small increase in pentapeptide species, suggesting that overexpression of CprR may cause changes in PG-related enzyme expression and/or their activity similar to those caused by deletion of the sensor

kinase. It is unknown whether these changes may have affected ability of either Δ*cprS* or *cpr*R^{OE} to transition to coccoid morphology like WT.

It was previously noted that supernatants of overnight cultures of Δ*cprS* contained more protein than those of WT (**FIG. 2.8**) and it was hypothesized that cell envelope differences in the mutant that lead to lysis may underlie this observation. The late stage culturability defect, together with PG changes may be consistent with lysis in the Δ*cprS* mutant. Increased amounts of the cytoplasmic protein CosR were observed in supernatants of Δ*cprS,* providing evidence that it was undergoing lysis. Since *C. jejuni* secretes a subset of proteins via the flagellar export apparatus [121], and because increased expression of flagellar was observed in Δ*cprS*, both by proteomics and microarrays, this finding does not completely rule out secretion as a mechanism for release of the protein species observed previously, especially as flagella appear to be required for *C. jejuni* biofilm formation [369, 378]. Measurement of proteins in the supernatant of Δ*cprS* following mutation of components of the flagellar export apparatus, such as *flhA*, will provide further support that release of proteins observed in this strain was lysis-dependent.

While moderate changes in PG structure were observed in Δ*cprS*, it is unclear whether this was directly related to increased lysis in the strain. Nonetheless, metabolism of PG is intimately related to mechanisms of autolysis [148, 540]. For example, β-lactam antibiotics stimulate autolysis [541], and autolytic mechanisms often involve either dedicated or housekeeping enzymes that cleave specific bonds within PG. These include lytic transglycosylases, amidases that cleave the peptide crosslink from Mur*N*Ac residues, and endopeptidases or carboxypeptidases that cleave linkages in peptide crosslinks [148]. An active autolytic mechanism in *C. jejuni* has not yet been described, and understanding of proteins required for routine maintenance and biogenesis of the PG layer in the ε-proteobacteria is also currently relatively limited. Decreased glycan chain length was not observed in Δ*cprS* PG, which would be suggestive of increased activity of lytic transglycosylases. Furthermore, an increase in shorter disaccharide-peptide species was not observed, which would be expected if endopeptidases were activated. Furthermore, lysis in Δ*cprS* may have been a passive process, resulting from an inability to maintain cell wall integrity under osmotic pressure due to changes in PG or other envelope structures. Thus, it is presently unclear whether lysis in Δ*cprS* represents upregulation of an active autolytic process or if the mutant was simply exhibiting passive lysis. Nonetheless, autolytic mechanisms commonly underlie either biofilm formation or dispersal in other bacteria [153], and a close relationship between both the envelope stress response and cell wall turnover with biofilm formation has also been reported in Gram-negative bacteria [419, 542]. Determining if both lysis and biofilm formation is suppressed in Δ*cprS* by addition of divalent cations will support a connection between these phenomena.

This TCRS was originally named CprRS after observing the enhanced biofilm phenotype of Δ*cprS*, and hypothesized that CprRS (*Campylobacter* planktonic growth regulation) was required for expression of genes required for planktonic growth. CprRS appeared to be expressed most highly during rapid growth in log

phase and rapidly shut off before entry into stationary phase (**FIG. 3.1**). Furthermore, *cprR* transcript levels and growth behaviour of Δ*cprS* and *cprR*^{OE} suggested that CprRS activity correlates with growth rate (**FIG. 3.1**). An increase in both the log phase growth rate and differences in PG structure were observed in the *cprR*^{OE} overexpressing strain, compared to both WT and Δ*cprS*. PG structure is altered during the helical to coccoid transition in *C. jejuni* [543, 544], and in *H. pylori*, this process is marked by an increase in Glc*N*Ac-Mur*N*Ac-dipeptide species [154, 545]. Interestingly, the *cprR*^{OE} strain showed an increase in dipeptide-containing muropeptides. However, *cprR*^{OE} showed a decrease, rather than an increase in, coccoid morphology. It is possible that this strain was eliciting PG changes such as those that occur during the helical-to-coccoid transition, but other envelope defects prevent progression to coccoid shape. Nonetheless, the increased growth rate of this mutant in log phase was consistent with earlier display of PG muropeptides that mark the progression to VBNC.

Thus, CprRS may be required to express envelope-related genes required for logarithmic growth. Specific environmental signals detected by CprS may be required to express genes necessary for taking advantage of high nutrient conditions, and the Δ*cprS* mutant may represent a bacterium that cannot escape the biofilm mode of growth. However, it is still unclear whether biofilms represent the default lifestyle for *C. jejuni*. Alternatively, adverse conditions may stimulate progression to a biofilm lifestyle, and it follows that biofilm formation may be a stress response of the Δ*cprS* mutant to the dysregulation of envelope genes that was observed. Generally, it has been observed that conditions that may affect the envelope (**FIG. 2.8**), as well as mutations that affect aspects of envelope biogenesis (for example, Δ*peb4*, Δ*kpsM*, and Δ*waaF*) result in enhanced, rather than defective, biofilm formation in *C. jejuni* [87, 368, 375]. This provides evidence for the notion that biofilm formation may be a response to stress, and specifically envelope stress, in *C. jejuni*. Confirmation that that biofilm lifestyle confers increased stress tolerance to *C. jejuni* will certainly provide support to this last hypothesis. Microarray analysis of Δ*cprS* suggests that reduction of CprRS activity has a pleiotropic effect on *C. jejuni* physiology. Combining microarray analysis of the *cprR*^{OE} strain and refinement the CprR binding consensus sequence with the Δ*cprS* expression data may allow more accurate definition of the CprRS regulon. This may help determine the exact role of CprRS in control of either self-preservation or nutritional competence.

4 FLAGELLA-MEDIATED ADHESION AND RELEASE OF EXTRACELLULAR DNA CONTRIBUTE TO BIOFILM FORMATION AND STRESS TOLERANCE OF *CAMPYLOBACTER JEJUNI*

4.1 Introduction and synopsis

Campylobacter jejuni is a leading cause of food and waterbourne gastroenteritis, despite displaying fragile and fastidious behaviour under standard laboratory conditions. In the natural environment, *C. jejuni* may survive mostly as part of biofilms – sessile communities of microorganisms encased in a protective polymeric matrix. This lifestyle is thought to impart resident bacteria with distinct characteristics from their planktonic counterparts grown in rich broth culture, such as enhanced stress tolerance. Despite importance of biofilms to *C. jejuni* survival in environments related to pathogenesis, little is understood about the mechanisms of biofilm formation in this organism. Furthermore, evidence for their role in providing stress tolerance in *C. jejuni* is weak. Previous characterization of the CprRS TCRS provided evidence for a possible relationship between flagellar expression, envelope stress, lysis, and biofilm formation in *C. jejuni* (**CHAPTER 2-3**). A further understanding of both the mechanisms underlying biofilm formation, and whether it imparts greater resilience to *C. jejuni*, were sought through continued analysis of the biofilm-enhanced Δ*cprS* two-component sensor kinase mutant. Specifically, roles for components such as flagella and eDNA were clarified through analysis of biofilm formation in flagellar mutants, as well as biofilms grown in DNase and under conditions that enhance biofilm formation, such as presence of DOC. Epistasis experiments with Δ*cprS* and flagellar mutations (Δ*flhA*, Δ*pflA*) suggested that biofilm formation was initiated by adherence to a surface into microcolonies in a process mediated by flagellar adhesion, and that motility may aid kinetics of biofilm formation. Enhanced lysis was also observed in Δ*cprS*, as well as in biofilm-enhanced WT bacteria grown in DOC, suggesting that adherence was followed by a lytic process that releases eDNA. This process was independent of the flagellar export apparatus, and allowed maturation into three-dimensional biofilm structures in a mechanism that was inhibited by DNase I and presumably mediated by eDNA. Finally, inhibiting biofilm formation by mutating flagella or removing eDNA with DNase decreased stress tolerance of *C. jejuni*, and release of eDNA during conditions promoting biofilm formation may also contribute to genetic plasticity of *C. jejuni* biofilm communities. Thus, the biofilm lifestyle may provide *C. jejuni* with resilience during transmission and pathogenesis that is not apparent in planktonic bacteria.

4.2 Materials and Methods

4.2.1 Routine and experimental bacterial culture.

Bacterial strains and routine culture conditions. Studies were performed using the *C. jejuni* WT strain 81-176. Mutant strains are listed in **TABLE A.1**, and have been described previously, such as the Δ*cprS* (**CHAPTER 2**), Δ*flhA*, and Δ*flgR* [408]. The Δ*pflA* mutant was isolated from a transposon mutant screen using the Mariner system developed for *C. jejuni* [103]. *C. jejuni* was cultured microaerobically in MH media

supplemented with vancomycin and trimethoprim, as in **CHAPTER 2**. Where appropriate, antibiotics Kan, Cm, and Str were added to a final concentration of 40 μg mL^{-1}, 15 μg mL^{-1}, and 100 mg mL^{-1}, respectively.

Crystal violet biofilm assay. Biofilm formation was assessed as previously described (**CHAPTER 2**). Where indicated, DNase (Invitrogen) was added to a final concentration of 90 U mL^{-1}, and antimicrobial agents (Sigma) were added to the following concentrations: DOC, 0.05%; PxB, 0.12 mg mL^{-1}; Amp, 1.25 mg mL^{-1}. DNA was added to biofilm cultures at a concentration of 500 ng $mL^{-1}$and was either isolated from WT *C. jejuni* using the Qiagen genomic tip 100/G kit (gDNA), or purchased from Sigma (salmon DNA; ssDNA).

Static culture growth. Standard biofilm cultures of each strain (WT, Δ*cprS*, Δ*flhA*, and Δ*cprS* Δ*flhA*) were set up at an OD_{600} of 0.002 in either MH broth alone, MH + 0.05% DOC, MH + 90 U mL^{-1} DNase I, or MH + 0.05% DOC + 90 U mL^{-1} DNase I. All cultures were supplemented with trimethoprim and vancomycin. Following 2 days of growth under microaerobic conditions, tubes were either stained with CV to assess biofilm formation, or vortexed for one min., followed by measuring OD_{600}.

Measurement of genetic recombination. Recombination was measured in mixed-strain shaking broth cultures. Each strain was marked (on the chromosome) with different antibiotic resistance markers, and recombination was determined by measuring appearance of doubly resistant recombinant clones. Briefly, WT (marked with Str^R) was grown in mixed culture (1:1) with either an isogenic WT strain (marked with Kan^R; insertion into an rRNA spacer via pRRK) or the Δ*cprS* hyperbiofilm mutant (marked with Kan^R; allelic replacement of the *cprS* locus). Cultures were grown in either MH alone or MH with 0.05% DOC. Cells were removed A) immediately following inoculation and B) following 8h growth and plated on MH agar with the appropriate antibiotics (Kan, Cm, and/or Str) for CFU determination.

4.2.2 Microscopy.

Confocal microscopy of biofilms. For confocal microscopy, a plasmid encoding green fluorescent protein (GFP) expressed from the strong *atpI*' promoter [512] was introduced into *C. jejuni* strains. Biofilm cultures were set up as in above, except a glass coverslip included in each tube. Following 12h, 24h, or 36h, media was removed and biofilms were fixed by the addition of 4% paraformaldehyde in PBS, pH 7.4 for 15 min. Fixing solution was removed and replaced with PBS and coverslips were stored at 4°C. Samples were mounted using Prolong Gold Antifade with DAPI (4',6-diamino-2-phenylindol; Invitrogen), and imaging was performed with an Olympus Fluoview FV1000 laser scanning confocal microscope with FV10-ASW 2.0 Viewer software to adjust images.

4.2.3 Molecular and biochemical analyses.

Detection of bacterial cell lysis. Bacterial lysis was assessed by Western blot analysis of culture supernatants, similarly to **CHAPTER 3**. Samples were then analyzed by SDS-PAGE followed by silver staining or Western blotting, using an anti-CosR (S. Thompson) or anti-FlgR [393] antibody.

Quantification of eDNA. Levels of eDNA were measured using QPCR. Culture supernatants were prepared as in above and were used as templates for QPCR reactions using primers *cprR*-QPCR-FWD/REV. QPCR was performed in triplicate using IQ SYBR Green Supermix and MyIQ Real-time PCR Detection System (Biorad, Mississauga, ON) according to the manufacturer's specifications.

4.3 Results

Culture supernatants of hyperbiofilm *C. jejuni*, such as Δ*cprS* and WT grown in DOC, contain more protein species, even in the absence of expression of the flagellar export apparatus. The Δ*cprS* mutant previously showed increased amounts of protein species in the media following routine culture (**FIG. 2.8**). Release of specific proteins via the flagellar export apparatus has also been reported in *C. jejuni*, and it was initially hypothesized that, because expression of proteins comprising the flagellum were increased in this mutant, appearance of protein species in Δ*cprS* supernatants may have been dependent on secretion by the flagellar export apparatus. However, Western blotting later demonstrated that both Δ*cprS* and *cprR*^{OE} mutants harboured cytoplasmic protein in culture supernatants (**FIG. 3.9**), suggesting that lysis was occurring, and that appearance of the proteins was independent of flagellar secretion. To confirm that appearance of these proteins was occurring independently of flagella-mediated secretion, the protein profile of culture supernatants from the Δ*cprS* mutant was compared to that of a Δ*cprS* Δ*flhA* double mutant (**FIG. 4.1A**). The Δ*flhA* mutation eliminates flagellar secretion [121]. A similar protein profile to Δ*cprS* was observed for conditioned media harvested from an aflagellate Δ*cprS* Δ*flhA* mutant, which suggested that flagella-mediated secretion was not responsible for the proteins in the media of Δ*cprS*. Sub-MIC levels of DOC are known to stimulate expression of flagellar secreted proteins [121], and like Δ*cprS*, WT bacteria grown in DOC form

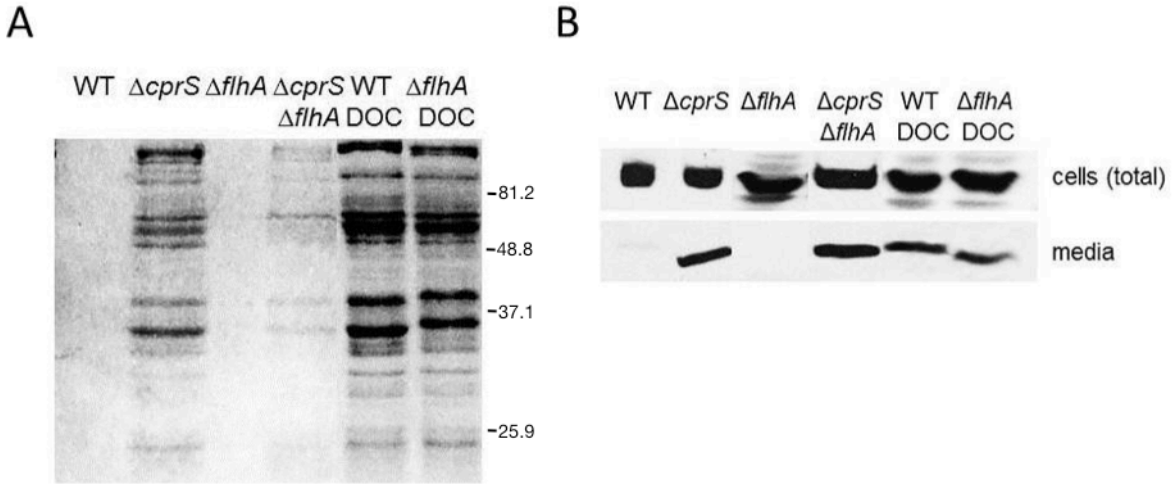


FIG. 4.1. An increase in lysis, occurring independently of the flagellar export apparatus, is seen in strains and under conditions that promote biofilm formation. **A**) Appearance of protein species in supernatants occurred independently of expression of flagella. Concentrated culture supernatants, isolated from cultures of the indicated strains at similar optical densities following 24h of growth, were analyzed by SDS-PAGE and silver staining. **B)** A cytosolic protein appeared in culture supernatants, independently of expression of the flagellar export apparatus, which was consistent with a lytic process. Culture were analysed by SDS-PAGE and Western blotting with antibodies to detect cytosolic response regulator CosR.

enhanced biofilms. It was hypothesized that such conditions, like the Δ*cprS* mutation, may also increase the appearance of protein species in the media following routine culture. Consistent with this, a similar protein profile to Δ*cprS* was noted in supernatants from both WT in MH+DOC. Moreover, an aflagellate Δ*flhA* mutant grown in MH+DOC also showed a similar increase in media protein species. Again, this suggested that the appearance of proteins in the supernatants of WT *C. jejuni* grown in the presence of DOC, like in Δ*cprS*, was also occurred independently of flagellar secretion.

As release of proteins appeared to be occurring independently of expression of the flagellar export apparatus, and we previously noted increased amounts of cytoplasmic protein in Δ*cprS* supernatants compared to WT, this suggested that lysis was occurring. However, to confirm that release of proteins into the supernatant of both Δ*cprS* and WT in MH+DOC was occurring through a non-selective lytic process that did not require flagellar expression, supernatants of flagellate and aflagellate *C. jejuni* strains were analyzed for the presence of the cytoplasmic protein CosR. As before, significant amounts of CosR were detected in Δ*cprS* supernatants, but not in WT supernatants (**FIG. 4.1B)**. Like WT, supernatants of Δ*flhA* also did not contain cytoplasmic protein. However, CosR was detected in supernatants of both Δ*cprS* and a Δ*cprS* Δ*flhA* mutant, confirming that flagella-mediated secretion was not responsible for appearance of extracellular cytoplasmic protein. Moreover, cytosolic protein was also seen in supernatants of both WT and Δ*flhA* bacteria grown in DOC. Together, this suggested that a non-selective lytic mechanism, occurring independently of flagella-mediated export, underlies appearance of protein in supernatants of both Δ*cprS* and WT bacteria grown in MH+DOC.

Amount and timing of eDNA release appear to correlate with biofilm formation. In addition to protein species that may mediate biofilm formation, lysis can also release eDNA [155, 342]. PI-staining fibres were previously observed surrounding *C. jejuni* biofilms (**FIG. 2.4**; **FIG. 2.8**), and these were especially apparent in biofilm-enhanced bacteria (Δ*cprS* in MH alone; WT in MH+DOC). However, while the amount of eDNA appeared to correlate with the level of biofilm formation, it was unclear whether it contributed mechanistically to biofilm formation or provided biofilms with distinct characteristics. Thus, attention was focused on the relationship between DNA release and biofilm formation. To determine if the amount of eDNA was in fact increased under conditions that enhance biofilm formation, QPCR was performed to measure the relative DNA concentration of culture supernatants. A significant ($p<0.0001$) threefold increase in the amount of DNA in the supernatant of Δ*cprS* compared to WT in MH broth alone (**FIG. 4.2A**). Furthermore, three- to fourfold more DNA was also measured in supernatants of WT in MH+DOC compared to WT grown in MH alone ($p=0.0015$). This suggested that bacteria growing in conditions that enhance both lysis and biofilm formation also showed increased amounts of eDNA.

To determine the temporal relationship between eDNA release and biofilm formation, confocal microscopy was used to observe formation of biofilms by green GFP-expressing bacteria. DNA was stained blue with DAPI. At early time points (i.e., 12h), WT bacteria appeared to be adhered to the slide in small microcolonies

(**FIG. 4.2B**). This was followed, at 24h, by the appearance of blue DNA. This appeared to be more prevalent in regions where bacteria were either lysing or no longer expressing GFP, as fewer green bacteria were observed near more prominent areas of DAPI-stained material. As time progressed, the DNA formed strand-

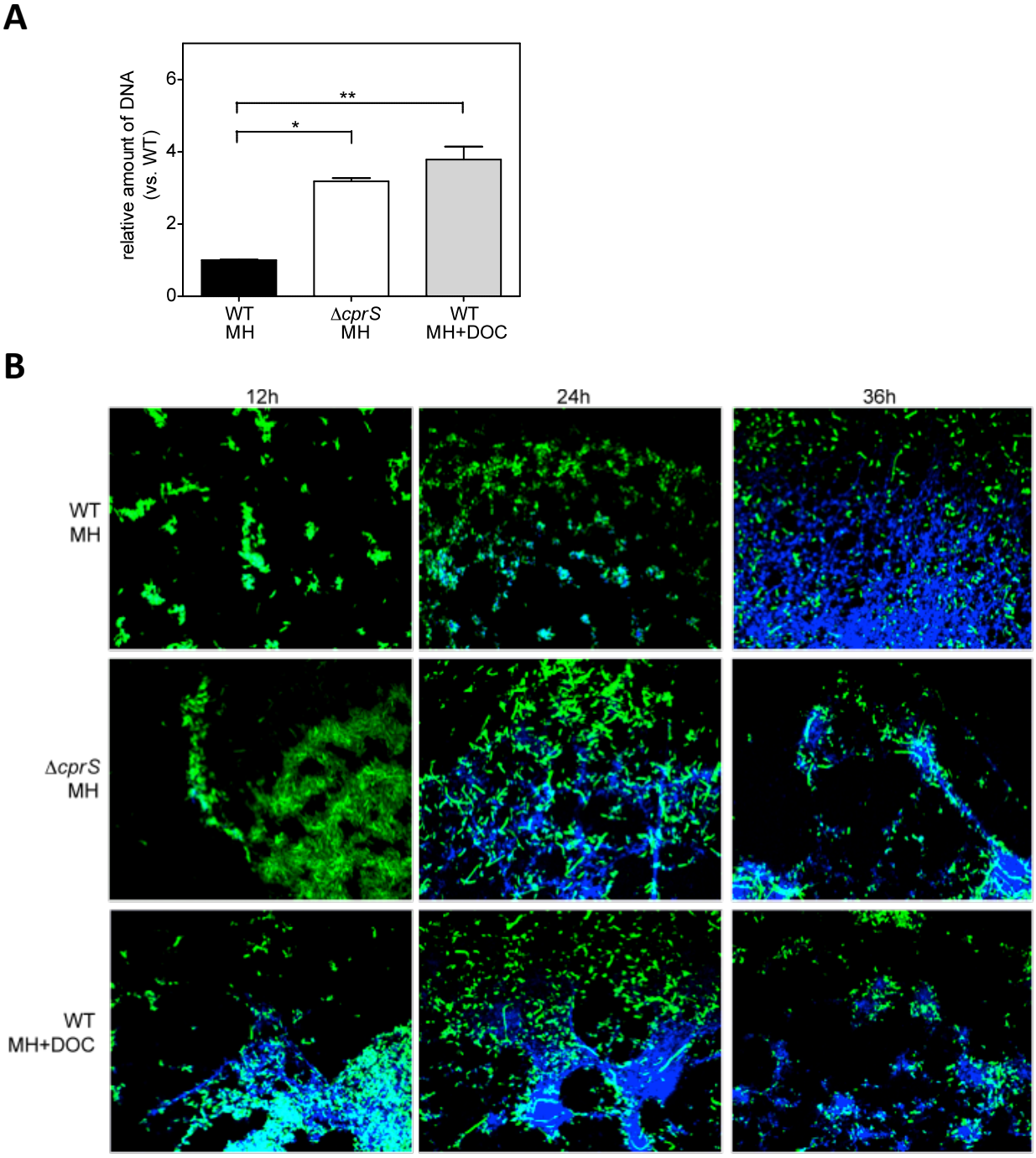


FIG. 4.2. Biofilm formation correlates with DNA release. A) Supernatants from biofilm-enhanced bacteria contain more eDNA than those from WT. Cell-free supernatants were isolated from WT or Δ*cprS* cells in plain MH, or WT in MH+0.05% DOC. Total DNA was quantified by QPCR. **p*<0.0001; ***p*=0.0015. **B)** DNA is observed following attachment and is enhanced in Δ*cprS* and WT in DOC. Bacteria expressing GFP were allowed to form biofilms on coverslops in plain MH or MH+0.05% DOC. At indicated times, slides were fixed, DNA was stained with DAPI, and biofilms were visualized by confocal microscopy.

like structures and biofilm thickness increased. For the Δ*cprS* mutant in MH alone, as well as for WT in MH+DOC, a similar progression was observed to WT in MH alone; however, in these cultures, the process appeared more rapid and pronounced. For example, at 12h, Δ*cprS* mutant bacteria were present as larger aggregates, already with minor foci of blue DNA and relatively extensive three-dimensional structure. By 24h, large amounts of DNA were observed in strand-like structures. Thus, eDNA seemed to appear following attachment, and release appeared to be more pronounced in biofilm-enhanced cultures, where it appeared to correlate with development of more elaborate three-dimensional structures.

Addition of exogenous DNA to standing cultures of *C. jejuni* enhances biofilm formation. *C. jejuni* biofilms can be disrupted with DNase I (**FIG. 2.4**; **FIG. 2.8**), suggesting that eDNA may play a structural role in *C. jejuni* biofilms. To confirm that appearance of eDNA was not simply a consequence of biofilm formation, but affects maturation of *C. jejuni* biofilms, it was determined if addition of DNA to standing cultures could enhance biofilm formation of WT (**FIG. 4.3**). First, supernatants concentrated for high molecular weight components, were added to standing cultures during a CV biofilm assay. WT grown in media to which supernatants were added ('MH+WT_{sup}') showed a significant increase in biofilm formation (p=0.08) compared to WT in MH alone, but not as much of an increase as Δ*cprS* in MH or WT in MH+DOC. As supernatant fractions likely contained multiple high molecular weight components, as well as potentially inhibitory metabolites, purified DNA was added to separate standing cultures in order to determine if the effect of supernatants on biofilm formation was specific to DNA contained in these fractions. WT biofilms grown in media with added *C. jejuni* gDNA (MH+gDNA) showed a significant increase in biofilm formation compared to the WT strain in MH broth alone (p=0.003). Finally, an equal amount of purified DNA from another organism (salmon; MH+ssDNA) also modestly stimulated biofilms. This was observed consistently, although it not reach statistical significance (p=0.17).

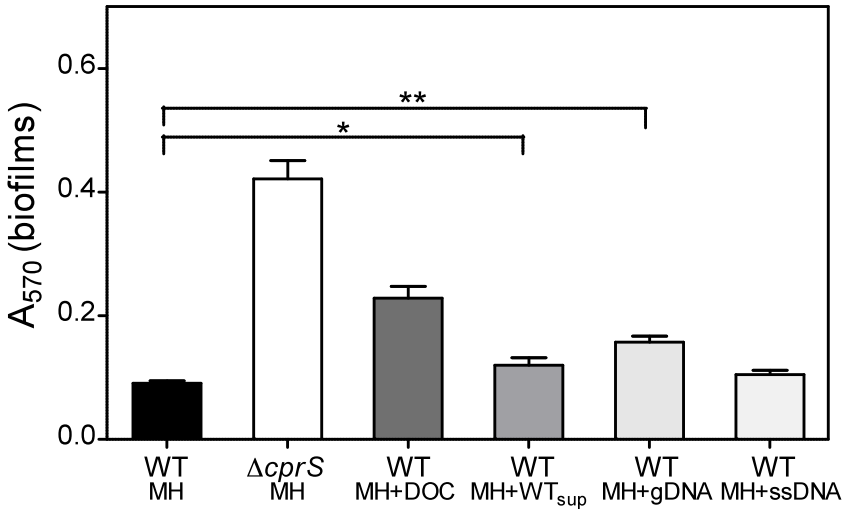


FIG. 4.3. Exogenous DNA enhances biofilms. Culture supernatants, concentrated for >3kDa components, gDNA from WT *C. jejuni* (500ng), or purified salmon DNA (500 ng) was added to fresh MH broth and inoculated with WT or Δ*cprS*. After 2 days, biofilms were stained with CV. *p=0.08; **p=0.003.

DNA released during biofilm formation is required for maturation of the *C. jejuni* biofilm. It was previously noted that DNase could disrupt biofilms. However, as addition of exogenous DNA to biofilm cultures appeared to enhance biofilm formation, it was next determined whether endogenous DNA was actually required for biofilm formation. The effect of DNase on biofilms formed by WT in MH alone, and on biofilms formed by biofilm-enhanced cultures that also showed increased lysis and eDNA release (Δ*cprS* in MH; WT in MH+DOC) was determined using the CV assay (**FIG. 4.4A**). Biofilm formation by WT grown in the presence of DNase ('MH+DNase') was reduced compared to that of WT in MH alone (p=0.0013). Furthermore, while biofilm formation was again increased in both Δ*cprS* in MH alone and WT in MH+DOC, when DNase was included in these biofilm cultures ('WT, MH+DOC+DNase'; 'Δ*cprS*, MH+DNase'), levels of biofilm formation were observed to be reduced to levels similar to those shown by WT in MH+DNase and were significantly different from their counterparts grown without DNase (p=0.0017, p=0.0025, respectively).

Macroscopic observation of biofilms using the CV assay suggested that biofilm formation in the presence of DNase was inhibited. To determine at which point biofilm formation was arrested by DNase, confocal microscopy was again used to observe the progression of biofilm formation by WT or biofilm-enhanced bacteria in the absence or presence of DNase (**FIG. 4.4B).** At 36h, compared to WT in MH alone (top left panel), when DNase was included in WT cultures (MH+DNase, bottom left panel), biofilm formation was observed to be reduced. However, although less mature biofilm was observed in MH+DNase (like the CV assay), bacteria did not appear to be significantly reduced for adherence to the glass slide. In DNase, green bacteria were observed to be attached to the slide, but unlike WT in MH alone, very little blue eDNA was noted. Moreover, at later time points (48h, data not shown), the three-dimensional structures that were seen in WT biofilms grown in MH alone were not observed in biofilms grown in MH+DNase, suggesting that biofilm formation may have been arrested at a single layer stage following attachment, rather than prior to adherence to the slide. The effect of DNase on biofilm formation in biofilm-enhanced bacteria (Δ*cprS* in MH; WT in MH+DOC) was also observed. Like the CV assay, microscopy also showed enhanced biofilm formation by Δ*cprS* in MH alone (top middle panel), or WT in MH+DOC (top right panel). Biofilm formation was again inhibited by inclusion of DNase into the media in these cultures. Again, no blue eDNA was observed surrounding bacteria on these slides. However, in contrast to what was observed for WT in MH+DNase (bottom left panel) very few bacteria were observed to be adhered to the slide for either Δ*cprS* in MH+DNase (bottom middle panel) or WT in MH+DOC+DNase (bottom right panel). The biofilm may provide *C. jejuni* with protection during stress, and may be required by Δ*cprS* in MH or WT in MH+DOC. This hypothesis was addressed in subsequent experiments (**TABLE 4.1**).

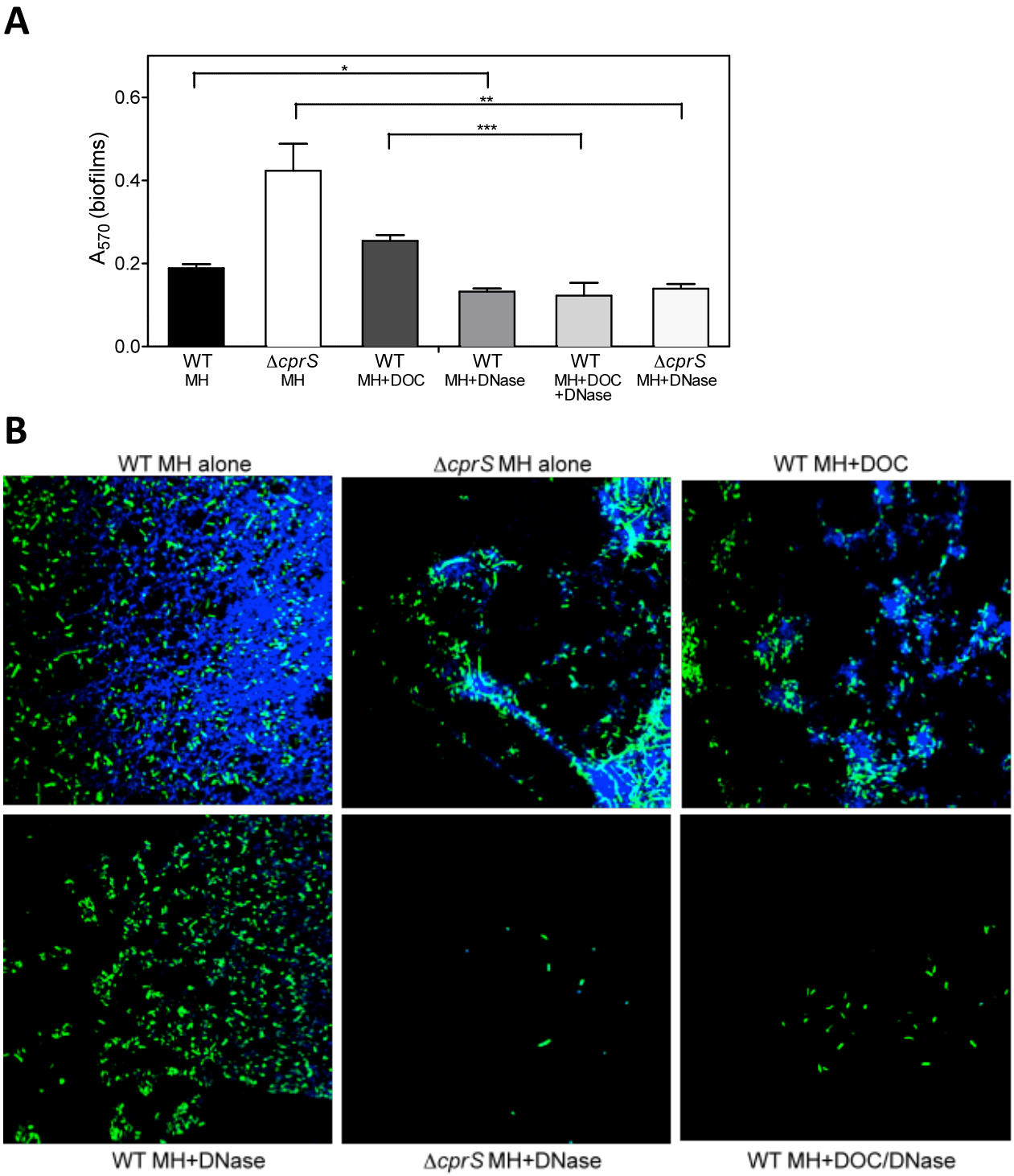


FIG. 4.4. Biofilm maturation is arrested after adherence by removing released DNA. **A)** WT biofilms, and enhanced Δ*cprS* or DOC biofilms, are reduced by the presence of DNase I. WT of Δ*cprS* biofilms were grown in either MH alone, MH broth with 0.05% DOC ('MH+DOC'), 90 U mL^{-1} DNase I ('MH+DNase'), or both ('MH+DOC+DNase'), followed by CV staining after 2 days growth. **p*=0.0013, ***p*=0.0017, ****p*=0.0025. **B)** Biofilms treated with DNase show adherence but reduced maturation into three-dimensional biofilms. Biofilms grown as in above were fixed after 36h, stained with DAPI, and visualized by confocal microscopy.

Flagella are required for biofilm formation by both WT and biofilm-enhanced *C. jejuni*. Previous observations suggested that release of eDNA through lysis may be part of biofilm formation and maturation in *C. jejuni*. However, these observations also suggested that eDNA may be released, and also required, in steps following adherence. Numerous reports suggest that flagella are absolutely required for *C. jejuni* to form a biofilm, although the stage they are required has not been determined. Based on microscopy observations, it was hypothesized that flagella may be required for initial steps of biofilm formation, before the appearance of eDNA. The requirement of flagella for biofilm formation was confirmed by assessing biofilm formation by flagellar mutants using a standard CV biofilm assay (**FIG. 4.5**). Mutations in regulatory (*rpoN*) and structural (*flhA*) components caused severely defective biofilm formation. Biofilm formation was also tested in other flagellar mutants, such as Δ*flhB*, Δ*flgS*, Δ*flgR*, and was found to be similarly defective (data not shown). Furthermore, when introduced into a Δ*cprS* background, mutations reducing flagellar expression also resulted in defective biofilm formation, suggesting they were epistatic to Δ*cprS*. Finally, when the Δ*flhA* mutant was grown in MH+DOC, which enhances biofilm formation in the WT strain, biofilm formation was still defective (data not shown).

Lysis in biofilm-enhanced bacteria, that release more eDNA than WT, appeared to be occurring independently of expression of the flagellar export apparatus (**FIG. 4.1**). However, while release of eDNA may also occur via lysis, the mechanism of eDNA release was unknown. To confirm that that eDNA release was not dependent on the flagellar export apparatus, the eDNA concentration of supernatants from WT, Δ*cprS*, Δ*flhA,* as well as a double Δ*flhA* Δ*cprS* mutant, cultures in MH alone, as well as WT and Δ*flhA* mutant

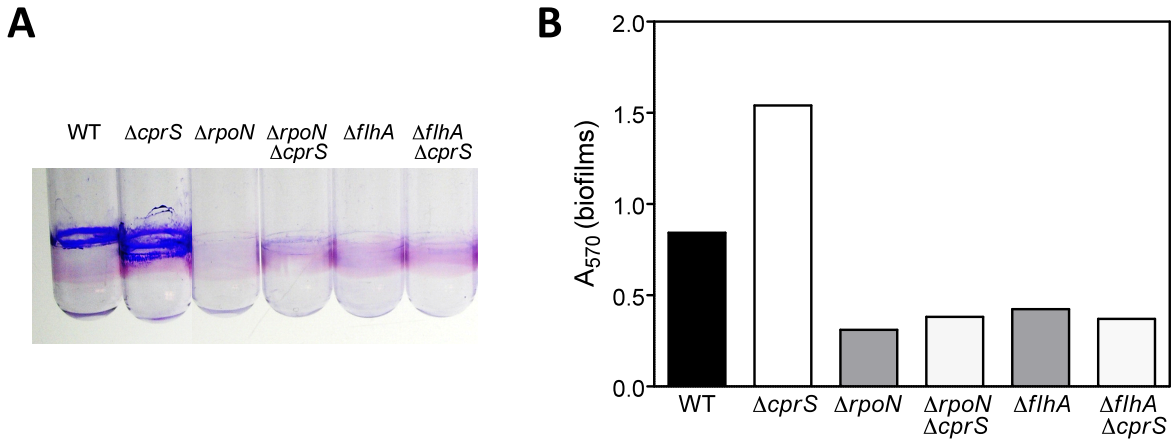


FIG. 4.5. Flagellar mutations cause defective biofilm formation and are epistatic to Δ*cprS*. Biofilm formation was measured in MH broth by WT, Δ*cprS*, single flagellar mutant strains (Δ*rpoN* or Δ*flhA*), and double Δ*cprS* flagellar mutants (Δ*cprS* Δ*rpoN* or Δ*cprS* Δ*flhA*). Following 2 days growth, biofilms were stained with CV and either photographed (**A**) or quantified by measuring A_{570} of dissolved CV (**B**).

in MH+DOC was measured by QPCR (**FIG. 4.6A**). A significantly smaller amount of DNA was measured in the supernatant of Δ*flhA* (p=0.004); however, this mutant did show DNA levels similar to the WT when both strains were grown in MH+DOC. Like Δ*cprS*, more DNA was also measured in supernatants of Δ*flhA* Δ*cprS*compared to WT. This was despite the fact that aflagellate strains were biofilm-defective. The amount of DNA in Δ*cprS* Δ*flhA* supernatants was less than that in those from Δ*cprS*. Nonetheless, these observations suggested that loss of flagella did not affect eDNA release.

All of the mutants assessed for biofilm formation in the above experiment were expected to be aflagellate. However, *C. jejuni* flagella are important for both motility and as adhesion organelles [109], and in order to determine whether flagella make a stronger contribution to biofilm formation through motility or adhesion, biofilm formation in the aflagellate Δ*flhA* mutant was compared to that of a non-motile flagellated Δ*pflA* mutant [which expresses paralyzed flagella [178]] using the CV assay (**FIG. 4.6B**). In MH alone, both Δ*flhA* and the Δ*pflA* mutant appeared to be highly defective for biofilm formation compared to both WT and Δ*cprS* (**FIG. 4.6B**, left). However, in MH+DOC, these strains behaved differently from each other (**FIG. 4.6B**, right). While the Δ*flhA* aflagellate mutant appeared to remain essentially completely biofilm-defective in MH+DOC, in contrast, Δ*pflA* was not as defective in MH+DOC as it was in MH alone, as it showed a significant increase in biofilm formation in MH+DOC compared to MH alone (p<0.0001). This suggested that while loss of motility could be partially rescued in conditions that promote biofilm formation, such conditions could not overcome complete absence of flagella.

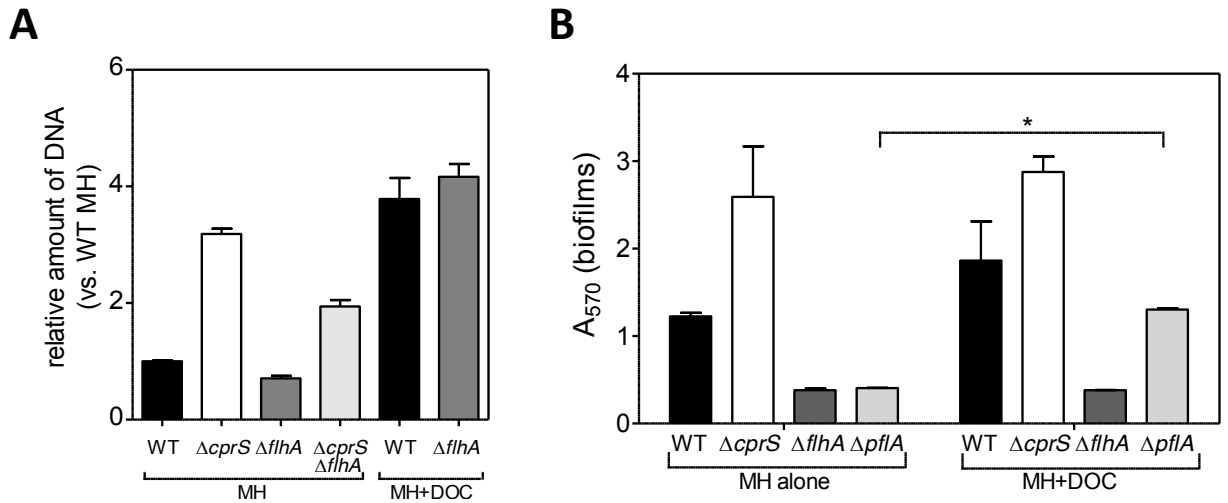


FIG. 4.6. Loss of flagella does not abolish release of eDNA; conditions that promote biofilm formation in WT partially rescue biofilm formation in a paralyzed flagella mutant. **A)** Aflagellate mutants are not defective for eDNA release. eDNA was quantified by QPCR from culture supernatants from bacteria grown in either MH alone or MH+0.05% DOC. **B)** Both aflagellate and non-motile flagellated bacteria are defective for biofilm formation in MH; biofilm formation is partially rescued by DOC in Δ*pflA* only. Biofilm formation (in MH alone or MH+ DOC) was compared for WT, Δ*cprS*, Δ*flhA*, and Δ*pflA*. Biofilms were grown in MH alone or MH+DOC and quantified with CV staining following 2 days growth. *p<0.0001.

Flagella may be required for attachment; motility may also aid kinetics of biofilm formation. To assess the relative importance of flagellar motility and adhesion to biofilm formation, and to expand observations made in the CV assay, microscopy was used to determine the stage of biofilm formation at which flagella were important. An aflagellate Δ*flgR* mutant (Kan^R), which was also biofilm defective (data not shown), was used in place of Δ*flhA* to allow introduction of GFP on a Cm^R plasmid for microscopy. In MH alone, compared to WT (top left panel) the aflagellate Δ*flgR* mutant (top middle panel) appeared to adhere poorly (**FIG. 4.7**). In contrast, the Δ*pflA* mutant in MH alone did not appear to be as defective for adherence (top right panel). However, Δ*pflA* biofilm formation did appear to be delayed compared to WT, as little DNA was observed surrounding this mutant. In MH+DOC, compared to WT (bottom left panel), Δ*flgR* was still observed to adhere poorly. Moreover, although very few adhered bacteria were observed for Δ*flgR*, DNA was still observed attached to Δ*flgR*-incubated slides. Finally, consistent with CV results, the non-motile Δ*pflA* mutant (bottom right panel) appered to form better biofilms in MH+DOC compared to MH alone, although still not to the levels of WT. Compared to MH alone, significant amounts DNA were observed surrounding Δ*pflA* in MH+DOC.

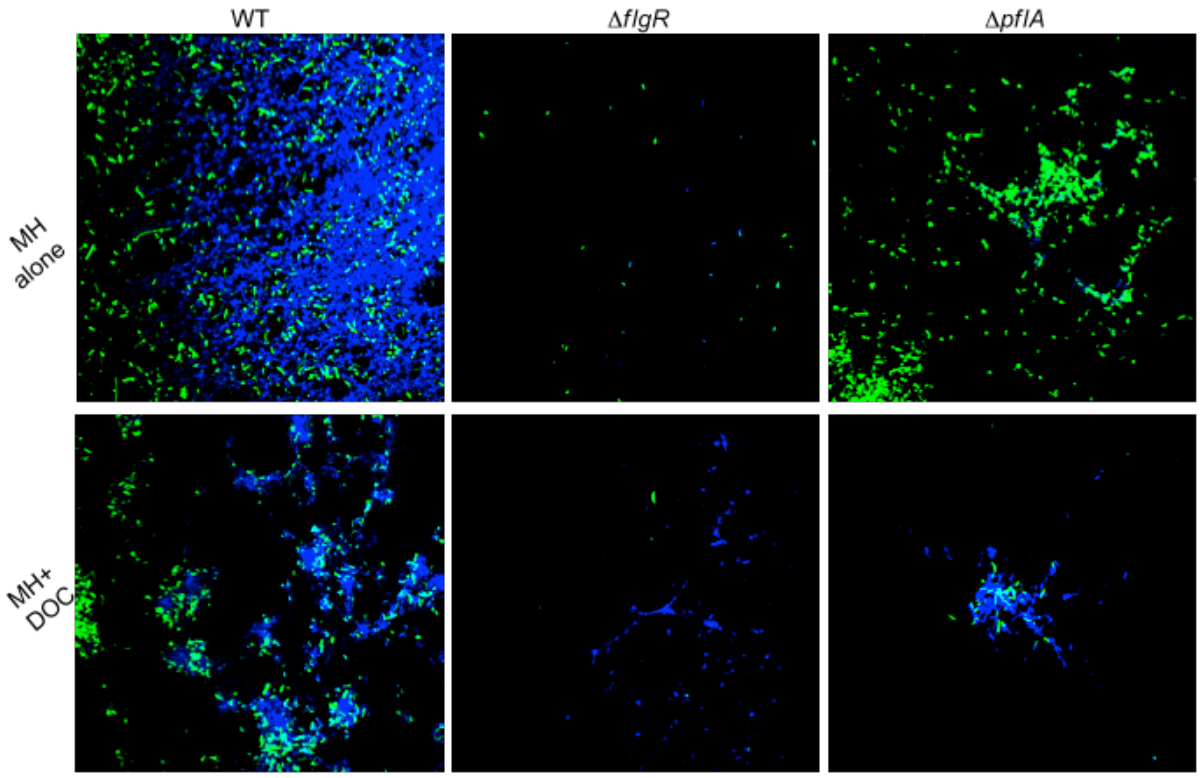
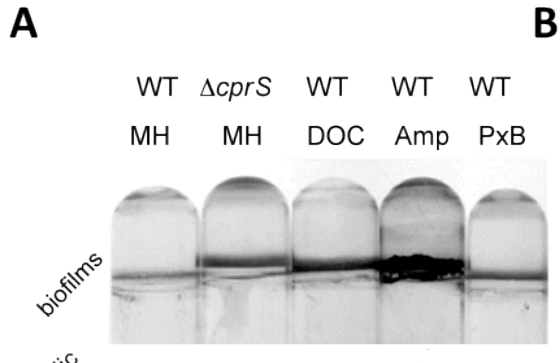


FIG. 4.7. Flagella, motility, and eDNA play distinct but interrelated roles in attachment and maturation of *C. jejuni* biofilms. Bacteria without flagella are defective for adherence; kinetics of biofilm formation is delayed in the absence of motility but presence of flagella. Biofilms of the aflagellate Δ*flgR* mutant or non-motile flagellated Δ*pflA* mutant compared to WT and Δ*cprS* at 36h by fixing, staining with DAPI, and visualization by confocal microscopy.

Conditions that may cause envelope stress increase both biofilm formation and lysis. The Δ*cprS* mutant exhibits phenotypes that may be consistent with envelope changes, possibly due to dysregulation of genes such as *htrA* (**TABLE 3.2**). Furthermore, in general, enhanced biofilms were observed when WT was grown in media containing detergent-like compounds, such as DOC (**FIG. 2.8**), that may interact with the cell envelope. Envelope stress may thus lead to formation of enhanced biofilms. Analysis of biofilm formation was expanded to include WT grown in sub-MIC levels of envelope-interacting compounds Amp and PxB, and like DOC, these compounds were observed to stimulate biofilm formation (**FIG. 4.8A,** top). Furthermore, like WT in MH+DOC or Δ*cprS* in MH alone, WT in MH+PxB or MH+Amp also displayed increased lysis compared to WT in MH alone (**FIG. 4.8A,** bottom). Biofilm formation was then measured in strains with mutations that affect aspects of the cell envelope, such as Δ*kpsM* (CPS), Δ*spoT* (CFW-reactive polysaccharide), Δ*waaF* (LOS), and Δ*rpoN* (flagella)(**FIG. 4.8B,** third column). Like Δ*cprS*, mutation of *kpsM* and *waaF* (A. Cameron, personal communication), *spoT*, and *rpoN* negatively affected growth in MH+150 mM NaCl. Furthermore, all of these mutants, except the aflagellate Δ*rpoN* mutant, showed increased biofilm formation (**FIG. 4.8B,** fourth column). Finally, mutants that showed decreased osmotolerance and enhanced biofilm formation (Δ*cprS*, Δ*kpsS*, and Δ*spoT*) also exhibited increased lysis, as indicated by increased cytoplasmic protein (CosR) in culture supernatants compared to the WT strain. Together, this suggested that conditions that may cause envelope stress may stimulate biofilm formation and associated phenotypes, such as lysis.



B

Strain	MH broth (growth)	MH+NaCl (growth)	Biofilms (vs. WT)	Lysis
WT	+	+	WT	-
Δ*cprS*	+	-	++	+
Δ*kpsM*	+	-*	++**	ND
Δ*kpsS*	+	ND	++	+
Δ*waaF*	+	-*	++	+
Δ*spoT*	+	-	++	+
Δ*rpoN*	+	-	-	-

*A. Cameron, pers. comm.
** McLennan *et al.* 2008

FIG. 4.8. Lysis and biofilm formation may be a response to envelope stress in *C. jejuni*. **A)** Mutations and conditions that cause envelope stress increase lysis and biofilm formation. Biofilm formation by either WT or Δ*cprS* bacteria, in MH broth alone or MH broth with the indicated additions, were stained with CV. DOC: sodium deoxycholate; Amp: ampicillin; PxB: polymyxin B. **B)** Salt tolerance, lysis, and biofilm phenotypes of envelope mutants. Salt tolerance was assessed by determining growth in MH broth supplemented with 150 mM NaCl. Biofilm formation was determined after 2 days by the CV assay. Lysis was determined by measuring release of the cytoplasmic protein CosR into supernatants after 24h of broth culture. ND, not determined.

Biofilms contribute to survival under conditions that may cause envelope stress. A general trend of enhanced biofilm formation was noted under conditions that were expected to cause envelope stress, such as those affecting the cell envelope, or mutations affecting the cell envelope that result in increased osmotic sensitivity (**FIG. 4.8**). However, it was also noted that strains that do not express flagella and cannot form biofilms, such as Δ*rpoN*, also showed increase salt sensitivity. Other work also suggests that Δ*rpoN* shows growth differences and stress sensitivity in standing culture [289]. It was also observed, while preparing and analyzing samples for microscopy, that generally, while bacteria experiencing envelope stress (Δ*cprS* in MH; WT in MH+DOC) formed enhanced biofilms, if biofilm formation in these bacteria was also inhibited (by deleting genes required for production of flagella or by incubation in MH+DNase), biofilms grew poorly and very few bacteria were observed adhered to the slide. Together, this suggested that first, biofilm formation may be a response to stress, and second, if *C. jejuni* cannot form a biofilm, it may exhibit reduced stress tolerance.

To explore the relationship between biofilm formation and stress tolerance, three phenotypes were compared for strains under combinations of conditions that cause envelope stress (Δ*cprS* mutation; growth in MH+DOC) and those that inhibit biofilm formation, such as loss of flagella (Δ*flhA* mutation) or loss of eDNA (growth in MH+DNase)] (**TABLE 4.1**). Different strains (WT, Δ*cprS*, Δ*flhA*, and Δ*cprS* Δ*flhA*) were thus grown under different static growth conditions (MH alone, MH+DOC, MH+DNase, and MH+DOC+DNase). Characteristics analyzed included biofilm formation (compared to WT in MH alone after 2 days growth) (**TABLE 4.1**, third column), whether bacteria may generally be experiencing envelope stress (**TABLE 4.1**, fourth column), and finally, total growth (as measured by OD_{600}, after vortexing a 1 day standing culture)(**TABLE 4.1**, last two columns). Total growth was measured after 1 day, rather than the 2 days used to measure biofilms, to avoid extensive clumping that interfered with accurately measuring biomass by reading optical density.

The first observation from this experiment was that generally, bacteria that form enhanced biofilms show higher total growth in standing culture. For this, total growth was compared for each strain in MH alone as a % of WT. The Δ*cprS* mutant, which forms enhanced biofilms under these conditions, grew to almost twice the density (184.9%) of the WT strain (**TABLE 4.1**, second-to-last column, starred entries). Furthermore, Δ*flhA*, which was biofilm-defective, reached 64.9% of the OD_{600} of WT. Finally, rather than reaching a higher density than WT like the Δ*cprS* single mutant, the Δ*cprS* Δ*flhA* double mutant grew even more poorly than Δ*flhA* (50.6% of WT). Together, this suggests that the ability to form a biofilm allows *C. jejuni* to reach a higher density in static culture than if allowed to grow only planktonically.

TABLE 4.1. *C. jejuni* biofilm formation is associated with stress tolerance. Three characteristics are listed for each strain: biofilm formation, presence of envelope stress, and total growth compared to WT (in MH alone). Biofilm formation was impaired by either mutation of flagella (Δ*flhA*) or addition of DNase to remove eDNA; envelope stress is represented by either the presence of the Δ*cprS* mutation or DOC in the media. *****: relative amount of growth between each strain in MH broth alone. **A**,**B**,**C**,**D**: relative amount of growth within a strain compared to that strain in MH broth alone.

	MH broth +:	Biofilm phenotype:	Envelope stress:	Total growth (OD_{600}, 1 day):	
				%vs. WT in MH	%vs. self in MH
WT	(-)	+	-	100.0*	100.0^{A}
	DOC	+	+	100.4	100.4^{A}
	DNase	-	-	95.5	95.5^{A}
	DOC+DNase	-	+	59.6	59.6^{A}
Δ*cprS*	(-)	+	+	184.9*	100.0^{B}
	DOC	+	+	143.1	77.4^{B}
	DNase	-	+	71.6	38.7^{B}
	DOC+DNase	-	+	52.7	28.5^{B}
Δ*flhA*	(-)	-	-	64.9*	100.0^{C}
	DOC	-	+	51.4	79.2^{C}
	DNase	-	-	65.7	101.2^{C}
	DOC+DNase	-	+	52.7	81.2^{C}
Δ*cprS* Δ*flhA*	(-)	-	+	50.6*	100.0^{D}
	DOC	-	+	54.5	107.7^{D}
	DNase	-	+	54.5	107.7^{D}
	DOC+DNase	-	+	42.7	84.4^{D}

The second observation progressed from the above comparisons of each strain in MH alone (**TABLE 4.1**, starred entries) - namely the fact that the Δ*cprS* Δ*flhA* double mutant grew even more poorly than Δ*flhA*. Compared to Δ*flhA,* Δ*cprS* Δ*flhA* may have been experiencing envelope stress, and unlike Δ*cprS* alone, this strain cannot form a biofilm. The conditions used to grow each strain were subsequently expanded in order to provide further evidence that stress tolerance requires biofilm formation. For these experiments, total growth under each condition was compared within each strain (**TABLE 4.1**, last column), rather than to WT under each growth condition (second column). Results of these analyses suggest that *C. jejuni* can tolerate envelope stress only if able to form a biofilm. For example, when WT (marked with '**A**') was grown in MH, MH+DOC, and MH+DNase, it reached almost the same density (100.0, 100.4, and 95.5%, respectively). However, if it was grown under conditions of both envelope stress and biofilm formation was inhibited (MH+DOC+DNase), it reached only 59.6% of WT density in MH alone. For Δ*cprS* (marked with '**B**'), added envelope stress (MH+DOC) reduced growth to 77.4% of that in MH. In contrast to WT, which does not harbour intrinsic envelope stress, growth of Δ*cprS* in DNase was markedly affected (38.7% of growth in MH alone). Growth of Δ*cprS* in MH+DOC+DNase was extremely poor (28.5%). Growth of the Δ*flhA* aflagellate biofilm-defective mutant (marked with '**C**'), unlike WT, was affected by growth in MH+DOC (79.2% of growth in MH alone). Consistent with absence of envelope stress in this strain, however, DNase did not affect growth of Δ*flhA*. Furthermore, as Δ*flhA* was already biofilm-defective (due to lack of flagella), it

showed a similar amount of growth in both MH+DOC and MH+DOC+DNase (79.2% and 81.2% of MH alone, respectively). Finally, growth of the double Δ*cprS* Δ*flhA* mutant (marked with '**D**') was assessed under each culture condition. As this strain was already quite defective for growth in MH broth alone (50.6% of the WT strain), a small effect of each condition on growth was observed. However, the culture of the double mutant in DOC+DNase showed the lowest growth of all the strains (42.7%).

Conditions that increase eDNA release and biofilm formation also promote genetic recombination. The rate of genetic exchange was measured under two conditions that promote biofilm formation and eDNA release: mutation of *cprS*, and growth in MH+DOC. Strains marked with antibiotic resistance (Kan^R or Str^R) on the chromosome were grown in mixed culture. When WT marked with Str^R was grown with an isogenic strain carrying Kan^R in mixed culture, the appearance of doubly resistant colonies, which were not present upon inoculation of the cultures, was noted (**FIG. 4.9**). When mixed cultures of WT Str^R and WT Kan^R were grown in MH+DOC, the appearance of these clones was observed at higher levels than for mixed cultures in MH alone (p=0.09). Moreover, when WT Str^R was co-cultured with Δ*cprS* Kan^R, a significant (p=0.02) increase in the appearance of doubly resistant clones was measured compared to co-cultures of the two WT background strains in MH alone.

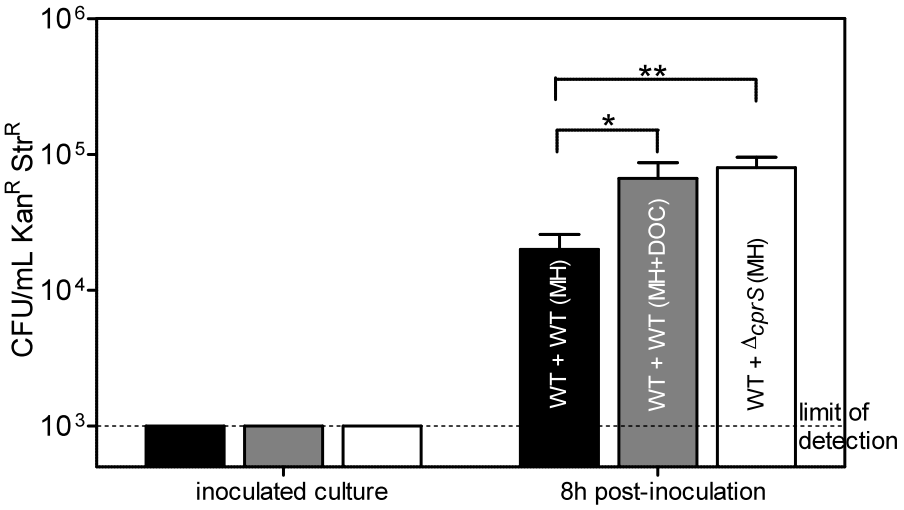


FIG. 4.9. Bacteria grown under conditions that promote DNA release and biofilm formation also show increased genetic exchange. WT bacteria, marked with Str^R, were grown in mixed culture (1:1) with either an isogenic WT strain marked with Kan^R or the Δ*cprS* mutant marked with Kan^R. Cultures were grown in either MH broth alone or MH+0.05% DOC. Cells were removed at indicated time points and plated on plates containing various antibiotics for CFUs. * p=0.09; ** p=0.02.

4.4 Discussion

Previous work has not identified dedicated virulence factors and specific stress response proteins that sufficiently explain why *C. jejuni* is such a successful zoonotic pathogen, surviving and thriving in numerous environments during transmission and pathogenesis. Analysis of a hyperbiofilm-forming mutant (Δ*cprS*) has allowed identification of stages and molecular factors involved in *C. jejuni* biofilm formation, a phenomenon that may explain the resilience of *C. jejuni* outside of the laboratory. The contribution of two specific phenomena has been determined: flagella and eDNA. Flagella appear to be necessary for initiation of biofilm formation on a surface by mediating adhesion. Furthermore, motility provided by flagella aids the kinetics of biofilm formation. A lytic process, following adherence, was also observed that may be responsible for release of eDNA. eDNA appeared to be required for maturation from microcolonies into a three-dimensional biofilm. Finally, observations made in this work were consistent with the hypothesis that the biofilm lifestyle confers *C. jejuni* with resilience needed as a zoonotic pathogen that has so far not been apparent from studies of planktonic bacteria growing in rich media.

The process of *C. jejuni* biofilm formation, like that of other bacteria, appeared to proceed in discrete steps, starting with adhesion. Flagella appeared to be required for adhesion, as aflagellate mutants were not observed to adhere to coverslips under any conditions (**FIG. 4.7**). It has previously been proposed that *C. jejuni* uses both flagellum-dependent and flagellum-independent mechanisms of biofilm formation [337]. Two studies noted that bacteria adhere to *ex vivo* tissue samples by flagella in microcolony-like structures [174, 546], and an *in vitro* exploration of *C. jejuni* biofilm formation [383] also found microcolonies formed on glass coverslips with flagella forming bridges between organisms. Autoagglutination behaviour (which is thought to be dependent on flagella) and biofilm formation also seem to be interrelated in *C. jejuni* [337, 379]. Mutant analyses suggest that while motility might aid the kinetics of biofilm formation and be required to allow WT levels of biofilm, unlike the flagellar structure, it was not absolutely required. While biofilm formation by Δ*pflA* was delayed in MH broth (when assessed generally by the CV assay) microscopy allowed observation of Δ*pflA* biofilms in more detail, and delayed, but intermediate, biofilm formation was displayed by the Δ*pflA* mutant (**FIG. 4.6B**; **FIG. 4.7**). This was consistent with previous observations of flagellar morphology, biofilm formation, and motility reported in the literature (**TABLE 5.1**). Biofilm formation is severely defective in aflagellate mutants (such as Δ*flhA*), but delayed in mutants such as Δ*flaA,* Δ*flaB,* Δ*fliA, and* Δ*flaC* [378], which express either normal or morphologically aberrant flagella, but are not aflagellate. Some of these strains have been reported to have reduced (~20% of WT) or absent motility [176, 378]. Finally, a Δ*flaG* mutant expresses long flagella, but retains full motility, suggesting that motility alone may be insufficient for biofilm formation [378]. Thus, flagellum structure appeared to be absolutely required for biofilm formation, and while motility seems to aid kinetics of biofilm formation, it may be insufficient for biofilm formation if flagellum morphology is altered. A central role for flagella in biofilm formation is also supported by previously reported expression data. Motility peaks during late log phase [76], and Class II and III flagellar genes show sustained or increasing expression through stationary phase. This suggests that components of the flagellum are

necessary for adaptation to stationary phase. Biofilms often exhibit characteristics of stationary phase cells and share similar expression profiles [547]. *C. jejuni* biofilm cells also display higher expression of flagellar genes compared to stationary phase cells grown planktonically [378]. Finally, proteomic and microarray expression analysis of the Δ*cprS* hyperbiofilm mutant (**FIG. 2.7**; **TABLE 3.2**) also suggested increased expression of flagellar genes. It was initially wondered if sustained expression of flagellar genes through stationary phase reflects their requirement for sessile growth, and if increased expression of flagella in Δ*cprS* was responsible for enhanced biofilms.

The stages of biofilm formation following adherence often include release of polymeric substances that form the matrix that encases the bacteria into a mature biofilm. Surface carbohydrates are common components of the biofilm matrix. The *C. jejuni* cell surface is highly glycosylated; thus, it is puzzling that a specific carbohydrate component of the *C. jejuni* matrix has yet to be identified. Previous work suggested that the Δ*cprS* mutant carries no defects in surface polysaccharides (**FIG. 2.4**). The Δ*cprS* mutant was thus used to try to identify carbohydrate-independent mechanisms of biofilm maturation. It was previously noted that DNA surrounded *C. jejuni* biofilms, especially in Δ*cprS* and in WT bacteria under conditions favouring biofilm formation (MH+DOC), and treatment of pre-formed biofilms with DNase was also found to disrupt them (**FIG. 2.4**). Other groups have shown visual evidence of an extracellular material that binds Ruthenium Red [383], a dye that stains carbohydrate matrices, but also binds double-helical DNA [548]. In this work, by measuring eDNA in culture supernatants, it was demonstrated that conditions that promoted biofilm formation increased the amount of eDNA (**FIG. 4.2**) and that biofilms grown with added purified *C. jejuni* gDNA show enhanced biofilm formation (**FIG. 4.3**). Finally, inclusion of DNase in standing cultures inhibited biofilm formation (**FIG. 4.4**).

The mechanism of eDNA release in biofilms is often autolytic in nature. Furthermore, examples of a connection between lysis and biofilm formation exist in other bacteria. In *P. aeruginosa*, autolysis appears to contribute to dispersal of organisms from the biofilm, whereas in other bacteria such as *E. faecalis*, *S. aureus*, and *Neisseria meningitidis*, it appears to be involved in both eDNA release and biofilm development [152, 342, 549, 550]. Lytic transglycosylases in *Salmonella* Typhimurium also link cell wall turnover to biofilm formation [542]. An increase in amounts of protein species (**FIG. 2.8**) and cytosolic proteins (**FIG. 3.9**) was previously noted in supernatants of biofilm-enhanced Δ*cprS* bacteria, and it was hypothesized that this was a result of lysis. A strong positive correlation was also observed between increased lysis of strains (such as Δ*kpsS*, Δ*waaF*, and Δ*spoT*) and tendency to form a biofilm (**FIG. 4.8B**). In this work, it was also found that numerous antimicrobial compounds that may interact with the cell envelope also caused both lysis and enhanced biofilm formation (**FIG. 4.8A**). Since increased amounts of eDNA were observed in strains that show lysis, and culture supernatants and purified DNA enhance biofilm formation, it may be concluded that eDNA released during lysis may promote biofilm formation. This hypothesis was supported by microscopy observations, which demonstrated that biofilm formation was arrested in the presence of DNase. However, the mechanism

of lysis is currently unknown. While an autolytic mechanism cannot be ruled out, which has not yet been described in *C. jejuni*, analysis of the PG structure of Δ*cprS* may be consistent with a passive lytic phenomenon, relating to subtle changes in PG structure. The Δ*cprS* mutant displays distinct growth differences from the WT strain in broth culture: following log phase, cultures of Δ*cprS* display a marked loss of culturability (**FIG. 2.2B**). A decrease in CFUs was also observed (approximately 2 logs) in WT cultures after exponential phase of growth. *C. jejuni* is thought to convert to a coccoid VBNC form; however, observations of Δ*cprS* shape through a growth curve were not consistent with a rapid progression into these forms (**FIG. 3.8**). It was previously thought that loss of culturability may represent an absence from the planktonic fraction during liquid culture due to aggregation (**CHAPTER 2**); however, because envelope genes were dysregulated in Δ*cprS* (**CHAPTER 3**), it is conceivable that absence of Δ*cprS* from the planktonic fraction could have, at least in part, been due to lysis. Indeed, it has been confirmed that proteins in the supernatant of Δ*cprS* cultures appeared independently of expression of the flagellar export apparatus and also harbour cytoplasmic proteins. Release of DNA was also observed in a Δ*flgR* mutant by microscopy (**FIG. 4.7**), even though this mutant was unable to adhere to the coverslip. This suggests that lysis, whether active or passive, does not require adherence.

Lysis also appeared to correlate with biofilm maturation, and lysis may contribute to maturation of the *C. jejuni* biofilm by releasing eDNA. Release of eDNA occurred following attachment of microcolonies (**FIG. 4.2**), and removal of eDNA with DNase arrested biofilm formation at the microcolony stage (**FIG. 4.4**). In *H. pylori*, eDNA has also been identified as a component of the biofilm matrix [551]. However, DNA fingerprinting suggested a marked difference between eDNA and intracellular DNA, suggesting that a non-specific lytic mechanism does not release of DNA in this pathogen. However, DNase does not affect biofilm formation by *H. pylori*, and thus, it was concluded that the main function of eDNA in this bacterium was to contribute to the genetic variation of this species. It is unknown whether *C. jejuni* eDNA is indistinguishable from gDNA, as QPCR was performed with primers specific for only one gene (*cprR*) and further analysis of released DNA was not undertaken. However, the presence of the *cprR* gene in eDNA suggests that this DNA may be derived from the chromosome. Moreover, as lysis correlates with appearance of eDNA in *C. jejuni*, this suggests a non-selective mechanism of total chromosomal DNA may exist in *C. jejuni*, unlike *H. pylori*. Furthermore, the distinct structural role played by eDNA in *C. jejuni* suggests different mechanisms of release may also exist in these related pathogens. However, further analysis of the nature *C. jejuni* eDNA is required to conclude that eDNA release in *C. jejuni* is non-specific. While DNA uptake appears to be mediated by a Type II secretion system, a putative DNA secretion apparatus has not been identified in *C. jejuni*. The pVIR plasmid harboured by some strains, including the robust biofilm former 81-176, encodes a putative Type IV secretion system which could presumably play this role. However, mutation of *virB11*, encoding an essential component of this secretion system, does not affect biofilm formation in strain 81-176 (S. Svensson and E. Gaynor, unpublished observations).

It was initially proposed that biofilm formation may be an envelope stress response (**CHAPTER 3**), and that demonstration of increased stress tolerance by biofilms would support this hypothesis. In this work, increased biofilm formation by WT was seen in various compounds that affect the envelope, such as DOC, Amp, and PxB (**FIG. 4.8A**). Furthermore, *C. jejuni* appeared to require biofilm formation to tolerate conditions of stress. In general, bacteria that cannot form a mature biofilm (either by genetic lesion of flagellar genes or enzymatic removal of eDNA) were less able to tolerate envelope stress conditions, such as DOC in standing culture (**TABLE 4.1**). A close relationship between envelope stress and biofilm formation exists in other pathogens. For example, it has been proposed that the Cpx-controlled envelope stress response of Gram-negative bacteria mediates biofilm formation [419]. The R1 conjugative plasmid is thought to induce biofilm formation through activation of envelope stress responses by conferring expression of pili [552], and envelope-interacting compounds such as bile stimulate biofilm formation in *V. cholerae* [504]. In *C. jejuni*, enhanced biofilm formation and autoagglutination has also been noted in a Δ*peb4* mutant, which is affected for expression of envelope proteins [375]. It was also recently reported that aerobic conditions stimulate biofilm formation in *C. jejuni* [337], and bile upregulates the *flaA* promoter (Allen and Griffiths 2001), suggesting that adverse conditions may promote biofilm formation in this pathogen.

The mechanism by which biofilms conferred *C. jejuni* with increased stress tolerance in this work is currently unknown. In general, the contribution of biofilms to stress tolerance is thought to be multifactorial, and may include altered metabolism, induction of stress response genes, decreased penetration of O_2 or toxic compounds (such as DOC), or specific contributions of the properties of matrix components, such as eDNA. When *C. jejuni* was growing in a biofilm, cultures were observed to reach a much higher total biomass than those growing solely planktonically, suggesting that this lifestyle may provide a niche that is well suited to growth and/or survival of this pathogen. In addition to possibly providing an undefined niche suitable for short-term growth, eDNA may contribute to longer-term survival of this pathogen in some reservoirs by contributing to the genetic plasticity and heterogeneity of *C. jejuni* populations. Increased rates of recombination were observed under conditions that promoted biofilm formation and lysis, which presumably also increased release of eDNA (**FIG. 4.9**). Autolysis can also be a trigger for natural transformation [553]. It follows that inappropriate application of antimicrobials may contribute to persistence by stimulating biofilms and/or natural transformation. Furthermore, as compounds such as bile salts may be encountered in the intestinal tract of commensal or susceptible hosts, this provides some support that biofilms may be relevant *in vivo*.

5 GENERAL DISCUSSION AND FUTURE DIRECTIONS

5.1 Summary

In order to aid understanding of gene regulation and adaptation to conditions encountered during pathogenesis, CprRS, a putative TCRS upregulated in the presence of live host cells *in vitro* [231] was characterized. It was originally hypothesized that CprRS mediates adaptation to the host environment, and subsequently it was proposed that this TCRS controls essential biological processes and biofilm formation. Demonstration of the role of this TCRS in *C. jejuni* biology and pathogenesis was achieved by determining the CprRS regulon, as well as phenotypes controlled by CprRS.

CprRS was found to be encoded in an operon, next to the *htrA* protease, in an organization broadly conserved in *Campylobacter* spp. CprRS was found to be expressed from a promoter upstream of *cprR*, which was most active during log phase and also showed characteristics of autoregulation. The CprR response regulator and CprS sensor kinase comprised a cognate system, as the CTD of the CprS interacted with the NTD of CprR, and genetic manipulation of either *cprR* or *cprS* also resulted in similar phenotypes. The CprR response regulator was essential for viability, suggesting that CprRS regulates essential biological processes in *C. jejuni*, and phosphorylation of the CprR protein, presumably by CprS, was also essential. One-hybrid data was consistent with binding of phospho-CprR to 5'-[C/G]TAAA[C/T]; however, the related response regulator RacR may share this consensus.

Initial characterization of a Δ*cprS* sensor kinase mutant suggested that this system may regulate phenotypes relating to osmotolerance, protein release, culturability, and biofilm formation. Proteomic and microarray analysis suggested Δ*cprS* enters stationary phase sooner than WT, as it exhibits increased expression of middle and late flagellar genes and decreased expression of metabolic genes. The CprRS regulon was putatively composed of genes involved in diverse aspects of the cell envelope, and CprRS directly regulates the adjacent *htrA* gene, encoding a periplasmic protease. The Δ*cprS* mutant also showed morphological differences from WT, and harboured PG with an increase in pentapeptides. Thus, it was concluded that CprRS controls aspects of the cell envelope, and that Δ*cprS* phenotypes were related to envelope changes.

Through subsequent analysis of the Δ*cprS* hyperbiofilm mutant and complementary strains, development of *C. jejuni* biofilms was explored *in vitro*. The role of flagella in biofilms was clarified, and a link between envelope stress, lysis, and eDNA release identified. Initiation, via adherence to a surface, required the flagellar structure, whereas motility aided kinetics of adhesion. Adherence was followed by lysis, which correlated with release of eDNA. Moreover, release of eDNA appeared to be required for maturation of the biofilm and maintenance of biofilm structure. Both adherence and lysis were enhanced under conditions that promote biofilm formation. Finally, evidence that the biofilm lifestyle confers resilience to *C. jejuni* that may not be apparent from either genome annotation or behaviour of bacteria under routine planktonic growth conditions in the laboratory was provided. It was found that *C. jejuni* could tolerate envelope-related stresses when

initiation or maturation of the biofilm was inhibited by genetic (i.e., mutation of flagellar genes) or biochemical (i.e., enzymatic digestion of eDNA) means, respectively. Finally, conditions that promoted biofilm formation also increased natural transformation, presumably at least in part through increase of eDNA release.

While initial characterization of CprRS was undertaken, which also provided insight into *C. jejuni* biofilm formation, questions have arisen during this work remain which are beyond the scope of this dissertation. This includes those regarding CprRS, mechanisms of biofilm formation, contribution of the biofilm lifestyle to *C. jejuni* pathogenesis, and the *C. jejuni* envelope stress response.

5.2 CprRS and two-component gene regulation in *C. jejuni*

Characterization of CprRS has provided insight into TCRS signaling in *C. jejuni*. However, CprRS signalling appear to be complex, and questions regarding possible interactions with other TCRS and regulatory proteins and the nature of the signal that CprS detects remain. This work suggests that cross-talk occurs in *C. jejuni* TCRS, as CprR appeared to receive phosphate from an unidentified source in Δ*cprS*. These observations may not be significant in WT bacteria; however, in a bacterium with such a small complement of regulatory proteins, some overlap of signalling circuits may exist. In addition to cross-talk between sensor kinases and CprR, it is possible that CprS can phosphorylate other response regulators, especially orphan regulators for which no obvious cognate sensor exists. While many of the phenotypes explored in this work were shared by Δ*cprS* and *cprR* loss-of-function (dominant negative or knockdown) strains, other aspects of *C. jejuni* biology may be controlled by CprS through interaction response regulators other than CprR. The good candidate for this, based on phenotypes of Δ*cprS*, may be CbrR. CbrR harbours two receiver domains, and does not appear to be encoded adjacent to an obvious cognate sensor kinase. CprRS may also regulate other TCRS proteins at the transcriptional level. Examples of phosphorylation-independent response regulators have been identified in the ε-proteobacteria, such as CosR of *C. jejuni*. These are often orphans that may represent degeneration of a prototypical TCRS, and are regulated transcriptionally, rather than by phosphorylation [469].

The same consensus was isolated for both CprR and RacR. It remains to be seen whether these response regulators bind the same consensus *in vivo*. Overlapping DNA binding sites can result in different regulatory logics that allow fine tuning of outputs through integration of numerous environmental inputs [554]. Paralogous response regulators – those that bind similar consensus sequences - do exist. For example, in the delta-proteobacterium *Desulfovibrio vulgaris*, paralogues DVU0946 and DVU0539 share binding site motifs, and fine-tune lactate utilization [555]. However, their corresponding sensor kinases do not appear to be related. *E. coli* encodes two TCRS, NarLX and NarQP, whose response regulators bind the same consensus [556], and the sensor kinases of these systems can cross-phosphorylate each response regulator [557]. It is possible that *C. jejuni* has similarly evolved two TCRSs, through gene duplication and divergence that also allows fine-tuning of regulation of some regulons in response to different environmental signals. Interestingly, the

cognate sensor kinases of CprR and RacR (CprS and RacS, respectively) showed less conservation than their response regulator counterparts (39% identity, 61% similarity), and thus, may detect different environmental signals. In addition to a high degree of sequence similarity, the CprRS and RacRS TCRS are both encoded adjacent to heat shock-related genes (*htrA* and *dnaJ*, respectively). RT-qPCR and promoter-*lux* fusions in Δ*cprS* and Δ*rac*R mutants may be used to show that similar regulons are dysregulated, and followed up with experiments in *E. coli* or gel-shift experiments to determine if RacR and CprR activate the same promoter-*lux* fusions.

It is currently unclear whether CprR activates or represses expression of target promoters. Phosphorylation was necessary for binding DNA, and in the absence of CprS activity, CprRS-regulated genes (such as *htrA*) were expressed at lower levels. This, taken together with placement of the putative CprR consensus in the *htrA* promoter (immediately upstream of the transcription start site), suggests that phospho-CprR may activate at least a subset of genes. However, it is possible that an alternative placement of the CprR consensus in other promoters could elicit repression upon binding of the response regulator. Moreover, response regulators like *H. pylori* ArsR activate different subsets of genes based on their phosphorylation state [469], and CprR may behave in a similar fashion. The *cprR* promoter itself was less active in Δ*cprS*, suggesting CprRS autoregulates and may be required to increase its own expression under activating conditions. TCRS operons are often expressed from two promoters – one constitutive, and one positively autoregulated [442]. Low levels of constitutive expression may occur from the *cprR* promoter, as basal levels of light production from the *cprR* promoter-*lux* fusion were observed in Δ*cprS*. Inducible expression of *cprRS* may also occur from the *cprR* promoter. Due to strong conservation of the position of *cprRS* next to *htrA*, it was originally thought that expression of *cprRS* may occur or via cotranscription with the upstream *htrA* gene. However, analysis of cDNA from log-phase bacteria did not detect cotranscription, and a transcriptional terminator is present between *htrA* and *cprR* [256]. The *cprR* promoter appeared to be rapidly repressed following peak expression. In contrast to the inverted repeat upstream of the *htrA* transcription start site, the putative CprR consensus repeats in the *cprR* promoter surrounded the transcription start site. This was more consistent with negative regulation. Unidentified CprR binding sites that activate expression may be present, however, as *cprR* promoter expression was observed to be lower in Δ*cprS*, and CprRS may both positively and negatively regulate its own expression. Refinement of the CprR consensus will allow clarification of the relationship between CprR binding site placement and regulation of *cprRS*, and in combination with activity of *lux* reporter fusions, this may allow construction of a clearer model of *cprRS* regulation.

Analysis of the Δ*cprS* sensor kinase mutant suggested that the CprRS regulon may be composed of genes relating to the cell envelope. Presence of these genes in the directly-regulated CprRS regulon may be confirmed by performing RT-qPCR analysis of transcription in WT, Δ*cprS*, and $cprR^{OE}$, followed by alignment of promoter regions to search for additional CprR-binding sites. Many of the putative CprRS regulon members appeared to be related to envelope protein expression. Gross differences in envelope protein

expression in Δ*cprS* (inner membrane, outer membrane, and periplasmic proteins) by one-dimensional PAGE were not observed. However, subsequent observation of dysregulation of genes such as *peb4* and *htrA* in this mutant raise the question of whether more subtle differences in envelope proteome exist in Δ*cprS*. A Δ*peb4* mutant shows relatively broad changes in outer membrane protein expression [375]. Two-dimensional PAGE, coupled with mass spectrometry, may identify such changes that were not observed in the first early analysis of envelope protein expression. Changes in PG structure and lysis were noted D*cprS*; however, envelope integrity was not assessed. The use of Live/Dead stains in *C. jejuni* has not yet been optimized, as it has been observed that motile bacteria, that are presumably viable, stain with PI – a dye that is only supposed to stain bacteria with compromised membranes (A. Cameron and E. Gaynor, unpublished observations). In lieu of this, membrane integrity may be assessed using the dye 1-*N*-phenylnaphthylamine (NPN), for which permeation of *E. coli* cells correlates inversely with membrane integrity [558]. Finally, major changes in LOS or CPS were not observed in the sensor kinase mutant; however, further biochemical analyses of LOS or CPS isolated from D*cprS* may identify more subtle changes not identified by the more crude methods used initially. Major expression changes in LOS- or CPS-related genes were not observed in Δ*cprS* at the RNA level. Envelope proteome analysis, mentioned above, may uncover changes in expression of enzymes required for synthesis of these structures that may be a result of dysregulation of proteins such as HtrA, which may affect expression and maturation of envelope proteins. However, it is currently unknown if HtrA plays a 'housekeeping' role in *C. jejuni* envelope protein expression, or if it is solely required under stress conditions. Thus, the most information may be uncovered initially by analysis of the envelope proteome of Δ*cprS* (and *cprR*^{OE}), together with analysis of mutants such as Δ*htrA* and Δ*peb4*, which may identify enzymes expressed differently in these strains from WT and help to direct analysis to specific envelope phenomena (such as PG, LOS, CPS, or proteins) that may be altered upon dysregulation of CprRS signalling. A Δ*htrA* mutant was modestly enhanced for biofilm formation, suggesting that while loss of HtrA function may contribute to phenotypes of Δ*cprS*, it was not solely responsible. This may be followed up with creation of targeted mutant strains in genes encoding dysregulated proteins to identify mutations that may further replicate the phenotypes of Δ*cprS*.

The cell envelope is the first line of interaction between a bacterium and the environment, and thus it is not surprising that well-conserved systems for detecting and responding to envelope stress exist. Many of these are TCRSs, including the general secretion stress response system CssRS of *B. subtilis*, and the BaeSR and CpxAR systems of Gram-negative bacteria [533, 559-561]. Specifically, CpxAR integrates numerous signals, such as adhesion, misfolded envelope proteins, and metabolic cues to promote survival by protecting the cell envelope [561], and the Cpx system has been proposed to mediate biofilm formation [419]. A similar system for regulating envelope physiology in *C. jejuni* has not yet been identified. As homologues of classical extracytoplasmic sigma factors, such as RpoE/σ^E, as well as an obvious CpxAR homologue, are absent from the *C. jejuni* genome [55], it is possible that CprRS may fill this role in *C. jejuni*. The strong conservation of the

location of *cprRS* downstream of *htrA* is consistent with a role for CprRS in regulating aspects of the cell envelope. However, it is unlikely that CprRS represents the top of the *C. jejuni* envelope stress response if biofilm formation represents a major adaptation to adverse conditions, as defects in CprRS signalling (i.e., Δ*cprS*) enhance biofilms. However, CprRS may repress this response in the absence of adverse conditions. Nonetheless, CprRS controls essential aspects of *C. jejuni* biology, such as envelope physiology, and understanding regulation of factors that control envelope metabolism may provide insight for design of infection control strategies. In support of this, Δ*cprS* was defective for both colonization of chickens and tolerance of conditions such as high salt that may present during food processing.

The inability to recover Δ*cprR* mutants suggests CprRS was essential for expression of genes necessary for routine laboratory culture. Based on the preliminary identification of the CprRS regulon, this TCRS may control genes required to adapt of the cell envelope to growth in rich media. However, the signal to which CprS responds has not yet been identified. Activity of the *cprR* promoter appeared to correlate with activation of CprRS and thus may indicate presence of the CprS-activating signal. Increased expression of the *cprR* promoter through log phase suggests that CprS may detect conditions that develop during growth in rich broth culture. CprRS-controlled envelope genes may be required for either rapid increases in cell numbers/biomass during logarithmic growth, or tolerance of potentially toxic metabolites that are the product of growth in rich media, such as ammonia from deamination of amino acids [67], or acetate from acetyl CoA recycling [433]. Consistent with this, the Δ*cprS* mutant enters stationary phase early. Growth in rich media can result in excretion of acetate [76], which could accumulate and affect the envelope. However, mutation of acetate switch pathway genes, such as *pta* or *ackA,* does not suppress Δ*cprS* phenotypes (S. Svensson and E. Gaynor, unpublished observations). Growth and biofilm phenotypes of Δ*cprS* were rescued by increasing broth concentration. This suggests cell envelope changes may be affecting either acquisition of nutrients, or causing envelope instability that can be rescued by increasing osmotic or divalent cation support.

Because of the identification of CprRS as upregulated in the presence of live host cells, the Δ*cprS* sensor kinase mutant was assessed for host-related phenotypes such as adherence, invasion, and intracellular survival. Interestingly, Δ*cprS* showed no difference from WT for either adherence or invasion into INT407 cells, but showed a modest (<10-fold) increase in survival following invasion. To the best of our knowledge, this was the first report of a *C. jejuni* mutant exhibiting enhanced intracellular survival (and no invasion differences from WT). More recently, mutants in the *paqPQ* ABC transporter have been found to survive better than WT within host cells [234], and cells infected with these mutants showed decreased activation of proinflammatory pathways, suggesting that increased survival of bacteria may be consequence of increased survival of host cell monolayers. It has also been proposed that *C. jejuni* uses a different metabolic program within epithelial cells [226], and the broad expression changes observed in Δ*cprS* may reflect broad metabolic changes that make the mutant more suited to survival inside host cells. Finally, exposure of clinical strains to an aerobic atmosphere

prior to infection of epithelial cells monolayers enhances intracellular survival [562]; thus, the stress of dysregulated essential processes may have 'primed' Δ*cprS* for survival within the intracellular environment.

5.3 Mechanisms of biofilm formation in Δ*cprS* and WT *C. jejuni*

Analysis of the Δ*cprS* hyperbiofilm mutant unexpectedly contributed to understanding of *C. jejuni* biofilms (**FIG. 5.1**). Reports in the literature suggested that flagella are maintained in, and required by, *C. jejuni* biofilm cells. However, the reason for this was unclear. Furthermore, the nature of the *C. jejuni* biofilm matrix was unknown, as mutation of many carbohydrate-related genes either had no effect on biofilm formation or enhanced biofilm formation, and genes responsible for biogenesis of a prototypical EPS are not apparent in the annotated genome. Finally, while some conditions that promote biofilm formation had been identified, specific signals that may trigger biofilm formation were unknown, and while reports suggested that biofilm-residing *C. jejuni* are more resilient than their planktonic counterparts, it had not yet been demonstrated that biofilms provide WT bacteria with enhanced fitness in the presence of pathogenesis-related stresses. Analysis of Δ*cprS* and complementary mutants, together with specific antimicrobial agents, has provided a clearer picture of the role of flagella in biofilms, demonstrate both the presence of and requirement for eDNA during biofilm maturation, and finally, demonstrate that *C. jejuni* requires biofilm formation to tolerate specific stresses.

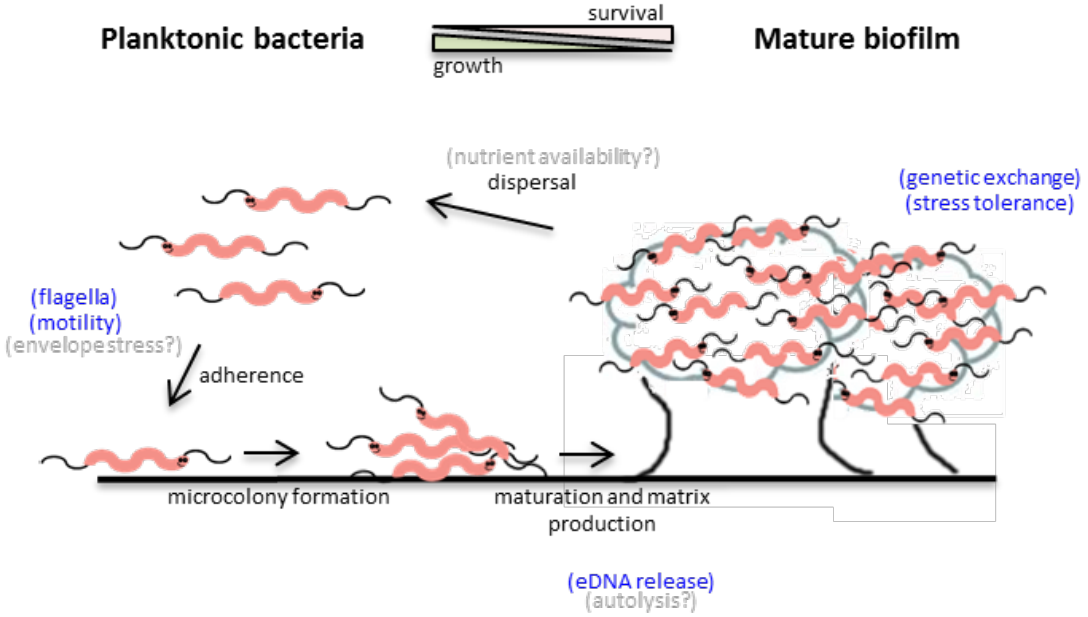


FIG. 5.1. General model of mechanisms of formation, and characteristics of *C. jejuni* biofilms. Evidence for the role of flagella and motility, eDNA release, and genetic exchange in *C. jejuni* biofilms has been provided. Biofilm formation also appears to confer tolerance of specific stresses, such as those that may be encountered during pathogenesis.

5.3.1 Flagella.

C. jejuni flagella have numerous roles beyond motility, including autoagglutination, adherence to host cells, and secretion of virulence factors [109](**TABLE 5.1**). Consistent with many previous reports, flagella were found to be absolutely required for biofilm formation. Furthermore, the contribution of flagella to biofilm formation may be two-fold: first, they may allow adhesion to a surface. While the flagellar structure was absolutely required for biofilm formation under all conditions, paralyzed flagella were sufficient to mediate at least modest levels of biofilm formation when conditions promoting enhanced biofilm also exist (such as DOC). Second, motility may allow cells to reach a surface, as delayed, but not completely defective, biofilms were observed for in Δ*pflA* paralyzed flagellum mutant. Development of a procedure for centrifuging bacteria onto coverslips before incubation, to allow motility-independent interaction with the glass surface, will allow more definitive statement regarding the specific contributions of motility and flagellar structure to biofilm formation. Furthermore, comparing biofilm formation in a double Δ*cprS* Δ*flhA* mutant to that of Δ*cprS* Δ*pflA* will also confirm that motility is, in general, conditionally dispensable.

Microarray analysis suggested the Δ*cprS* mutant shows higher expression of many flagellar genes. Since flagella were absolutely required for biofilm formation, even by Δ*cprS*, it is unclear whether overexpression of flagellar genes in the sensor kinase mutant was solely responsible for its hyperbiofilm phenotype. Biofilm-defective mutants (Δ*carB*, Δ*luxS, and* Δ*pgp1*) with mild motility defects have been reported [368, 428, 563], and Δ*cprS* appears to be dominant over such mutations for biofilm phenotype (data not shown). It was initially hypothesized that increased biofilm formation may have been due to increased secretion of biofilm mediators through the flagellar export apparatus. This was consistent with observation of proteins in supernatants of Δ*cprS* cultures. It was also supported by the fact that synthesis of flagellar-secreted Cia proteins is increased in DOC [123], which also enhanced biofilm formation. However, increased protein release was observed to occur independently of the flagellar export apparatus in both WT and Δ*cprS* genetic backgrounds. Comparison of this analysis to published work suggests that the biofilm defect of flagellar mutants was not

TABLE 5.1. Summary of phenotypes of relevant *C. jejuni* flagellar mutants.

Strain	Gene product	Flagellum morphology [A,C]	Motility [A,D]	Secretion [B]	Autoagglutination [D,E,F]	Biofilms
WT	N/A	WT	+	+	+	+
Δ*flaA*	major flagellin	stubby	reduced	+	-	delayed[A]
Δ*flaB*	minor flagellin	~WT	+	N/A	N/A	delayed[A]
Δ*rpoN*	s factor (s^{54})	aflagellate	-	N/A	-	-
Δ*flaA*Δ*flaB*	major/minor flagellins	aflagellate	-	-	-	-[G]
Δ*flg*R	response regulator	aflagellate	-	N/A	N/A	-
Δ*flhA*	export apparatus	aflagellate	-	N/A	-	-
Δ*pflA*	unknown	paralyzed	-	N/A	+	delayed
Δ*flaG*	possible flagellar protein	long flagella	+	N/A	N/A	-[A]
Δ*fliA*	s factor (s^{28})	stubby	reduced	N/A	N/A	delayed
Δ*flaC*	minor flagellin	~WT	+	N/A	N/A	delayed[A]

[A]Kalmokoff *et al.* 2006. [E]Guerry *et al.* 2006.
[B]Konkel *et al.* 2004. [F]Misawa *et al.* 2000.
[C]Jagannathan *et al.* 2001. [G]Reeser *et al.* 2007.
[D]Golden *et al.* 2002.

due to loss of secretion. Secretion of Cia proteins is dependent on a functional export apparatus, and mutations affecting filament assembly cause a secretion-negative phenotype [121]. In contrast, a Δ*flaA* mutant, which contains stubby flagella and is non-motile, is secretion positive. Importantly, this mutant has also been reported to be biofilm defective [174, 378, 383]. Cytosolic proteins were also observed in culture supernatants, which was consistent with lysis. Moreover, it is likely that increased flagellar expression in Δ*cprS* was not causing lysis, as a double Δ*cprS* Δ*flhA* mutant, which should not express flagella, still showed increased lysis. Furthermore, mutation of *flhA* in a WT genetic background did not affect DOC-stimulated lysis. Analysis of levels of Cia secretion in Δ*cprS*, in the presence of envelope stressors other than DOC, or mutation of specific Cia proteins in the Δ*cprS* strain, may confirm that increased secretion does not underlie the Δ*cprS* enhanced biofilm phenotype. Investigation of the epistatic relationships between *cprS* and flagellar genes, such as *fliA*, *flaA*, and *flaB* would also be interesting, as would isolation of *C. jejuni* mutants that are motile but biofilm defective.

5.3.2 Biofilm matrix and eDNA.

Surface carbohydrates are intimately related to biofilm formation in most bacteria. However, no difference was found for Δ*cprS* for surface carbohydrate expression, including LOS, CPS, and the CFW-reactive polysaccharide, suggesting that mechanisms of enhanced biofilm formation in the Δ*cprS* mutant were distinct from those previously characterized. Nonetheless, more extensive analysis of LOS and CPS by biochemical methods may be warranted. Surface hydrophobicity of *C. jejuni* is affected by mutations affecting the CPS and LOS [564], and cell surface hydrophobicity can mediate surface interactions leading biofilm formation [565, 566]. The method commonly used to determine hydrophobicity of *C. jejuni* is 'salting out' with ammonium sulfate, and the tendency of cultures of the Δ*cprS* mutant to aggregate interfered with this procedure. Use of solvent partitioning may be more suited to analysis of Δ*cprS*. Certainly, the extensive expression differences in cell-envelope-related gene expression in Δ*cprS* warrant further exploration of its surface characteristics.

An EPS component of the *C. jejuni* matrix has not been definitively identified, and this remains a significant open question. Overexpression of putative matrix carbohydrates in Δ*cprS* was not observed. However, it appeared that eDNA may be a key component of the matrix, and it contributed to both structure and function of the *C. jejuni* biofilm. Biofilms formed by WT bacteria grown in DNase resembled those formed by strains without flagella (i.e. Δ*flhA*) when stained with CV and quantified or observed macroscopically. However, when these biofilms were observed under the microscope, the biofilm defect exhibited by cells grown in DNase was distinct from that of aflagellate mutants. Specifically, while aflagellate mutants adhered very poorly to the slide, in DNase, bacteria adhered to the slide, but were arrested at the microcolony stage. This suggests that flagellar mutations and removal of eDNA act at different stages in the biofilm formation program. Release of eDNA was also observed following adherence. Both culture supernatants and gDNA promoted biofilm formation, suggesting that eDNA may promote biofilm maturation (following adherence). Highly purified DNA from an unrelated organism (salmon) did not enhance biofilm formation as strongly as

that extracted from *C. jejuni*. It has been shown that an immune response directed towards a bacterial nucleoid protein could disperse biofilms [567]; therefore, eDNA alone may not be sufficient to promote biofilm maturation, and chromatin-like material might actually be required.

The mechanism by which eDNA was released in both WT and Δ*cprS* is unclear. The amount of eDNA was increased in Δ*cprS*, which also exhibited dysregulation of envelope-related genes, PG structure changes, and sensitivities that were consistent with envelope alterations. Furthermore, biofilm formation was also increased in WT bacteria grown under envelope stress conditions, or other mutant strains with lesions in envelope-related genes. The modest changes in PG structure of Δ*cprS* were not consistent with a defect in transpeptidation, but more with decreased trimming of muropeptides. It is still possible that this mutant contained localized regions of defective crosslinking that cannot be observed by the methods used in this study that may lead to lysis. It thus is unclear whether lysis in Δ*cprS* was passive, or due to triggering of an active autolytic mechanism. The osmotic sensitivity phenotype displayed by Δ*cprS* is certainly consistent with passive lysis due to loss of cell wall integrity under differences in osmotic pressure. However, inhibition of PG crosslinking by mutation of PG modifying enzymes and antibiotics can trigger autolytic mechanisms in other bacteria [568]. Other modes of envelope stress can also trigger autolysis, and autolytic mechanisms are commonly observed during biofilm formation [342, 569, 570]. Increased adherence of Δ*cprS* to slides prior to lysis was observed, suggesting that lysis may occur following phenomena that initiate biofilm formation. Thus, lysis may be a regulated response to envelope stress that occurs in later steps of biofilm formation, rather than passive lysis.

Autolytic phenomena often result from activation of PG hydrolases [148]. Homologues of accessory autolysins were not identified in the genome; however, it is possible that a biofilm-related autolytic mechanism uses core PG modification enzymes. Many of these may be essential, and are likely to not be uncovered in transposon suppressor screens. The Δ*cprS* mutant did not show changes in muropeptide species that were consistent with altered activity of lytic transglycosylases, such as an increase in shorter chain muropeptides or an increase in Anhydro species. The overall degree of PG crosslinking was also not different in Δ*cprS* compared to WT. Modest changes in peptide chain length were observed; thus a putative autolytic mechanism may arise in Δ*cprS* as a result of altered activity of carboxy- or endopeptidases. However, specific players in this putative mechanism are currently unknown. Interestingly, autolysis can also be affected by HtrA-like proteases, which can control the activity of specific autolysins. In *E. faecalis,* proteases regulate biofilm formation through control of eDNA release [342], and in *Lactococcus lactis*, HtrA degrades the AcmA autolysin, and decreased activity of HtrA increases autolysis [571]. Morphological changes have also been reported in a Δ*htrA* mutant, which was also found to be mildly biofilm-enhanced; however, the nature of these changes was not described [256]. It cannot be definitively concluded that the mechanism of DNA release was the same in WT bacteria under envelope stress and in Δ*cprS*; however, observations presented here suggest both involve a lytic mechanism. Development of zymogram protocols for *C. jejuni* has not been

successful (E. Frirdich, personal communication); however, such assays may help to identify changes in autolysin activity in Δ*cprS*. Finally, although a strong correlation between lysis and biofilm formation was observed, it is still unclear whether lysis either caused, or was required for biofilm formation. Stabilization of the cell envelope with divalent cations suppressed the Δ*cprS* biofilm phenotype. It will certainly be interesting to observe if supplementation of MH broth with Mg^{2+} also rescues lysis in the mutant. Adaptations of Live/Dead assays [572], or experiments to assess the integrity of the *C. jejuni* membrane may also solidify a connection between these phenomena and more finely define the mechanism of autolysis in *C. jejuni*.

The small colony phenotype and lysis in broth culture of Δ*cprS* was reminiscent of *P. aeruginosa* mutants that develop in biofilms [573]. Such strains have changes in LPS structure that make them more sensitive to a self-produced colicin-like molecule, pyocin. Thus, when grown in planktonic broth culture, mutants lyse at a lower culture density than the WT strain. It was proposed that such mutations confer fitness in biofilms, but decreased fitness on plates or in planktonic culture. Phages also contribute to biofilm-related autolysis in Gram-negative bacteria [574, 575]. Colicin-like molecules were not found, and the 81-176 genome does not appear to harbour phages that may contribute to lysis. Of note, the Δ*cprS* mutant has also been observed to be defective for growth in mixed culture with WT (S. Svensson and E. Gaynor, unpublished observations), although it is unclear whether this observation was due to competition for resources or sensitivity of the mutant to metabolites or lytic agents produced by WT.

5.4 Contribution of the biofilm lifestyle to stress tolerance of *C. jejuni*

In the absence of the large repertoire of survival factors that are expected for a zoonotic pathogen, it was hypothesized that global changes in physiology may underlie adaptation of *C. jejuni* to stressful environments. Furthermore, as phenotypes required for rapid growth are often expressed at the expense of stress tolerance [330], such phenotypes may not be present during planktonic broth culture, explaining the apparent fastidiousness of *C. jejuni* in the lab. It was hypothesized that stress tolerance may be a consequence of the biofilm lifestyle, and consistent with this, biofilms are thought to be a response to stressful conditions, such as decreased nutrient availability [336]. Stress conditions both increase survival [243, 576] and promote biofilm formation [337, 420] of *C. jejuni*. Biofilm formation may be a compensatory response observed upon mutation of key global regulators [368], and such mutants often do not show as strong defects as would be expected for tolerance of *in vitro* stresses or colonization. For example, a Δ*phoX* mutant forms enhanced biofilms, and despite a marked decrease in PolyP levels, shows surprisingly increased resistance to antibiotics and osmotic stress [117]. Moreover, enhanced biofilm-forming mutants such as Δ*ppk1* and Δ*spoT* show either a dose-dependent or no defect in chick colonization [229, 246](E. Gaynor, personal communication), like Δ*cprS*. Thus, in addition to an understanding of mechanisms of biofilm formation, evidence to support the hypotheses that biofilms confer stress tolerance to *C. jejuni* was also sought. Biofilm formation was enhanced in WT bacteria grown in a range of envelope-stressing compounds, and in mutants with envelope-related defects, suggesting that biofilm formation correlated with the presence of stressful conditions. Perhaps most

significantly, however, if biofilm formation was inhibited, survival and/or growth of bacteria in the presence of stress conditions was markedly affected. Consistent with behaviour of aflagellate bacteria in this study, a Δ*rpoN* mutant cannot tolerate osmotic and acid stress in static culture [289]. Mutation of a phosphoethanolamine transferase required for flagellar biosynthesis causes sensitivity to PxB [323]; however, as this mutation also affects LOS modification, it cannot be concluded whether a biofilm defect is solely responsible for PxB sensitivity in this mutant.

This work raises questions about experiments that have used biofilm-defective strains, such as flagellar mutants. For example, Cia protein secretion appears to be abolished in flagellar mutants. However, secretion can be stimulated by treating bacteria with sub-MIC levels of DOC, and flagellar mutants, which cannot form a biofilm like WT, may be more sensitive to DOC than the WT strain. Therefore, reductions in expression of certain proteins may be a byproduct of reduced fitness under these conditions, rather than a direct loss of flagellar secretion. Similar effects may be observed in cell infection experiments where detergents (or water) are used to lyse eukaryotic cells. It follows that mutants should be assessed for both survival and biofilm formation under conditions used in the experiment. Development of conditions that enhance biofilm formation, either through genetic or chemical means, without compromising the cell envelope, will allow examination of biofilm physiology and resilience in the absence of pleiotropic effects on physiology of bacteria. Nonetheless, the data obtained in this work supports the original hypothesis that biofilm formation does in fact impart *C. jejuni* with resilience not seen in planktonic cells, and has provided evidence that more global physiological responses may provide pathogens with fitness not apparent from either behaviour in the laboratory, or annotation of genome sequences.

Natural transformation is common in *C. jejuni* strains, and recombination and acquisition of horizontally-acquired elements contributes to virulence [56, 78, 577-579]. Exchange and recombination of genetic markers both *in vitro* and during colonization of chickens has been observed [130]. This work provides preliminary evidence that lifestyles such as biofilm formation may also contribute to survival by increasing dissemination of virulence- and/or survival- related genes. Whether DNA uptake and recombination mechanisms are upregulated in biofilms remains to be seen, and investigation of mutation and recombination rates in biofilm cells will also be interesting. The rate of flagellar phase variation may be lower *in vivo*, due to the importance of flagella for colonization [75, 395]. It would be interesting to determine if emergence of flagellar phase variants is also lower in biofilms. Furthermore, a mechanism of *C. jejuni* biofilm dispersal has also not yet been identified, and phase variation of flagellar expression could presumably contribute to this.

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APPENDIX A. Primers.

TABLE A.1. Primers used in this study.

Primer name	Sequence (5' → 3')	Restriction site	Source
cprS-TOPO-FWD	GTTTCAAGCGCAAGACAT	-	this study
cprS-TOPO-REV	GAGCTTAAGGAGCGTTTGGA	-	this study
cprS-INV-FWD	TAAGCCACAAAAAGCCAAGT	*Mfe*I	this study
cprS-INV-REV	AATCAAGTTTTGGATTAGGGCTTT	*Mfe*I	this study
cprS-pGEM-FWD	GTCTGGATCCGTTTTGCGACTTTGCTTGTGC	*Bam*HI	this study
cprS-pGEM-REV	GTGTCTCGAGTACTCTACCGCTGAGCTAATCCG	*Xho*I	this study
cprS-INV2-FWD	GCAGTCTAGAGAAATGATAGAAGATAATAAATATAAAGAGCG	*Xba*I	this study
cprS-INV2-REV	GTAAGGTACCGCCAAGTATAACACTAACCCCAGC	*Kpn*I	this study
cprS-pRRC-FWD	GCTCTAGAGGTTATAAGCTTACTCAATGAATAAATC	*Xba*I	this study
cprS-pRRC-REV	GCCAATTGTTACTCCTTAACAATAACACTTTTTAAATTTC	*Mfe*I	this study
ak233	GCAAGAGTTTTGCTTATGTTAGCAC	-	Karlyshev and Wren 2005
PKanF	CAAGTGGTATGACATTGCCTTCTG	-	J. Ketley
*cpr*R-BT-FWD	GATCGGATCCATGACAAATATTCTTATGATAGAAGATGATT	*Bam*HI	this study
*cpr*R-BT-REV	GTACACTAGTTCATTGAGTAAGCTTATAACCTATTCC	*Spe*I	this study
*dcc*R- BT-FWD	GATCGGATCCATGGCTGCTAAAATTTTACTTTTAG	*Bam*HI	this study
*dcc*R-BT-REV	GTACACTAGTTTAGCCATAGCAATATCCCCTG	*Spe*I	this study
cprS^{CTD}-TRG-FWD	GATCGGATCCATGGGGGTGGATGAAATTTC	*Bam*HI	this study
cprS^{CTD}-TRG- REV	GTACCTCGAGTTACTCCTTAACAATAACACTTTTTAAATTT	*Xho*I	this study
htrA-3'	GTTCAAGTGCTGATGAAGCAGG	-	this study
*cpr*R-pRY112 FWD	GCCAATTGGGGTGATATTATCATAGG	*Mfe*I	this study
*cpr*R-pRY112-REV	GCGCTCTAGAATCATTGAGTAAGCTT	*Xba*I	this study
*cpr*R^{Asp52Ala}-FWD	GCTTATCATTTTAGCGCTTTCTTTGC	*Afe*I	this study
*cpr*R^{Asp52Ala}-REV	GCAAAGAAAGCGCTAAAATGATAAGC	*Afe*I	this study
*cpr*R^{Asp52Glu}-FWD	GCTTATCATTTTAGCGCTTTCTTTGC	-	this study
*cpr*R^{Asp52Glu}-REV	GCAAAGAAAGCTCTAAAATGATAAGC	-	this study
*cpr*R^{KO}-FWD	TCACTGATGTTCAAAAAGGTTCAAG	-	this study
*cpr*R^{KO}-REV	CCAGTGCTTACATCTTCTATATCACC	-	this study
*cpr*R-INV-FWD	GCGGTACCGCTTAGAAAGACCTATAT	*Kpn*I	this study
*cpr*R-INV-REV	GCGGATCCGCAGTAGAATTCGTCAAA	*Bam*HI	this study
*cpr*R-QPCR-FWD	GACCTTTCTTTGCCAGGGCTTGAT	-	this study
*cpr*R-QPCR-REV	GGTAGGTAATCATCTGCTCCAAGCTC	-	this study
htrA-QPCR-FWD	AATCCTGCTGCTGGAAATGCAGTG	-	this study
htrA-QPCR-REV	ATCCAAAGGGCTTGGACGATTTGC	-	this study
gyrA-QPCR-FWD	CTTTGCCTGACGCAAGAGATGGTT	-	this study
gyrA-QPCR-REV	AGCACCCACTATACGGGCTGATTT	-	this study
*cpr*R-GSP1	TGTCCTTTCATGGTAATAATATGC	-	this study
*cpr*R-GSP2	CTTTTACACTTTTTGCTATGGCG	-	this study
*cpr*R-GSP3	AAGGTTTTGGTAGGTAATCATCTGC	-	this study
P_{cprR}-*lux*-FWD	GTACGCGGCCGCGGGTGATATTATCA	*Not*I	this study
P_{cprR}-*lux*-REV	TTATTTTAAACTTAATTTTAATTTTAAA	-	this study

TABLE A.1. cont.

Primer name	Sequence (5' -> 3')	Restriction site	Source
*cpr*R^{CTD} B1H-FWD	GTACGCGGCCGCGATAGCAAAAAGT	*Not*I	this study
*cpr*R^{CTD} B1H-REV	GTACCCTAGGTCATTGAGTAAGCTTA	*Avr*II	this study
HU100	CAAATATGTATCCGCTCATGAC	-	Meng *et al.* 2006
htrA-FWD	CCAAATCCTCCAAAACCACTGC	-	this study
htrA-REV	GATCTTTTACACTTTTTGCTATGGC	-	this study
htrA-INV-5'	GATCGAATTCAGGATTCACACGATTAGCCG	*Eco*RI	this study
htrA-INV-3'	GATCGGATCCTTTTGCGACTTTGATTGTGC	*Bam*HI	this study
P$_{htrA}$-A-FWD	GATCCCCGGGTTATTTACAATTTTGTAAATTTTCGGTTTTAT	*Xma*I	this study
P$_{htrA}$-A--REV	GATCGAATTCATAAAACCGAAAATTTACAAAATTGTAAATAA	*Eco*RI	this study
P$_{htrA}$-B-FWD	GATCCCCGGGACTATGAAGACAGTAAAATATTTTACGAGATA	*Xma*I	this study
P$_{htrA}$-B-REV	GATCGAATTCTATCTCGTAAAATATTTTACTGTCTTCATAGT	*Eco*RI	this study
P$_{htrA}$-C-FWD	GATCCCCGGGATTTATCTTTAAGAACAAAAGGAAATGAAATG	*Xma*I	this study
P$_{htrA}$-C-REV	GATCGAATTCCATTTCATTTCCTTTTGTTCTTAAAGATAAAT	*Eco*RI	this study
*cpr*S^{CTD}-FWD	GCTCTAGAAAGGAAGATAAATGATAAATTTGCC	*Mfe*I	this study
*cpr*S^{CTD}-REV	GCCAATTGTTACTCCTTAACAATAACACTTTTTAAATTTC	*Xba*I	this study
*cpr*R^{NTD}-FWD	GCTCTAGAAAAGGAAAACTATGACAAATATTCTTATG	*Xba*I	this study
*cpr*R^{NTD}-REV	GATCTCTAGATCAGCTTTTTGTATTTGAAATACGTCTTAAAT	*Xba*I	this study
P$_{peb1a}$-FWD	TCGGGCCCTAGGTGGAGCTGAGTTAATGCAT	*Apa*I	this study
P$_{peb1a}$-REV	CTCTCGAGTTAATGTCAGAAATAAACCTTGCAT	*Xho*I	this study
*cpr*R^{OE}-P$_{peb1a}$-FWD	ACGATATCAAATAAAGGAAAACTATGACAAATATTCTTAT	*Pst*I	this study
*cpr*R^{OE}-P$_{peb1a}$-REV	ACTGTACTGCAGATCGAAGATTTATTCATTGAGTAAGC	*Sma*I	this study
*cpr*R^{KD}-P$_{peb1a}$-FWD	ATCGTACTGCAGATGACAAATATTCTTATGATAGAAGATGAT	*Eco*RV	this study
*cpr*R^{KD}-P$_{peb1a}$-REV	GTACATCCCGGGTATAACCTATTCCACGTATAGAGTG	*Pst*I	this study

APPENDIX B. Strains and plasmids.

TABLE B.1. Strains and plasmids used in this study.

Name	Relevant characteristics	Source
***C. jejuni* strains**		
81-176	Wild type strain	Korlath *et al.* 1985
Δ*cprS*	Δ*cprS*::Cm^R	this study
Δ*cprS* (Kan^R)	Δ*cprS*::Kan^R	this study
Δ*cprS*^{C}	Δ*cprS*::Cm^R *rrn*::*cprS*	this study
Δ*spoT*	*spoT*::Kan^R	Gaynor *et al.* 2005
DRH461	*rpsL*^{Sm} Δ*astA*	Hendrixson and DiRita 2003
Δ*cpr*R pSS82	Δ*cprR*::Kan^R pSS82	this study
Δ*flhA*	*flhA*::*cat-rpsL*	Hendrixson and DiRita 2003
Δ*flg*R	*flgR*::*kan-rpsL*	Hendrixson and DiRita 2003
Δ*pflA*	*pflA*::*solo* (Kan^R) (transposon mutant)	laboratory collection
Δ*cprS* Δ*flhA*	Δ*cprS*::Kan^R, Δ*flhA*::Cm^R	this study
*cpr*R^{OE}	pRY112-*cprR*^{OE} (*peb1a* promoter)	this study
cprS^{CTD}	*rrn*::*cprS*	this study
Δ*cprS cprS*^{CTD}	Δ*cprS*::Kan^R *rrn*::*cprS*	this study
*cpr*R^{NTD}	*rrn*::*cprR*^{NTD}	this study
Δ*cprS cpr*R^{NTD}	Δ*cprS*::Kan^R *rrn*::*cprR*^{NTD}	this study
*cpr*R^{KD}	pRY112-*cprR*^{KD}	this study
Δ*cprS cpr*R^{KD}	Δ*cprS*::Kan^R pRY112-*cprR*^{KD}	this study
Δ*kpsS*	*kpsS*::*solo* (Kan^R) (transposon mutant)	laboratory collection
Δ*kpsM*	*kpsM*::Kan^R	Bacon *et al.* 2001
Δ*waaF*	81-176 Δ*waaF*::Kan^R	Naito *et al.* 2010
Δ*cprS* Δ*waaF*	Δ*cprS*::Cm^R Δ*waaF*::Kan^R	this study
Δ*rpoN*	Δ*rpoN*::*cat-rpsL*	Hendrixson and DiRita 2003
Δ*cprS* Δ*rpoN*	Δ*cprS*::Kan^R Δ*rpoN*::*cat-rpsL*	this study
Δ*flhB*	*flhB*::*cat-rpsL*	Hendrixson and DiRita 2003
Δ*flgS*	Δ*flgS*::*cat-rpsL*	Hendrixson and DiRita 2003
Δ*htrA*	Δ*htrA*::Kan^R	this study
***E. coli* strains**		
DH5a	general cloning strain, *recA1 endA1*	Invitrogen
Bacteriomatch II	two-hybrid reporter strain, *HIS3 aad*, Kan^R	Stratagene
US0	one-hybrid reporter strain, Δ*hisB* Δ*pyrF* [F′*lacI*^{q}Z DMI5 Tn10 (Tet^R)]	Meng *et al.* 2006
Plasmids		
pCR-XL-TOPO®	PCR cloning vector, Kan^R	Invitrogen
pGEM-T	PCR cloning vector, Amp^R	Promega
pJM1	*cprS*::Cm^R in pCR-XL-TOPO®, Kan^R Cm^R	this study
pJM2	*cprR*::Cm^R in pCR-XL-TOPO®, Kan^R Cm^R	this study
pSS3	*cprS*::Kan^R in pGEM-T, Kan^R Amp^R	this study
pRK600	conjugation helper plasmid, Cm^R	Keen *et al.* 1988
pRRK	*C. jejuni* rRNA spacer integration vector, Kan^R Amp^R	J. Ketley
pSS50	*cprS* in pRRK, Kan^R Amp^R	this study

TABLE B.1 cont.

Name	Relevant characteristics	Source
pBT	two-hybrid lambda cI fusion vector, Tet^R	Stratagene
pTRG	two-hybrid RNAPα fusion vector, Cm^R	Stratagene
pSS38	*cprS*^{CTD} in pTRG, Cm^R	this study
pSS39	*dccR* in pBT, Tet^R	this study
pSS41	*cprR* in pBT, Tet^R	this study
pSS27	*cprR*^{NTD} in pRRC, Cm^R	this study
pSS55	*cprS*^{CTD} in pRRC, Cm^R	this study
pSS56	*cprR*::Kan^R in pGEM-T, Amp^R Kan^R	this study
pB1H1	RNAPα-fusion vector, Cm^R	Meng *et al.* 2006
pU3H3	One-hybrid library reporter vector, Kan^R	Meng *et al.* 2006
pSS83	*cprR*^{CTD} in pB1H1, Cm^R	this study
pRY112	*C. jejuni*/*E. coli* shuttle vector, Cm^R	Yao *et al.* 1993
pSS82	*cprR*^{WT} in pRY112, native promoter, Cm^R	this study
pSS84	*cprR*^{Asp52Ala} in pRY112, native promoter, Cm^R	this study
pSS85	*cprR*^{Asp52Glu} in pRY112, native promoter, Cm^R	this study
pRY112-*lux*	promoterless *luxCDABE* in pRY112, Cm^R	Apel *et al.* 2012
pSS81	*cprR* promoter in pRY112-*lux*, Cm^R	this study
pRY112-*gfp*	GFP driven from *atpF'* promoter in pRY112, Cm^R	Apel *et al.* 2012
pSS87	*cprR*^{WT} in pGEM-T, native promoter, Amp^R	this study
pSS88	*cprR*^{Asp52Ala} in pGEM-T, native promoter, Amp^R	this study
pSS95	*htrA* promoter fragment A in pU3H3, Kan^R	this study
pSS96	*htrA* promoter fragment B in pU3H3, Kan^R	this study
pSS97	*htrA* promoter fragment C in pU3H3, Kan^R	this study
pRY112-P_{peb1a}	*peb1a* promoter in pRY112, Cm^R	this study
pRY112-*cprR*^{KD}	*cprR* antisense expressed from the *peb1* promoter, Cm^R	this study
pRY112-*cprR*^{OE}	*cprR* overexpressed from the *peb1* promoter, Cm^R	this study

APPENDIX C. Microarray data.

TABLE C.1 Δ*cprS* microarray data. The fold change in expression for each gene at four time points in an MH broth growth curve are shown for WT vs. the Δ*cprS* mutant. Reported differences are significant by a parametric statistical *t* test ($p<0.05$) as described in the Methods.

	Downregulated in Δ*cprS*	Fold change (WT vs. Δ*cprS*)	
		Experiment 1	Experiment 2
3h	Cj1170c Omp50 porin	-2.56	-3.02
	Cj1169c periplasmic protein	-2.50	-2.14
	Cj1357c NrfA cytochrome c552 nitrite reductase catalytic subunit	-2.01	-2.14
6h	Cj1170c Omp50 porin	-2.50	-4.82
	Cj1169c periplasmic protein	-2.22	-3.43
12h	Cj0671 DcuB C4-dicarboxylate transporter, anaerobic	-13.90	-3.01
	Cj0699c GlnA glutamine synthetase	-3.53	-14.17
	Cj0009 GltD glutamate synthase small subunit	-2.79	-4.92
	Cj0264c molybdopterin-containing oxidoreductase	-8.06	-6.21
	Cj0265c putative cytochrome C-type haem-binding periplasmic protein	-6.04	-5.77
	Cj0414 Conserved hypothetical protein	-2.25	-3.27
	Cj0415 putative gluconate dehydrogenase	-2.70	-3.09
	Cj0449c conserved hypothetical protein	-3.90	-2.11
	Cj0604 conserved hypothetical protein	-2.64	-2.14
	Cj0780 NapA Periplasmic nitrate reductase, large subunit	-3.00	-2.46
	Cj0873c hypothetical protein	-2.11	-2.19
	Cj0903 Sodium:alanine symporter family protein	-2.34	-3.01
	Cj1199 Putative iron/ascorbate-dependent oxidoreductase	-2.03	-4.36
24h	Cj1228c HtrA serine protease	-2.16	-2.87
	Cj0087 AspA aspartate ammonia lyase	-2.65	-3.03
	Cj1359 Ppk1 polyphosphate kinase	-2.81	-2.26
	Cj0078 CdtB cytolethal distending toxin subunit	-2.05	-3.08
	CJJ81176_0064 putative periplasmic protein	-2.02	-2.61
	CJJ81176_0064 cytochrome C family protein	-2.13	-2.38
	Cj0185c PhnA-like protein	-3.25	-3.51
	Cj0236c integral membrane protein	-2.24	-3.49
	Cj0256 putative integral membrane sulfatase	-2.13	-3.92
	Cj0298c PanB 3-methyl-2-oxobutanoate hydroxymethyltransferase	-2.18	-2.72
	Cj0358 Cytochrome c551 peroxidase	-2.06	-2.94
	Cj0361 LspA Lipoprotein signal peptidase	-2.13	-2.62
	Cj0362 integral membrane protein	-2.23	-2.83
	Cj0367c CmeA membrane fusion protein	-2.12	-2.93
	Cj0413 probable periplasmic protein	-2.21	-2.03
	Cj0414 conserved hypothetical protein	-3.00	-2.58
	Cj0415 gluconate dehydrogenase	-2.51	-2.24
	Cj0434 Pgm 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	-2.05	-3.78
	Cj0900c hypothetical protein	-2.35	-2.86
	Cj0940 GlnP Amino acid ABC transporter, His/Glu/Gln/Arg/opine family	-2.06	-2.03
	Cj0950 conserved hypothetical lipoprotein	-2.02	-3.58
	Cj0951 Methyl-accepting chemotaxis protein, putative, (pseudogene)	-3.65	-4.47
	Cj0955c PurL Phosphoribosylformylglycinamidine synthase	-2.34	-2.10
	Cj0968 putative periplasmic protein	-3.31	-2.16
	Cj0982 CjaA Amino acid transporter, periplasmic solute-binding protein	-3.17	-2.75
	Cj1127c WlaE Polysaccharide biosynthesis protein	-2.77	-2.12
	Cj1130c WlaB ABC transporter ATP-binding/permease protein	-2.77	-2.41
	Cj1131c GalE UDP-glucose 4-epimerase	-3.40	-3.87
	Cj1132c WlaX Polysaccharide biosynthesis protein	-2.36	-9.25
	Cj1134 WaaM Lipid A biosynthesis lauroyl acyltransferase	-2.10	-8.67
	Cj1153 Cytochrome-related conserved hypothetical protein	-2.31	-2.28
	Cj1160c Small hydrophobic protein	-2.93	-2.49
	Cj1360c putative proteolysis tag for 10Sa_RNA	-2.69	-4.06
	Cj1365 Probable secreted serine protease (subtilase family)	-2.29	-2.29
	Cj1366c GlmS Glucosamine-fructose-6-phosphate aminotransferase	-2.10	-2.77

TABLE C.1. cont.

	Upregulated in Δ*cprS*	Fold change (WT vs. Δ*cprS*)	
		Experiment 1	Experiment 2
3h	Cj1228c HtrA serine protease	3.05	2.84
	Cj1227c CprR response regulator	2.60	2.27
	Cj0420 YceI-like lipid binding periplasmic protein	2.17	3.01
	Cj0012 Rrc rubrerythrin non-haeme iron protein	2.47	3.13
	Cj0409 FrdA fumarate reductase	2.42	2.30
	Cj0408 FrdC fumarate reductase	2.10	2.34
	Cj0074c hypothetical iron-sulfur protein	2.53	2.35
	Cj0075c probable oxidoreductase iron-sulfur subunit	2.74	2.31
	Cj0076c LctP L-lactate permease	2.69	2.24
	Cj0168c conserved hypothetical protein	4.91	15.20
	Cj0853c HemL Glutamate-1-semialdehyde 2,1-aminomutase	2.66	2.03
6h	Cj0168c conserved hypothetical protein	2.66	10.24
	Cj0012 Rrc rubrerythrin non-haeme iron protein	2.43	6.55
	Cj0073c conserved hypothetical protein	2.59	6.35
	Cj0074c hypothetical iron-sulfur protein	2.53	4.78
	Cj0075c probable oxidoreductase iron-sulfur subunit	3.36	7.55
	Cj0076c LctP L-lactate permease	2.12	6.66
	Cj0409 FrdA fumarate reductase	2.08	2.83
	CjPVIRORF6 Cjp07	2.34	5.97
	CjPVIRORF7 Cjp08	2.29	6.60
	CjPVIRORFf2 VirB9	2.12	3.56
12h	Cj0073c conserved hypothetical protein	4.58	4.98
	Cj0074c hypothetical iron-sulfur protein	3.18	3.51
	Cj0075c probable oxidoreductase iron-sulfur subunit	4.97	3.65
	Cj0076c LctP L-lactate permease	3.54	3.77
	Cj0168c conserved hypothetical protein, periplasmic	5.31	2.78
	CJJ81176_0063 conserved hypothetical protein, putative cytochrome C	2.59	2.56
24h	Cj0199c Conserved hypothetical protein	6.42	8.91
	Cj0200c Conserved hypothetical protein	13.36	5.61
	Cj0201c putative integral membrane protein	2.01	3.71
	Cj0390 Conserved hypothetical integral membrane protein (TPR domain protein)	2.74	2.04
	Cj0391c Conserved hypothetical protein, flagellin homologue	8.77	4.22
	Cj0395 Conserved hypothetical protein	2.74	4.45
	Cj0516 PlsC 1-acyl-sn-glycerol-3-phosphate acyltransferase	2.27	2.27
	Cj0520 Conserved hypothetical protein	2.95	2.60
	Cj0887c FlaD Flagellin	8.89	4.31
	Cj0045c putative iron-binding protein	3.12	3.55
	Cj0524 Possible Na-dependent transporter	2.59	4.17
	Cj0525c PbpB Penicillin-binding protein	2.04	2.12
	Cj0526c FliE Flagellar hook-basal body protein	3.87	2.79
	Cj0527c FlgC Flagellar basal body rod protein	4.66	2.62
	Cj0528c FlgB Flagellar basal body rod protein	3.86	4.34
	Cj0571 Conserved hypothetical protein, putative transcriptional regulator	5.59	4.38
	Cj0580 Oxygen-independent coproporphyrinogen III oxidase	4.13	5.04
	Cj0583 Conserved hypothetical protein	2.30	4.19
	Cj0585 FolP 7,8-dihydropteroate synthase	4.44	2.76
	Cj0142 Cation ABC transporter, ATP-binding protein	3.37	2.45
	Cj0126c Conserved hypothetical protein	2.20	4.48
	Cj1001 RpoD RNA polymerase sigma 70	2.67	2.52
	Cj0082 CydB Cytochrome *bd* oxidase, subunit II	3.53	4.04
	Cj0080 Conserved hypothetical protein	9.47	7.93
	Cj0070c very hypothetical protein	4.05	2.25
	Cj0073c conserved hypothetical protein	5.75	3.39
	Cj0074c hypothetical iron-sulfur protein	3.62	2.12

TABLE C.1. cont.

	Upregulated in Δ*cprS*	Fold change (WT vs. Δ*cprS*)	
		Experiment 1	Experiment 2
24h cont.	Cj0075c probable oxidoreductase iron-sulfur subunit	8.39	3.13
	Cj0076c LctP L-lactate permease	5.04	2.15
	Cj0040 hypothetical protein	2.37	5.14
	Cj0041 FliK Hook length control protein	3.45	6.82
	Cj0042 FlgD Flagellar hook assembly protein	10.92	15.48
	Cj0043 FlgE2 Flagellar hook protein	7.87	7.38
	Cj0055c hypothetical protein	2.24	2.05
	Cj0056c hypothetical protein	4.36	2.45
	Cj0062 Conserved hypothetical membrane protein	9.52	11.04
	Cj0063 ATP-binding protein, ParA family	2.41	2.17
	Cj0067 Chlorohydrolase family protein	3.12	4.54
	Cj0617 Conserved hypothetical protein (617 family), contains poly-GC tract	2.59	2.66
	Cj0624 HypC Hydrogenase expression/formation protein	2.43	2.61
	Cj0659 Conserved hypothetical protein, possible periplasmic protein	2.92	3.56
	Cj0687c FlgH Flagellar basal body L-ring protein	4.40	3.17
	Cj0693c MraW S-adenosyl-methyltransferase	2.33	2.19
	Cj0697 FlgG2 Flagellar distal rod protein	4.97	3.87
	Cj0698 FlgG Flagellar distal rod protein	4.05	3.07
	Cj0825 Conserved hypothetical protein, possible peptidase	7.33	7.28
	Cj0826 Conserved hypothetical integral membrane protein	2.36	8.02
	Cj0836 Ogt Methylated-DNA--protein-cysteine methyltransferase	2.34	2.29
	Cj0846 Hypothetical integral membrane protein, Ser/Thr protein phosphatase	3.97	2.48
	Cj0853c HemL Glutamate-1-semialdehyde 2,1-aminomutase	2.95	2.27
	Cj0859c Conserved hypothetical protein	3.79	3.09
	Cj0865 DsbB Disulfide bond formation protein B	2.25	2.66
	Cj0887 Flagellin family protein	8.89	2.50
	Cj0888 possible ABC transporter (ATP-binding protein)	2.21	2.08
	Cj0889 putative two-component sensor histidine kinase	3.63	4.31
	Cj1026 Conserved hypothetical lipoprotein	5.35	4.42
	Cj1031 putative outer membrane component of efflux system	2.23	5.08
	Cj1032 Conserved hypothetical protein	2.58	4.23
	Cj1034 DnaJ homolog	5.63	4.95
	Cj1040 probable transmembrane transport protein	3.46	6.91
	Cj1241 putative transporter	2.03	6.48
	Cj1242 Conserved hypothetical protein	8.02	63.36
	Cj1244 putative radical SAM domain protein	2.06	4.55
	Cj1293 FlmA/PglF/PseB UDP-GlcNAc C6-dehydratase/C4-reductase	8.06	9.53
	Cj1294 PseC putative aminotransferase (DegT family)	7.90	7.39
	Cj1312 PseG nucleotidase	9.52	2.65
	Cj1314 HisF Imidazoleglycerol phosphate synthase, cyclase subunit	3.58	7.56
	Cj1315c HisH Amidotransferase	2.62	4.52
	Cj1316c PseA Flagellin pseudaminic acid biosynthesis protein	3.73	2.30
	Cj1339c FlaB Flagellin	4.03	2.01
	Cj1343c CtsG Campylobacter transformation system protein	2.64	2.73
	Cj1351 PldA Outer membrane phospholipase A	3.11	3.90
	Cj1352 CeuB Enterochelin ABC transporter, permease protein	2.60	2.59
	Cj1371 Conserved hypothetical lipoprotein (VacJ family)	3.45	3.35
	Cj1389 DcuD Cryptic C4-dicarboxylate transporter pseudogene	2.03	3.42
	Cj1423c HddC putative sugar-phosphate nucleotidyltransferase (capsule)	2.43	3.03
	Cj1458c ThiL Thiamine monophosphate kinase	10.47	8.26
	Cj1460 Conserved hypothetical protein (CheY/NtrC-like)	2.65	2.29
	Cj1461 Conserved hypothetical protein, predicted DNA methyltransferase	3.93	10.43
	Cj1462 FlgI Flagellar P-ring protein	31.43	8.28
	Cj1463 FlgJ Flagellar biosynthesis-related muramidase	6.73	13.80
	Cj1464 FlgM Flagellar (s^{28}) anti sigma factor	4.45	2.27
	Cj1465 FlgN secretion chaperone for hook-associated proteins	6.63	4.13
	Cj1466 FlgK Flagellar hook-associated protein	7.05	3.58

TABLE C.1. cont.

	Upregulated in Δ*cprS*	Fold change (WT vs. Δ*cprS*)	
		Experiment 1	**Experiment 2**
24h cont.	Cj1467 Conserved hypothetical protein, DNA-glycosylase	2.98	4.14
	Cj1470c Type II protein secretion system F protein pseudogene	4.77	6.10
	Cj1599 HisB Imidazoleglycerol-phosphate dehydratase/histidinol-phosphatase	2.67	4.56
	Cj1613 Putative pyridoxamine 5'-phosphate oxidase	5.88	2.10
	Cj1616 ChuC Hemin ABC transporter, ATP-binding protein	6.62	2.01
	Cj1628 MotA/TolQ/ExbB biopolymer transport protein	2.90	5.58
	Cj1630 TonB putative transport protein	4.67	3.07
	Cj1631c Conserved hypothetical protein	3.57	2.73
	Cj1632c putative periplasmic protein	9.20	2.83
	Cj1635c Rnc Ribonuclease III	2.89	3.81
	Cj1640 Conserved hypothetical protein, CheY-like	3.06	3.29
	Cj1662 Possible ABC transporter (permease protein)	2.39	3.85
	Cj1668c putative periplasmic protein	2.66	2.38
	Cj1731c RuvC Crossover junction endodeoxyribonuclease	2.37	5.24
	CJ81176_0025 FlgE flagellar hook protein	4.40	2.67
	CjPVIRORF6 Cjp07	2.09	2.33
	CjPVIRORF7 Cjp08	3.34	3.79
	HS23,36CJ1334	3.65	3.03