RESEARCH ARTICLE

Campylobacter jejuni CsrA Regulates Metabolic and Virulence Associated Proteins and Is Necessary for Mouse Colonization

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Abstract

Campylobacter jejuni infection is a leading bacterial cause of gastroenteritis and a common antecedent leading to Gullian-Barré syndrome. Our previous data suggested that the RNA-binding protein CsrA plays an important role in regulating several important phenotypes including motility, biofilm formation, and oxidative stress resistance. In this study, we compared the proteomes of wild type, csrA mutant, and complemented csrA mutant C. jejuni strains in an effort to elucidate the mechanisms by which CsrA affects virulence phenotypes. The putative CsrA regulon was more pronounced at stationary phase (111 regulated proteins) than at mid-log phase (25 regulated proteins). Proteins displaying altered expression in the csrA mutant included diverse metabolic functions, with roles in amino acid metabolism, TCA cycle, acetate metabolism, and various other cell processes, as well as pathogenesis-associated characteristics such as motility, chemotaxis, oxidative stress resistance, and fibronectin binding. The csrA mutant strain also showed altered autoagglutination kinetics when compared to the wild type. CsrA specifically bound the 5’ end of flaA mRNA, and we demonstrated that CsrA is a growth-phase dependent repressor of FlaA expression. Finally, the csrA mutant exhibited reduced ability to colonize in a mouse model when in competition with the wild type, further underscoring the role of CsrA in C. jejuni colonization and pathogenesis.

Introduction

Campylobacter jejuni is a leading bacterial cause of gastroenteritis throughout the world. As a pathogen of significant public health importance, C. jejuni has been extensively studied; however, our understanding of the exact mechanisms by which it causes disease remain incomplete. Although most frequently associated with poultry due to a commensal relationship with avian species and the frequent occurrence of disease following the ingestion of undercooked chicken,
C. jejuni inhabits a broad range of habitats, both enteric and environmental, requiring it to regulate gene expression accordingly. Analysis of the C. jejuni genome indicates that gene regulation is limited to a fraction of the regulatory elements found in other bacteria [1]. However, C. jejuni has a homolog of the E. coli post-transcriptional regulator, CsrA. In E. coli, CsrA regulates the translation of target proteins by binding to sequences containing the motif, ANGGA, which is often found overlapping or adjacent to the ribosome binding site (RBS) of target mRNAs [2]. This action of CsrA upon its target is capable of repressing translation by blocking the availability of the RBS for the ribosome as well as either decreasing or increasing mRNA stability [3, 4]. We previously reported that mutation of C. jejuni csrA disrupts a number of important phenotypes [5]. In the absence of CsrA, C. jejuni cells display reduced motility, decreased ability to accumulate biofilms when grown in static culture, an increased sensitivity to oxidative stresses, and defective adherence to human intestinal epithelial cells in vitro. The csrA mutant was also hyperinvasive in the same intestinal epithelial cell model.

The majority of bacteria in nature exist not as planktonic cells, but as biofilm communities of one or more species held together by an extracellular polymeric substance (EPS)[6] and/or DNA [7]. Campylobacter-containing biofilms occur in numerous niches in nature, including poultry houses and various environmental water locations [8, 9]. Furthermore, biofilm formation appears important for C. jejuni survival in slaughterhouses and on contaminated meats [10]. These mono- or multi-species biofilms may contribute to survival of the fastidious and oxygen-sensitive Campylobacter in nutrient-poor, aerobic environments, and may play a role in the transmission of C. jejuni both within poultry farms, and to humans from the environment. Biofilms can also form on mucosal surfaces during infections; biofilms allow the bacteria within them to become highly resistant to elimination by the host immune system or by antibiotic therapy. Campylobacter biofilms form readily on ex vivo primary human intestinal tissue and thus may be important during human infection [11]. Although the formation of C. jejuni biofilms is not fully understood, as with many other bacteria they contain DNA [7, 12] and polysaccharide [13].

In this study, we used proteomics to identify both direct and indirect targets of CsrA, in an effort to define the C. jejuni CsrA regulon and elucidate molecular mechanisms for the phenotypes observed in the csrA mutant. We found that the effect of CsrA on the expression of whole cell proteins was more profound at stationary phase growth as compared to mid-log. While the expression of 25 proteins was altered in csrA mutant cells grown to mid-log phase, when grown to stationary phase, 111 proteins were differentially expressed in the absence of CsrA. We also demonstrated a direct role for the growth-phase-dependent regulation of FlaA (flagellin) expression via specific binding of CsrA to the 5′ end of flaA mRNA. The csrA mutant also displayed altered autoagglutination kinetics in comparison to the wild type strain, and decreased ability to experimentally colonize mice.

Materials and Methods

Bacterial strains and routine growth conditions

All bacterial strains used in this study are listed in Table 1. C. jejuni strain 81–176 [14], its isogenic csrA mutant, and complemented mutant strain csrA/pJF11 [5] were stored at -80°C in Mueller Hinton (MH) broth (0.2% beef extract, 1.75% acid digest of casein, 0.15% starch) containing 20% (v/v) glycerol and grown on MH agar at 42°C in a tri-gas incubator (85% N2, 10% CO2, 5% O2) or in microaerophilic atmospheres generated within Mitsubishi AnaeroPack jars using AnaeroPouch microaerophilic gas generator sachets (Remel). For some experiments, C. jejuni strains were grown in MH biphasic cultures. To select for C. jejuni mutant and complemented strains, chloramphenicol (30 μg/ml) and kanamycin (30 μg/ml) were used. E. coli
strains were stored at -80°C in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1% NaCl) containing 20% (v:v) glycerol, and routine growth was carried out at 37°C on LB agar or in LB broth with shaking. When appropriate, *E. coli* strains were selected in LB medium using ampicillin (100 μg/ml) or kanamycin (50 μg/ml).

**Two dimensional protein gels**

Proteomics experiments were performed as previously described [16] employing differential in-gel electrophoresis (DIGE) methodology and a GE Biosystems semi-automated workstation. Briefly, wild type, csrA mutant, and complemented *C. jejuni* cells were grown to mid-log (OD$_{600} = 0.5$) or stationary phase (OD$_{600} = 1.0$) in shaking cultures at 42°C. The cultures where then harvested on ice and RNA and protein synthesis were halted as described [16]. Cells were collected by centrifugation, and cell lysates prepared as described [16]. The protein concentrations of the lysates were determined using the BCA assay kit (Pierce). Next, 25 μg of protein each from wild type, csrA mutant, and complemented strains were labeled in the dark with 1 μl Cy2, Cy3, or Cy5 dye conjugates respectively according to the manufacturer’s instructions (GE Biosystems). The labeled proteins were mixed with the corresponding unlabeled proteins in equal amounts (to total 50 μg of each protein sample), subjected to isoelectric focusing (IEF) (IPGPhor strips, range 3–10, non-linear), and then separated by size on 12% SDS polyacrylamide gels.

Following SDS-PAGE, the gel was scanned on a Typhoon fluorescent flatbed scanner (GE Biosystems) at the appropriate wavelengths for Cy2, Cy3, and Cy5 [16]. These images were then overlaid and proteins exhibiting altered expression in the csrA mutant compared to either the wild type or complemented strains were identified using Decyder Differential In-Gel Analysis (DIA) software (version 4.0, GE Biosystems). Proteins of interest were excised, digested

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**Table 1. Bacterial strains, plasmids, and primers used in this study.**

| Strain or Plasmid | Description | Resistance | Source or Reference |
|-------------------|-------------|------------|---------------------|
| **Strain** | | | |
| *Campylobacter jejuni* | | | |
| 81–176 | Wild type | | [14] |
| 81–176ΔcsrA | *C. jejuni* csrA mutant | Cm | [5] |
| 81–176 ΔcsrA/pJF11 | complemented csrA mutant | Cm, Kan | [5] |
| **Escherichia coli** | | | |
| JM109 | Cloning host | | Promega |
| BL21(DE3) | Protein expression strain | | Promega |
| **Plasmids** | | | |
| pCRII-TOPO | Cloning vector | Amp, Kan | Invitrogen |
| pET-20b(+) | Protein expression vector | Amp | Novagen |
| pJAF50 | csrA cloned into pCRII-TOPO | Amp, Kan | This study |
| pJAF51 | csrA cloned into pET-20b(+) | Amp | This study |
| **Primers** | | | |
| JAFcsrANdel | | | This study |
| JAFcsrAXhol | | | This study |
| JL107 | | | This study |
| JL108 | | | This study |
| *phoB* T7-F | | | [15] |
| *phoB* T7-R | | | [15] |

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with trypsin (Invitrogen), and analyzed by using MALDI-ToF/ToF spectrometry (Applied Biosystems). Protein identifications were a result of searching protein databases with tryptic fingerprint data and primary amino acid sequences of fragmented peptides following MS/MS. Proteomics experiments were performed on three biological replicates. Proteins were mapped to functional categories using the KEGG database [17] or according to known functions (e.g. motility/chemotaxis) to emphasize their roles in C. jejuni biology.

**Purification of recombinant C-terminally His-tagged CsrA**

Recombinant *C. jejuni* CsrA containing a C-terminal His tag was purified by affinity chromatography. PCR primers and plasmids used in this work are listed in Table 1. Primers JAFcsrANdel and JAFcsrAXhoI (Table 1) were used in PCR reactions to amplify the csrA coding sequence (lacking the stop codon) from 81–176 genomic DNA. The resulting amplicon was then cloned into the TA-cloning vector pCRII-TOPO (Invitrogen) to generate the plasmid pJAF50. The insert was excised by restriction endonuclease digestion with the enzymes XhoI and Ndel, gel purified and subcloned into the *E. coli* expression vector pET-20b(+) (Novagen) to yield plasmid pJAF51. Plasmid pJAF51 was then transformed into *E. coli* expression strain BL21(DE3) for use in overexpression and purification. This strain was grown in two liters of LB broth supplemented with ampicillin (50 μg/ml) to mid-log (OD600 = 0.5), then was induced to express recombinant His-tagged CsrA by the addition of 1 mM IPTG for three hours. After the induction period, the culture was pelleted and washed with STE buffer (0.1 M NaCl, 10 mM Tris-HCL, pH 8, 1 mM EDTA, pH 8) and resuspended in lysis buffer (50 mM Tris, pH 7.8, 2 mM β-mercaptoethanol, 5% glycerol, 2 mM EDTA, pH 8) and homogenized. The lysate was then further incubated with an additional 4 ml of lysis buffer for 30 minutes followed by sonication on ice ten times at ten second intervals punctuated by 10 second pauses to prevent overheating of the lysate. The sonicated lysate was centrifuged at 12,000 rpm, retaining the supernatant for further purification steps. The supernatant was fractionated by incubation for 1 hour in the presence of ammonium sulfate (0.56 g/ml) and centrifuged as above. The pellet was resuspended in buffer CJA (125 mM Tris, pH 7.5, 5 mM β-mercaptoethanol, 2.5% glycerol, 2 mM EDTA, pH 8) and dialyzed overnight at 4°C against buffer CJA. Imidazole (50 mM) was added to the dialyzed lysate for binding to a Nickel-Sepharose column. After washing, bound protein was eluted by the addition of buffer CJA containing 300 mM imidazole, and fractions were collected for further analysis. Fractions containing purified His-tagged CsrA were then pooled and dialyzed against a storage buffer (50 mM Tris, pH 8, 50% glycerol, 2 mM EDTA, 100 mM NaCl) to concentrate the sample. Purified and concentrated CsrA-His<sub>6</sub> aliquots were stored at -20°C for future experiments. Rabbit polyclonal antiserum to CsrA-His<sub>6</sub> was prepared by a commercial vendor (Cocalico Biologicals).

**FlaA expression during growth curve**

To test for CsrA- and growth-phase-dependent expression of FlaA, we grew WT and csrA mutant *C. jejuni* as follows. Strains 81–176 and the csrA mutant were grown overnight in MH biphasic cultures at 42°C. Overnight cultures were then diluted into fresh MH broth to an initial OD<sub>600</sub> = 0.1, and incubated at 37°C. Samples were removed at 5, 6, 8, and 10 hours of incubation and subjected to western blots using FlaA-specific polyclonal antiserum. Samples for western blots were normalized for protein content, determined by BCA assay.

**Electrophoretic Mobility Shift Assay (EMSA)**

EMSA experiments were performed essentially as described [18]. DNA templates to be used for *in vitro* RNA transcription were generated by using PCR and primers containing T7
promoters (Table 1), designed to correspond to the region encompassing the 5’ end of mRNA for either C. jejuni flaA or E. coli phoB (as a CsrA-non-binding control)\cite{15}. RNA was then synthesized from these purified PCR products using the MEGAscript kit (Ambion). The transcripts were purified via phenol:chloroform extraction, followed by ethanol precipitation. Purified RNA was resuspended in TE buffer, heated to 85°C, and allowed to cool to room temperature on the bench. The transcripts were then incubated with concentrations of purified, recombinant CsrA-His\textsubscript{6} ranging from 0–300 nM, in the presence of yeast RNA in 10 μl reactions (10 mM Tris-HCl, pH 7.5, 10 mM MgCl\textsubscript{2}, 100 mM KCl, 32.5 ng total yeast RNA, 20 mM DTT, 7.5% glycerol) and 4 U of RNase inhibitor (Ambion) for 30 minutes at 37°C. The reactions were resolved on 15% native polyacrylamide gels and scanned on a Bio-Rad phosphorimager. The dissociation constant (K\textsubscript{d}) was calculated as described \cite{19}.

**Autoagglutination assay**

Autoagglutination of C. jejuni wild type, csrA mutant and complemented csrA mutant strains was performed as previously described \cite{20}. Overnight, liquid cultures of C. jejuni were diluted to an OD\textsubscript{600} of 1.0 in phosphate buffered saline (PBS), distributed into 2 ml aliquots and incubated at room temperature. The OD\textsubscript{600} of the top 1 ml of the culture was measured at 2, 4, 6, and 24 hours to determine the kinetics of autoagglutination. These assays were performed in triplicate with three separate biological replicates.

**Electron microscopy**

Electron microscopy of wild type and csrA mutant C. jejuni was performed essentially as described \cite{21}. Briefly, cells were grown on MH agar for 16 hours. After growth, wild-type and csrA mutant strains were resuspended from MH agar into PBS, pelleted for 3 min at 13,200 r.p.m. in a microcentrifuge, resuspended in 2% gluteraldehyde, and then incubated on ice for 1 h. Samples were then stained with 2% uranyl acetate and visualized with an FEI Technai G2 Spirit Bio TWIN transmission electron microscope.

**Mouse colonization**

Competition mouse colonization studies were carried out as previously described \cite{16}. Wild-type and csrA mutant were mixed in equal proportions (approximately 5 x 10\textsuperscript{8} CFU each) prior to inoculation and administered to BALB/cByJ mice (The Jackson Laboratory) by oral gavage. Fecal pellets where collected from each infected mouse at 0, 7, 14, and 21 days post-inoculation and suspended in PBS. Shed bacteria were then enumerated via serial dilution of fecal suspensions on MH agar containing 5% sheep blood, cephalazone (20 μg/ml), vancomycin (10 μg/ml) and amphotericin B (2 μg/ml) without or with chloramphenicol (15 μg/ml) to differentiate between wild-type and mutant bacteria recovered. Mouse colonization experiments were approved by the Augusta University Institutional Animal Care and Use Committee (IACUC)–protocol 09-02-168. Mice do not suffer any symptoms or disease resulting from the colonization, and were observed daily to ensure that no adverse events occurred (e.g. changes in behavior, activity, posture, or ability to move). At the conclusion of the experiments, mice were euthanized by CO\textsubscript{2} overdose as recommended by the IACUC, in accordance with the recommendations of the American Veterinary Medical Association Panel on Euthanasia.
Statistical analysis

Results are presented as means ± standard error of means. Statistical analysis was determined using one-way analysis of variance (ANOVA) or student’s t-test. P values of less than 0.05 were considered significant.

Results

Mutation of CsrA alters protein expression in both mid-log and stationary phase

Considering the pleiotropic phenotypes of the C. jejuni csrA mutant [5], we performed proteomics experiments to examine the protein expression profiles of the wild type, csrA mutant, and complemented mutant strains. C. jejuni cells were grown overnight at 42°C diluted into fresh MH media and then allowed to grow in parallel to either mid-log phase (OD$_{600}$ = 0.5) or to stationary phase (OD$_{600}$ = 1.0) at 42°C. These cultures were then harvested and subjected to 2D-DIGE analysis followed by MALDI-ToF/ToF mass spectrometry to enumerate and identify proteins differentially expressed more than ±1.5 fold in the csrA mutant bacteria, as compared to the wild type, at both growth phases. We observed that when grown to mid-log phase, 25 proteins showed altered expression in the csrA mutant as compared to the wild type; 9 were more highly expressed (Table 2, Table A in S1 File) and 16 were less abundant (Table 2, Table B in S1 File). In contrast, when grown to stationary phase the expression of 111 proteins was altered—54 proteins were more abundant in the csrA mutant as compared to the wild type (Table 3, Table C in S1 File), while 57 proteins were less abundant (Table 3, Table D in S1 File). Expression of all of the proteins whose abundance was altered in the csrA mutant was restored to normal in the complemented strain. All differentially expressed proteins were excised and subjected to MALDI-ToF/ToF mass spectrometry for identification. The magnitude of change for the majority of the differentially expressed proteins was less than three-fold, consistent with the degree of change of E. coli CsrA-regulated proteins [22].

Table 2. Proteins with altered expression ΔcsrA at mid-log phase.

| Functional category$^1$ | Higher (9) | Lower (16) |
|-------------------------|------------|------------|
| **Metabolism**          |            |            |
| Amino acid              | HisD       | AnsA       |
| Carbohydrate            | AcnB       | OorA, SucC |
| Energy                  | NuoG, Ppa  |            |
| Nucleotide              | GuaB       | Adk        |
| **Genetic information processing** | | |
| Translation             | Tig        | EF-P, EF-Tu|
| Folding, sorting, degradation | HtrA, PEB4 | DnaK, GroEL|
| **Environmental information processing** | | |
| Membrane transport      | CjaA       | CosR       |
| **Cellular processes**  |            |            |
| Cell motility           | FlaA, FlaB | Tlp6       |
| Oxidoreductase          | AhpC, TrxB |            |
| Hypothetical            | Cjij81176_0443, Cjij81176_0107 |

$^1$KEGG category

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The proteins whose expression was altered in the csrA mutant fell into a number of functional classes, including central metabolism (amino acid metabolism, TCA cycle, acetate metabolism, glycolysis, gluconeogenesis), respiration, transporters, and heat shock proteins (Tables 2 and 3). Other functional categories included those related to pathogenesis, such as motility / chemotaxis, oxidative stress resistance, and cell wall factors including adhesins. At mid-log phase, two of the proteins most overexpressed in the csrA mutant were FlaA and FlaB flagellins, along with HtrA protease and the TCA cycle protein AcnB (Table 2). Proteins with lower expression in the csrA mutant include the oxidative stress proteins AhpC and TrxB, the response regulator CosR, and the chemoreceptor Tlp6.

A much greater number of proteins showed differential expression in the csrA mutant at stationary phase. Among the more highly expressed proteins were several related to amino acid metabolism (AnsA, GGT, PEB1a, AspA, GlnH, and MetY), TCA cycle (AcnB, FumC, OorABC, and SucC), acetate metabolism (AckA and Acs), and several respiration-related proteins. A number of proteins related to heat shock or cell processes were also overexpressed. Three global regulators (CosR, Fur, and RacR) were also more abundant in the csrA mutant, as well as

### Table 3. Proteins with altered expression ΔcsrA at stationary phase.

| Functional category | Higher (54) | Lower (57) |
|---------------------|------------|------------|
| **Metabolism**      |            |            |
| Amino acid          | AnsA, GGT, PEB1a, AspA, GlnH, MetY | PheA, IlvC, IlvE, Asd, ArgG, DapA |
| Carbohydrate        | AckA, AcnB, Acs, Cjj81176_0110, FbaA, FumC, Mez, OorA, OorB, OorC, PFOR, Pyk, SucC | AccA, Ena, FrdA, GltA, OorD, Pta, SucD |
| Energy              | Nuol       | AtpA, AtpD, Cj0414, MfrA, TorA |
| Nucleotide          | GuaB       | Adk, PurA, PurB |
| Glycan              |            | LpxB, LgF, WaaF |
| Cofactors/vitamins  | PabB       | Cj81176_0265, CoaE |
| **Genetic information processing** |            |            |
| Transcription       | Rho        |            |
| Translation         | EF-Tu, HisS, RpmB, SerS, Tig | FusA, LepA, RpsA |
| Folding, sorting, degradation | HtrA, PEB4 | FtsH, GroEL, HtpG, MogA |
| Replication and repair | Ogt, RecA, RuvA | |
| **Environmental information processing** |            |            |
| Membrane transport  | Cjj81176_1525, Cjj81176_1566, ModC, PorA | Cjj81176_0211, PstB |
| Signal transduction | CosR, Fur, RacR | |
| **Cellular processes** |            |            |
| Cell motility       | FlaA, FlaB, FlID, PseI, Tlp6, Tlp8 | CheV, CheY |
| Oxidoreductase      | Cjj81176_0382 | AhpC, KatA, Tpx, TrxA, TrxB |
| Adhesins²           | PEB3       | PEB2, CadF, FlpA, Cjj81176_1348 |
| Hypothetical        | pVir08, Cjj81176_1382, Cjj81176_1458, Cjj81176_0107, Cjj81176_0176, Cjj81176_1344 | Cjj81176_1215, Cjj81176_0435, Cjj81176_0977, Cjj81176_0792, Cjj81176_0793, Cjj81176_0828, Cjj81176_0443, Cjj81176_0729, Cjj81176_0266, Cjj81176_1062 |

1KEGG category; 2Known C. jejuni adhesins

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several motility/chemotaxis proteins including FlaA and FlaB, which were the most highly overexpressed proteins in the csrA mutant (Table 3).

Among proteins whose expression was lower in the csrA mutant were several other amino acid metabolism (PheA, IlvC, IlvE, Asd, ArgG, DapA) and TCA cycle proteins (FrdA, GltA, OorD, and SucD), as well as the Pta protein, involved in acetate metabolism. Several respiration-related proteins (TorA, MfrA, and Cj0414) were also less abundant. Expression of proteins involved in chemotaxis (CheV, CheY), oxidative stress (AhpC, Tpx, TrxA, TrxB, and KatA) and cell wall structures including fibronectin-binding adhesins (CadF, FlpA, and Cjj81176_1348) was also lower in the csrA mutant.

**FlaA expression is regulated by growth-phase and by CsrA**

Proteomics results showed increased expression of FlaA in the csrA mutant, at both mid-log and stationary phase (Tables 2 and 3). To confirm and extend these results, we used western blots to assess FlaA expression at several growth time points. The expression of FlaA in WT cells increased significantly throughout the growth curve, with much greater FlaA levels at 10 hours of incubation as compared to 5 hours (Fig 1A). Like WT, expression of FlaA in the csrA mutant was growth-phase regulated, with highest amounts of FlaA seen in later stages of growth. Consistent with proteomics results, the expression of FlaA was greater in the csrA mutant than in the WT strain (Fig 1A). This suggests that CsrA is an inhibitor of FlaA expression.

**CsrA binds directly to flaA RNA**

Because proteomics and western blots both implicated CsrA in regulation of flagellar expression, we performed EMSAs to determine whether CsrA directly regulated FlaA expression by binding to its mRNA. We generated RNA transcripts corresponding to the 5' end of flaA mRNA, as well as to E. coli phoB as a negative control that does not bind CsrA [15]. These transcripts were incubated with purified CsrA-His6 at concentrations ranging from 0–300 nM. CsrA binding was observed for flaA but not phoB RNA (Fig 1B). CsrA bound to flaA mRNA with a dissociation constant of 6.53 +/- 3.11 nM, indicating a specific, high-affinity interaction.

**Autoagglutination is altered in the absence of CsrA**

Proteomic analysis of the csrA mutant compared to the wild type strain revealed altered expression of PEB4. Previous studies in our laboratory revealed that mutation of PEB4 changed the ability of C. jejuni cells to autoagglutinate, a phenotype dependent on full length, glycosylated flagella [20]. We examined the kinetics of autoagglutination over a 24 hour period and found that the rate of autoagglutination was different between the wild type, csrA mutant, and complemented mutant strains (Fig 2). Although all three strains autoagglutinated to similar levels after 24 hours, at 2, 4 and 6 hours, the csrA mutant autoagglutinated to a lesser extent compared to the wild type and complemented strains (p<0.05), indicating that mutation of csrA resulted in delayed autoagglutination. Electron microscopy of WT and csrA mutant strains showed no overt differences in flagellar number, structure, or length (data not shown) that would explain differences in autoagglutination or motility [5](Fig 3).

**Mutation of csrA renders C. jejuni defective in its ability to colonize mice**

Numerous lines of evidence suggest defective pathogenicity of the csrA mutant [5]. Therefore, we tested the ability of the csrA mutant to colonize mice using a competitive infection model as previously described [16]. Wild type and csrA mutant bacteria were mixed at a 1:1 ratio and
orogastrically administered to BALB/c-ByJ mice. Fecal pellets were collected at 7, 14, and 21 days post-inoculation, the pellets were homogenized in PBS and bacteria shed were serially diluted and enumerated on selective and non selective MH agar (Fig 4). The mean colonization densities of both strains were compared and a significant decrease in the ability of the csrA mutant strain to compete against the wild type was observed. This deficiency was observed at 7, 14, and 21 days post-infection (p < 0.01 for each) and became more striking as the experiment progressed. The parent cultures from both strains used for mouse infection were tested for motility on the day of infection to rule out phase variation in flagellar synthesis as a factor in mouse colonization (data not shown) and both strains have been shown to grow at similar rates [5], indicating that the colonization defect of the csrA mutant also was not due to a general growth defect.

Fig 1. C. jejuni CsrA regulates FlaA expression by binding to the 5' end of flaA mRNA. (A) WT and csrA mutant C. jejuni were diluted to an initial OD$_{600}$ = 0.1 and grown at 42°C. Cells were removed at 5, 6, 8 and 10 hours, and were used in western blots with antibodies against FlaA. (B) Electrophoretic Mobility Shift Assay (EMSA) analysis was used to assess the interaction between purified C. jejuni CsrA-His$_6$ and the 5’ end of flaA mRNA. Purified CsrA-His$_6$ at concentrations varying from 0–300 nM were incubated with 100 ng of purified $^{32}$P-end-labeled RNA corresponding to the 5’ ends of either C. jejuni flaA (top panel) or E. coli phoB (bottom panel) as a control that does not bind CsrA [15]. The positions of bound (B) and free (F) RNA are shown.

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Discussion

Paradoxically, *C. jejuni* survives in diverse environments despite its relative small number of regulatory proteins. While several transcriptional regulators have been characterized, our understanding of how gene regulation affects *C. jejuni* disease is still incomplete, prompting us to investigate the post-transcriptional regulator CsrA and its role in pathogenesis. Previously, we reported that mutation of *csrA* in the highly virulent *C. jejuni* strain 81–176 altered motility,

![Figure 2](image)

**Fig 2.** The kinetics of autoagglutination are altered in the *csrA* mutant strain. Static suspensions of *C. jejuni* wild type, *csrA* mutant, and complemented *csrA* mutant strains were incubated and OD_{600} measurements were taken at 2, 4, 6, and 24 hours. The assay was performed in triplicate on three separate occasions. Statistical significance (p<0.05) is represented by an asterisk. Error bars are present; however, they are too small to be seen.

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![Figure 3](image)

**Fig 3.** Mutation of *csrA* does not affect flagellar structure. Transmission electron microscopy of wild type (top) and *csrA* mutant (bottom) strains showed that flagellar structure is not affected by the *csrA* mutation. The bar at the lower right of the figure is 1 μm.

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biofilm formation, oxidative stress responses, and in vitro adherence and invasion of intestinal epithelial cells [5]. In the current study, we examined the proteomes of WT, isogenic csrA mutant and complemented csrA mutant strains, and identified a role for CsrA in the expression of a number of proteins important to C. jejuni central metabolism, motility, oxidative stress resistance, and pathogenesis.

A total of 117 unique proteins were differentially expressed in the csrA mutant relative to wild type strain 81–176, and these comprise the putative CsrA regulon. A greater number of proteins showed altered abundance at stationary phase (111 proteins) compared to mid-log phase (25 proteins); a total of 19 proteins were differentially expressed in the csrA mutant at both stages of growth. Expression of each of these proteins was restored to wild type levels in the complemented mutant. Together, these data suggest that CsrA regulation is more predominant during stationary phase, consistent with the role of CsrA as a stationary phase regulator in other bacteria [23]. The degree of regulation (generally 1.5- to 4-fold) noted for proteins of the presumptive CsrA regulon is similar to those of proteins regulated by E. coli CsrA [22]. This is consistent with the role of CsrA in fine-tuning protein expression; this level of regulation can be superimposed on transcriptional and/or allosteric regulation. In this work we examined differential protein expression in the csrA mutant at 42°C, the relevant growth temperature for the thermophilic C. jejuni, however, it is certainly possible that varying the growth temperature or other environmental conditions could have an effect on CsrA regulation.

Proteins of the putative CsrA regulon fall into a number of different functional classes. Consistent with proteins regulated by CsrA in E. coli [22, 24] and other bacteria, by far the largest class of proteins putatively regulated by C. jejuni CsrA is related to central metabolism. Among C. jejuni CsrA targets, many were involved in carbon metabolism. While the effect of CsrA on the expression of amino acid metabolizing proteins is modest at mid-log phase (two proteins), at stationary phase there are 12 such proteins whose expression is altered. Proteins with higher expression (i.e. normally repressed by CsrA) are members of pathways for acquiring and metabolizing aspartate, glutamate, asparagine, and glutamine, which are some of the preferred carbon sources for C. jejuni [25, 26]. Proteins involved in metabolism of aromatic and

![Fig 4. CsrA is involved in mouse colonization. BALB/c-ByJ mice were orally inoculated with a 1:1 mixture of wild type (closed circles) and csrA mutant (open circles) C. jejuni and fecal pellets were collected at 7, 14, and 21 days post-infection for enumeration of wild type and mutant bacteria. The geometric means of colonization of each group are represented by horizontal lines. The limit of detection (represented by a dashed line) was 10^2 CFU/g. doi:10.1371/journal.pone.0156932.g004](image-url)
branched-chain amino acids, lysine, and arginine are expressed at lower amounts in the csrA mutant, suggesting that in wild-type cells CsrA stimulates their expression.

C. jejuni lacks a complete glycolytic pathway due to the absence of 6-phosphofructokinase [25, 26]. However, the FbaA protein, which functions in both glycolysis and gluconeogenesis, is more highly expressed in the csrA mutant. In the csrA mutant, several proteins involved in the flow of carbon through the lower end of glycolysis and into the TCA cycle are altered in stationary phase—Pyk (pyruvate kinase)[27] and Mez (malic enzyme)[27] are more highly expressed, while Eno (enolase) is less abundant. Expression of 10 proteins of the TCA cycle was altered in the csrA mutant. AcnB, FumC, OorA, OorB, OorC, and SucC levels were elevated at stationary phase, while those of FrdA, GltA, OorD, and SucD were lower. The expression of OorA is lower in the csrA mutant during mid-log phase, suggesting that control of TCA cycle enzymes by CsrA is complex. Abundance of a number of proteins that are involved in providing electrons for respiration is also altered in the csrA mutant, some with greater and some with lesser expression. NuoI [28] and PFOR [29] are more highly expressed during stationary phase, while the respiratory proteins MfrA [30–32], TorA [32, 33], and Cj0414 [16] are less abundant. The altered expression of these proteins suggests that CsrA controls aspects of cellular respiration, especially at stationary phase.

Three putative CsrA targets (AckA, Acs, and Pta) are related to acetate metabolism (see also below). Like E. coli, C. jejuni has an acetate switch [34], whereby excess carbon accumulated during log-phase growth is converted to acetate via the Pta and AckA proteins. Upon consumption of preferred carbon sources, at the transition into stationary phase C. jejuni switches from acetate production to consumption via either AckA-Pta or Acs. In the csrA mutant, AckA and Acs are more highly expressed at stationary phase while Pta abundance is lower. Because this pathway is reversible, there exists the possibility that CsrA could regulate production and/or consumption of acetate. Together, these results indicate that CsrA controls a number of aspects of central metabolism, primarily during stationary phase, and may allow the fine-tuning of nutrient assimilation, carbon flux, and respiration.

CsrA also appears to regulate a number of other cellular processes (Tables 2 and 3 and Tables A-D in S1 File), including predicted transporters, biosynthesis of cofactors and vitamins, transcription and translation, nucleotide metabolism, DNA repair, and energy homeostasis. Heat shock proteins or chaperones such as GroEL, HtpG, HtrA, and PEB4 are also among the CsrA regulon, suggesting CsrA regulation of general stress responses at both mid-log and stationary phases. Some of these have also been shown to play a role in virulence. PEB4 is a periplasmic peptidyl cis-trans isomerase, and mutation of the peb4 gene results in C. jejuni that are more motile and more invasive of INT407 human intestinal epithelial cells, and are less able to colonize mice than 81–176 [35]. C. jejuni HtrA is associated with aerotolerance, adherence to and invasion of INT407 cells [36], altered host cell apoptosis and intestinal immune responses [37] and cleaves E-cadherin [38]. It is possible that alteration of one or more of these proteins impact the phenotypes previously shown for the csrA mutant [5]. Clearly a full understanding of the influence of CsrA in central metabolism and other cellular processes will be a substantial undertaking and the subject of future research.

Three proteins (Fur, CosR, and RacR) whose expression is increased in the csrA mutant are transcriptional regulators. These proteins direct large regulatory networks related to C. jejuni iron availability, oxidative stress resistance, and growth temperature, respectively. Fur regulates both Fe homeostasis and oxidative stress resistance responses; the Fur regulon includes KatA, AhpC, and TrxB [39] whose expression is also altered in the csrA mutant. However, other than these proteins, there is not significant overlap of the Fur regulon with that of CsrA, suggesting that CsrA regulation of KatA, AhpC, and TrxB is not mediated simply through Fur. CosR is an essential orphan response regulator that is responsible for regulating the expression of 93 genes.
including several that are also differentially expressed in the *csrA* mutant such as KatA, AhpC, TrxB, SucCD, and GroEL [40]. AhpC transcription is not significantly affected by CosR knockdown, leading Hwang et al. to conclude that AhpC is likely also regulated post-transcriptionally [40]; our present data suggests that this may be mediated by CsrA. RacR is a transcriptional regulator that controls the expression of a number of genes in response to growth temperature [41–43]. Although some of the RacR targets are also members of the CsrA regulon, including catalase, cytochrome c peroxidase, AnsA, AspA, Tlp6, and GGT [41–43], the majority are not. As with Fur, the lack of overlap among the majority of proteins in the CosR, RacR and CsrA regulons shows that CsrA effects are not manifest solely through CosR or RacR. Therefore, while altered expression of these regulators in the *csrA* mutant raises the possibility that some of the effects of CsrA on whole-cell protein expression are indirect (i.e. mediated through secondary regulators), the lack of significant overlap among members of these regulons suggest that most CsrA-related changes in protein expression are direct. This is consistent with results found with *E. coli* CsrA [22]. However, it is possible that the expression of some of the targets of these transcriptional regulators is modulated by CsrA.

Another class of targets putatively regulated by CsrA and directly relevant to pathogenesis is motility and chemotaxis. FlaA and FlaB are the major and minor flagellins, respectively, that make up *C. jejuni* flagella [44]. The expression of both FlaA and FlaB is significantly higher at both mid-log and stationary phase (3.4- to 5.4-fold), and this magnitude of regulation is among the highest noted for any CsrA target in these experiments. Greater expression of FlaA in the *csrA* mutant at various stages of growth was confirmed using western blots (Fig 1A). We also observed growth-phase regulation of FlaA expression, with FlaA abundance increasing significantly throughout the growth curve (Fig 1A) in both WT and *csrA* mutant strains. Using EMSA (Fig 1B), we showed that CsrA directly binds to the 5′end of *flaA* mRNA containing the *flaA* RBS; binding of CsrA in this region is consistent with the model of CsrA function in other bacteria [23]. Together, these data show that CsrA directly represses FlaA synthesis by binding to the 5′end of *flaA* mRNA, and that FlaA abundance is growth-phase-dependent. The greater expression of FlaA and FlaB seems paradoxical with respect to the decreased motility of the *csrA* mutant [5]. Electron microscopy of the *csrA* mutant shows that the *csrA* mutant has flagella that appear normal in number, length, and morphology (Fig 3), suggesting that there are no obvious flagellar defects that would explain the lesser motility of the *csrA* mutant. It is possible that there are subtle differences in the flagella of the *csrA* mutant that are not apparent by TEM that could result in the altered autoagglutination kinetics of this strain (Fig 2), which depend on glycosylated flagella [45]. The apparent regulation of *C. jejuni* flagellins is consistent with CsrA being an ancestral regulator linking motility and cellular metabolic processes, as proposed for *B. subtilis* [46] and with the proteomics results described in this work.

The expression of several chemotaxis-related proteins is also altered in the *csrA* mutant, including two methyl-accepting chemotaxis proteins, Tlp6 and Tlp8. The abundance of Tlp6 is lower at mid-log phase, but the expression of both Tlp6 and Tlp8 is higher at stationary phase. Both Tlp6 and Tlp8 are group C sensors [47], which are thought to recognize cytoplasmic signals, such as redox status in the case of Tlp8 [48]. Furthermore, the chemotaxis signaling proteins CheV and CheY are both expressed at lower levels in the *csrA* mutant, but only at stationary phase. CheY is a response regulator that controls the direction of flagellar rotation in response to chemotactic signals [44]. CheV is another component of the *C. jejuni* chemotaxis apparatus, mediating adaptation to chemoattractants [44]. CheV has affinity for both Tlp6 and Tlp8 [49], suggesting that interplay between these proteins and downstream chemotaxis signaling events could be influenced by CsrA. Due to changes in the expression of multiple chemotaxis proteins, it is therefore possible that chemotaxis is altered in the *csrA* mutant, which could contribute to the altered motility of the *csrA* mutant. Alternatively, the altered expression of
characteristics not typically associated with motility could play a role, including oxidative stress resistance.

Several proteins whose expression is altered in the *csrA* mutant are related to oxidative stress, and could explain the increased oxidative stress sensitivity of the mutant strain [5]. A number of antioxidant pathways are known to be important for oxidative stress responses, particularly to aerotolerance [50–56], and here we show that the abundance of some of these is altered in the absence of CsrA. The expression of KatA, Tpx, AhpC, TrxA, and TrxB is lower in the *csrA* mutant at stationary phase, while AhpC and TrxB are less abundant at mid-log phase as well. KatA [57], and Tpx [55] are the primary defense mechanisms against hydrogen peroxide [57], to which the *C. jejuni csrA* mutant shows increased sensitivity [5]. AhpC is involved in defense against organic peroxides [50]. We did not identify the important oxidative stress resistance protein superoxide dismutase as being CsrA-regulated in these experiments. Together, the lowered expression of these antioxidant proteins could explain the sensitivity of the *csrA* mutant to oxidative stress. However, Flint et al. identified a number of other pathways that may be involved indirectly in oxidative stress resistance, including motility, electron transport, energy metabolism, cation transport, and general bacterial physiology [58]. It is conceivable that the decreased motility noted for the *csrA* mutant [5], or changes in respiratory proteins, is associated with the increased sensitivity of the mutant to various oxidative stressors.

Another defect in the *csrA* mutant is a decreased ability to bind to intestinal epithelial cells in vitro [5]. *Campylobacter* adherence has been extensively characterized, and a number of adhesins including the fibronectin-binding proteins CadF, FlpA and Cjj81176_1348 have been identified [59,60]. In the absence of CsrA, all of these adhesins have reduced expression when compared to the wild type, suggesting that these may influence the decreased adherence phenotype observed in the *csrA* mutant. PEB3 is another adhesin [61] that is altered in the *csrA* mutant, however, its expression is increased at stationary phase and is therefore unlikely to be the cause of the adherence defect of the *csrA* mutant [5].

The *csrA* mutant also exhibits a defect in biofilm production [5], and one of our goals is to determine the role of CsrA in biofilm formation. The effects of CsrA on biofilm accumulation in *E. coli* and other bacteria have been well documented [62–66]. While the study of biofilms in *C. jejuni* is still emerging and the precise role of CsrA remains unknown, a number of factors and proteins are known to contribute to *C. jejuni* biofilm formation. While the biofilm-related *E. coli pgaABCD* operon [66] is absent from *C. jejuni*, recently an α-dextran was reported to play a role in forming *C. jejuni* biofilm [13], although the genes responsible for the synthesis of this polysaccharide have not been defined. Other known glycans are not required for *C. jejuni* biofilm accumulation, as mutants lacking capsule, protein glycosylation, or full-length lipooligosaccharide formed biofilms at levels equal to or greater than wild type [45,67].

A previous proteome study from Kalmokoff et al. examined *C. jejuni* NCTC 11168 proteins that were upregulated in biofilms compared to planktonic bacteria and identified a major role for motility-related proteins in biofilm production [68]; the role of motility in biofilm formation was confirmed and extended in subsequent work [12]. Of the biofilm-related proteins identified by Kalmokoff [68], 13 are also in the CsrA regulon identified in the current work: FlaA, FlaB, FlfD, AhpC, Tpx, GroEL, CosR, RacR, SucD, PEB1a, PEB4, Cjj81176_0443, and Cjj81176_0793. There are several possibilities that could explain the overlap in the subset of proteins with altered expression in biofilms and those regulated by *csrA*. The most simple explanation is that CsrA plays a role in regulating protein expression in biofilms, as in other bacteria CsrA is a central regulator coordinating the transition from planktonic (motile) to biofilm (sessile) growth [23,63]. In *C. jejuni*, the regulation of major flagellin FlaA (as well as minor flagellin FlaB), is also consistent with previous observations on the role of flagella and
motility in biofilm formation [12, 68]. Combined with the observation that C. jejuni CsrA protein regulation is more active at stationary phase than at mid-log, this suggests that CsrA may play a direct role in biofilm development upon entering stationary phase.

As discussed previously, during the C. jejuni acetate switch, excess carbon produced during logarithmic growth is converted to acetate, which is then used as an alternate carbon source upon consumption of preferred carbon sources [34]. Because the timing of the onset of C. jejuni biofilm production coincides with the period of acetate consumption [34], it is possible that CsrA-mediated modulation of the expression of acetate metabolizing enzymes such as AckA, Pta, and Acs could influence biofilm formation. As acetate metabolism is involved in the production of E. coli biofilms [69, 70], the altered expression of C. jejuni proteins related to acetate metabolism is intriguing. Acetate metabolism could play a role in C. jejuni biofilms either as a stationary phase carbon source, required for synthesizing a biofilm polysaccharide [13] via gluconeogenesis (including CsrA-regulated FbaA) or via the acetate pathway intermediate acetyl phosphate (AcP). In E. coli and other bacteria, AcP can phosphorylate response regulators, resulting in changes of the downstream targets of these response regulators, including genes involved in biofilm formation [71, 72]. Interestingly, AckA is elevated considerably in the csrA mutant and wild type, and did not colonize the mice to the same extent as wild type. Given the pleiotropic nature of the csrA mutant strain, it is difficult to assign this colonization deficiency.
to one specific pathway or phenotype. It is possible that the competitive colonization defect of the csrA mutant reflects an overall fitness of the mutant, although its in vitro growth is similar to that of wild type. However, csrA is upregulated in a rabbit ileal loop model, suggesting that the expression of CsrA is involved in C. jejuni colonization of the mammalian intestine [74]. We also note that the body temperature of the mouse (37°C) is different than the temperature at which the proteomics experiments were done (42°C), so we cannot preclude that growth temperature of the thermophilic C. jejuni has an effect on the colonization results.

In summary, our data indicate that CsrA participates in a complex remodeling of C. jejuni central metabolism and general stress responses during stationary phase. In addition to metabolic proteins, CsrA affects the expression of a number of proteins with roles in pathogenesis-related functions, such as in motility/chemotaxis, oxidative stress resistance, and host cell interactions. In particular, CsrA is a direct regulator of FlaA expression in a growth-phase dependent manner, and affects autoagglutination without causing a gross defect in flagellar structure. These data suggest that C. jejuni CsrA plays a role in stationary phase physiology, potentially by fine-tuning the metabolic changes involved in the transition to stationary phase and to biofilm formation.

Supporting Information

S1 File. Proteins with altered expression in the csrA mutant. Table A. Proteins with increased expression in the csrA mutant—mid-log. Table B. Proteins with decreased expression in the csrA mutant—mid-log. Table C. Proteins with increased expression in the csrA mutant—stationary. Table D. Proteins with decreased expression in the csrA mutant—stationary. (PDF)

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Author Contributions

Conceived and designed the experiments: JAF JL CJG DRH SAT. Performed the experiments: JAF JL CJG. Analyzed the data: JAF JL CJG DRH SAT. Contributed reagents/materials/analysis tools: JAF JL CJG DRH SAT. Wrote the paper: JAF JL CJG DRH SAT.

References

1. Parkhill J, Wren BW, Mungall K, Ketley JM, Churcher C, Basham D, et al. (2000) The genome sequence of the food-borne pathogen Campylobacter jejuni reveals hypervariable sequences. Nature 403:665–668. PMID: 10688204
2. Liu MY, Romeo T. (1997) The global regulator CsrA of Escherichia coli is a specific mRNA-binding protein. J Bacteriol 179:4639–4642. PMID: 9226279
3. Baker CS, Morozov I, Suzuki K, Romeo T, Babitzke P. (2002) CsrA regulates glycogen biosynthesis by preventing translation of glgC in Escherichia coli. Mol Microbiol 44:1599–1610. PMID: 12067347
4. Wei BL, Brun-Zinkernagel AM, Simecka JW, Pruss BM, Babitzke P, Romeo T. (2001) Positive regulation of motility and flhDC expression by the RNA-binding protein CsrA of Escherichia coli. Mol Microbiol 40:245–256. PMID: 11298291
5. Fields JA, Thompson SA. (2008) *Campylobacter jejuni* CsrA mediates oxidative stress responses, biofilm formation, and host cell invasion. J Bacteriol 190:3411–3416. doi: 10.1128/JB.01928-07 PMID: 18310331

6. Goller CC, Romeo T. (2008) Environmental influences on biofilm development. Current topics in microbiology and immunology 322:37–66. PMID: 18453271

7. Svensson SL, Davis LM, MacKichan JK, Allan BJ, Pajaniappan M, Thompson SA, 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 71:253–272. doi: 10.1111/j.1365-2958.2008.06534.x PMID: 19017270

8. Trachoo N, Frank JF, Stern NJ. (2002) Survival of *Campylobacter jejuni* in biofilms isolated from chicken houses. Journal of food protection 65:1110–1116. PMID: 12117243

9. Bronowski C, James CE, Winstanley C. (2014) Role of environmental survival in transmission of *Campylobacter jejuni*. FEMS Microbiol Lett 356:8–19. doi: 10.1111/1574-6968.12488 PMID: 24888326

10. Kudirkienė E, Cohn MT, Stabler RA, Strong PC, Semiene L, Wren BW, et al. (2012) Phenotypic and genotypic characterizations of *Campylobacter jejuni* isolated from the broiler meat production process. Curr Microbiol 65:398–406. doi: 10.1007/s00284-012-0170-z PMID: 22735984

11. Haddock G, Mullin M, MacCallum A, Sherry A, Tetley L, Watson E, et al. (2010) *Campylobacter jejuni* 81–176 forms distinct microcolonies on in vitro infected human small intestinal tissue prior to biofilm formation. Microbiology 156:3079–3084. doi: 10.1099/mic.0.039867-0 PMID: 20616103

12. Svensson SL, Pryjma M, Gaynor EC. (2014) Flagella-mediated adhesion and extracellular DNA release contribute to biofilm formation and stress tolerance of *Campylobacter jejuni*. PLoS One 9: e106063. doi: 10.1371/journal.pone.0106063 PMID: 25166748

13. Jowiya W, Brunner K, Abouelhadid S, Hussain HA, Nair SP, Sadiq S, et al. (2015) Pancreatic amylase is an environmental signal for regulation of biofilm formation and host interaction in *Campylobacter jejuni*. Infect Immun 83:4884–4895. doi: 10.1128/IAI.0064-15 PMID: 26438798

14. Black RE, Levine MM, Clements ML, Hughes TP, Blaser MJ. (1988) *Campylobacter jejuni* infection in humans. J Infect Dis 157:472–479. PMID: 3343522

15. Patterson-Fortin LM, Vakulskas CA, Yakhnin H, Babitzke P, Romeo T. (2013) Dual posttranscriptional regulation via a cofactor-responsive mRNA leader. J Mol Biol 425:3662–3677. doi: 10.1016/j.jmb.2012.12.010 PMID: 23274138

16. Pajaniappan M, Hall JE, Cawthraw SA, Newell DG, Gaynor EC, Fields JA, et al. (2008) A temperature-regulated *Campylobacter jejuni* gluconate dehydrogenase is involved in respiration-dependent energy conservation and chicken colonization. Mol Microbiol 68:474–491. doi: 10.1111/j.1365-2958.2008.06161.x PMID: 18284594

17. Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M. (2016) KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res 44:D457–D462. doi: 10.1093/nar/gkv1070 PMID: 26476454

18. Baker CS, Eory LA, Yakhnin H, Mercante J, Romeo T, Babitzke P. (2007) CsrA inhibits translation initiation of *Escherichia coli* hsf by binding to a single site overlapping the Shine-Dalgarno sequence. J Bacteriol 189:5472–5481. PMID: 17526692

19. Yakhnin AV, Babitzke P. (2010) Mechanism of NusG-stimulated pausing, hairpin-dependent pause site selection and intrinsic termination at overlapping pause and termination sites in the *Bacillus subtilis* trp leader. Mol Microbiol 76:690–705. doi: 10.1111/j.1365-2958.2010.07126.x PMID: 20384694

20. Rathbun KM, Thompson SA. (2009) Mutation of PEB4 alters the outer membrane protein profile of *Campylobacter jejuni*. FEMS Microbiol Lett 300:188–194. doi: 10.1111/j.1574-6968.2009.01795.x PMID: 19824902

21. Joslin SN, Hendrixson DR. (2008) Analysis of the *Campylobacter jejuni* FlgR response regulator suggests integration of diverse mechanisms to activate an NtrC-like protein. J Bacteriol 190:2422–2433. doi: 10.1128/JB.01827-07 PMID: 18223079

22. Edwards AN, Patterson-Fortin LM, Vakulskas CA, Mercante JW, Potrykus K, Vinella D, et al. (2011) Circuitry linking the Csr and stringent response global regulatory systems. Mol Microbiol 80:1561–1580. doi: 10.1111/j.1365-2958.2010.07063.x PMID: 21488981

23. Vakulskas CA, Potts AH, Babitzke P, Ahmer BM, Romeo T. (2015) Regulation of bacterial virulence by Csr (Rsm) systems. Microbiol Mol Biol Rev 79:193–224. doi: 10.1128/MMBR.00052-14 PMID: 25833324

24. Morin M, Ropers D, Letisse F, Laguerre S, Portais JC, Cocaign-Bousquet M, et al. (2016) The post-transcriptional regulatory system CSR controls the balance of metabolic pools in upper glycolysis of *Escherichia coli*. Mol Microbiol published online February 2, 2016:
25. Hofreuter D. (2014) Defining the metabolic requirements for the growth and colonization capacity of Campylobacter jejuni. Front Cell Infect Microbiol 4:137. doi: 10.3389/fcimb.2014.00137 PMID: 25325018

26. Stahl M, Butcher J, Stintzi A. (2012) Nutrient acquisition and metabolism by Campylobacter jejuni. Front Cell Infect Microbiol 2:5. doi: 10.3389/fcimb.2012.00005 PMID: 22919597

27. Velayudhan J, Kelly DJ. (2002) Analysis of gluconeogenic and anaplerotic enzymes in Campylobacter jejuni: an essential role for phosphoenolpyruvate carboxykinase. Microbiology 148:685–694. PMID: 11882702

28. Weerakoon DR, Olson JW. (2008) The Campylobacter jejuni NADH:ubiquinone oxidoreductase (complex I) utilizes flavodoxin rather than NADH. J Bacteriol 190:915–925. PMID: 18065531

29. St Maurice M, CremaDES N, Croxen MA, Sissons G, Sancho J, Hoffman PS. (2007) Flavodoxin:quinone reductase (FqrB): a redox partner of pyruvate:ferredoxin oxidoreductase that reversibly couples pyruvate oxidation to NADPH production in Helicobacter pylori and Campylobacter jejuni. J Bacteriol 189:4764–4773. PMID: 17468253

30. Kassem II, Khatri M, Sanad YM, Wolboldt M, Saif YM, Olson JW, et al. (2014) The impairment of methylmenaquinol:fumarate reductase affects hydrogen peroxide susceptibility and accumulation in Campylobacter jejuni. Microbiologyopen 3:168–181. doi: 10.1002/mbo3.158 PMID: 24515965

31. Guccione E, Hitchcock A, Hall SJ, Mulholland F, Shearer N, van Vliet AH, et al. (2010) Reduction of fumarate, mesaconate and crotonate by Mfr, a novel oxygen-regulated periplasmic reductase in Campylobacter jejuni. Environ Microbiol 12:576–591. doi: 10.1111/j.1462-2920.2009.02096.x PMID: 19919540

32. Liu YW, Hitchcock A, Salmon RC, Kelly DJ. (2014) It takes two to tango: two TatA paralogues and two redox enzyme-specific chaperones are involved in the localization of twin-arginine translocase substrates in Campylobacter jejuni. Microbiology 160:2053–2066. doi: 10.1099/mic.0.080713-0 PMID: 24961951

33. Weingarten RA, Grimes JL, Olson JW. (2008) Role of Campylobacter jejuni respiratory oxidases and reductases in host colonization. Appl Environ Microbiol 74:1367–1375. doi: 10.1128/AEM.0261-07 PMID: 18192421

34. Wright JA, Grant AJ, Hurd D, Harrison M, Guccione EJ, Kelly DJ, et al. (2009) Metabolite and transcriptome analysis of Campylobacter jejuni in vitro growth reveals a stationary-phase physiological switch. Microbiology 155:80–94. doi: 10.1099/mic.0.021790-0 PMID: 19118349

35. Rathbun KM, Hall JE, Thompson SA. (2009) Cj0596 is a periplasmic peptidyl prolyl cis-trans isomerase involved in Campylobacter jejuni motility, invasion, and colonization. BMC microbiology 9:160. doi: 10.1186/1471-2180-9-16 PMID: 19664234

36. Brandstøl L, Andersen MT, Parker M, Jorgensen K, Ingmer H. (2005) The HtrA protease of Campylobacter jejuni is required for heat and oxygen tolerance and for optimal interaction with human epithelial cells. Appl Environ Microbiol 71:3205–3212. PMID: 15933023

37. Heimesaat MM, Fischer A, Alutis M, Grundmann U, Boehm M, Tegtmeyer N, et al. (2014) The impact of serine protease HtrA in apoptosis, intestinal immune responses and extra-intestinal histopathology during Campylobacter jejuni infection of infant mice. Gut Pathog 6:16. doi: 10.1186/1757-4749-6-16 PMID: 24883112

38. Boehm M, Hoy B, Rohde M, Tegtmeyer N, Baek KT, Oyarzabal OA, et al. (2012) Rapid paracellular transmigration of Campylobacter jejuni across polarized epithelial cells without affecting TER: role of proteolytic-active HtrA cleaving E-cadherin but not fibronectin. Gut Pathog 4:3. doi: 10.1186/1757-4749-4-3 PMID: 22534208

39. Butcher J, Handley RA, van Vliet AH, Stintzi A. (2015) Refined analysis of the Campylobacter jejuni iron-dependent/independent Fur- and PerR-transcriptomes. BMC Genomics 16:498. doi: 10.1186/s12864-015-1661-7 PMID: 26141822

40. Wang S, Zhang Q, Ryu S, Jeon B. (2012) Transcriptional regulation of the CmeABC multidrug efflux pump and the KatA catalase by CosR in Campylobacter jejuni. J Bacteriol 194:6883–6891. doi: 10.1128/JB.01636-12 PMID: 23065977

41. Brás AM, Chatterjee S, Wren BW, Newell DG, Ketley JM. (1999) A novel Campylobacter jejuni two-component regulatory system important for temperature-dependent growth and colonization. J Bacteriol 181:3296–3302. PMID: 10322038

42. Apel D, Ellermeier J, Priyama M, Dritia VJ, Gaynor EC. (2012) Characterization of Campylobacter jejuni RacRS reveals roles in the heat shock response, motility, and maintenance of cell length homogeneity. J Bacteriol 194:2342–2354. doi: 10.1128/JB.06041-11 PMID: 22343300

43. van der Stel AX, van Mourik A, Laniewski P, van Putten JP, Jaguszyn-Krynicka EK, Wosten MM. (2013) The Campylobacter jejuni RacRS two-component system activates the glutamate synthesis by
directly upregulating gamma-glutamyltranspeptidase (GGT). Front Microbiol 6:567. doi: 10.3389/fmicb.2015.00567 PMID: 26097472

44. Lertsetthakam P, Ottemann KM, Hendrixson DR. (2011) Motility and chemotaxis in Campylobacter and Helicobacter. Annual review of microbiology 65:389–410. doi: 10.1146/annurev-micro-090110-102908 PMID: 21939377

45. Guerry P. (2007) Campylobacter flagella: not just for motility. Trends in microbiology 15:456–461. PMID: 17920274

46. Mukherjee S, Yakhnin H, Kysela D, Sokoloski J, Babitzke P, Kearns DB. (2011) CsrA-FliW interaction governs flagellar homeostasis and a checkpoint on flagellar morphogenesis in Bacillus subtilis. Mol Microbiol 82:447–461. doi: 10.1111/j.1365-2958.2011.07822.x PMID: 21895793

47. Marchant J, Wren B, Ketley J. (2002) Exploiting genome sequence: predictions for mechanisms of Campylobacter chemotaxis. Trends in microbiology 10:155–159. PMID: 11912013

48. Reuter M, van Vliet AH. (2013) Signal balancing by the CetABC and CezZ chemoreceptors controls energy taxis in Campylobacter jejuni. PLoS One 8:e54390. doi: 10.1371/journal.pone.0054390 PMID: 23382896

49. Parrish JR, Yu J, Liu G, Hines JA, Chan JE, Mangiola BA, et al. (2007) A proteome-wide protein interaction map for Campylobacter jejuni. Genome Biol 8:R130. PMID: 17615063

50. Baillon ML, van Vliet AH, Ketley JM, Constantinidou C, Penn CW. (1999) An iron-regulated alkyl hydroperoxide reductase (AhpC) confers aerotolerance and oxidative stress resistance to the microaerophilic pathogen Campylobacter jejuni. J Bacteriol 181:4798–4804. PMID: 10438747

51. Elvers KT, Wu G, Gilberthorpe NJ, Poole RK, Park SF. (2004) Role of an inducible single-domain hemoglobin in mediating resistance to nitric oxide and nitrosative stress in Campylobacter jejuni and Campylobacter coli. J Bacteriol 186:5332–5341. PMID: 15292134

52. van Vliet AH, Baillon MA, Penn CW, Ketley JM. (2001) The iron-induced ferredoxin FdxA of Campylobacter jejuni is involved in aerotolerance. FEBS Microbiol Lett 196:189–193. PMID: 11267778

53. van Vliet AH, Baillon ML, Penn CW, Ketley JM. (1999) Campylobacter jejuni contains two fur homologs: characterization of iron-responsive regulation of peroxide stress defense genes by the PerR repressor. J Bacteriol 181:6371–6376. PMID: 10515927

54. van Vliet AH, Ketley JM, Park SF, Penn CW. (2002) The role of iron in Campylobacter gene regulation, metabolism and oxidative stress defense. FEMS microbiology reviews 26:173–186. PMID: 12069882

55. Atack JM, Harvey P, Jones MA, Kelly DJ. (2008) The Campylobacter jejuni thiol peroxidases Tpx and Bcp both contribute to aerotolerance and peroxide-mediated stress resistance but have distinct substrate specificities. J Bacteriol 190:5279–5290. doi: 10.1128/JB.00100-08 PMID: 18515414

56. Kim JC, Oh E, Kim J, Jeon B. (2015) Regulation of oxidative stress resistance in Campylobacter jejuni, a microaerophilic foodborne pathogen. Front Microbiol 6:751. doi: 10.3389/fmicb.2015.00751 PMID: 26294041

57. Stead D, Park SF. (2000) Roles of Fe superoxide dismutase and catalase in resistance of Campylobacter coli to freeze-thaw stress. Appl Environ Microbiol 66:3110–3112. PMID: 10877819

58. Flint A, Sun YQ, Butcher J, Stahl M, Huang H, Stintzi A. (2014) Phenotypic screening of a targeted mutant library reveals Campylobacter jejuni defenses against oxidative stress. Infect Immun 82:2266–2275. doi: 10.1128/IAI.00152-13 PMID: 24643543

59. Flanagan RC, Neal-McKinney JM, Dhillon AS, Miller WG, Konkel ME. (2009) Examination of Campylobacter jejuni putative adhesins leads to the identification of a new protein, designated FlpA, required for chicken colonization. Infect Immun 77:2399–2407. doi: 10.1128/IAI.01528-13 PMID: 19349427

60. Monteville MR, Yoon JE, Konkel ME. (2003) Maximal adherence and invasion of INT 407 cells by Campylobacter jejuni requires the CadF outer-membrane protein and microfilament reorganization. Microbiology 149:153–165. PMID: 12576589

61. Rubinichik S, Seddon AM, Karlyshev AV. (2014) A negative effect of Campylobacter capsule on bacterial interaction with an analogue of a host cell receptor. BMC microbiology 14:141. doi: 10.1186/1471-2180-14-141 PMID: 24985441

62. Agladze K, Wang X, Romeo T. (2005) Spatial periodicity of Escherichia coli K-12 biofilm microstructure initiates during a reversible, polar attachment phase of development and requires the polysaccharide adhesin PGA. J Bacteriol 187:8237–8246. PMID: 16321928

63. Jackson DW, Suzuki K, Oakford L, Simecka JW, Hart ME, Romeo T. (2002) Biofilm formation and dispersal under the influence of the global regulator CsrA of Escherichia coli. J Bacteriol 184:290–301. PMID: 11741870

64. Cerca N, Jefferson KK. (2008) Effect of growth conditions on poly-N-acetylglicosamine expression and biofilm formation in Escherichia coli. FEMS Microbiol Lett 283:36–41. doi: 10.1111/j.1574-6968.2008.01142.x PMID: 18445167
65. Jones MK, Warner EB, Oliver JD. (2008) CsrA inhibits the formation of biofilms by *Vibrio vulnificus*. Appl Environ Microbiol 74:7064–7066. doi: 10.1128/AEM.01810-08 PMID: 18820071

66. Wang X, Dubey AK, Suzuki K, Baker CS, Babitzke P, Romeo T. (2005) CsrA post-transcriptionally represses pgaABCD, responsible for synthesis of a biofilm polysaccharide adhesin of *Escherichia coli*. Mol Microbiol 56:1648–1663. PMID: 15916613

67. Joshua GW, Guthrie-Irons C, Karlyshev AV, Wren BW. (2006) Biofilm formation in *Campylobacter jejuni*. Microbiology 152:387–396. PMID: 16436427

68. Kalmokoff M, Lanthier P, Tremblay TL, Foss M, Lau PC, Sanders G, et al. (2006) Proteomic analysis of *Campylobacter jejuni* 11168 biofilms reveals a role for the motility complex in biofilm formation. J Bacteriol 188:4312–4320. PMID: 16740937

69. Irsfeld M, Pruss BM, Stafslien SJ. (2014) Screening the mechanical stability of *Escherichia coli* biofilms through exposure to external, hydrodynamic shear forces. J Basic Microbiol 54:1403–1409. doi: 10.1002/jobm.201400054 PMID: 25042085

70. Lynnes T, Pruss BM, Samanta P. (2013) Acetate metabolism and *Escherichia coli* biofilm: new approaches to an old problem. FEMS Microbiol Lett 344:95–103. doi: 10.1111/1574-6968.12174 PMID: 23651469

71. Prüss BM, Verma K, Samanta P, Sule P, Kumar S, Wu J, et al. (2010) Environmental and genetic factors that contribute to *Escherichia coli* K-12 biofilm formation. Archives of microbiology 192:715–728. doi: 10.1007/s00203-010-0599-z PMID: 20559621

72. Wolfe AJ. (2005) The acetate switch. Microbiol Mol Biol Rev 69:12–50. PMID: 15755952

73. Li J, Fields JA, Thompson SA. (2016) Manuscript in preparation.

74. Stintzi A, Marlow D, Palyada K, Naikare H, Panciera R, Whitworth L, et al. (2005) Use of genome-wide expression profiling and mutagenesis to study the intestinal lifestyle of *Campylobacter jejuni*. Infect Immun 73:1797–1810. PMID: 15731981