Campylobacter jejuni CsrA Mediates Oxidative Stress Responses, Biofilm Formation, and Host Cell Invasion

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The putative global posttranscriptional regulator csrA was mutated in Campylobacter jejuni 81-176. The csrA mutant was attenuated in surviving oxidative stress. CsrA also contributed to biofilm formation and adherence to and invasion of INT407 intestinal epithelial cells, suggesting a regulatory role for CsrA in C. jejuni pathogenesis.

Diarrheal diseases represent an immense burden to both the developing and the industrial world, and the gram-negative pathogen Campylobacter jejuni is recognized around the world as a leading bacterial cause of gastroenteritis (3, 13, 16). Although C. jejuni requires very specific growth conditions in the laboratory, it persists in the environment. As it passes from host (commonly avian species) to human, C. jejuni must survive a great range of hostile environmental stresses, including limited carbon sources, suboptimal growth temperatures, and exposure to atmospheric oxygen. During infection, C. jejuni must withstand changes in pH and the host inflammatory response. In order to survive these stresses, C. jejuni must be able to sense these changes and respond accordingly. However, relatively little is known about the molecular mechanisms of Campylobacter pathogenesis and even less is known about how its virulence properties are regulated. While C. jejuni possesses several predicted global regulatory systems, including regulators of flagellar assembly and function (28, 67), iron homeostasis (58), heat shock (33), cold shock (45; W. A. Agee and S. A. Thompson, unpublished data), and the stringent response (19), its complement of regulators is dramatically less than that of enteric pathogen such as Salmonella enterica. Furthermore, C. jejuni has only three sigma factors (σ^70 [rpoD], σ^54 [rpoN], and σ^24 [filA]), seven histidine kinases, and 10 response regulators (44, 45). The small number of sigma factors and other global regulators in C. jejuni suggests that there may be other uncharacterized mechanisms of gene regulation.

C. jejuni genome sequences (18, 45) revealed orthologs of the Escherichia coli global posttranscriptional regulator csrA (carbon starvation regulator). In E. coli, CsrA was identified as a posttranscriptional regulator of translation (49, 50) responsible for repression or activation of many important processes. CsrA is a homodimeric RNA-binding protein that typically binds the 5’ untranslated regions of target mRNAs at one or more sites that are often adjacent to or overlapping the ribosome binding site, thus inhibiting ribosome access to the ribosome binding site and inhibiting translation initiation, which can either increase or decrease mRNA half-life (5, 7, 15, 39, 40, 48, 61).

In E. coli, CsrA is involved in regulating stationary-phase metabolism, represses glycerogen biosynthesis, gluconeogenesis, peptide transport, and biofilm formation (2, 15, 27, 37, 51, 52, 61), and activates glycolysis, acetate metabolism, and motility (52, 63, 64). Analysis of bacterial genomes has revealed widespread distribution of csrA throughout the eubacteria (65). Subsequently, the role of CsrA in the life cycles of several pathogenic bacteria has been studied, revealing that CsrA not only regulates stationary-phase metabolism but also is an important regulator of virulence determinants, including host cell invasion, quorum sensing, biofilm formation, iron acquisition, type III secretion systems, and outer membrane protein expression (4, 11, 12, 17, 25, 26, 34, 37, 38, 42, 43, 46, 47, 66). In the gastric pathogen Helicobacter pylori, a close relative of C. jejuni (21), CsrA is reported to play a role in the regulation of several virulence phenotypes, including motility, oxidative stress resistance, and mouse colonization (8).

Considering the limited contingent of regulatory effectors found in C. jejuni genomes, we suspected that CsrA might play a vital role in the regulation of stress responses and virulence determinants in this enteric pathogen. In this study, we sought to examine the role of CsrA in C. jejuni pathogenesis. We therefore constructed a C. jejuni 81-176 csrA mutant and complemented mutant strains for use in studies of survival and virulence-related phenotypes. We report that mutation of csrA reveals a potential role for CsrA in the regulation of C. jejuni genes required for survival of oxidative stress. Furthermore, CsrA plays a role in the activation of biofilm formation, motility, and adherence to host cells in vitro; however, it contributes to the repression of invasion of human cells.

Mutation of csrA in C. jejuni 81-176. A nonpolar mutation in csrA was constructed by inverse-PCR mutagenesis (68). Briefly, by use of primers JAF44 and JAF45, Cj1103 (csrA) including 500 bp upstream and downstream was amplified using PCR and cloned into pCRII-TOPO (Invitrogen). The new construct, pJF06, was then subjected to inverse PCR using primers JAF50A and JAF51, digested with NheI, and self-ligated to yield pJF07. pJF07 was digested with NheI and XmaI and ligated with the chloramphenicol acetyltransferase (cat) cassette amplified from pRY111 (69) by use of primers JAF52 and JAF53 and digested with the same enzymes to generate

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the plasmid pJF09. This plasmid contained a deletion of 75% of the csrA gene (replaced with csi) while maintaining the translation initiation signals of the downstream Cj1104 gene to avoid polarity. This construct was then introduced into C. jejuni 81-176 by electroporation (62), and a chloramphenicol-resistant (20 μg/ml) csrA mutant was verified by PCR and DNA sequencing (data not shown).

**Complementation of the csrA mutant in trans.** Complementation of the csrA mutant was accomplished by introducing the csrA gene under the control of its native promoter on the Campylobacter shuttle vector pRY107 (69). Briefly, csrA was amplified with primers JAF60 and JAF64 and cloned into pCRII-TOPO, producing pJF10A. Next, the csrA promoter (upstream of Cj1097) was amplified with primers JAF61 and JAF62, digested with Xmal and NdeI, and cloned upstream of csrA in pJF10A to create pJF10B. The csrA promoter cassette was then digested with EcoRI and subcloned into pRY107, giving the csrA complementation vector pJF11. pJF11 was then introduced into the csrA mutant by triparental mating (36). Transconjugants were recovered on chloramphenicol (15 μg/ml) and kanamycin (50 μg/ml), and the presence of pJF11 was confirmed by plasmid midi-prep (Qiagen) (data not shown).

**Mutation of csrA decreases swarming ability.** The swarming ability of the csrA mutant was determined on Mueller-Hinton (MH) media containing 0.4% agar (22) and confirmed via light microscopy of wet mounts (data not shown). After inoculation, the strains were incubated at 37°C for 24 h (Fig. 1A) and 48 h (Fig. 1B). The swarming ability of the mutant was >30% less than that of the parent strain after 24 h (P = 0.009) and 48 h (P = 0.0007), despite highly similar growth characteristics in MH broth (Fig. 1C). This was consistent with reported observations for E. coli and H. pylori (8, 64) and suggests that C. jejuni CsrA contributes to the regulation of motility or chemotaxis, as either can affect swarming ability.

**CsrA is required for resistance to oxidative stress.** Resistance of the 81-176, 81-176 csrA, and 81-176 csrA/pJF11 strains to oxidative stress was determined by assessing killing by atmospheric oxygen (19) and hydrogen peroxide (60). Aerotolerance was determined by transferring bacteria grown in MH broth to early log phase (optical density at 600 nm [OD600] of ~0.1) from a microaerobic environment to atmospheric and microaerobic growth conditions and incubating the bacteria for 9 h at 37°C. At 0, 3, 6, and 9 h, viable counts were measured by serial dilution and plating on MH plates. This experiment (Fig. 2A) showed that the csrA mutant was highly sensitive to atmospheric oxygen, resulting in greater than 99% loss of viability by 9 h (P = 0.0005). The strains grown under microaerobic conditions remained viable and grew to stationary phase (data not shown), indicating that the loss of viability under atmospheric conditions was specific to atmospheric oxygen exposure. For hydrogen peroxide resistance, cells were grown on blood agar overnight at 37°C, harvested in phosphate-buffered saline, and diluted to an OD600 of 1.0. A 100-μl portion of each strain was spread on MH agar, onto which filter discs (6 mm) inoculated with 10 μl of 1 mM, 10 mM, 100 mM, or 1 M hydrogen peroxide were placed and then incubated at 37°C for 9 h at 37°C. At 0, 3, 6, and 9 h, viable counts were measured by serial dilution and plating on MH plates. This experiment (Fig. 2A) showed that the csrA mutant was highly sensitive to atmospheric oxygen, resulting in greater than 99% loss of viability by 9 h (P = 0.0005) with error bars.

**Table 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Resistance</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td>Strains</td>
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<tr>
<td>E. coli JM109</td>
<td>Cloning host</td>
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<td>Invitrogen</td>
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<tr>
<td>C. jejuni 81-176</td>
<td>Wild type</td>
<td></td>
<td>10</td>
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<tr>
<td>Plasmids</td>
<td>Cloning vector</td>
<td>Amp, Kan</td>
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<tr>
<td>pCRRI-TOPO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRY107</td>
<td>C. jejuni shuttle vector</td>
<td>Kan</td>
<td>69</td>
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<tr>
<td>pRY111</td>
<td>C. jejuni shuttle vector</td>
<td>Cm</td>
<td>69</td>
</tr>
<tr>
<td>pJF06</td>
<td>1.2-kb csrA locus in pCRRI-TOPO</td>
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<td>This study</td>
</tr>
<tr>
<td>pJF07</td>
<td>Self-ligated inverse-PCR product of pJF06</td>
<td>Amp, Kan</td>
<td>This study</td>
</tr>
<tr>
<td>pJF09</td>
<td>pJF07::csrAcat</td>
<td>Amp, Kan, Cm</td>
<td>This study</td>
</tr>
<tr>
<td>pJF10A</td>
<td>csrA in pCRRI-TOPO</td>
<td>Amp, Kan</td>
<td>This study</td>
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<tr>
<td>pJF10B</td>
<td>pJF10A and pcaA</td>
<td>Amp, Kan</td>
<td>This study</td>
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<tr>
<td>pJF11</td>
<td>csrA and promoter in pRY107</td>
<td>Kan</td>
<td>This study</td>
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*AMP, ampicillin; Kan, kanamycin; Cm, chloramphenicol.*
under microaerobic conditions for 48 h. These studies (Fig. 2B) revealed greater sensitivity of the csrA mutant to all doses tested ($P < 0.01$). Taken together these data suggest that, as in H. pylori, CsrA contributes to the regulation of oxidative stress responses in C. jejuni.

CsrA is an activator of biofilm formation. By use of previously described methods (14), biofilms were quantitated via crystal violet (CV) staining of static biofilm formation in 24-well, flat-bottomed polystyrene tissue culture dishes at 48 h. Briefly, strains were inoculated in MH broth at an OD$_{600}$ of 0.05 and incubated statically at 37°C for 48 h. Biofilms were visualized by staining with CV, washed with distilled H$_2$O, and photographed, and CV binding was quantitated by determining the OD$_{570}$ after solubilization in 80% dimethyl sulfoxide for 24 h (Fig. 3). The csrA mutant formed a very sparse biofilm on the bottoms and sides of the wells (Fig. 3A). Conversely, both the wild type and the complement formed dense biofilms; however, a great deal of the matrix formed by the complemented mutant was present on the sides of the wells and is not represented in the aspect shown. Quantification of CV staining (Fig. 3B) revealed that the csrA mutant formed nearly 50% less biofilm than 81-176 ($P = 0.0001$); however, the complemented mutant formed twice as much biofilm as the wild type. It has been demonstrated that flagellar function and responses to both general and oxidative stress are critical to biofilm formation (24, 30, 31, 57, 59). These results suggest that CsrA is an activator of biofilm formation, possibly via regulation of motility and oxidative stress responses in C. jejuni. This conclusion is noteworthy considering that CsrA represses biofilm formation in several gammaproteobacteria (1, 2, 27, 54, 61). Biofilm

![FIG. 2. Deletion of CsrA results in reduced resistance to sources of oxidative stress. The 81-176 (●), 81-176 csrA (○), and 81-176 csrA/pJF11 (◆) strains were subjected to oxidative stress by exposure to atmospheric oxygen, whereby the strains were inoculated in flasks at an OD$_{600}$ of $\sim 0.1$ and a 6:1 surface-to-volume ratio and then incubated at 37°C and 100 rpm in an air incubator (A), and various concentrations of hydrogen peroxide in filter discs on MH agar plates (B). One representative, in triplicate, of three experiments is shown (*, $P \leq 0.05$; **, $P \leq 0.005$) with error bars.](http://jb.asm.org/)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′ → 3′)*</th>
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<tr>
<td>JAF43</td>
<td>TCA TTT GAT TAG TTT TTT GC</td>
</tr>
<tr>
<td>JAF44</td>
<td>ATG CAA GGA ATT ATC TCC TA</td>
</tr>
<tr>
<td>JAF45</td>
<td>GGT ATG TCA TCT TCA AAT TC</td>
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<tr>
<td>JAF50A</td>
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</tr>
<tr>
<td>JAF51</td>
<td>AGA GGC TAG CTT AAC ATT TTT CAA CCT TAT T</td>
</tr>
<tr>
<td>JAF52</td>
<td>CTC TGC TAG CCG AGG AGA AAT GAT GCA ATT</td>
</tr>
<tr>
<td>JAF53</td>
<td>AGA GCC CGG GTT ATT TAT TCA GCA AGT CTT</td>
</tr>
<tr>
<td>JAF60</td>
<td>CTA CCC GGG ATT CAT ATG TTA ATA TTA TCA</td>
</tr>
<tr>
<td>JAF61</td>
<td>GAT CCC GGG TAA TCA GCT TTA CTA AGT TIG TGA TTT GAC</td>
</tr>
<tr>
<td>JAF62</td>
<td>GCT CAT ATG AAA AAC CTT ATT AAA TAT TTA TAT CAA AAG</td>
</tr>
</tbody>
</table>

*a Underlined nucleotides indicate restriction sites introduced for cloning purposes.

![FIG. 3. CV staining of C. jejuni biofilm. CV-stained biofilms were solubilized in 80% dimethyl sulfoxide (A) and quantitated by determining the OD$_{570}$ (B). One representative, in triplicate, of three experiments is shown (**, $P \leq 0.005$) with error bars.](http://jb.asm.org/)
formation in C. jejuni is poorly understood but is certainly complex and requires flagellar function (30). Therefore, reduced biofilm formation by the C. jejuni CsrA mutant is consistent with the observation of reduced motility (Fig. 1) and also suggests that CsrA-mediated control of biofilm formation may be inherently different in C. jejuni and E. coli.

**Adherence and invasion of intestinal epithelial cells.** The role of CsrA in adherence and invasion of host cells in vitro was determined as previously described (9, 41, 62). The csrA mutant exhibited a 5.4-fold decrease in the ability to adhere to INT407 cells at a multiplicity of infection of 15 were examined in vitro. (A) Adherence is expressed as the percentage of bacteria which had either adhered to or invaded cultured intestinal epithelial cells after a 3-h incubation, compared to the inoculum. (B) Invasiveness is expressed as the percentage of intracellular bacteria surviving gentamicin treatment of the INT407 cells after an additional 2-h incubation, compared to the number of adherent bacteria (to account for differences in adherence among strains). One representative, in triplicate, of three experiments is shown (*, \(P \leq 0.05\)) with error bars.

**Fig. 4.** Adherence and invasion of INT407 cells. The capacities of the 81-176, 81-176 csrA, and 81-176 csrA/pJF11 strains to adhere to and invade INT407 cells at a multiplicity of infection of 15 were examined in vitro (A). Adherence is expressed as the percentage of bacteria which had either adhered to or invaded cultured intestinal epithelial cells after a 3-h incubation, compared to the inoculum. (B) Invasiveness is expressed as the percentage of intracellular bacteria surviving gentamicin treatment of the INT407 cells after an additional 2-h incubation, compared to the number of adherent bacteria (to account for differences in adherence among strains). One representative, in triplicate, of three experiments is shown (*, \(P \leq 0.05\)) with error bars.

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**REFERENCES**


