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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assembly and function (28, 67), iron homeostasis (58), heat shock (33), cold shock (45; W. A. Agee and S. A. Thompson, unpublished data), and the stringent response (19), its complement of regulators is dramatically less than that of enteric pathogens such as *Salmonella enterica*. Furthermore, *C. jejuni* has only three sigma factors (σ^{70} [*rpoD*], σ^{54} [*rpoN*], and σ^{28} [*fliA*]), 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* (*c*arbon *s*tarvation *r*egulator). 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 glycogen 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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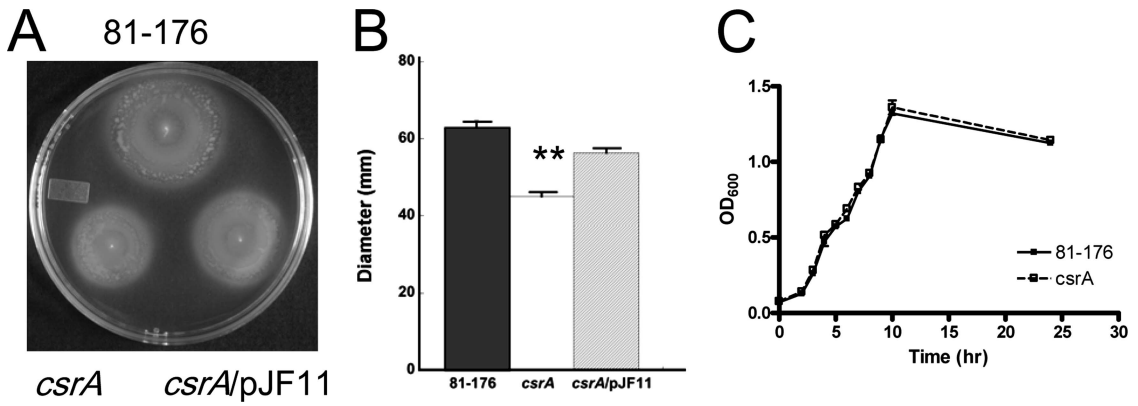

FIG. 1. CsrA is required for full motility. Swarming ability was assessed on MH agar containing 0.4% agar. Strains were inoculated into MH motility agar and incubated for 24 h (A) and 48 h (B) at 37°C under microaerobic conditions. (C) Growth of the 81-176 and 81-176 *csrA* strains was observed in MH broth and measured by determining the OD_{600}. The assay was carried out in triplicate, and one representative of three experiments is shown (**, $P \leq 0.005$) with error bars.

the plasmid pJF09. This plasmid contained a deletion of 75% of the *csrA* gene (replaced with *cat*) 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 JAF43 and cloned into pCRII-TOPO, producing pJF10A. Next, the *csrA* promoter (upstream of Cj1097) was amplified with primers JAF61 and JAF62, digested with XmaI 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 [OD_{600}] 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 OD_{600} 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

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Resistance[a]	Source or reference
Strains			
E. coli JM109	Cloning host		Promega
C. jejuni 81-176	Wild type		10
Plasmids			
pCRII-TOPO	Cloning vector	Amp, Kan	Invitrogen
pRY107	*C. jejuni* shuttle vector	Kan	69
pRY111	*C. jejuni* shuttle vector	Cm	69
pJF06	1.2-kb *csrA* locus in pCRII-TOPO	Amp, Kan	This study
pJF07	Self-ligated inverse-PCR product of pJF06	Amp, Kan	This study
pJF09	pJF07::*csrA*Δ*cat*	Amp, Kan, Cm	This study
pJF10A	*csrA* in pCRII-TOPO	Kan	This study
pJF10B	pJF10A and p*csrA*	Kan	This study
pJF11	*csrA* and promoter in pRY107	Kan	This study

[a] Amp, ampicillin; Kan, kanamycin; Cm, chloramphenicol.

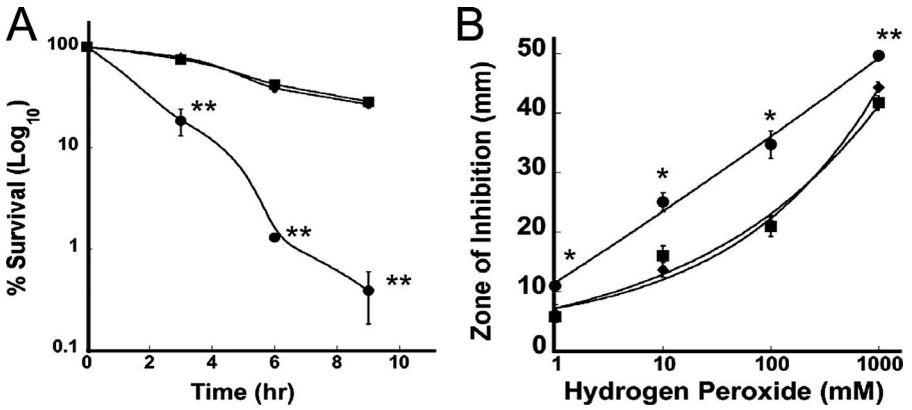

FIG. 2. Deletion of CsrA results in reduced resistance to sources of oxidative stress. The 81-176 (■), 81-176 *csrA* (●), and 81-176 *csrA*/pJF11 (◆) strains were subjected to oxidative stress by exposure to atmospheric oxygen, whereby the strains were inoculated in flasks at an OD_{600} of ~0.1 and 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.

under microaerobic conditions for 48 h. These studies (Fig. 2B) revealed greater sensitivity of the *csrA* mutant to all doses tested ($P \leq 0.01$). 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_2O, 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

TABLE 2. Primers used in this study

Primer	Sequence (5′ → 3′)[a]
JAF43	TCA TTT GAT TAG TTT TTT GC
JAF44	ATG CAA GGA ATT ATC TCC TA
JAF45	GGT ATG TCA TCT TCA AAT TC
JAF50A	CTC TGC TAG CAC CCG GGT GTT GTT CAG AAT GAT ATT AAA C
JAF51	AGA GGC TAG CTT AAC ATT TTT CAA CCT TAT T
JAF52	CTC TGC TAG CGG AGG ATA AAT GAT GCA ATT
JAF53	AGA GCC CGG GTT ATT TAT TCA GCA AGT CTT
JAF60	CTA CCC GGG ATT CAT ATG TTA ATA TTA TCA
JAF61	GAT CCC GGG TAA TCA GCT TTA CTA AGT TTG TGA TTT GAC
JAF62	GCT CAT ATG AAA AAC CTT ATT AAA TAT TTT TTA TAT CAA AAG

[a] Underlined nucleotides indicate restriction sites introduced for cloning purposes.

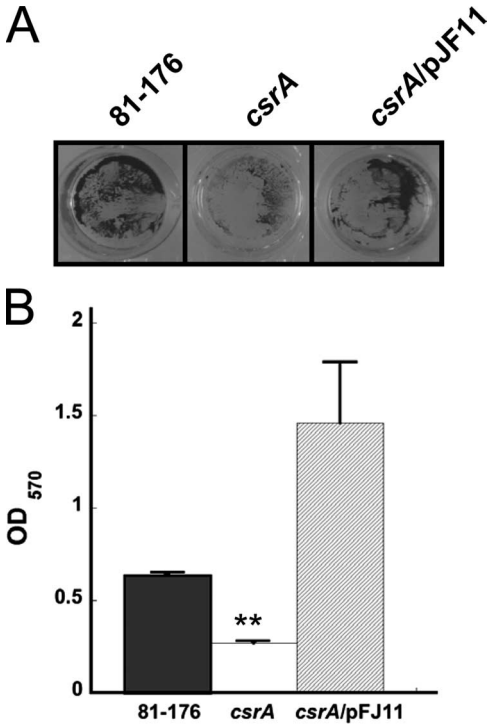

FIG. 3. CV staining of *C. jejuni* biofilms. CV-stained biofilms were solubilized in 80% dimethyl sulfoxide (A) and quantitated by determining the OD_{570} (B). One representative, in triplicate, of three experiments is shown (**, $P \leq 0.005$) with error bars.

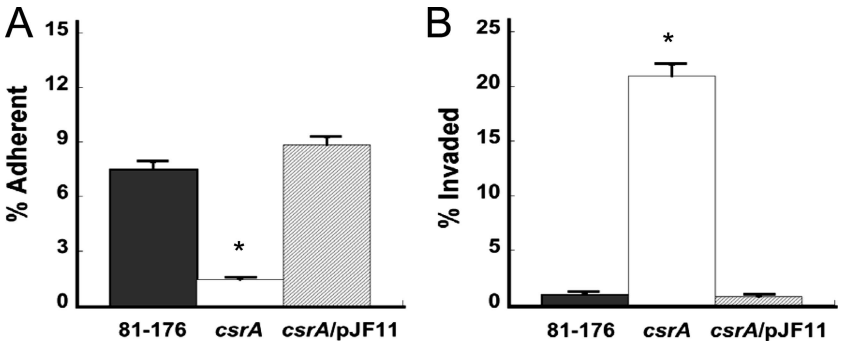

FIG. 4. Adherence and invasion of INT407 cells. The capacities of the 81-176, 81-176 *csrA*, and 81-176 *csrA*/pJF11 strains to adhere to and invade INT407 cells at a multiplicity of infection of 15 were examined in vitro. (A) Adherence is expressed as the percentage of bacteria which had either adhered 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.

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 (Fig. 4A) ($P = 0.002$). This attenuation of adherence was contrasted by a 20-fold increase in invasion by adherent *C. jejuni csrA* mutant cells (Fig. 4B) ($P = 0.01$) despite reduced motility, a factor known to influence invasion (20, 23, 29, 70). There was no difference in susceptibility to gentamicin among the strains. This is the first report to implicate CsrA in the regulation of host cell adherence. Previous studies have reported that CsrA functions in both the activation and the repression of invasion (4, 17, 37). Our data suggest that in *C. jejuni* the role of CsrA in epithelial cell invasion is primarily carried out via repression of invasion-specific genes. This conclusion introduces a paradox because both motility and adherence are important for host cell invasion in *C. jejuni* (20, 23, 29, 62, 70); however, the *csrA* mutant displays defects in both. However, while both motility and adherence are certainly prerequisites for invasion, the adherence and invasion processes involve different proteins. For example, molecules that are involved uniquely in the invasion step but not in adherence include the *Campylobacter* invasion antigens, gamma-glutamyl transpeptidase, and the polysaccharide capsule (6, 9, 32). CsrA may therefore directly or indirectly regulate these or other invasion-specific *Campylobacter* proteins, and changes in the expression of these proteins may override any effect of the decrease in motility and result in the observed increase in invasion.

Conclusions. Posttranscriptional regulation in *C. jejuni* has not been studied previously, and many questions remain to be considered in future studies to address how CsrA works in *Campylobacter* and other epsilonproteobacteria. Presently, it is not known how CsrA is regulated in *C. jejuni*. In *E. coli* and many other bacteria, CsrA has been shown to be regulated by the small noncoding RNAs *csrB* and *csrC* (5), which have not been identified in *Campylobacter* (35). Furthermore, regulation of *E. coli csrBC* is directed by the BarA/UvrY two-component regulatory system (17, 53–56), which does not appear to have an ortholog in *C. jejuni* (45). These data, therefore, represent an important first step in elucidating the role of CsrA in *C. jejuni* physiology and pathogenesis. In summary, we have constructed a *C. jejuni* mutant lacking the predicted posttranscriptional regulator CsrA. The *csrA* mutant exhibits changes in several virulence-related properties, including oxidative stress resistance, motility, adherence, and invasion. These pleiotropic effects suggest that CsrA is an important regulator involved in *C. jejuni* pathogenesis.

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