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The *Campylobacter jejuni* Transcriptional Regulator Cj1556 Plays a Role in the Oxidative and Aerobic Stress Response and Is Important for Bacterial Survival *In Vivo*[▽]

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Campylobacter jejuni **is the leading bacterial cause of human gastroenteritis worldwide. Despite stringent microaerobic growth requirements, *C. jejuni* is ubiquitous in the aerobic environment and so must possess regulatory systems to sense and adapt to external stimuli, such as oxidative and aerobic (O_2) stress. Reannotation of the *C. jejuni* NCTC11168 genome sequence identified Cj1556 (originally annotated as a hypothetical protein) as a MarR family transcriptional regulator, and further analysis indicated a potential role in regulating the oxidative stress response. A *C. jejuni* 11168H *Cj1556* mutant exhibited increased sensitivity to oxidative and aerobic stress, decreased ability for intracellular survival in Caco-2 human intestinal epithelial cells and J774A.1 mouse macrophages, and a reduction in virulence in the *Galleria mellonella* infection model. Microarray analysis of gene expression changes in the *Cj1556* mutant indicated negative autoregulation of *Cj1556* expression and downregulation of genes associated with oxidative and aerobic stress responses, such as *katA*, *perR*, and *hspR*. Electrophoretic mobility shift assays confirmed the binding of recombinant Cj1556 to the promoter region upstream of the *Cj1556* gene. *cprS*, which encodes a sensor kinase involved in regulation of biofilm formation, was also upregulated in the *Cj1556* mutant, and subsequent studies showed that the mutant had a reduced ability to form biofilms. This study identified a novel *C. jejuni* transcriptional regulator, Cj1556, that is involved in oxidative and aerobic stress responses and is important for the survival of *C. jejuni* in the natural environment and *in vivo*.**

Campylobacter jejuni infection is one of the most commonly identified bacterial causes of acute human gastroenteritis worldwide (1). The symptoms of campylobacteriosis are malaise, fever, severe abdominal pain, and diarrhea (12). *C. jejuni* infection has also been associated with postinfectious sequelae, including septicemia and neuropathies, such as Guillain-Barré syndrome (GBS) (52). *C. jejuni* is a commensal in avian species, and the consumption and handling of poultry products is a major source of human infection (4, 39). However, a diverse range of environmental sources, such as untreated water, raw or unpasteurized milk, vegetables, and transmission from pets, are also all recognized sources of infection (55). Despite specific microaerobic growth requirements, *C. jejuni* is ubiquitous in the aerobic environment and appears capable of withstanding different stresses, including suboptimal carbon source growth, temperature changes, and exposure to atmospheric oxygen (26). *C. jejuni* can also persist in the environment through survival in biofilms (36). During human infection, *C. jejuni* has to withstand a further range of stresses, including changes in pH and the host innate immune response (11). The last decade has seen major advances in our understanding of *C. jejuni* physiology, yet many unanswered questions remain regarding the pathogenesis and survival mechanisms of the bacterium. A more complete understanding of the regulation of *C. jejuni* response mechanisms to the diverse stresses encountered both during the infection cycle and within the natural environment is required to facilitate appropriate intervention strategies to reduce the burden of *C. jejuni*-associated disease (64).

Oxidative, nitrosative, and aerobic (O_2) stresses are major factors that pathogens must counteract in order to survive within the host (4, 25, 57, 87). *C. jejuni* is a microaerophilic organism optimally suited to low levels of atmospheric oxygen; however, the bacterium is able to survive oxidative stresses *in vivo* (4). The incomplete reduction of oxygen to water creates reactive oxygen species (ROS) molecules, such as hydrogen peroxide (H_2O_2), that are used by the host against invading pathogens (20). ROS are also released by the immune system to combat invading microorganisms (4). An example of ROS release is the deposition of various oxygen species generated by the respiratory burst oxidase as the bacterium remains bound within an endosome (41). ROS can damage bacterial DNA (33). Reactive nitrogen species (RNS), such as nitric oxide, are a family of antimicrobial molecules produced by the enzymatic activity of inducible nitric oxide synthase 2 (iNOS) (34). Acidified nitrite kills *C. jejuni*, and expression of the NOS2 isoform is increased in macrophages upon exposure to the bacterium (34). RNS tend to interfere with respiration and DNA replication through inactivation of zinc metalloproteins (25). Both ROS and RNS are also derived from phagocytosis through the generation of superoxide and nitric oxide radicals via NADPH phagocyte oxidase and inducible nitric oxide synthase path-

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ways, which are important pathways within polymorphonuclear phagocytes, including white blood cells and mononuclear phagocytes (25). Aerobic stress is caused by bacterial exposure to raised oxygen levels. Even though oxygen is considered a stress for *C. jejuni*, few studies have described specific phenotypic consequences of aerobiosis, and those that have vary in their conclusions (72). Exposure of *C. jejuni* to oxygen for 24 h accelerated the transition to the viable but nonculturable (VBNC) state or coccoid form (40). In contrast, another study identified the increased culturability of *C. jejuni* when exposed to oxygen for 15 h (48). Recently, it has been demonstrated that aerobic stress conditions promoted the production of *C. jejuni* biofilms (65).

C. jejuni possesses a variety of mechanisms for reacting to nitrosative, oxidative, and aerobic stresses. *C. jejuni* possesses a truncated hemoglobin (Ctb), along with a single-domain hemoglobin (Cgb). Both Ctb and Cgb have been characterized as part of the *C. jejuni* nitrosative stress response regulon (23, 78). This regulon is under the control of NssR (49). Previous studies have also implicated Ctb with a role in oxygen metabolism (77, 78). *C. jejuni* contains several genes encoding important oxidative stress response proteins. The superoxide dismutase SodB is involved in the breakdown of superoxide to H_2O_2 and O_2 (61). The catalase KatA converts H_2O_2 to H_2O and O_2. In addition, the alkyl hydroperoxide reductase AhpC confers resistance to cumene hydroperoxide and aerobic stress (6). However, *C. jejuni* lacks an OxyR ortholog, which regulates *ahpC* and *katA* expression in response to oxidative stress in many enteric bacteria, such as *Salmonella* species and *Escherichia coli* (14). *C. jejuni* also lacks the classical SoxRS system, which mediates transcriptional activation of the oxidative stress regulon in response to superoxide-generating agents (2). In *C. jejuni*, the Fur homolog PerR was found to repress *ahpC* and *katA* transcription in an iron-dependent manner, thus making PerR a functional but not homologous substitute for OxyR (57, 75). In addition, *C. jejuni* proteins involved in responding to aerobic stress have also been identified. SodB and KatA have been shown to counteract the detrimental effects of aerobic stress (69). The heat shock protease HtrA and the regulator HspR have been shown to be important for short-term aerobic tolerance (3, 12). The *fdxA* gene upstream of *ahpC* encodes a ferrodoxin that has been identified as important for aerotolerance (74). Also, SpoT, which regulates the *C. jejuni* stringent response, was found to be important for low CO_2 growth and aerobic survival (28). Cj1556 was identified as a member of the MarA family of transcriptional regulators through reannotation of the NCTC11168 genome sequence (29). In this study, further bioinformatics analysis indicated a role for Cj1556 in the *C. jejuni* stress responses, and a defined isogenic *C. jejuni* 11168H *Cj1556* mutant was constructed in order to investigate this hypothesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *C. jejuni* wild-type strain used in this study was 11168H (38), a hypermotile derivative of the original sequenced strain NCTC11168 that shows higher levels of cecal colonization in a chick colonization model (35). *C. jejuni* was grown at 37°C in a microaerobic chamber (Don Whitley Scientific, United Kingdom) containing 85% N_2, 10% CO_2, and 5% O_2 either on blood agar plates containing Columbia agar base (Oxoid, United Kingdom) supplemented with 7% (vol/vol) horse blood (TCS Microbiology, United Kingdom) and *Campylobacter* Selective Supplement (Oxoid) or in brucella broth (Oxoid) with shaking at 75 rpm. *C. jejuni* strains were grown on blood agar plates for 24 h prior to use in coculture experiments. *E. coli* XL-2 Blue MRF′ competent cells (Stratagene) were used for cloning experiments and were grown at 37°C under aerobic conditions either on Luria-Bertani (LB) agar plates or in LB broth with shaking at 200 rpm. Appropriate antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and chloramphenicol, 50 μg/ml for *E. coli* studies and 10 μg/ml for *C. jejuni* studies. All reagents were obtained from Invitrogen (United Kingdom) unless otherwise stated.

TABLE 1. Oligonucleotide primers used in this study

Primer name	Sequence
Cj1556-F	ATCATTCTCTTTGTCCTAT
Cj1556-R	TAAGATGGATTCTAAACTATTG
Km^r forward-out	TGGGTTTCAAGCATTAGTCCATGCAAG
Km^r reverse-out	GTGGTATGACATTGCCTTCTGCG
Comp-*Cj1556*-F	CCCCCATGGATAAGGATTTATAATGAAAAAATATCATTCTCT
Comp-*Cj1556*-R	CCCGCTAGCTTAAACGATATTTTTATAGCTAT
Comp-*Cj1556*-R-HIS	CCCGCTAGCTTAATGATGATGATGATGATGAACGATATTTTTATAGCTAT
Upstream *Cj1556*-F	ATGCAATCTAGAAATTAT
Upstream *Cj1556*-R	GGACAAAGAGAATGATATT
Upstream *flaA*-F	ATCACAGCTTATATTAAAG
Upstream *flaA*-R	GTGTTAATACGAAATCCCAT
Upstream *flgK*-F	ATTTGTTCTTATTGTCAA
Upstream *flgK*-R	ATGTTCCAAAAATACCCAT

Construction of the *C. jejuni* 11168H *Cj1556* mutant. A defined isogenic 11168H *Cj1556* mutant was constructed using previously published methods (35, 38, 43). Briefly, the primers *Cj1556*-F and *Cj1556*-R were designed for PCR detection of *Cj1556* (Table 1). Using the pUC library from the *C. jejuni* NCTC11168 genome-sequencing project (58), plasmid cam25a2 (1489074.0.1490567), which contains a 1.494-kb insert including the coding sequences (CDSs) *Cj1555c* to *Cj1560*, was selected and designated pUC-*Cj1556*. The *Cj1556* open reading frame (ORF) in pUC-*Cj1556* was inactivated by insertion of an *aph-3* (aminoglycoside 3′-phosphotransferase) kanamycin resistance (Km^r) cassette (73). The Km^r cassette was excised from pJMK30 (76) using BamHI. pUC-*Cj1556* was digested with BclI and ligated with the Km^r cassette to form pUC-*Cj1556*-Km^r. pUC-*Cj1556*-Km^r was transformed into XL-2 Blue MRF′ competent cells, and transformants were selected on LB agar supplemented with ampicillin and kanamycin after 48 h of growth at 37°C. Transformants were screened by PCR using *Cj1556*-specific and Km^r-specific primers (Table 1). pUC-*Cj1556*-Km^r plasmids with the Km^r cassette in the same orientation as the *Cj1556* gene were selected and electroporated into wild-type 11168H as described previously (35, 38). The electroporated bacteria were plated onto blood agar plates and incubated at 37°C under microaerobic conditions for 2 days. Cells were harvested and resuspended in 0.5 ml phosphate-buffered saline (PBS). Two hundred microliters of this suspension was spread onto blood agar plates containing kanamycin. Putative *Cj1556* mutants were screened using PCR and sequencing.

Complementation of the *C. jejuni Cj1556* mutant. Complementation procedures were performed by inserting a copy of the *Cj1556* gene into the *Cj1556* mutant chromosome using a *C. jejuni* NCTC11168 complementation vector (31). The coding region for *Cj1556* was amplified by PCR using primers Comp-*Cj1556*-F and Comp-*Cj1556*-R (Table 1), which introduced an NcoI site at the 5′ end and an NheI site at the 3′ end, as well as the native ribosome binding site of *Cj1556* (72, 83). Following digestion with NheI and NcoI, this PCR product was ligated into the pDENNIS vector. The construct was checked by sequencing and electroporated into the *Cj1556* mutant. Putative clones were selected on blood agar plates containing kanamycin and chloramphenicol. Confirmation of the presence of copies of both *Cj1556* and *Cj1556*-Km^r was performed by PCR using the Comp-*Cj1556*-F and Comp-*Cj1556*-R primers, as well as the *Cj1556*-F and *Cj1556*-R primers, and also by sequencing. For isolation of recombinant Cj1556 protein, a 6×His tag sequence was cloned into a second construct using the primers Comp-*Cj1556*-F and Comp-*Cj1556*-R-HIS (Table 1).

Nitrosative, oxidative, and heat stress assays. *C. jejuni* was grown on blood agar plates for 24 h at 37°C under microaerobic conditions. Bacterial cells were harvested into 1 ml PBS and adjusted to an optical density at 600 nm (OD_{600}) of 1. For nitrosative stress assays, bacterial cells were exposed to acidified sodium

nitrite ($NaNO_2$) at a final concentration of 100 mM $NaNO_2$ for 30 min and 10 mM $NaNO_2$ for 75 min. For nitrosative stress assays, all media used were at pH 5 to allow formation of acidified $NaNO_2$ to promote the production of nitric oxide radicals (22, 34). For oxidative-stress assays, bacterial cells were exposed to hydrogen peroxide (H_2O_2) at a final concentration of 10 mM for 15 min. Heat stress assays were performed at 42°C for 1 h, 55°C for 15 min, and 60°C for 5 min. Serial dilutions were prepared, 10 μl of the 10^{-1} to 10^{-6} dilutions were spotted onto blood agar plates and incubated for 48 h at 37°C under microaerobic conditions, and colonies were counted.

Cell culture procedures. The Caco-2 human intestinal epithelial and J774A.1 mouse macrophage cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (FCS) (Sigma-Aldrich, United Kingdom), 1% (vol/vol) nonessential amino acids, 100 μg/ml streptomycin, and 100 U/ml penicillin. The T84 human colonic epithelial cell line was maintained in a 1:1 mixture of DMEM and Ham's F-12 medium containing Glutamax, 2.5 mM L-glutamine, 15 mM HEPES, and 0.5 mM sodium pyruvate supplemented with 10% (vol/vol) FCS, 100 μg/ml streptomycin, and 100 U/ml penicillin. The cells were maintained at 37°C in 5% CO_2 and 95% air. For Caco-2 cell coculture experiments, cells were seeded at 1×10^5/ml and grown in 24-well plates to >90% confluence (~1×10^6 cells/ml). For T84 cell coculture experiments, cells were seeded at 5×10^5/ml and grown in 24-well plates to >90% confluence (~5×10^6 cells/ml). For coculture experiments involving J774A.1 mouse macrophages, cells were seeded at 5×10^5/ml and grown in 24-well plates for 24 h. For enzyme-linked immunosorbent assay (ELISA) experiments, T84 cells were maintained in low-serum 1% (vol/vol) and antibiotic-free medium overnight prior to coculture. Infections were terminated by removing the supernatant from the cells, followed by two washes in PBS. Cell culture supernatants were stored at −80°C.

Interaction, invasion, and intracellular survival assays. Interaction (adhesion and invasion) and invasion assays were performed using Caco-2 cells as described previously (12). Bacterial cells were harvested into 1 ml brucella broth and adjusted to an OD_{600} of 0.1. Serial dilutions were prepared, and 200-μl volumes were plated onto blood agar plates and incubated for 72 h at 37°C under microaerobic conditions. Colonies were counted to calculate the initial inoculum. *C. jejuni* (approximately 1×10^8 cells) in DMEM was added to a monolayer of approximately 1×10^6 Caco-2 cells (multiplicity of infection [MOI], 100:1) and incubated for 3, 6, or 24 h. The number of interacting bacteria was determined by washing the monolayers three times with PBS and then lysing the cells by addition of 0.2% (vol/vol) Triton X-100. The number of intracellular bacteria was determined by further incubating the monolayers after the initial interaction time point with DMEM containing gentamicin (150 μg/ml) for 2 h at 37°C to allow killing of extracellular bacteria. The monolayers were then washed three times in PBS, and the epithelial cells were lysed as described above. For intracellular survival assays, bacterial cells were cocultured with a monolayer of Caco-2 cells for 3 h, followed by washing the monolayers three times with PBS. The monolayers were then incubated in DMEM containing gentamicin (150 μg/ml) for 2 h and incubated in DMEM containing a reduced concentration of gentamicin (10 μg/ml) for 19 h. The monolayers were then washed three times in PBS, and the epithelial cells were lysed as described above. To ascertain whether the above results were due to a genuine *Cj1556* mutant phenotype and not to increased sensitivity to Triton X-100, stress assays were performed on all three strains with 0.2% (vol/vol) Triton X-100. No difference was observed between the levels of sensitivity to Triton X-100 in the 11168H wild-type strain, the *Cj1556* mutant, and the *Cj1556* complement strains (data not shown). Experiments with survival in tissue culture medium from coculture were performed as described above, but after 24 h of coculture, the tissue culture medium alone was removed, followed by plating of serial dilutions to determine the CFU/ml. In all cases, serial dilutions, plating, and determination of bacterial numbers were performed as stated above.

Macrophage survival assay. Macrophage survival assays were performed as described previously (80) using J774A.1 mouse macrophages (67). Briefly, *C. jejuni* was harvested into 1 ml brucella broth and adjusted to an OD_{600} of 0.1. *C. jejuni* cells (approximately 1×10^8 cells) in DMEM were added to a culture of approximately 5×10^5 J774A.1 mouse macrophages (MOI, 200:1) and incubated for 3 h. The cells were washed three times in PBS, followed by incubation in DMEM containing gentamicin (150 μg/ml) for 2 h to allow killing of extracellular bacteria. The macrophages were incubated in DMEM containing a reduced concentration of gentamicin (10 μg/ml), and bacterial survival was determined at 0, 4, and 16 h. At each time point, the macrophages were washed three times with PBS and lysed by adding 0.2% (vol/vol) Triton X-100 in PBS. Serial dilutions, plating, and enumeration of bacteria were performed as stated above.

IL-6 and IL-8 ELISAs. Supernatants from uninfected T84 cells and T84 cells infected with *C. jejuni* at an MOI of 20:1 for 24 h were collected. The levels of interleukin-6 (IL-6) and IL-8 secretion were assessed using a commercially available sandwich ELISA kit according to the manufacturer's instructions (Peprotech, United Kingdom). Detection was performed using a Dynex MRX II 96-well plate reader at an absorbance of 405 nm (A_{405}) and analyzed using Revelation software (Dynex).

TABLE 2. DNA fragments used as promoter probes for electrophoretic mobility shift assays

Fragment region	Purpose of selection	Location within genome (nucleotides)	Size of fragment (bp)
Upstream of *Cj1556*	Proposed area of binding	1489630–1489800	170
Upstream of *flaA*	Negative control	1271120–1270940	180
Upstream of *flgK*	Negative control	1400460–1400600	140

Transcriptome studies: experimental design, template labeling, microarray hybridizations, data acquisition, and microarray data analysis. Gene expression profiling of *C. jejuni* 11168H from the late log growth phase (16 h) was performed using an indirect-comparison method or type 2 experimental design (86). Replicate test sets of Cy5-labeled *C. jejuni* 11168H total RNA samples were combined with a common reference sample (Cy3-labeled *C. jejuni* 11168H genomic DNA) as described in previous studies (24, 44, 84). *C. jejuni* 11168H genomic DNA was isolated from bacteria grown on blood agar for 24 h using the Puregene DNA purification kit (Gentra, United Kingdom) and used as the common reference sample in all microarray experiments. *C. jejuni* RNA was isolated from 16-h cultures using the RNeasy Mini purification kit (Qiagen) and RNAprotect Bacteria Reagent (Qiagen) as described previously (37). Whole-genome *C. jejuni* NCTC11168 microarrays printed on UltraGAPS glass slides (Corning) constructed by the BμG@S Microarray Group (http://www.bugs.sgul.ac.uk/) were used in this study (37). The procedures used for Cy5 labeling of total RNA samples (37) and Cy3 labeling of 11168H genomic DNA (21) were as described previously. All hybridizations were performed as described previously (21, 37) with the following modifications. For probe hybridization, Cy5-labeled probes of *C. jejuni* 11168H total RNA (test) and Cy3-labeled common reference samples of *C. jejuni* 11168H DNA (control) were combined and purified using a MinElute PCR Purification kit (Qiagen). The final elution was made up to a volume of 50 μl with a final concentration of 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.3% (wt/vol) SDS. The hybridization mixture was denatured at 98°C for 2 min and cooled slowly to room temperature. A 22- by 25-mm LifterSlip coverslip (Erie Scientific) was placed over the reporter element area on the microarray, and the hybridization mixture was applied underneath the coverslip. The microarray slide was placed in a humidified hybridization cassette (Telechem International) and incubated in a water bath for 18 h at 65°C without shaking. The microarray slides were then washed as described previously (37). The microarray slides were scanned with an Affymetrix 418 array scanner (MWG Biotech, Germany) according to the manufacturer's guidelines. Signal and local background intensity readings for each spot were quantified using ImaGene software v8.0 (BioDiscovery). The quantified data were analyzed using GeneSpring GX software v7.3 (Agilent). Statistically significant up- and downregulated genes were selected when comparing gene expression against the 11168H wild-type strain using analysis of variance (ANOVA) with a Benjamini and Hochberg false-discovery rate as the multiple-testing correction (5, 18).

Electrophoretic mobility shift assays. *E. coli* strains were grown overnight for 16 h at 37°C with shaking at 200 rpm. The cultures were spun at 4,000 rpm for 10 min at 4°C. The bacterial pellet was resuspended in 1 ml equilibration buffer (Sigma-Aldrich). The cells were sonicated according to the manufacturer's instructions (Diagenode, Belgium), followed by centrifugation for 5 min at 13,000 rpm. The supernatant containing lysed cell content was poured into a new 1.5-ml microcentrifuge tube. The lysed cells were incubated with Ni-nitrilotriacetic acid (NTA) (Qiagen) for 1 h at 4°C on a rotator. Elution was performed using a His-Select spin column (Sigma-Aldrich). To demonstrate the DNA binding properties of Cj1556, purified recombinant protein was hybridized to PCR-amplified fragments (140 to 180 bp) located upstream of the translation initiation sites of the *Cj1556*, *flaA*, and *flgK* genes (Tables 1 and 2). Recombinant native protein (2.5 μg) was hybridized with 20 ng of purified DNA, along with 2 μl hybridization solution (20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiothreitol [DTT], 250 mM NaCl, 50 mM Tris-HCl, pH 7.5) and incubated at room temperature for 40 min. Samples were resuspended in Tris-glycine native

sample buffer (Invitrogen) up to 10 μl and analyzed using a Tris-glycine gel under nondenaturing conditions (Invitrogen), followed by Western blot analysis.

Biofilm assays. *C. jejuni* was grown on blood agar plates for 24 h at 37°C under microaerobic conditions. Brucella broth (10 ml) was preincubated in a 50-ml flask at 37°C under microaerobic conditions 24 h prior to inoculation and then inoculated with the bacterial cells harvested into brucella broth to an OD_{600} of 0.1; 1 ml was then added to 24-well polystyrene plates (Corning) and incubated under microaerobic conditions with gentle agitation for 3 days. The wells were washed three times with PBS, followed by addition of 0.2% (wt/vol) crystal violet (Sigma-Aldrich) for 10 min. The wells were then washed three times with PBS, followed by dissolving the biofilm with 20% acetone-80% ethanol. Detection was performed using a Dynex MRX II 96-well plate reader at A_{595}.

***G. mellonella* infection model.** *Galleria mellonella* larvae were obtained from LiveFoods Direct (United Kingdom) and kept on wood chips at 16°C. The larvae were injected with a 10-μl inoculum of a 24-h *C. jejuni* culture diluted to an OD_{600} of 0.1 by microinjection (Hamilton, Switzerland) in the right foremost leg, giving an infectious dose of approximately 10^6 CFU (17). Injections with PBS and no-injection controls were also performed. The larvae were incubated at 37°C, with survival and percentage survival recorded at 24-h intervals. For each experiment, 10 *G. mellonella* larvae were infected, and experiments were repeated three times.

Statistical analyses. Data are presented as mean ± standard deviation (SD). All experiments represent at least three biological replicates performed in triplicate in each experiment. Statistical analyses were performed using Prism software (GraphPad Software). Variables were compared using Student's *t* test.

Microarray data accession numbers. The array design is available in BμG@Sbase (accession no. A-BUGS-9) (http://bugs.sgul.ac.uk/A-BUGS-9) and also ArrayExpress (accession no. A-BUGS-9). Fully annotated microarray data have been deposited in BμG@Sbase (accession number E-BUGS-119) (http://bugs.sgul.ac.uk/E-BUGS-119) and also ArrayExpress (accession number E-BUGS-119).

RESULTS

Bioinformatics analysis indicates Cj1556 has a role in regulation of stress responses. The 333-nucleotide predicted CDS of *Cj1556* was originally annotated as a hypothetical protein in the genome sequence of *C. jejuni* NCTC11168 (29). Following reannotation, the updated product function indicated that Cj1556 is a transcriptional regulator based on the identification of a new Pfam motif (PF01638), defined as an HxlR-like helix-turn-helix motif (29). The HxlR-like helix-turn-helix motif is located 45 nucleotides into the CDS and encompasses the remainder of the CDS. The HxlR-like helix-turn-helix motif is part of the MarR family of transcriptional regulators, which includes proteins that control virulence factor production, bacterial responses to both antibiotics and oxidative stress, and also catabolism of environmental aromatic compounds (82, 85). The predicted function of Cj1556 was investigated further using the Campylobacter Protein Interaction Database (60), and putative interactions with Ctb (Cj0465c) were identified. Ctb is a group III truncated hemoglobin, and characterization studies in *C. jejuni* have already shown Ctb to be part of the nitrosative stress response regulon (49). Ctb has also been linked with moderating oxygen metabolism within *C. jejuni* (49). Collectively, these bioinformatics analyses suggest that Cj1556 has an important role as a stress response regulator.

Construction and characterization of a *C. jejuni* 11168H *Cj1556* mutant. To investigate the function of Cj1556, a defined isogenic 11168H *Cj1556* mutant was constructed by insertion of a Km^r cassette, using standard mutagenesis techniques (35, 38), with Km^r in the same orientation as the *Cj1556* CDS to obviate potential polar effects. To further confirm phenotypic changes, the *Cj1556* mutant was complemented, verified by PCR/sequencing, and termed *Cj1556* complement.

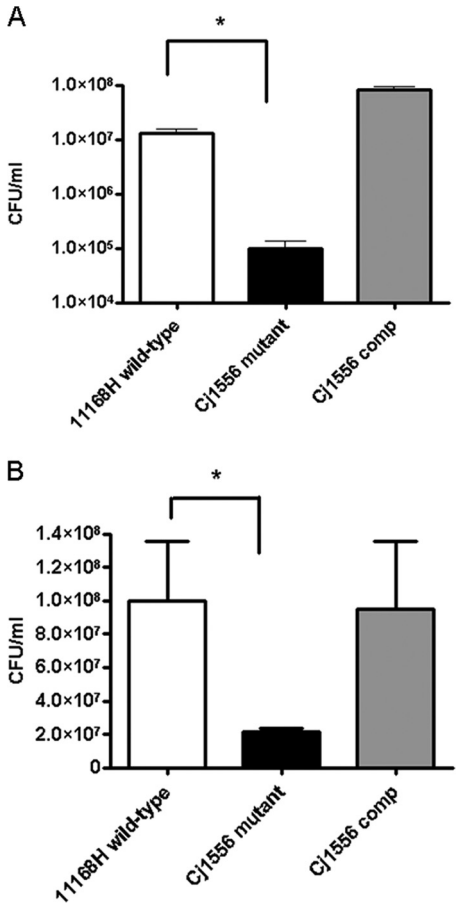


FIG. 1. Effects of oxidative (A) and heat (B) stresses on the survival of the *C. jejuni* 11168H wild-type, *Cj1556* mutant, and *Cj1556* complement (*Cj1556* comp) strains. The *C. jejuni* strains were incubated with 10 mM H_2O_2 for 15 min at 37°C (A) or at 60°C for 5 min (B), and bacterial survival was assessed. The asterisks denote a statistically significant difference ($P < 0.05$) for the *Cj1556* mutant compared to the 11168H wild-type strain.

Motility assays demonstrated that there were no significant differences in the motility of the *Cj1556* mutant or *Cj1556* complement compared to the wild-type strain 11168H at 24, 48, and 72 h (data not shown).

The Cj1556 mutant exhibits increased sensitivity to both oxidative and heat stresses. Nitrosative stress assays were performed using acidified $NaNO_2$. However, no differences between the survival of the wild-type strain 11168H and of the *Cj1556* mutant were observed (data not shown). Oxidative stress assays were performed using H_2O_2. The *Cj1556* mutant exhibited increased sensitivity to H_2O_2 compared to the 11168H wild-type strain (Fig. 1A). In addition, the *Cj1556* complement restored the wild-type H_2O_2 sensitivity phenotype (Fig. 1A). Previous research has suggested a link between aerobic and heat stresses (12, 63). In order to investigate this further, a range of heat stress experiments were performed. No significant differences in survival were observed at 42°C/60 min or 55°C/15 min. However, the *Cj1556* mutant displayed increased sensitivity compared to the wild-type strain at 60°C/5 min, and the *Cj1556* complement restored the wild-type phenotype (Fig. 1B).

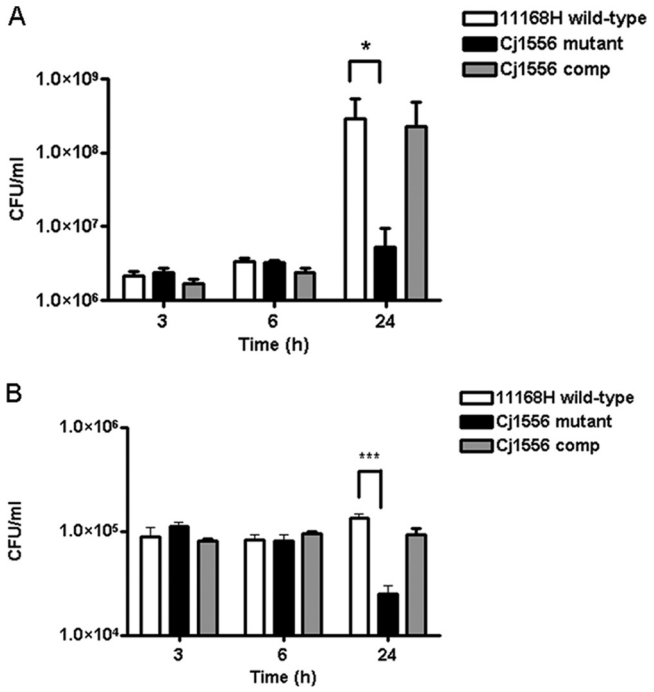


FIG. 2. Interaction (adhesion and invasion) and invasion assays. The 11168H wild-type, *Cj1556* mutant, and *Cj1556* complement (*Cj1556* comp) strains were cocultured with Caco-2 intestinal epithelial cells for 3, 6, or 24 h. The Caco-2 cells either were lysed and the numbers of interacting bacteria were assessed (A) or were incubated with gentamicin (150 μg/ml) for 2 h to kill extracellular bacteria and then lysed, and the numbers of intracellular bacteria were assessed. The asterisks denote a statistically significant difference (*, $P < 0.05$; ***, $P < 0.001$) for the *Cj1556* mutant compared to the 11168H wild-type strain.

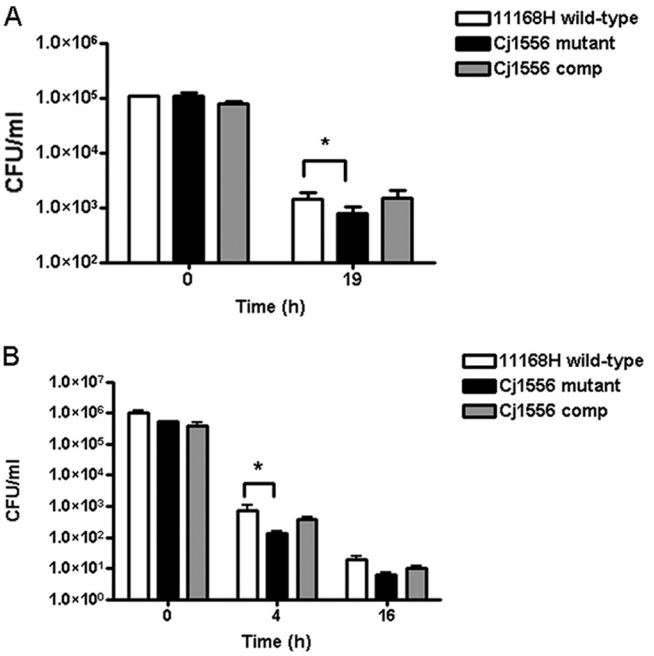
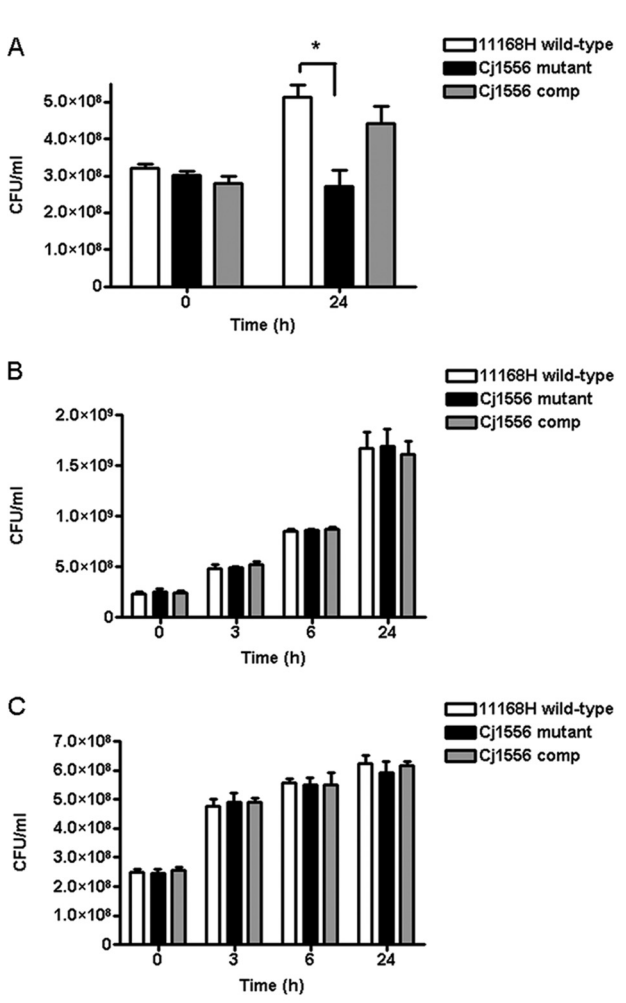


FIG. 3. Intracellular survival assays. The 11168H wild-type, *Cj1556* mutant, and *Cj1556* complement (*Cj1556* comp) strains were cocultured with Caco-2 intestinal epithelial cells (A) or J774A.1 mouse macrophages (B) for 3 h and then incubated with gentamicin (150 μg/ml) for 2 h to kill extracellular bacteria, followed by further incubation with gentamicin (10 μg/ml). The cells were lysed, and the numbers of intracellular bacteria were assessed. The asterisks denote a statistically significant difference ($P < 0.05$) for the *Cj1556* mutant compared to the 11168H wild-type strain.

The Cj1556 mutant displays reduced ability to interact with and invade Caco-2 intestinal epithelial cells. Interaction (adhesion and invasion) and invasion assays were performed using 11168H wild-type, *Cj1556* mutant, and *Cj1556* complement strains. No significant differences were observed when the levels of interaction at 3 h and 6 h were compared; however, the *Cj1556* mutant displayed a reduced ability to interact with Caco-2 cells after 24 h of coculture compared with the 11168H wild-type and *Cj1556* complement strains (Fig. 2A). The *Cj1556* mutant also displayed a reduced ability to invade Caco-2 cells after 24 h of coculture compared with the 11168H wild-type and *Cj1556* complement strains (Fig. 2B). No significant differences were observed when the levels of invasion at 3 h and 6 h were compared.

The *Cj1556* mutant exhibits reduced intracellular survival in Caco-2 intestinal epithelial cells and in J774A.1 macrophage cells. A modification of the interaction and invasion assays was used to analyze the level of intracellular survival in Caco-2 intestinal epithelial cells (53) in order to investigate the ability of *C. jejuni* to survive when exposed to intracellular stress conditions, such as ROS. There was a statistically significant reduction in the level of intracellular survival of the *Cj1556* mutant compared to the 11168H wild-type and *Cj1556* complement strains (Fig. 3A). Intracellular survival assays using macrophages were also performed to further investigate the survival rates of the 11168H wild-type, *Cj1556* mutant, and *Cj1556* complement strains. Macrophages internalize and destroy *C. jejuni* (80), and previous studies have shown that *C. jejuni* is killed within 24 h of internalization (80). There was a statistically significant reduction in the level of intracellular survival of the *Cj1556* mutant compared to the 11168H wild-type strain (Fig. 3B).

The *Cj1556* mutant exhibits reduced survival in both coculture media and an aerobic environment. A further variation of the intracellular survival assay was used to assess the survival of *C. jejuni* in tissue culture medium. There was a statistically significant increase in the number of viable bacterial cells obtained from the supernatant after 24 h of coculture with Caco-2 cells when the 11168H wild-type and *Cj1556* complement strains were compared to the *Cj1556* mutant (Fig. 4A). Following the identification of significant differences between the 11168H wild-type strain and the *Cj1556* mutant in response to oxidative stress and intracellular survival, further investigations of the abilities of these strains to survive aerobic stress were performed. The difference in the level of *Cj1556* mutant survival between the interaction and intracellular assays suggested that additional stresses might affect *C. jejuni* during these assays. Survival assays with the 11168H wild-type, *Cj1556* mutant, and *Cj1556* complement strains were performed under either microaerobic or aerobic conditions in either brucella broth or tissue culture medium with no shaking to replicate the conditions for the coculture assays. A statistically significant reduction in the number of viable bacterial cells with the *Cj1556* mutant compared to the 11168H wild-type strain in



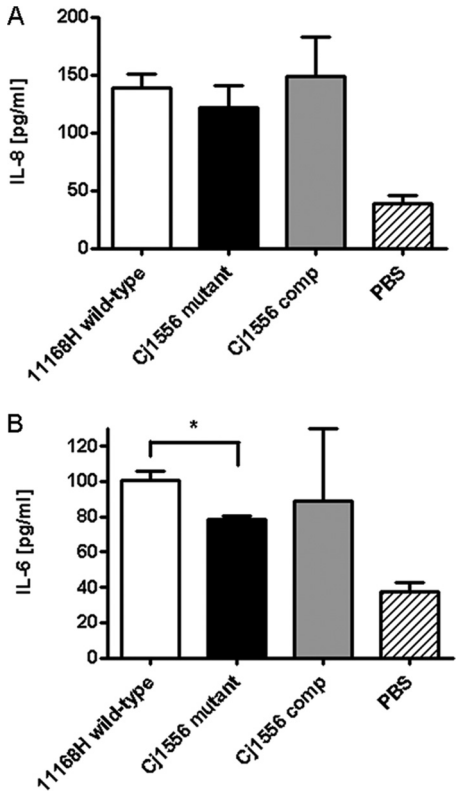


FIG. 5. T84 intestinal epithelial cell responses to 24 h of coculture with the 11168H wild-type, *Cj1556* mutant, and *Cj1556* complement (*Cj1556* comp) strains were assessed. The levels of IL-8 and IL-6 secreted during *C. jejuni* interaction with T84 cells were quantified using either a human IL-8 ELISA (A) or IL-6 ELISA (B). The asterisk denotes a statistically significant difference ($P < 0.05$) for the *Cj1556* mutant compared to the 11168H wild-type strain.

both types of media was observed after 24 h of incubation under aerobic conditions (Fig. 4D and E), but not under microaerobic conditions (Fig. 4B and C).

The *Cj1556* mutant induces a reduced IL-6 response from T84 cells. IL-6 and IL-8 are well-characterized markers denoting a host immune response against pathogens (56). Only minimal secretion of IL-6 and IL-8 was detected when the 11168H wild-type and *Cj1556* mutant strains were cocultured with Caco-2 cells (data not shown). However, using the T84 cell line, significant levels of induction of both IL-6 and IL-8 by the 11168H wild-type strain and the *Cj1556* mutant were observed (Fig. 5). There was no significant difference in the level of IL-8 induction by the *Cj1556* mutant compared to that by the

FIG. 4. Survival assays. (A) The 11168H wild-type, *Cj1556* mutant, and *Cj1556* complement strains (*Cj1556* comp) were cocultured with Caco-2 intestinal epithelial cells for 24 h, followed by assessment of the number of bacteria in the coculture medium. (B to E) Further survival assays were performed, in which the *C. jejuni* strains were grown under microaerobic (B and C) and aerobic (D and E) conditions in brucella broth (B and D) or tissue culture medium (C and E). Then, the numbers of viable bacteria were assessed. The asterisks denote a statistically significant difference ($P < 0.05$) for the *Cj1556* mutant compared to the 11168H wild-type strain.

TABLE 3. Changes in expression of genes linked to the *C. jejuni* oxidative and aerobic stress responses in the *Cj1556* mutant compared to the 11168H wild-type strain[a]

Gene name	Fold change	Product function
spoT	+1.26	Putative guanosine-3′,5′-bis(diphosphate) 3′-pyrophosphohydrolase
sodB	+1.24	Superoxide dismutase (Fe)
htrA	+1.21	Serine protease (protease DO)
fdxA	+1.07	Ferredoxin
dcuA	−1.17	Anaerobic C_4-dicarboxylate transporter
ahpC	−1.27	Alkyl hydroperoxide reductase
dps	−1.36	Putative bacterioferritin
hspR	−2.07	Heat shock transcriptional regulator
perR	−5.05	Peroxide stress regulator
katA	−5.13	Catalase

[a] *htrB* showed no hybridization during microarray studies and was not included in this analysis.

11168H wild-type strain (Fig. 5A); however, a significant reduction in the level of IL-6 induction by the *Cj1556* mutant was observed (Fig. 5B).

Microarray analysis indicates negative autoregulation of *Cj1556* expression. To analyze the gene expression profile of the *Cj1556* mutant compared to the 11168H wild-type strain, microarray experiments were performed using total-RNA samples isolated from *C. jejuni* grown to late log phase (16 h). A total of 91 genes were differentially expressed in the *Cj1556* mutant compared to the 11168H wild type, with 73 genes upregulated and 18 genes downregulated based on an ANOVA selection methodology (5, 18). Interestingly, the gene with the most pronounced upregulation (10.4-fold) was *Cj1556*. Sequence analysis of the *Cj1556* reporter element used on the arrays showed that this particular sequence was present upstream of the Km^r cassette in the *Cj1556* mutant (data not shown). Usually, the mutated gene in a defined mutant would be expected to appear downregulated; however, the microarray data indicate that in the absence of the Cj1556 protein, *Cj1556* gene expression is dramatically increased. This suggests that Cj1556 represses further expression of the *Cj1556* gene, acting as a negative autoregulator. Further analysis of genes associated with oxidative and aerobic stress responses showed that many were downregulated in the *Cj1556* mutant, including *katA* (5.13-fold), *perR* (5.05-fold), and *hspR* (2.07-fold) (Table 3), indicating potential reasons for the increased sensitivity of the *Cj1556* mutant to these stresses.

Electrophoretic mobility shift assays indicate binding of Cj1556 to a DNA promoter probe upstream of the *Cj1556* gene. To investigate whether Cj1556 acts as a DNA binding protein and could potentially bind to the promoter region of the *Cj1556* gene to repress further expression, as indicated by the microarray data, electrophoretic mobility shift assays were performed. The full-length Cj1556 protein was expressed and purified from *E. coli*. Binding of this recombinant Cj1556 protein to a 170-bp DNA fragment upstream of the *Cj1556* gene was observed, indicating a protein-DNA complex (Fig. 6). Such binding of recombinant Cj1556 was not observed with DNA fragments representing the promoter regions of the negative-control genes *flaA* and *flgK*. These data indicate that Cj1556 acts as a DNA binding protein and also supports the microarray data that suggest a negative autoregulation system for the expression of Cj1556. Negative autoregulation is often a feature of the MarR family of transcriptional regulators (82).

The Cj1556 mutant exhibits reduced biofilm formation. Biofilms are commonly defined as matrix-enclosed bacterial populations adherent to each other and/or to surfaces of interfaces (19). Studies have shown that *C. jejuni* can form biofilms (36) and that this may be an important factor in the survival of *C. jejuni* in the environment. Recent studies have also shown increased biofilm formation under aerobic stress conditions (65). The microarray data identified *cprS* as being 2.0-fold upregulated in the *Cj1556* mutant compared to the 11168H wild-type strain. A *cprS* mutant has been shown to have enhanced and accelerated biofilm formation (71). Therefore, an increase in CprS production in the *Cj1556* mutant was predicted to result in a decrease in biofilm formation. Analysis of the 11168H wild-type and *Cj1556* mutant strains indicated a significant reduction in relative biofilm formation by the *Cj1556* mutant (Fig. 7). Complementation of the Cj1556 mutation restored the wild-type phenotype (Fig. 7).

***G. mellonella* larvae exhibit increased survival after infection with the *Cj1556* mutant.** *G. mellonella* larvae have been used as a model to study infection by *C. jejuni* and other enteric pathogens (15, 17). Insect larvae are favorable to use as nonmammalian infection models, as they can be infected at 37°C and possess specialized phagocytic cells, termed hemocytes (8, 51). Hemocytes mimic the functions of phagocytic cells in mammals and are able to degrade bacterial pathogens and also generate bactericidal compounds, such as superoxide, via a respiratory burst (8, 42). Infection with the *Cj1556* mutant resulted in a statistically significant increase in the survival of *G. mellonella* larvae compared to infection with the 11168H wild-type strain (Fig. 8). Complementation of the Cj1556 mutation restored the wild-type phenotype (Fig. 8). This suggests the *Cj1556* mutant is more susceptible to the host immune mechanisms, resulting in reduced bacterial survival within *G. mellonella*.

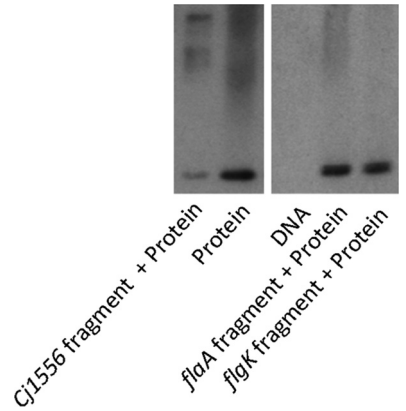


FIG. 6. Electrophoretic mobility shift assays indicate that Cj1556 binds to a DNA promoter sequence upstream of the *Cj1556* gene. Shown is a native Western blot for recombinant 6×His-tagged Cj1556 protein hybridized to DNA fragments representing the upstream promoter sequences of *Cj1556* and *flaA* and *flgK* (both negative controls) following separation on a Tris-glycine gel under nondenaturing conditions.

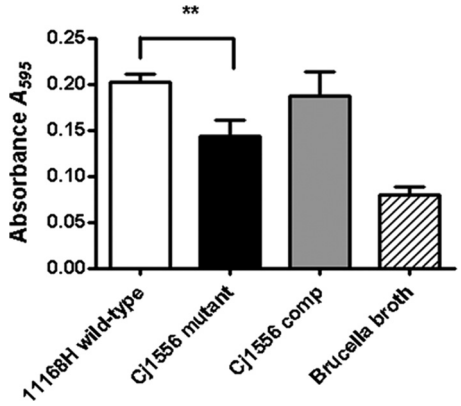


FIG. 7. Biofilm assay of the 11168H wild-type, *Cj1556* mutant, and *Cj1556* complement (*Cj1556* comp) strains. *C. jejuni* biofilms were grown for 3 days and rinsed three times with PBS, followed by crystal violet staining. The asterisks denote a statistically significant difference ($P < 0.01$) for the *Cj1556* mutant compared to the 11168H wild-type strain.

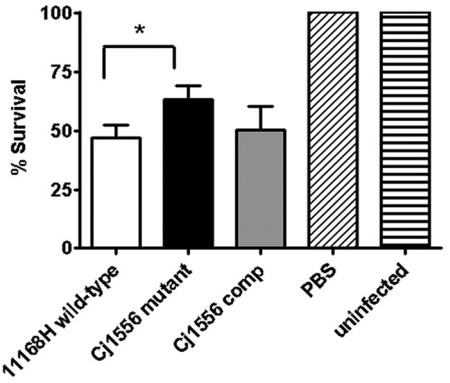


FIG. 8. *G. mellonella* larvae were injected with a 10-μl inoculum of a 24-h *C. jejuni* culture diluted to an OD_{600} of 0.1 by microinjection in the right foremost leg, giving an infectious dose of approximately 10^6 CFU. The larvae were incubated at 37°C, with survival and appearance recorded at 24-h intervals. Brucella broth and no-injection controls were used. For each experiment, 10 *G. mellonella* larvae were infected, and the experiments were repeated in triplicate. The asterisk denotes a statistically significant difference ($P < 0.05$) for the *Cj1556* mutant compared to the 11168H wild-type strain.

DISCUSSION

The human intestinal pathogen *C. jejuni* must survive diverse conditions in different hosts and also in the environment. The ability of *C. jejuni* to survive both oxidative and aerobic stress conditions is fundamental, considering the ubiquity of the bacterial pathogen. During reannotation of the *C. jejuni* NCTC11168 genome sequence (29), Cj1556 was identified as a putative transcriptional regulator. Based on motif and protein interaction data, we hypothesized that Cj1556 was an important *C. jejuni* stress response regulator and therefore investigated the ability of a *Cj1556* mutant to survive different stresses and further explored the role of Cj1556 during host-pathogen interactions.

In addition to Cj1556, the *C. jejuni* NCTC11168 genome contains another CDS (Cj1546) with the MarR family motif. Cj1546 was also reannotated as a putative transcriptional regulator with 43.6% identity and 58.4% similarity to Cj1556. Analysis of a comparative genomics microarray data set containing 111 *C. jejuni* strains (16) identified *Cj1546* in over 95% and *Cj1556* in approximately 50% of these *C. jejuni* strains. One hypothesis as to the function of these MarR motif-containing proteins is that both perform similar roles in relation to aerobic and oxidative stresses; however, while all *C. jejuni* strains contain Cj1546, strains such as *C. jejuni* NCTC11168 and 81-176 that also contain Cj1556 may have a greater ability for survival within the human host due to greater resistance to oxidative stresses.

Oxidative stress assays showed that the *Cj1556* mutant has an increased sensitivity to oxidative stress compared to the 11168H wild-type strain and that the wild-type level of sensitivity to oxidative stress was fully restored with complementation of the *Cj1556* mutation. In fact, the *Cj1556* complement demonstrated even greater resistance to H_2O_2 than the 11168H wild-type strain, possibly due to the strength of the promoter, as the complementation vector utilizes the constitutive chloramphenicol cassette promoter to express the *Cj1556* gene and not the native *Cj1556* promoter. *C. jejuni* proteins associated with heat stress responses, such as HspR, have also been linked to oxidative and aerobic stress (3). The *Cj1556* mutant showed a greater level of sensitivity to 60°C stress than the wild-type strain. Heat stress above 55°C has been noted to accelerate the spiral-to-coccoid transition and results in cell death (54). Previous studies have identified numerous *C. jejuni* genes involved in heat shock response, and HtrA and HspR have also been shown to have roles in aerobic survival, host cell adherence, and invasion (12). Transcriptional analysis identified *hspR* as being approximately 2.0-fold downregulated in the *Cj1556* mutant compared to the 11168H wild-type strain. It is interesting that the *Cj1556* mutant has increased sensitivity to heat stress, and this may be due to Cj1556 interacting with HspR, suggesting a connection between the heat shock response and aerobic tolerance (3, 12).

The ability of the *Cj1556* mutant to interact with (adhere and invade) and invade Caco-2 cells was investigated at 3-, 6-, and 24-h time points. Significant differences in both interaction and invasion were observed only at 24 h postinfection. This indicates that the *Cj1556* mutant does not appear to have any defect in the ability to adhere to or invade Caco-2 cells but may have a reduced ability to survive contact with host cells over time. To further investigate longer-term survival, intracellular survival assays were performed. These assays indicated that the *Cj1556* mutant has a reduced ability to survive within Caco-2 cells compared to the 11168H wild-type strain. The difference in the level of survival between the *Cj1556* mutant and the 11168H wild-type strain in the intracellular survival assay at 24 h postinfection was approximately 0.5 log units (Fig. 3A), very similar to the difference in the number of invasive bacteria between the *Cj1556* mutant and the 11168H wild-type strain at 24 h postinfection (Fig. 2B). However, the difference in the number of interacting bacteria between the *Cj1556* mutant and the 11168H wild-type strain at 24 h postinfection was approximately 1.5 log units (Fig. 2A). This suggested that, in addition to a reduced ability for intracellular survival, the *Cj1556* mutant was also more susceptible to extracellular

stresses than the 11168H wild-type strain. The *Cj1556* mutant exhibits increased sensitivity to H_2O_2 *in vitro*, so it is reasonable to suggest ROS released by Caco-2 cells during these experiments have an effect on *C. jejuni* survival. Standard coculture assays result in exposure of *C. jejuni*, not only to ROS released by Caco-2 cells, but also to aerobic stress, as the assays are performed in a CO_2 incubator. The approximate atmospheric O_2 and CO_2 levels are around 21% and 0.04%, respectively. During coculture experiments, the level of CO_2 is around 5%, so the O_2 level will be around 16 to 18%. Based on the relative levels of survival between the interaction, invasion, and intracellular survival assays, we hypothesized that the greater level of sensitivity exhibited by the *Cj1556* mutant during the interaction assay may be due in part to increased exposure of extracellular *C. jejuni* to aerobic stress. Aerobic survival assays were performed to replicate the conditions during the interaction, invasion, and intracellular survival assays by incubating *C. jejuni* in tissue culture medium, but in the absence of Caco-2 cells. A reduction in survival was observed for the *Cj1556* mutant compared to the 11168H wild-type strain under these aerobic stress conditions, but not under microaerobic conditions. *C. jejuni* typically loses viability within intestinal epithelial cells over 24 h with no evidence of intracellular replication (41). Evidence to date suggests that *C. jejuni* resides in membrane-bound compartments termed *C. jejuni*-containing vacuoles (CCV), avoiding entry into lysosomes (81). *C. jejuni* engulfed by macrophages must resist a combination of unfavorable conditions, such as ROS. There are contradictory reports regarding the ability of *C. jejuni* to survive within macrophages, depending on the macrophage cell type and *C. jejuni* strain used (20, 79). In this study, the *Cj1556* mutant exhibited reduced intracellular survival within the mouse macrophage J774A.1 cell line. Taken together, these data indicate that Cj1556 plays a multifactorial role in bacterial survival during adhesion to and invasion of human intestinal epithelial cells.

In this study, there was no significant difference in the levels of IL-8 induction by the *Cj1556* mutant and the 11168H wild-type strain; however, a significant reduction in the level of IL-6 induction by the *Cj1556* mutant compared to the 11168H wild-type strain was observed. IL-8 acts as a chemoattractant, allowing the recruitment of lymphocytes and neutrophils (32, 62), whereas IL-6 is believed to be important for epithelial cell integrity (27). It is possible that less IL-6 was induced when T84 cells were cocultured with the *Cj1556* mutant than with the 11168H wild-type strain due to the decreased survival characteristic of the *Cj1556* mutant strain. Based on data from this study, coculturing the *Cj1556* mutant for 24 h in a 37°C CO_2 incubator would result in decreased survival of the *Cj1556* mutant based on the increased sensitivity of the strain compared to the 11168H wild-type strain. This may be a possible reason for the decreased IL-6 production. This result also suggests that IL-8 may be important for an extracellular response, as both the *Cj1556* mutant and the 11168H wild-type strain elicited similar levels of IL-8 from T84 intestinal epithelial cells. However, IL-6 may be more important for an intracellular response, as the *Cj1556* mutant was shown to invade less than the 11168H wild-type strain and so elicited less IL-6 from T84 intestinal epithelial cells.

The digestive secretion bile consists of around 50% bile salts, such as cholates and deoxycholates. Bile salts exhibit potent antibacterial properties, acting as detergents to disrupt cell membranes and as DNA-damaging agents (7). Although bacteria inhabiting the gastrointestinal tract are able to resist the antimicrobial effects of bile, a number of studies have also shown that bile increases the virulence potential of enteric pathogens (7). The bile salt sDOC has been shown to increase the virulence of *C. jejuni*, enhancing bacterial ability to invade epithelial cells (45). Growing *C. jejuni* in the presence of a physiologically relevant concentration of sDOC (0.1% [wt/vol]) changes the invasion kinetics so that maximal invasion of INT 407 cells occurs in under 30 min compared to 3 h for *C. jejuni* grown in the absence of sDOC (45). Microarray analysis has shown that a number of *C. jejuni* virulence factor genes are upregulated in the presence of 0.1% (wt/vol) sDOC, including *ciaB*, *cmeABC*, *dccR*, and *tlyA* (45). Interestingly, *Cj1556* was also upregulated in the presence of sDOC, with transcription increased 2.8-fold (45). The transcriptional response of *E. coli* O157:H7 to bile treatment has also been investigated using microarrays and has identified bile-induced changes in transcription for genes encoding proteins affecting membrane structure and permeability, bile resistance, adhesion, and virulence potential (30). Most interestingly, these data indicate that bile induces expression of the *marRAB* operon by binding to the repressor protein MarR and thus preventing binding of MarR to the *marRAB* promoter site (30). Cj1556 is a member of the MarR family of transcriptional regulators, and further studies will be required to confirm whether bile can bind to the Cj1556 protein and thus prevent binding to the *Cj1556* promoter site, resulting in the upregulation of Cj1556 in the presence of bile observed previously (45).

Microarray analysis of the *Cj1556* mutant identified *Cj1556* as the most upregulated gene. Analysis of the *Cj1556* nucleotide sequence upstream of the Km^r cassette in the *Cj1556* mutant confirmed that this was the sequence printed on the oligonucleotide array, suggesting that expression of *Cj1556* is controlled by a negative autoregulation feedback mechanism. In the wild-type strain, basal levels of Cj1556 would block further expression of *Cj1556* by inhibiting the binding of RNA polymerase to the *Cj1556* promoter site. However, in the absence of Cj1556 in the *Cj1556* mutant, expression of *Cj1556* can continue. Such negative autoregulation is a feature of the MarR family of transcriptional regulators. In this study, experiments confirmed the binding of recombinant Cj1556 to a 170-bp DNA fragment upstream of the *Cj1556* gene, confirming the DNA binding ability of Cj1556. To confirm that this was not a nonspecific artifact, two random negative-control promoter regions were selected (upstream of *flaA* and *flgK*). Both of the negative controls showed bands for the Cj1556 recombinant protein alone. The microarray data also indicated downregulation of *katA*, *perR*, and *hspR* in the *Cj1556* mutant (Table 3). Reduced expression of KatA, PerR, and HspR would provide an explanation for the increased sensitivity of the *Cj1556* mutant to oxidative, aerobic, and heat stresses observed in this study; however, further experiments are required to confirm this hypothesis.

The ability of *C. jejuni* to form biofilms goes some way to explain how a bacterium with such fastidious growth requirements remains ubiquitous in the environment (13, 36). *C. jejuni* can form three distinct forms of biofilm: cell-cell aggregates,

pellicles at the air-liquid interface, and glass-attached flocs (36). Our understanding of the specific mechanisms underlying biofilm formation in *C. jejuni* is still limited (72). *C. jejuni* lacks the classical two-component regulatory systems involved in biofilm formation that are present in other bacteria, such as GacSA in *Pseudomonas aeruginosa* (59). Genes involved in biofilm formation have been linked to responses to oxidative and aerobic stress, and *C. jejuni* biofilm formation is increased under aerobic conditions (65). A *C. jejuni spoT* mutant has been found to overproduce a novel calcofluor white-reactive exopolysaccharide and to demonstrate enhanced biofilm formation (46). Interestingly, a *C. jejuni cprS* mutant has been shown to display growth defects and enhanced and accelerated biofilm formation and also to exhibit decreased oxidative stress tolerance (71). Transcriptional analysis of the *Cj1556* mutant identified *cprS* as being upregulated, and the decrease in biofilm formation observed in this study indicates a potential link between CprS and Cj1556.

The *G. mellonella* insect model has been developed for potential identification of *C. jejuni* virulence determinants and was used to investigate the pathogenicity of the *Cj1556* mutant (8). *G. mellonella* larvae possess specialized phagocytic cells, termed hemocytes. The insect immune system is subdivided into humoral and cellular defense responses. Humoral defenses include the production of antimicrobial peptides (47), reactive intermediates of oxygen or nitrogen (9), and the complex enzymatic cascades that regulate coagulation or melanization of hemolymph (50). Cellular defense refers to hemocyte-mediated immune responses, like phagocytosis, nodulation, and encapsulation (66, 70). Hemocytes perform many of the functions of phagocytic cells in mammals and are capable of ingesting bacterial pathogens and generating bactericidal compounds, such as superoxide, via a respiratory burst (8, 15). After infection of *G. mellonella* with *Yersinia pseudotuberculosis*, bacteria accumulate in hemocytes, thus suggesting that *G. mellonella* may be useful for the identification of other genes associated with intracellular survival (15). Infection with the *Cj1556* mutant resulted in increased survival of *G. mellonella* larvae compared to survival after infection with the 11168H wild-type strain. This suggests the *Cj1556* mutant is more susceptible to the host immune mechanisms, resulting in reduced bacterial survival within *G. mellonella*. At least six types of hemocytes have been identified in insects such as *G. mellonella*, with plasmatocytes and granulocytes the most abundant (10). Production of ROS has also been detected in hemocytes, with evidence of oxygen radicals and H_2O_2 both found in plasmatocytes of *G. mellonella* (68). These data link the increased sensitivity of the *Cj1556* mutant to H_2O_2 stress *in vitro* with attenuation of virulence *in vivo* using the *G. mellonella* model of infection.

In summary, the basis of *C. jejuni* survival is dependent upon the ability to sense and respond to the different environments encountered within hosts and in the environment. This study has identified a novel *C. jejuni* transcriptional regulator, Cj1556, that is involved in oxidative and aerobic stress responses and is important for the survival of *C. jejuni* in the natural environment and *in vivo*.

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