The *Campylobacter jejuni* transcriptional regulator Cj1556 plays a role in the oxidative and aerobic (O_2) stress response and is important for bacterial survival *in vivo*.

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Abstract

Campylobacter jejuni is the leading bacterial cause of human gastroenteritis worldwide. Despite stringent microaerobic growth requirements, *C. jejuni* is ubiquitous in the aerobic environment, so must possess regulatory systems to sense and adapt to external stimuli such as oxidative and aerobic (O_2) stress. Re-annotation 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 (O_2) 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 down-regulation of genes associated with oxidative and aerobic (O_2) 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 up-regulated in the *Cj1556* mutant and subsequent studies showed that this mutant had a reduced ability to form biofilms. This study has identified a novel *C. jejuni* transcriptional regulator Cj1556 that is involved in oxidative and aerobic (O_2) stress responses and is important for the survival of *C. jejuni* in the natural environment and *in vivo*.

Introduction

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 diarrhoea (12). *C. jejuni* infection has also been associated with post-infectious sequelae, including septicaemia 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 recognised 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 there remain many unanswered questions regarding the pathogenesis and survival mechanisms of this bacterium. A more complete understanding of the regulation of *C. jejuni* response mechanisms to the diverse stresses encountered during both 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 pathways which are important pathways within polymorphonuclear phagocytes including white blood cells and mononuclear phagocytes (25). Aerobic (O_2) 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 hours accelerated the transition to the viable but non-culturable state (VBNC) or coccoid form (40). In contrast, another study identified the increased culturability of *C. jejuni* when exposed to oxygen for 15 hours (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 (O_2) stress. *C. jejuni* possesses a truncated haemoglobin (Ctb), along with a single domain haemoglobin (Cgb). Both Ctb and Cgb have been characterised 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 (O_2) stress have also been identified. SodB and KatA have been shown to counteract the detrimental effects of aerobic (O_2) stress (69). The heat shock protease HtrA and regulator HspR have been shown to be important for short-term aerobic tolerance (3, 12). The *fdxA* gene upstream of *ahpC* encodes a ferrodoxin which 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 re-annotation of the NCTC11168 genome sequence (29). In this study, further bioinformatic 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 caecal colonisation in a chick colonisation model (35). *C. jejuni* was grown at 37°C in a microaerobic chamber (Don Whitley Scientific, U.K), containing 85% N_2, 10% CO_2 and 5% O_2 either on blood agar plates containing Columbia agar base (Oxoid, U.K), supplemented with 7% (v/v) horse blood (TCS Microbiology, U.K) and *Campylobacter* Selective Supplement (Oxoid) or in Brucella broth (Oxoid) shaking at 75 rpm. *C. jejuni* strains were grown on blood agar plates for 24 h prior to use in co-culture experiments. *E. coli* XL-2 Blue MRF' competent cells (Stratagene, U.S.A) were used for cloning experiments and were grown at 37°C in 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, (10 μg/ml) for *C. jejuni* studies. All reagents were obtained from Invitrogen (UK) unless otherwise stated.

Construction of *C. jejuni* 11168H *Cj1556* mutant

Construction of a defined isogenic 11168H *Cj1556* mutant was performed using previously published methods (35, 38, 43). Briefly, *Cj1556*-F and *Cj1556*-R primers 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..1490567) was selected which contains a 1.494 kb insert included the coding sequences (CDSs) *Cj1555c – Cj1560* and designated pUC-*Cj1556*. The *Cj1556* ORF in pUC-*Cj1556* was inactivated by insertion of an *aph-3* (aminoglycoside 3'-phosphotransferase, Km^R) cassette (73). The Km^R cassette was excised from pJMK30 (76) using *Bam*HI. pUC-*Cj1556* was digested with *Bcl*I, 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 selected on LB agar supplemented with ampicillin and kanamycin after 48 h 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 11168H wild-type as described previously (35, 38). 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). 200 µl of this suspension was spread onto blood agar plates containing kanamycin. Putative *Cj1556* mutants were screened using PCR and sequencing.

Complementation of *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 *Nco*I site at the 5' end and a *Nhe*I site at the 3' end as well as the native ribosome binding site of *Cj1556* (72, 83). Following digestion with *Nhe*I and *Nco*I, this PCR product was ligated into the pDENNIS vector. This construct was checked by sequencing and electroporated into the *Cj1556* mutant. Putative clones were selected on blood agar plates containing kanamycin and chloramphenicol. Confirmation for the presence of copies of both *Cj1556* and *Cj1556*- Km^R was performed by PCR using Comp-*Cj1556*-F and Comp-*Cj1556*-R primers and also *Cj1556*-F with *Cj1556*-R primers and also by sequencing. For isolation of recombinant Cj1556 protein, a 6XHis-tag sequence was cloned into a second construct using primers Comp-*Cj1556*-F and Comp-*Cj1556*-R-HIS (Table 1).

Nitrosative, oxidative and heat stress assays

C. jejuni were 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 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 minutes and 10 mM $NaNO_2$ for 75 minutes. For nitrosative stress assays, all media used was 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 minutes. Heat stress assays were performed at 42°C for 1 h, 55°C for 15 mins and 60°C for 5 mins. Serial dilutions were prepared and 10 µl of the 10^{-1} - 10^{-6} dilutions were spotted onto blood agar plates and incubated for 48 h at 37°C under microaerobic conditions and colonies counted.

Cell culture procedures

The Caco-2 human intestinal epithelial and J774A.1 mouse macrophage cell lines were maintained in Dulbecco's modified essential media (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS) (Sigma-Aldrich, UK), 1% (v/v) non-essential 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 Dulbecco's modified Eagle medium 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% (v/v) FCS, 100 µg/ml streptomycin and 100 U/ml penicillin. Cells were maintained at 37°C in 5% CO_2 and 95% air. For Caco-2 cell co-culture experiments, cells were seeded at $1x10^5$ cells/ml and grown in 24-well plates to >90% confluence (≈ $1x10^6$ cells/ml). For T84 cell co-culture experiments, cells were seeded at $5x10^5$ cells/ml and grown

in 24-well plates to >90% confluence (≈ $5x10^6$ cells/ml). For co-culture experiments involving J774A.1 mouse macrophage, cells were seeded at $5x10^5$ cells/ml and grown in 24-well plates for 24 h. For ELISA experiments, T84 cells were maintained in low serum 1% (v/v) and antibiotic-free media overnight prior to co-culture. Infections were terminated by removing the supernatant from the cells followed by two washes in PBS. Cell culture supernatants were stored at -80°C until required.

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 $1x10^8$ cells) in DMEM were added to a monolayer of approximately $1x10^6$ Caco-2 cells (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, then lysing the cells by addition of 0.2% (v/v) 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. Monolayers were then washed three times in PBS and the epithelial cells lysed as above. For intracellular survival assays, bacterial cells were co-cultured 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 then incubated in DMEM containing a reduced concentration of gentamicin (10 µg/ml) for 19 h. Monolayers were then washed three times in PBS and the epithelial cells lysed as 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% (v/v) Triton X-100. No difference in the level of sensitivity to Triton X-100 was observed between 11168H wild-type strain, *Cj1556* mutant and the *Cj1556* complement (data not shown). Survival in tissue culture medium from co-culture experiments were performed as above, but after 24 h co-culture, 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 enumeration 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* were harvested into 1 ml Brucella broth and adjusted to an OD_{600} of 0.1. *C. jejuni* cells (approximately $1x10^8$ cells) in DMEM were added to a culture of approximately $5x10^5$ J774A.1 mouse macrophage cells (MOI 200:1) and incubated for 3 h. 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. Macrophages were incubated in DMEM containing a reduced concentration of gentamicin (10 µg/ml) and bacterial survival 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% (v/v) Triton X-100 in PBS. Serial dilutions, plating and enumeration of bacterial numbers were performed as stated above.

Interleukin-6 and interleukin-8 enzyme-linked immunosorbant assay (ELISA)

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 IL-6 and IL-8 secretion were assessed using a

commercially available sandwich ELISA kit according to manufacturer's instructions (Peprotech, U.K). Detection was performed using a Dynex MRX II 96 well plate reader (Dynex, U.S.A) at an absorbance of 405 nm (A_{405}) and analysed using Revelation software (Dynex).

Transcriptome studies: experimental design, template labeling, microarray hybridizations, data acquisition and microarray data analysis

Gene expression profiling of *C. jejuni* 11168H from late-log growth phase (16 h) was performed using an indirect comparison method or type 2 experimental design (86). Replicate test sets of Cy5-labelled *C. jejuni* 11168H total RNA samples were combined with a common reference sample (Cy3-labelled *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, U.K) 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, U.S.A), constructed by the BμG@S Microarray Group (http://www.bugs.sgul.ac.uk/) were used in this study (37). The procedures used for Cy5-labelling of total RNA samples (37) and Cy3-labelling 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-labelled 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 4X SSC and 0.3% (w/v) SDS. The hybridization mixture was denatured at

98°C for 2 mins and cooled slowly to room temperature. A 22 x 25 mm LifterSlip coverslip (Erie Scientific, U.S.A) was placed over the reporter element area on the microarray and the hybridization mixture applied underneath the coverslip. The microarray slide was placed in a humidified hybridization cassette (Telechem International, U.S.A) and incubated in a water bath for 18 h at 65°C without shaking. 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, U.S.A). Quantified data were analysed using GeneSpring GX software v7.3 (Agilent, U.S.A). Statistically significant up- and down-regulated genes were selected when comparing gene expression against 11168H wild-type using ANOVA (ANalysis Of VAriance) with a Benjamini and Hochberg False Discovery Rate as the Multiple Testing Correction (5, 18). 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).

Electrophoretic mobility shift assays

E. coli strains were grown overnight for 16 h at 37°C shaking at 200 rpm. Cultures were spun at 4,000 rpm for 10 minutes at 4°C. The bacterial pellet was resuspended in 1 ml equilibration buffer (Sigma-Aldrich). Cells were sonicated using manufacturers instructions (Diagenode, Belgium), followed by centrifuged for 5 minutes at 13,000 rpm. The supernatant containing lysed cell content was poured into a new 1.5 ml microcentrifuge tube. Lysed cells were incubated with Ni-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 hybridised to PCR amplified fragments (140 – 180 bp) located upstream of the translation initiation sites of the *Cj1556*, *flaA* and *flgK* genes (Table 1 & 2). 2.5 µg recombinant native protein was hybridised with 20 ng of purified DNA along with 2 µl hybridisation solution (20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCL (pH 7.5) and incubated at room temperature for 40 minutes. Samples were resuspended in Tris-Glycine native sample buffer (Invitrogen) up to 10 µl and analysed using a Tris-Glycine gel under non-denaturing conditions (Invitrogen) followed by Western blot analysis.

Biofilm assays

C. jejuni were grown on blood agar plates for 24 h at 37°C under microaerobic conditions. 10 ml Brucella broth was preincubated in a 50 ml flask at 37°C under microaerobic conditions 24 h prior to inoculation, 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% (w/v) crystal violet (Sigma-Aldrich) for 10 minutes. 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 (Dynex, U.S.A) at an A_{595}.

***Galleria mellonella* infection model**

G. mellonella larvae were obtained from LiveFoods Direct (U.K) and kept on wood chips at 16°C. Larvae were injected with a 10 µl inoculum of a 24 h *C. jejuni* culture diluted to OD_{600} 0.1 by micro-injection (Hamilton, Switzerland) in the right foremost leg, giving an infectious

dose of approximately 10^6 CFU (17). Injection with PBS and no injection controls were also performed. Larvae were incubated 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 is presented as mean ± SD. All experiments represent at least three biological replicates performed in triplicate in each experiment. Statistical analyses were performed using Prism software (GraphPad Software, USA). Variables were compared using a student's *t*-test.

Results

Bioinformatic 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 re-annotation, 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 that includes proteins that control virulence factor production, bacterial responses to both antibiotics and oxidative stress and also catabolism of environmental aromatics 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 haemoglobin and characterisation 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 this bioinformatic analyses suggest that Cj1556 has an important role as a stress response regulator.

Construction and characterisation 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 kanamycin resistant cassette (Km^R) 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. Motility assays demonstrated

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

Cj1556 mutant exhibits increased sensitivity to both oxidative and heat stress

Nitrosative stress assays were performed using acidified $NaNO_2$. However no differences between the survival of the 11168H wild-type strain and 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 stress (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 mins or 55°C / 15 mins. However the *Cj1556* mutant displayed increased sensitivity compared to the wild-type strain at 60°C / 5 minutes and the *Cj1556* complement restored the wild-type phenotype (Fig. 1B).

Cj1556 mutant displays a 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 comparing the levels of interaction at either 3 h or 6 h, however the *Cj1556* mutant displayed a reduced ability to interact with Caco-2 cells after 24 h co-culture, 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 co-culture, compared with the 11168H wild-type and *Cj1556* complement strains (Fig. 2B). No significant differences were observed when comparing the levels of invasion at 3 h or 6 h.

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 analyse 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 macrophage cells were also performed to further investigate the survival rates of the 11168H wild-type, *Cj1556* mutant and *Cj1556* complement strains. Macrophages internalise and destroy *C. jejuni* (80) and previous studies have shown that *C. jejuni* are killed within 24 h of internalisation (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).

Cj1556 mutant exhibits reduced survival in both co-culture media and in 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 co-culture with Caco-2 cells when comparing the 11168H wild-type and *Cj1556* complement strains to the *Cj1556* mutant (Fig. 4A). Following the identification of significant differences between the 11168H wild-type strain and *Cj1556* mutant in response to oxidative stress and intracellular survival, further investigations on the ability of these strains to survive aerobic (O_2) 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 11168H wild-type strain, *Cj1556* mutant and *Cj1556* complement strains were performed under either microaerobic or aerobic conditions in either Brucella broth or tissue culture media with no shaking to replicate the conditions for the co-culture assays. A statistically significant reduction in the number of viable bacterial cells with the *Cj1556* mutant compared to the 11168H wild-type strain in both types of media was observed after 24 h incubation under aerobic conditions (Fig. 4D and E) but not under microaerobic conditions (Fig. 4B and C).

_Cj1556_ mutant induces a reduced IL-6 response from T84 cells

IL-6 and IL-8 are well-characterised 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 were co-cultured 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 *Cj1556* mutant was observed (Fig. 5). There was no significant difference in the level of IL-8 induction by the *Cj1556* mutant compared to that by the 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 analyse 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 up-regulated and 18 genes down-regulated, based on an ANOVA selection methodology (5, 18). Interestingly the

gene with the most pronounced up-regulation (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 expect to appear down-regulated, however the microarray data indicates 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 (O_2) stress responses showed that many were down-regulated 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*. This data indicates that Cj1556 acts as a DNA binding protein and also supports the microarray data that suggests a negative autoregulation system for the expression of Cj1556. Negative autoregulation is often a feature of the MarR family of transcriptional regulators (82).

446

447 ***Cj1556* mutant exhibits reduced biofilm formation**

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

459

460 ***Galleria mellonella* larvae exhibit increased survival after infection with *Cj1556* mutant**

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

471

472

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 (O_2) stress conditions is fundamental considering the ubiquity of this bacterial pathogen. During re-annotation of the *C. jejuni* NCTC11168 genome sequence (29), Cj1556 was identified as a putative transcriptional regulator. Based on motif and protein interaction data, we hypothesised 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 explore 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 re-annotated as a putative transcriptional regulator with 43.6% identity and 58.4% similarity to Cj1556. Analysis of a comparative genomics microarray dataset containing 111 *C. jejuni* strains (16) identified *Cj1546* in over 95% of these *C. jejuni* strains 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 (O_2) and oxidative stress, however whilst 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 utilises the constitutive chloramphenicol cassette promoter to

498 express the *Cj1556* gene and not the native *Cj1556* promoter. *C. jejuni* proteins associated
499 with heat stress responses such as HspR have also been linked to oxidative and aerobic stress
500 (3). The *Cj1556* mutant showed a greater level of sensitivity to 60°C stress compared to the
501 wild-type strain. Heat stress above 55°C has been noted to accelerate the spiral-to-coccoid
502 transition and result in cell death (54). Previous studies have identified numerous *C. jejuni*
503 genes involved in heat shock response and HtrA and HspR also have been shown to have
504 roles in aerobic survival, host cell adherence and invasion (12). Transcriptional analysis
505 identified *hspR* as being approximately 2.0 fold down-regulated in the *Cj1556* mutant
506 compared to the 11168H wild-type strain. It is interesting to note that the *Cj1556* mutant has
507 increased sensitivity to heat stress and this may be due to the Cj1556 interacting with HspR,
508 suggesting a connection between the heat shock response and aerobic tolerance (3, 12).
509 The ability of the *Cj1556* mutant to interact with (adhere and invade) and invade Caco-2 cells
510 was investigated at 3, 6 and 24 h time points. Significant differences in both interaction and
511 invasion were observed only at 24 h post-infection. This indicates that the *Cj1556* mutant
512 does not appear to have any defect in the ability to adhere to or invade Caco-2 cells, but may
513 have a reduced ability to survive contact with host cells over time. To further investigate
514 longer term survival, intracellular survival assays were performed. These assays indicated
515 that the *Cj1556* mutant has a reduced ability to survive within Caco-2 cells compared to the
516 11168H wild-type strain. The difference in the level of survival between the *Cj1556* mutant
517 and the 11168H wild-type strain in the intracellular survival assay at 24 h post-infection was
518 approximately 0.5 log (Fig. 3A), very similar to the difference between the number of
519 invasive bacteria between the *Cj1556* mutant and the 11168H wild-type strain at 24 h post-
520 infection (Fig. 2B). However, the difference in the number of interacting bacteria between the
521 *Cj1556* mutant and the 11168H wild-type strain at 24 h post-infection was approximately 1.5
522 log (Fig. 2A). This suggested that in addition to a reduced ability for intracellular survival,

the *Cj1556* mutant was also more susceptible to extracellular stresses when compared to 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 will have an effect on *C. jejuni* survival. Standard co-culture assays will not only result in exposure of *C. jejuni* to ROS released by Caco-2 cells but also to aerobic (O_2) 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 co-culture experiments, the level of CO_2 will be around 5% and so the O_2 level will be around 16-18%. Based on the relative levels of survival between the interaction, invasion and intracellular survival assays, we hypothesised the greater level of sensitivity exhibited by the *Cj1556* mutant during the interaction assay may be in part due to increased exposure of extracellular *C. jejuni* to aerobic (O_2) 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 media 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 (O_2) 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* reside in membrane bound compartments termed *C. jejuni* containing vacuole (CCV), avoiding entry into lysosomes (81). *C. jejuni* engulfed by macrophages must resist a combination of unfavourable 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, this data indicates that Cj1556 plays a multi-factorial role in bacterial survival during adhesion to and invasion of human intestinal epithelial cells.

548 In this study, there was no significant difference in the level of IL-8 induction by the *Cj1556*
549 mutant compared to the 11168H wild-type strain, however a significant reduction in the level
550 of IL-6 induction by the *Cj1556* mutant compared to the 11168H wild-type strain was
551 observed. IL-8 acts as a chemoattractant allowing the recruitment of lymphocytes and
552 neutrophils (32, 62), whereas IL-6 is believed to be important for epithelial cell integrity (27).
553 It is possible that less IL-6 was induced when T84 cells were co-cultured with *Cj1556* mutant
554 compared to the 11168H wild-type strain due to the decreased survival characteristic of the
555 *Cj1556* mutant strain. Based on data from this study, co-culturing the *Cj1556* mutant for 24 h
556 in a 37°C CO_2 incubator would result in decreased survival of the *Cj1556* mutant based on
557 the increased sensitivity of this strain compared to the 11168H wild-type strain. This may be
558 a possible reason for the decreased IL-6 production. This result also suggests that IL-8 may
559 be important for an extracellular response as both *Cj1556* mutant and the 11168H wild-type
560 strain elicited similar levels of IL-8 from T84 intestinal epithelial cells. However, IL-6 may
561 be more important for an intracellular response as the *Cj1556* mutant was shown to invade
562 less compared to the 11168H wild-type strain and so elicited less IL-6 from T84 intestinal
563 epithelial cells.
564 The digestive secretion bile consists of around 50% bile salts, such as cholates and
565 deoxycholates. Bile salts exhibit potent antibacterial properties, acting as detergents to disrupt
566 cell membranes and as DNA-damaging agents (7). Although bacteria inhabiting the
567 gastrointestinal tract are able to resist the antimicrobial effects of bile, a number of studies
568 have also shown that bile increases the virulence potential of enteric pathogens (7). The bile
569 salt sDOC has been shown to increase the virulence of *C. jejuni*, enhancing bacterial ability
570 to invade epithelial cells (45). Growing *C. jejuni* in the presence of a physiologically relevant
571 concentration of sDOC (0.1% w/v) changes the invasion kinetics such that maximal invasion
572 of INT 407 cells occurs in under 30 minutes 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 factors are up-regulated in the presence of 0.1% (w/v) sDOC, including *ciaB*, *cmeABC*, *dccR* and *tlyA* (45). Interestingly, *Cj1556* was also up-regulated 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 gene encoding proteins affecting membrane structure and permeability, bile resistance, adhesion and virulence potential (30). Most interestingly this data indicates 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 up-regulation of Cj1556 in the presence of bile observed previously (45).

Microarray analysis of the *Cj1556* mutant identified *Cj1556* as the most up-regulated 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 this was not a non-specific artefact, two random negative control promoter regions were selected (upstream of *flaA* and *flgK*). Both the negative controls only showed bands for the Cj1556

598 recombinant protein alone. The microarray data also indicated down-regulation of *katA*, *perR*
599 and *hspR* in the *Cj1556* mutant (Table 3). Reduced expression of KatA, PerR and HspR
600 would provide an explanation for the increased sensitivity of the *Cj1556* mutant to oxidative,
601 aerobic (O_2) and heat stress observed in this study, however further experiments are required
602 to confirm this hypothesis.
603 The ability of *C. jejuni* to form biofilms goes some way to explain how a bacterium with such
604 fastidious growth requirements remains ubiquitous in the environment (13, 36). *C. jejuni* can
605 form three distinct forms of biofilm: cell-cell aggregates, pellicles at the air-liquid interface
606 and glass-attached flocs (36). Our understanding of the specific mechanisms underlying
607 biofilm formation in *C. jejuni* is still limited (72). *C. jejuni* lacks the classical 2CRSs
608 involved in biofilm formation that are present in other bacteria such as GacSA in
609 *Pseudomonas aeruginosa* (59). Genes involved in biofilm formation have been linked to
610 responses to oxidative and aerobic (O_2) stress and *C. jejuni* biofilm formation is increased
611 under aerobic conditions (65). A *C. jejuni spoT* mutant has been found to overproduce a
612 novel calcofluor white reactive exopolysaccharide and demonstrate enhanced biofilm
613 formation (46). Interestingly a *C. jejuni cprS* mutant has been shown to display growth
614 defects, enhanced and accelerated biofilm formation and also to exhibit decreased oxidative
615 stress tolerance (71). Transcriptional analysis of the *Cj1556* mutant identified *cprS* as being
616 up-regulated and the decrease in biofilm formation observed in this study indicates a potential
617 link between CprS and Cj1556.
618 The *G. mellonella* insect model has been developed for potential identification of *C. jejuni*
619 virulence determinants and was used to investigate pathogenicity of the *Cj1556* mutant (8).
620 *G. mellonella* larvae possess specialised phagocytic cells, termed haemocytes. The insect
621 immune system is subdivided into humoral and cellular defence responses. Humoral defences
622 include the production of antimicrobial peptides (47), reactive intermediates of oxygen or

nitrogen (9) and the complex enzymatic cascades that regulate coagulation or melanisation of haemolymph (50). Cellular defence refers to haemocyte-mediated immune responses like phagocytosis, nodulation and encapsulation (66, 70). Haemocytes perform many of the functions of phagocytic cells in mammals, 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 haemocytes, 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 haemocytes 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 haemocytes with evidence of both oxygen radicals and H_2O_2 both found in plasmatocytes of *G. mellonella* (68). This data links the increased sensitivity of the *Cj1556* mutant to H_2O_2 stress *in vitro* with an 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 (O_2) stress responses and is important for the survival of *C. jejuni* in the natural environment and *in vivo.*

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936 # Tables

937

938 **Table 1. Oligonucleotide primers used in this study**

939

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	CCCGCTAGCTTAATGATGATGATGATGATGAACGATATTTTT ATAGCTAT
Upstream *Cj1556* - F	ATGCAATCTAGAAATTAT
Upstream *Cj1556* - R	GGACAAAGAGAATGATATT
Upstream *flaA* - F	ATCACAGCTTATATTAAAG
Upstream *flaA* - R	GTGTTAATACGAAATCCCAT
Upstream *flgK* - F	ATTTGTTCTTATTGTCAA
Upstream *flgK*- R	ATGTTCCAAAAATACCCAT

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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

947 **Table 3. Changes in expression of genes linked to the *C. jejuni* oxidative and aerobic**

948 (O_2) **stress responses in the *Cj1556* mutant compared to the 11168H wild-type strain**

949

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 C4-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

950

951 * *htrB* showed no hybridisation during microarray studies and was not included in this

952 analysis

Figure Legends

Fig. 1. Effect of oxidative (A) and heat stress (B) on the survival of *C. jejuni* 11168H wild-type, *Cj1556* mutant and *Cj1556* complement strains (*Cj1556* comp). *C. jejuni* strains were incubated with 10 mM H_2O_2 for 15 minutes at 37°C (A) or at 60°C for 5 minutes (B) and bacterial survival assessed. Asterisks (*) denote a statistically significant difference ($p < 0.05$) for *Cj1556* mutant compared to the 11168H wild-type strain.

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

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

Fig. 4. Survival assays. 11168H wild-type, *Cj1556* mutant and *Cj1556* complement strains (*Cj1556* comp) were co-cultured with Caco-2 intestinal epithelial cells for 24 h followed by assessing the number of bacteria in the co-culture media (A). Further survival assays were performed where these *C. jejuni* strains were grown under microaerobic (B and C) and aerobic conditions (D and E) in Brucella broth (B and D) or tissue culture media (C and E), then the numbers of viable bacteria were assessed. Asterisks denote a statistically significant difference (* = p <0.05) for the *Cj1556* mutant compared to the 11168H wild-type strain.

Fig. 5. T84 intestinal epithelial cell responses to 24 h co-culture with 11168H wild-type, *Cj1556* mutant and *Cj1556* complement strains (*Cj1556* comp) were assessed. 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.

Fig. 6. Electrophoretic mobility shift assays indicate that Cj1556 binds to a DNA promoter sequence upstream of the *Cj1556* gene. Native Western blot for recombinant 6XHis-tagged Cj1556 protein hybridised to DNA fragments representing the upstream promoter sequences of *Cj1556*, *flaA* and *flgK* (both negative controls), following separation on a Tris-Glycine gel under non-denaturing conditions.

Fig. 7. Biofilm assay on 11168H wild-type, *Cj1556* mutant and *Cj1556* complement strains (*Cj1556* comp). *C. jejuni* biofilms were grown for 3 days, rinsed three times with PBS, followed 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 OD_{600} 0.1 by micro-injection in the right foremost leg, giving an infectious dose of approximately 10^6 CFU. 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 experiments were repeated in triplicate. The asterisk denotes a statistically significant difference (* = p <0.05) for *Cj1556* mutant compared to the 11168H wild-type strain.

Figures

Fig. 1 (A)

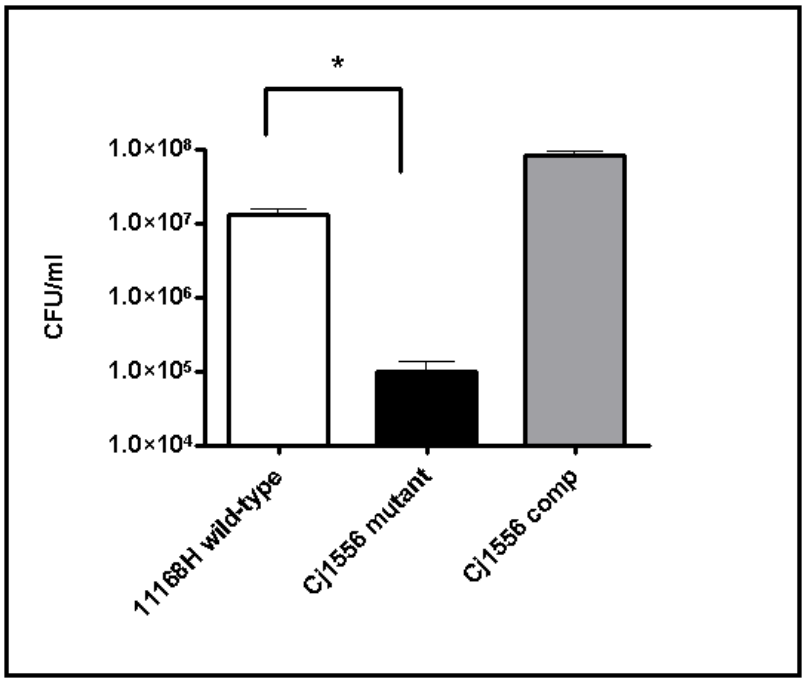
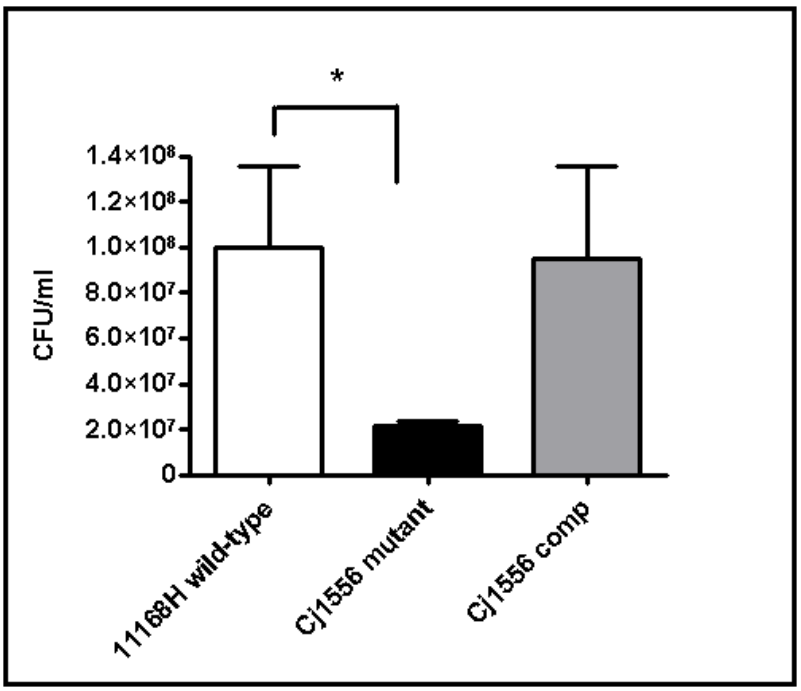


Fig. 1 (B)



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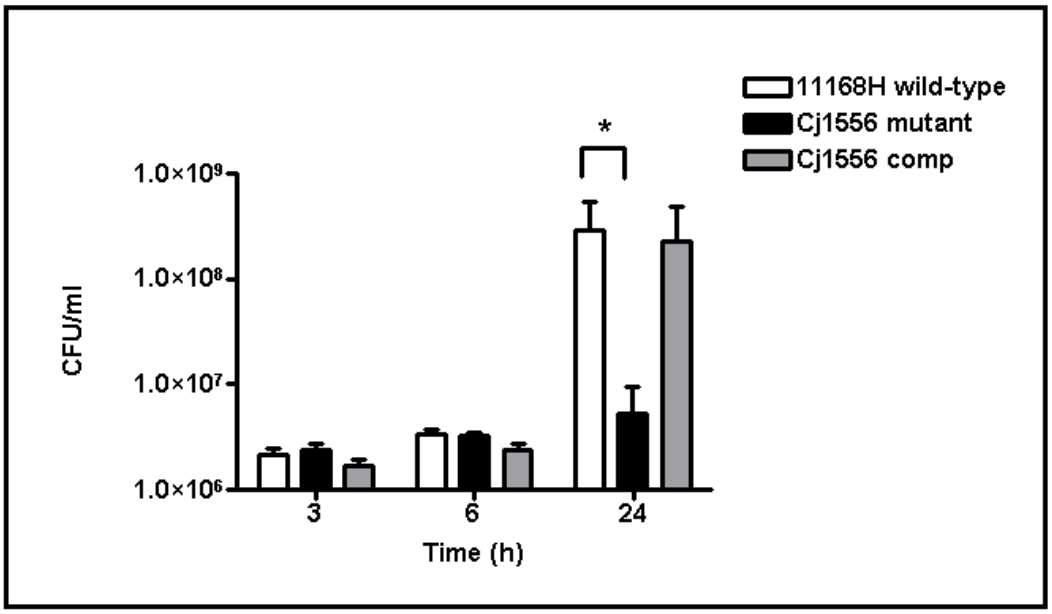

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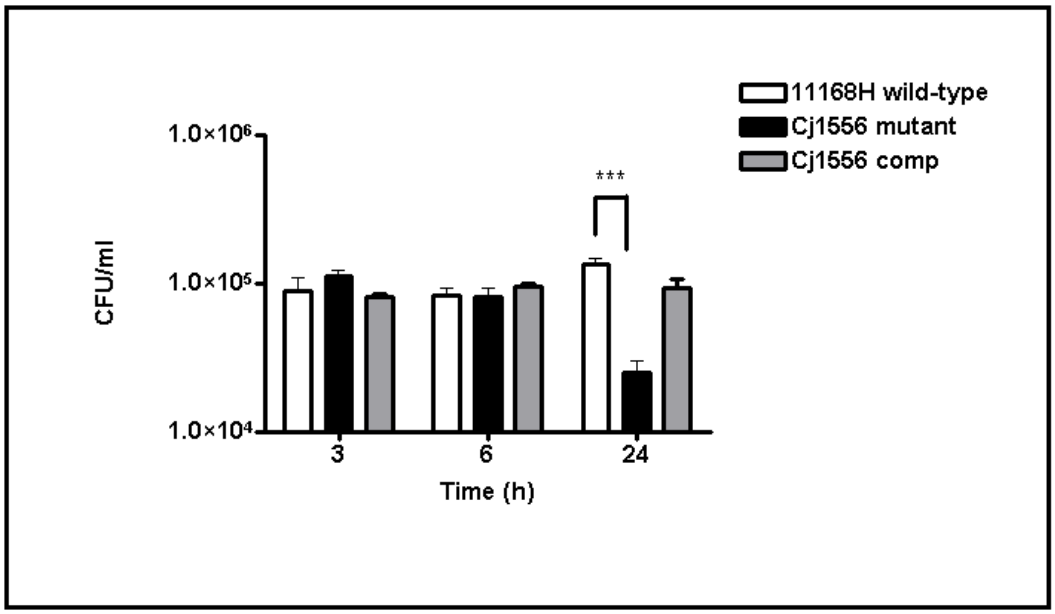

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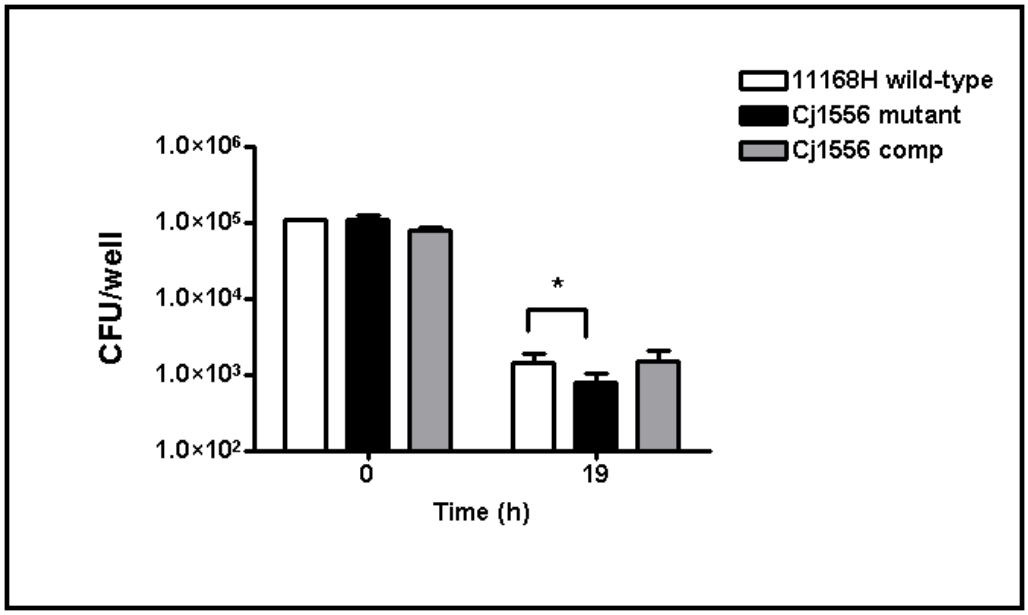

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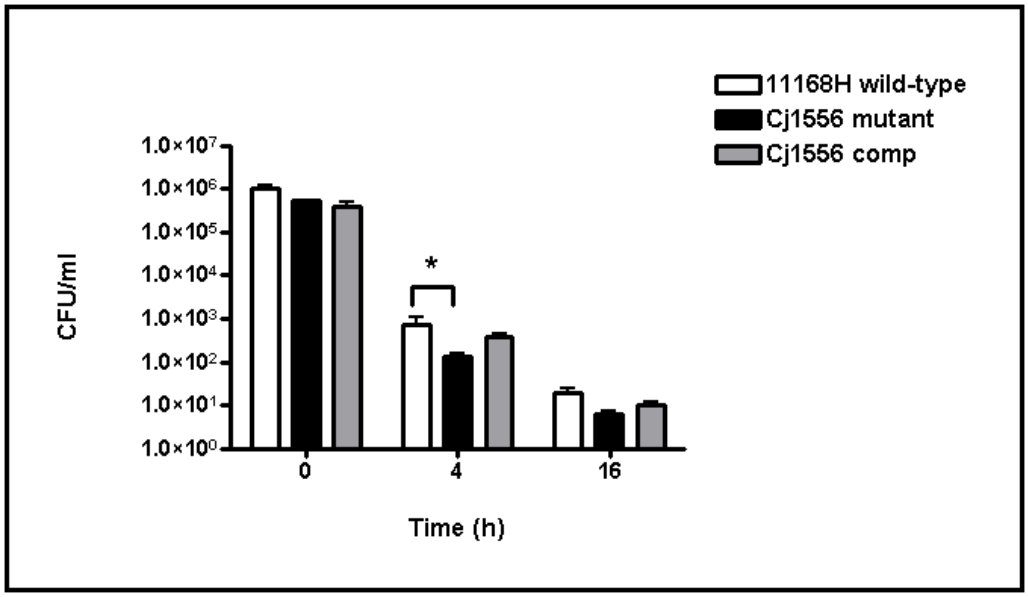

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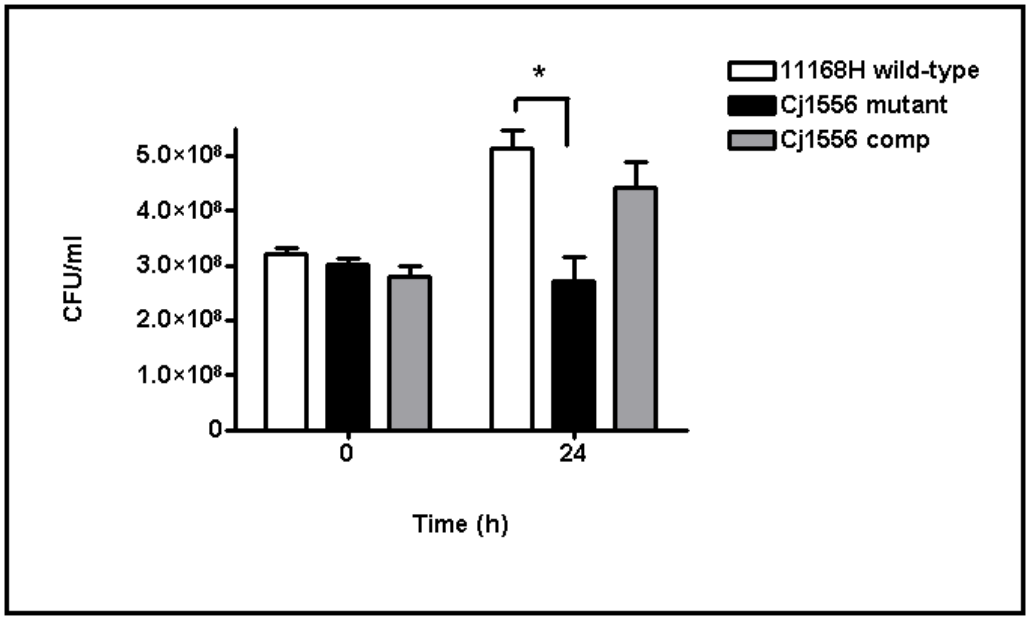
Fig. 4 (A)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Cj1556 mutant
Cj1556 comp
*
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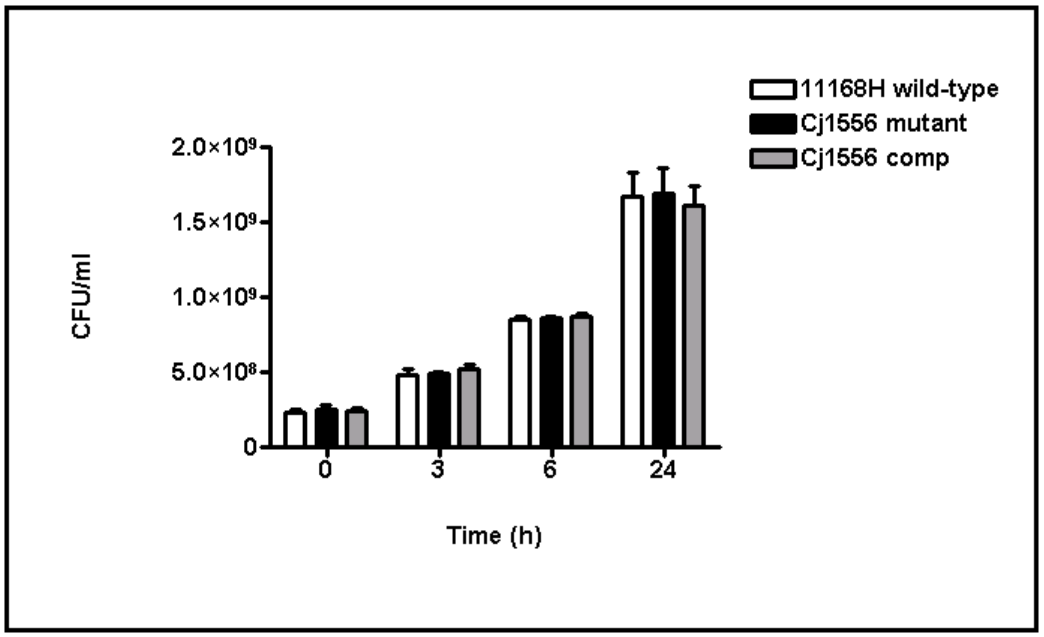
0
0
24
Time (h)

Fig. 4 (B)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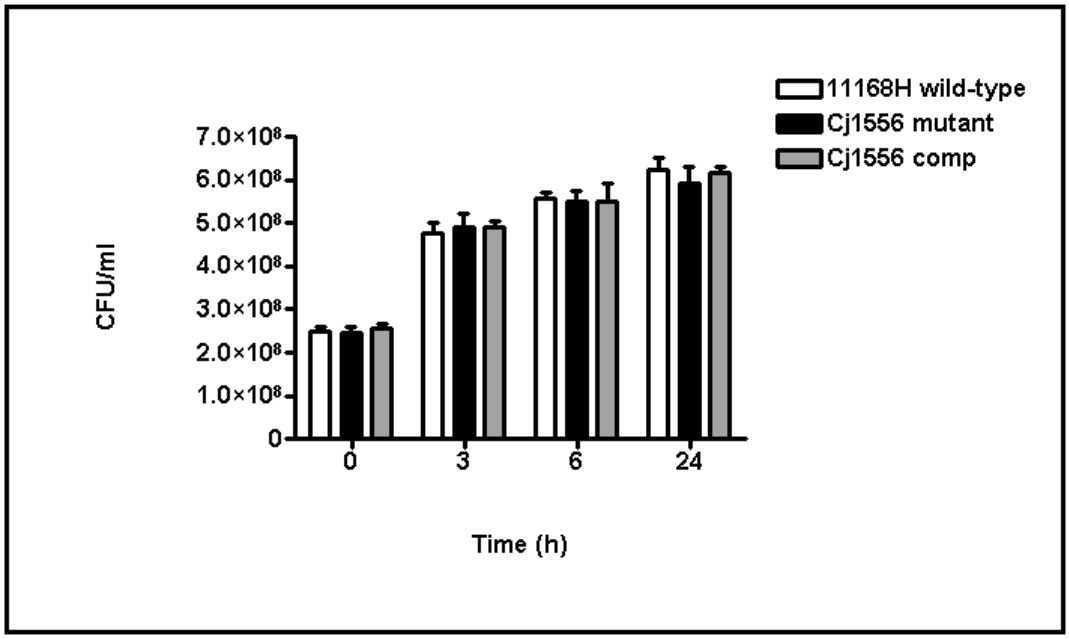
3
6
24
Time (h)

Fig. 4 (C)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Cj1556 mutant
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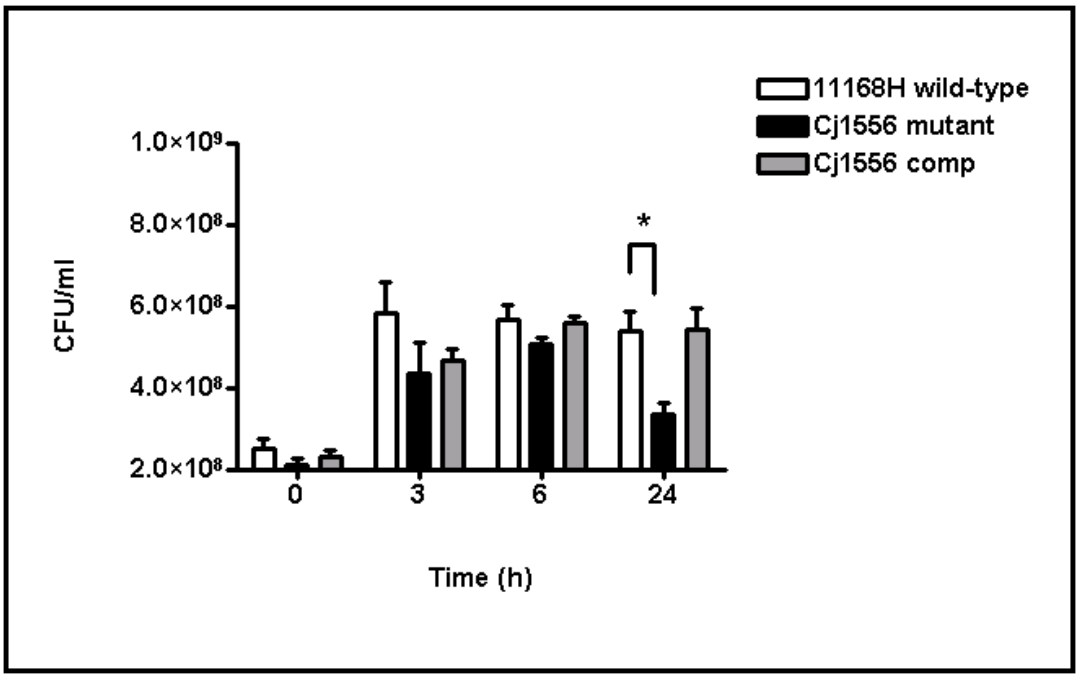
1.0×10⁸
0
0
3
6
24
Time (h)

Fig. 4 (D)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2.0×10⁸
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0
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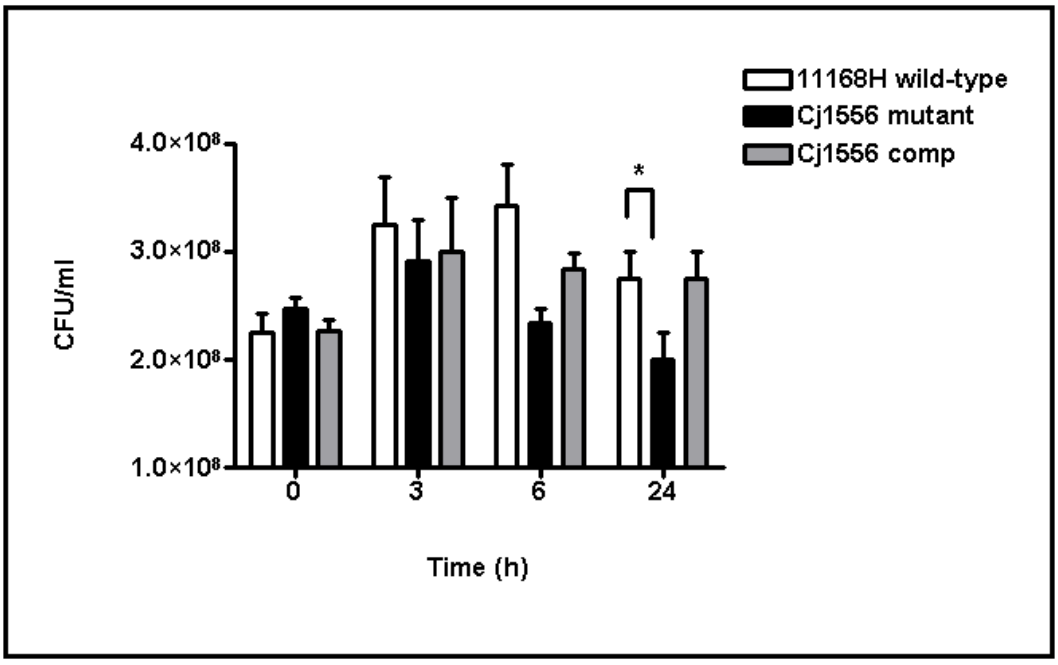
24
Time (h)

1039 Fig. 4 (E)



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1042 Fig. 5 (A)

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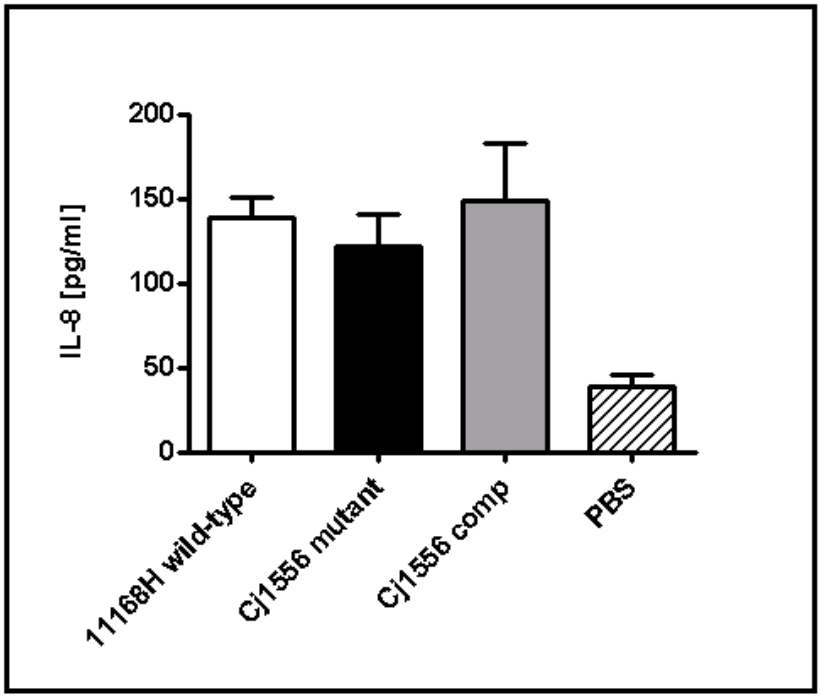

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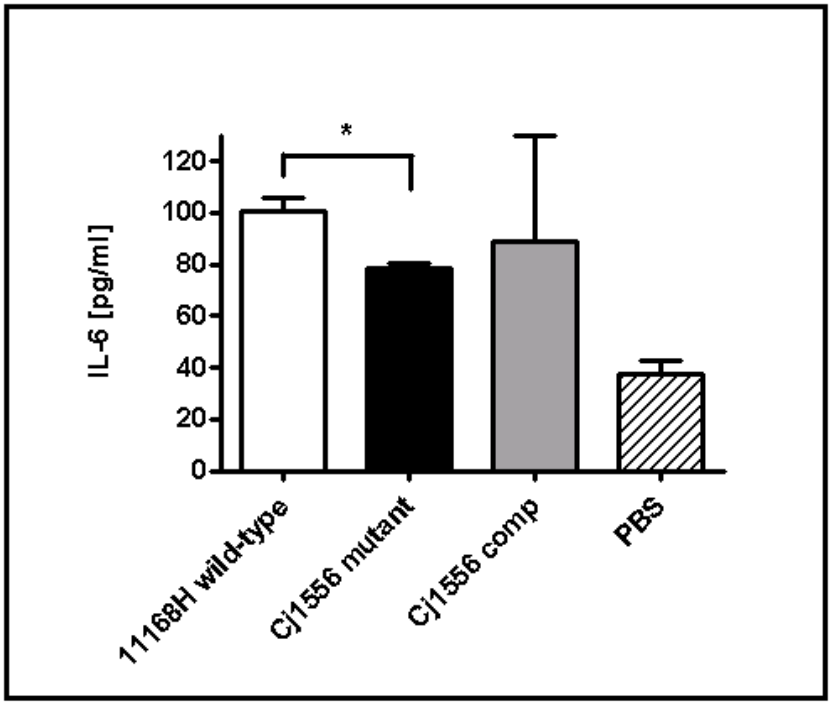

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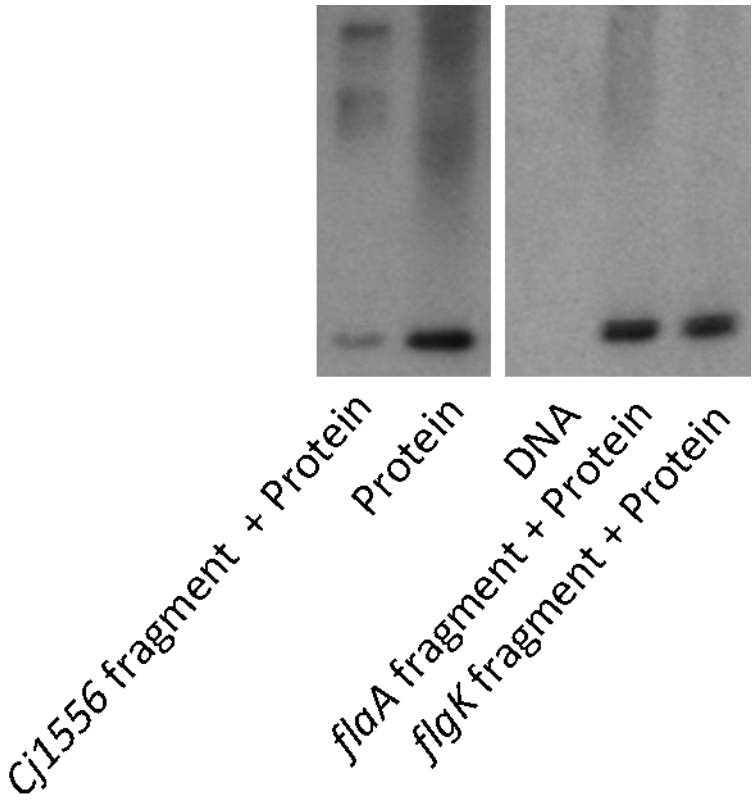

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1052 Fig. 7

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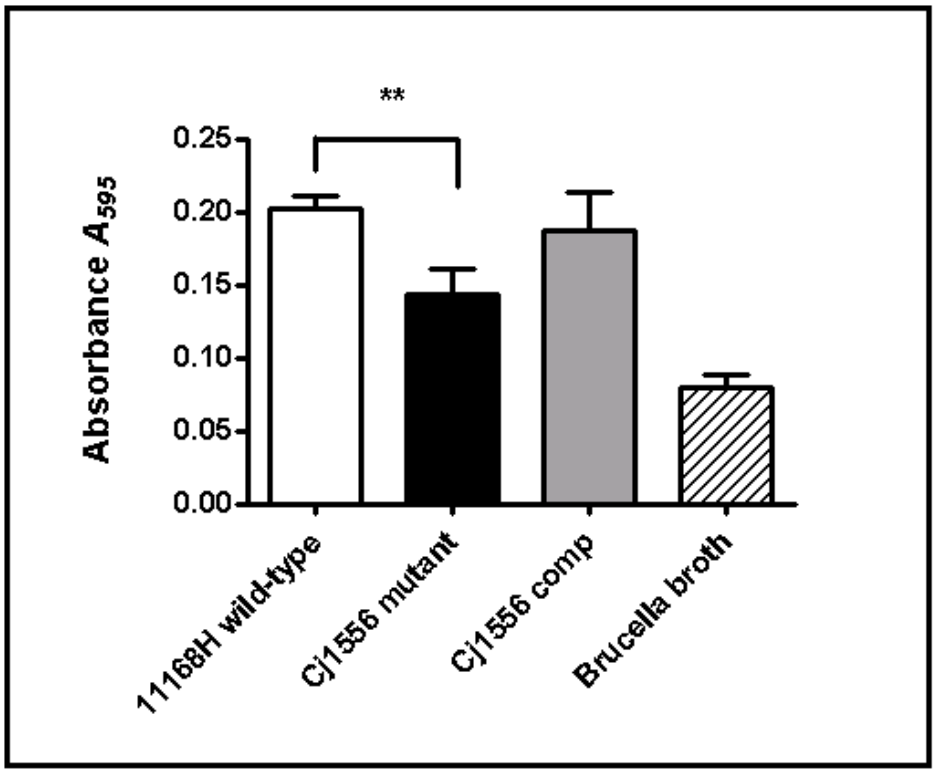

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1055 Fig. 8

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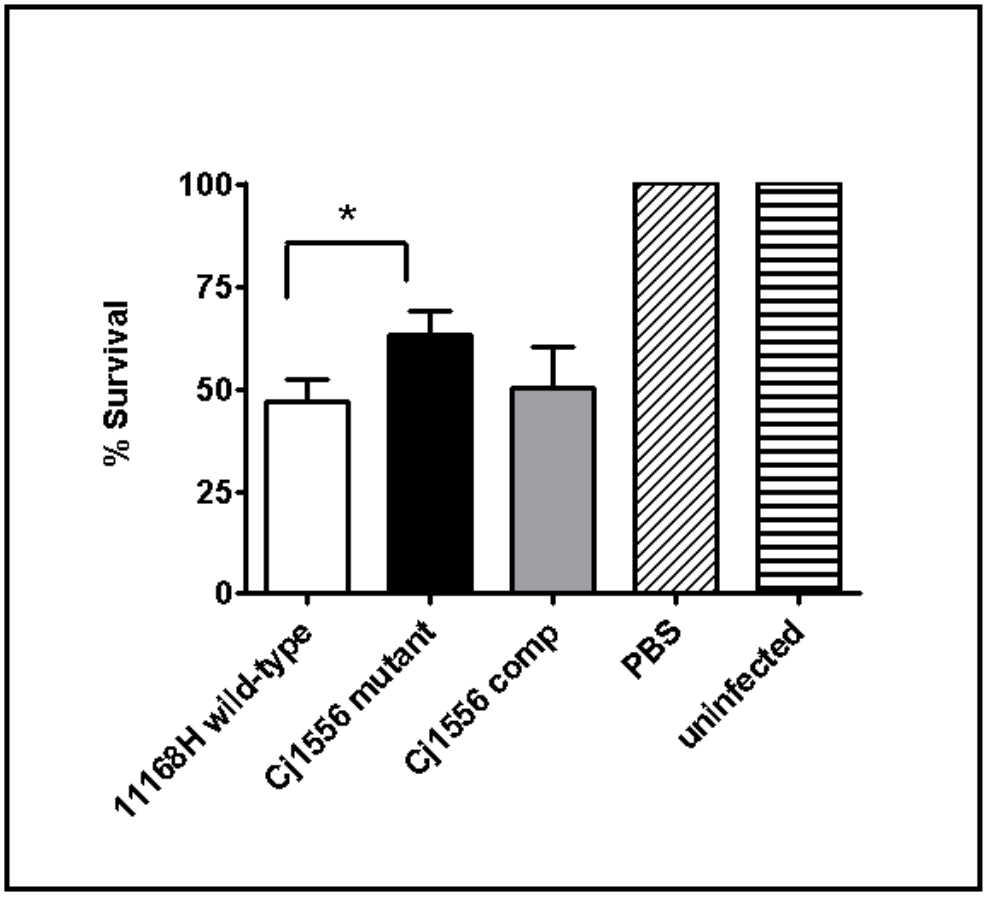

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