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Gundogdu, O; Mills, DC; Elmi, A; Martin, MJ; Wren, BW; Dorrell, N; (2011) The *Campylobacter jejuni* transcriptional regulator Cj1556 plays a role in the oxidative and aerobic (O₂) stress response and is important for bacterial survival in vivo. *Journal of bacteriology*, 193 (16). pp. 4238-49. ISSN 0021-9193 DOI: <https://doi.org/10.1128/JB.05189-11>

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1 **The *Campylobacter jejuni* transcriptional regulator Cj1556 plays a role in the oxidative**
2 **and aerobic (O₂) stress response and is important for bacterial survival *in vivo*.**

3

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15

16

17 **Abstract**

18 *Campylobacter jejuni* is the leading bacterial cause of human gastroenteritis worldwide.
19 Despite stringent microaerobic growth requirements, *C. jejuni* is ubiquitous in the aerobic
20 environment, so must possess regulatory systems to sense and adapt to external stimuli such
21 as oxidative and aerobic (O₂) stress. Re-annotation of the *C. jejuni* NCTC11168 genome
22 sequence identified Cj1556 (originally annotated as a hypothetical protein) as a MarR family
23 transcriptional regulator and further analysis indicated a potential role in regulating the
24 oxidative stress response. A *C. jejuni* 11168H *Cj1556* mutant exhibited increased sensitivity
25 to oxidative and aerobic (O₂) stress, decreased ability for intracellular survival in Caco-2
26 human intestinal epithelial cells and J774A.1 mouse macrophages and a reduction in
27 virulence in the *Galleria mellonella* infection model. Microarray analysis of gene expression
28 changes in the *Cj1556* mutant indicated negative autoregulation of *Cj1556* expression and
29 down-regulation of genes associated with oxidative and aerobic (O₂) stress responses, such as
30 *katA*, *perR* and *hspR*. Electrophoretic mobility shift assays confirmed the binding of
31 recombinant Cj1556 to the promoter region upstream of the *Cj1556* gene. *cprS*, which
32 encodes a sensor kinase involved in regulation of biofilm formation, was also up-regulated in
33 the *Cj1556* mutant and subsequent studies showed that this mutant had a reduced ability to
34 form biofilms. This study has identified a novel *C. jejuni* transcriptional regulator Cj1556 that
35 is involved in oxidative and aerobic (O₂) stress responses and is important for the survival of
36 *C. jejuni* in the natural environment and *in vivo*.

37

38

39 **Introduction**

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

60 Oxidative, nitrosative and aerobic (O₂) stresses are major factors that pathogens must
61 counteract in order to survive within the host (4, 25, 57, 87). *C. jejuni* is a microaerophilic
62 organism optimally suited to low levels of atmospheric oxygen, however the bacterium is
63 able to survive oxidative stresses *in vivo* (4). The incomplete reduction of oxygen to water

64 creates Reactive Oxygen Species (ROS) molecules, such as hydrogen peroxide (H₂O₂), that
65 are used by the host against invading pathogens (20). ROS are also released by the immune
66 system to combat invading microorganisms (4). An example of ROS release is the deposition
67 of various oxygen species generated by the respiratory burst oxidase, as the bacterium
68 remains bound within an endosome (41). ROS can damage bacterial DNA (33). Reactive
69 Nitrogen Species (RNS), such as nitric oxide, are a family of antimicrobial molecules
70 produced by the enzymatic activity of inducible nitric oxide synthase 2 (iNOS) (34).
71 Acidified nitrite kills *C. jejuni* and expression of the NOS2 isoform is increased in
72 macrophages upon exposure to the bacterium (34). RNS tend to interfere with respiration and
73 DNA replication through inactivation of zinc metalloproteins (25). Both ROS and RNS are
74 also derived from phagocytosis through the generation of superoxide and nitric oxide radicals
75 via NADPH phagocyte oxidase and inducible nitric oxide synthase pathways which are
76 important pathways within polymorphonuclear phagocytes including white blood cells and
77 mononuclear phagocytes (25). Aerobic (O₂) stress is caused by bacterial exposure to raised
78 oxygen levels. Even though oxygen is considered a stress for *C. jejuni*, few studies have
79 described specific phenotypic consequences of aerobiosis and those that have, vary in their
80 conclusions (72). Exposure of *C. jejuni* to oxygen for 24 hours accelerated the transition to
81 the viable but non-culturable state (VBNC) or coccoid form (40). In contrast, another study
82 identified the increased culturability of *C. jejuni* when exposed to oxygen for 15 hours (48).
83 Recently it has been demonstrated that aerobic stress conditions promoted the production of
84 *C. jejuni* biofilms (65).

85 *C. jejuni* possesses a variety of mechanisms for reacting to nitrosative, oxidative and aerobic
86 (O₂) stress. *C. jejuni* possesses a truncated haemoglobin (Ctb), along with a single domain
87 haemoglobin (Cgb). Both Ctb and Cgb have been characterised as part of the *C. jejuni*
88 nitrosative stress response regulon (23, 78). This regulon is under the control of NssR (49).

89 Previous studies have also implicated Ctb with a role in oxygen metabolism (77-78). *C. jejuni*
90 contains several genes encoding important oxidative stress response proteins. The superoxide
91 dismutase SodB is involved in the breakdown of superoxide to H₂O₂ and O₂ (61). The
92 catalase KatA converts H₂O₂ to H₂O and O₂. In addition, the alkyl hydroperoxide reductase
93 AhpC confers resistance to cumene hydroperoxide and aerobic stress (6). However *C. jejuni*
94 lacks an OxyR ortholog, which regulates *ahpC* and *katA* expression in response to oxidative
95 stress in many enteric bacteria such as *Salmonella* species and *Escherichia coli* (14). *C. jejuni*
96 also lacks the classical SoxRS system which mediates transcriptional activation of the
97 oxidative stress regulon in response to superoxide-generating agents (2). In *C. jejuni*, the Fur
98 homolog PerR was found to repress *ahpC* and *katA* transcription in an iron-dependent
99 manner, thus making PerR a functional, but not homologous substitute for OxyR (57, 75). In
100 addition, *C. jejuni* proteins involved in responding to aerobic (O₂) stress have also been
101 identified. SodB and KatA have been shown to counteract the detrimental effects of aerobic
102 (O₂) stress (69). The heat shock protease HtrA and regulator HspR have been shown to be
103 important for short-term aerobic tolerance (3, 12). The *fdxA* gene upstream of *ahpC* encodes a
104 ferredoxin which has been identified as important for aerotolerance (74). Also SpoT, which
105 regulates the *C. jejuni* stringent response, was found to be important for low CO₂ growth and
106 aerobic survival (28). Cj1556 was identified as a member of the MarA family of
107 transcriptional regulators through re-annotation of the NCTC11168 genome sequence (29). In
108 this study, further bioinformatic analysis indicated a role for Cj1556 in the *C. jejuni* stress
109 responses and a defined isogenic *C. jejuni* 11168H *Cj1556* mutant was constructed in order to
110 investigate this hypothesis.

111

112 **Materials and Methods**

113 **Bacterial strains and growth conditions**

114 The *C. jejuni* wild-type strain used in this study was 11168H (38), a hypermotile derivative of
115 the original sequenced strain NCTC11168 that shows higher levels of caecal colonisation in a
116 chick colonisation model (35). *C. jejuni* was grown at 37°C in a microaerobic chamber (Don
117 Whitley Scientific, U.K), containing 85% N₂, 10% CO₂ and 5% O₂ either on blood agar
118 plates containing Columbia agar base (Oxoid, U.K), supplemented with 7% (v/v) horse blood
119 (TCS Microbiology, U.K) and *Campylobacter* Selective Supplement (Oxoid) or in Brucella
120 broth (Oxoid) shaking at 75 rpm. *C. jejuni* strains were grown on blood agar plates for 24 h
121 prior to use in co-culture experiments. *E. coli* XL-2 Blue MRF' competent cells (Stratagene,
122 U.S.A) were used for cloning experiments and were grown at 37°C in aerobic conditions
123 either on Luria-Bertani (LB) agar plates or in LB broth with shaking at 200 rpm. Appropriate
124 antibiotics were added at the following concentrations; ampicillin (100 µg/ml), kanamycin
125 (50 µg/ml) and chloramphenicol (50 µg/ml) for *E. coli* studies, (10 µg/ml) for *C. jejuni*
126 studies. All reagents were obtained from Invitrogen (UK) unless otherwise stated.

127

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

129 Construction of a defined isogenic 11168H *Cj1556* mutant was performed using previously
130 published methods (35, 38, 43). Briefly, *Cj1556*-F and *Cj1556*-R primers were designed for
131 PCR detection of *Cj1556* (Table 1). Using the pUC library from the *C. jejuni* NCTC11168
132 genome sequencing project (58), plasmid cam25a2 (1489074..1490567) was selected which
133 contains a 1.494 kb insert included the coding sequences (CDSs) *Cj1555c* – *Cj1560* and
134 designated pUC-*Cj1556*. The *Cj1556* ORF in pUC-*Cj1556* was inactivated by insertion of an
135 *aph-3* (aminoglycoside 3'-phosphotransferase, Km^R) cassette (73). The Km^R cassette was
136 excised from pJMK30 (76) using *Bam*HI. pUC-*Cj1556* was digested with *Bcl*II, ligated with

137 the Km^R cassette to form pUC-*Cj1556*-Km^R. pUC-*Cj1556*-Km^R was transformed into XL-2
138 Blue MRF⁺ competent cells and transformants selected on LB agar supplemented with
139 ampicillin and kanamycin after 48 h growth at 37°C. Transformants were screened by PCR
140 using *Cj1556*-specific and Km^R-specific primers (Table 1). pUC-*Cj1556*-Km^R plasmids with
141 the Km^R cassette in the same orientation as the *Cj1556* gene were selected and electroporated
142 into 11168H wild-type as described previously (35, 38). Electroporated bacteria were plated
143 onto blood agar plates and incubated at 37°C under microaerobic conditions for 2 days. Cells
144 were harvested and resuspended in 0.5 ml phosphate buffered saline (PBS). 200 µl of this
145 suspension was spread onto blood agar plates containing kanamycin. Putative *Cj1556*
146 mutants were screened using PCR and sequencing.

147

148 **Complementation of *C. jejuni* *Cj1556* mutant**

149 Complementation procedures were performed by inserting a copy of the *Cj1556* gene into the
150 *Cj1556* mutant chromosome using a *C. jejuni* NCTC11168 complementation vector (31). The
151 coding region for *Cj1556* was amplified by PCR using primers Comp-*Cj1556*-F and Comp-
152 *Cj1556*-R (Table 1), which introduced an *Nco*I site at the 5' end and a *Nhe*I site at the 3' end
153 as well as the native ribosome binding site of *Cj1556* (72, 83). Following digestion with *Nhe*I
154 and *Nco*I, this PCR product was ligated into the pDENNIS vector. This construct was
155 checked by sequencing and electroporated into the *Cj1556* mutant. Putative clones were
156 selected on blood agar plates containing kanamycin and chloramphenicol. Confirmation for
157 the presence of copies of both *Cj1556* and *Cj1556*-Km^R was performed by PCR using
158 Comp-*Cj1556*-F and Comp-*Cj1556*-R primers and also *Cj1556*-F with *Cj1556*-R primers and
159 also by sequencing. For isolation of recombinant *Cj1556* protein, a 6XHis-tag sequence was
160 cloned into a second construct using primers Comp-*Cj1556*-F and Comp-*Cj1556*-R-HIS
161 (Table 1).

162

163 **Nitrosative, oxidative and heat stress assays**

164 *C. jejuni* were grown on blood agar plates for 24 h at 37°C under microaerobic conditions.
165 Bacterial cells were harvested into 1 ml PBS and adjusted to an OD₆₀₀ of 1. For nitrosative
166 stress assays, bacterial cells were exposed to acidified sodium nitrite (NaNO₂) at a final
167 concentration of 100 mM NaNO₂ for 30 minutes and 10 mM NaNO₂ for 75 minutes. For
168 nitrosative stress assays, all media used was at pH 5 to allow formation of acidified NaNO₂ to
169 promote the production of nitric oxide radicals (22, 34). For oxidative stress assays, bacterial
170 cells were exposed to hydrogen peroxide (H₂O₂) at a final concentration of 10 mM for 15
171 minutes. Heat stress assays were performed at 42°C for 1 h, 55°C for 15 mins and 60°C for 5
172 mins. Serial dilutions were prepared and 10 µl of the 10⁻¹ - 10⁻⁶ dilutions were spotted onto
173 blood agar plates and incubated for 48 h at 37°C under microaerobic conditions and colonies
174 counted.

175

176 **Cell culture procedures**

177 The Caco-2 human intestinal epithelial and J774A.1 mouse macrophage cell lines were
178 maintained in Dulbecco's modified essential media (DMEM) supplemented with 10% (v/v)
179 foetal calf serum (FCS) (Sigma-Aldrich, UK), 1% (v/v) non-essential amino acids, 100 µg/ml
180 streptomycin and 100 U/ml penicillin. The T84 human colonic epithelial cell line was
181 maintained in a 1:1 mixture of Dulbecco's modified Eagle medium and Ham's F-12 medium
182 containing Glutamax[®], 2.5 mM l-glutamine, 15 mM HEPES, and 0.5 mM sodium pyruvate,
183 supplemented with 10% (v/v) FCS, 100 µg/ml streptomycin and 100 U/ml penicillin. Cells
184 were maintained at 37°C in 5% CO₂ and 95% air. For Caco-2 cell co-culture experiments,
185 cells were seeded at 1x10⁵ cells/ml and grown in 24-well plates to >90% confluence (≈ 1x10⁶
186 cells/ml). For T84 cell co-culture experiments, cells were seeded at 5x10⁵ cells/ml and grown

187 in 24-well plates to >90% confluence ($\approx 5 \times 10^6$ cells/ml). For co-culture experiments
188 involving J774A.1 mouse macrophage, cells were seeded at 5×10^5 cells/ml and grown in 24-
189 well plates for 24 h. For ELISA experiments, T84 cells were maintained in low serum 1%
190 (v/v) and antibiotic-free media overnight prior to co-culture. Infections were terminated by
191 removing the supernatant from the cells followed by two washes in PBS. Cell culture
192 supernatants were stored at -80°C until required.

193

194 **Interaction, invasion and intracellular survival assays**

195 Interaction (adhesion and invasion) and invasion assays were performed using Caco-2 cells as
196 described previously (12). Bacterial cells were harvested into 1 ml Brucella broth and
197 adjusted to an OD_{600} of 0.1. Serial dilutions were prepared and 200 μl volumes were plated
198 onto blood agar plates and incubated for 72 h at 37°C under microaerobic conditions.
199 Colonies were counted to calculate the initial inoculum. *C. jejuni* (approximately 1×10^8 cells)
200 in DMEM were added to a monolayer of approximately 1×10^6 Caco-2 cells (MOI 100:1) and
201 incubated for 3, 6 or 24 h. The number of interacting bacteria was determined by washing the
202 monolayers three times with PBS, then lysing the cells by addition of 0.2% (v/v) Triton X-
203 100. The number of intracellular bacteria was determined by further incubating the
204 monolayers after the initial interaction time point with DMEM containing gentamicin (150
205 $\mu\text{g/ml}$) for 2 h at 37°C to allow killing of extracellular bacteria. Monolayers were then
206 washed three times in PBS and the epithelial cells lysed as above. For intracellular survival
207 assays, bacterial cells were co-cultured with a monolayer of Caco-2 cells for 3 h followed by
208 washing the monolayers three times with PBS. The monolayers were then incubated in
209 DMEM containing gentamicin (150 $\mu\text{g/ml}$) for 2 h and then incubated in DMEM containing
210 a reduced concentration of gentamicin (10 $\mu\text{g/ml}$) for 19 h. Monolayers were then washed
211 three times in PBS and the epithelial cells lysed as above. To ascertain whether the above

212 results were due to a genuine *Cj1556* mutant phenotype and not to increased sensitivity to
213 Triton X-100, stress assays were performed on all three strains with 0.2% (v/v) Triton X-100.
214 No difference in the level of sensitivity to Triton X-100 was observed between 11168H wild-
215 type strain, *Cj1556* mutant and the *Cj1556* complement (data not shown). Survival in tissue
216 culture medium from co-culture experiments were performed as above, but after 24 h co-
217 culture, the tissue culture medium alone was removed followed by plating of serial dilutions
218 to determine the CFU/ml. In all cases, serial dilutions, plating and enumeration of bacterial
219 numbers were performed as stated above.

220

221 **Macrophage survival assay**

222 Macrophage survival assays were performed as described previously (80) using J774A.1
223 mouse macrophages (67). Briefly, *C. jejuni* were harvested into 1 ml Brucella broth and
224 adjusted to an OD₆₀₀ of 0.1. *C. jejuni* cells (approximately 1×10^8 cells) in DMEM were added
225 to a culture of approximately 5×10^5 J774A.1 mouse macrophage cells (MOI 200:1) and
226 incubated for 3 h. Cells were washed three times in PBS followed by incubation in DMEM
227 containing gentamicin (150 µg/ml) for 2 h to allow killing of extracellular bacteria.
228 Macrophages were incubated in DMEM containing a reduced concentration of gentamicin
229 (10 µg/ml) and bacterial survival determined at 0, 4 and 16 h. At each time point, the
230 macrophages were washed three times with PBS and lysed by adding 0.2% (v/v) Triton X-
231 100 in PBS. Serial dilutions, plating and enumeration of bacterial numbers were performed as
232 stated above.

233

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

235 Supernatants from uninfected T84 cells and T84 cells infected with *C. jejuni* at an MOI of
236 20:1 for 24 h were collected. The levels of IL-6 and IL-8 secretion were assessed using a

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

241

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

244 Gene expression profiling of *C. jejuni* 11168H from late-log growth phase (16 h) was
245 performed using an indirect comparison method or type 2 experimental design (86). Replicate
246 test sets of Cy5-labelled *C. jejuni* 11168H total RNA samples were combined with a common
247 reference sample (Cy3-labelled *C. jejuni* 11168H genomic DNA) as described in previous
248 studies (24, 44, 84). *C. jejuni* 11168H genomic DNA was isolated from bacteria grown on
249 blood agar for 24 h using the Puregene® DNA purification kit (Gentra, U.K) and used as the
250 common reference sample in all microarray experiments. *C. jejuni* RNA was isolated from 16
251 h cultures using the RNeasy Mini purification kit (Qiagen) and RNeasy Protect Bacteria Reagent
252 (Qiagen) as described previously (37). Whole genome *C. jejuni* NCTC11168 microarrays
253 printed on UltraGAPSTTM glass slides (Corning, U.S.A), constructed by the BμG@S
254 Microarray Group (<http://www.bugs.sgu.ac.uk/>) were used in this study (37). The procedures
255 used for Cy5-labelling of total RNA samples (37) and Cy3-labelling of 11168H genomic
256 DNA (21) were as described previously. All hybridizations were performed as described
257 previously (21, 37) with the following modifications. For probe hybridization, Cy5-labelled
258 probes of *C. jejuni* 11168H total RNA (test) and Cy3-labeled common reference samples of
259 *C. jejuni* 11168H DNA (control) were combined and purified using a MinElute PCR
260 Purification kit (Qiagen). The final elution was made up to a volume of 50 μl with a final
261 concentration of 4X SSC and 0.3% (w/v) SDS. The hybridization mixture was denatured at

262 98°C for 2 mins and cooled slowly to room temperature. A 22 x 25 mm LifterSlip coverslip
263 (Erie Scientific, U.S.A) was placed over the reporter element area on the microarray and the
264 hybridization mixture applied underneath the coverslip. The microarray slide was placed in a
265 humidified hybridization cassette (Telechem International, U.S.A) and incubated in a water
266 bath for 18 h at 65°C without shaking. Microarray slides were then washed as described
267 previously (37). The microarray slides were scanned with an Affymetrix 418 array scanner
268 (MWG Biotech, Germany) according to the manufacturer's guidelines. Signal and local
269 background intensity readings for each spot were quantified using ImaGene software v8.0
270 (BioDiscovery, U.S.A). Quantified data were analysed using GeneSpring GX software v7.3
271 (Agilent, U.S.A). Statistically significant up- and down-regulated genes were selected when
272 comparing gene expression against 11168H wild-type using ANOVA (ANalysis Of
273 VAriance) with a Benjamini and Hochberg False Discovery Rate as the Multiple Testing
274 Correction (5, 18). The array design is available in BμG@Sbase (Accession No. A-BUGS-9;
275 <http://bugs.sgul.ac.uk/A-BUGS-9>) and also ArrayExpress (Accession No. A-BUGS-9). Fully
276 annotated microarray data have been deposited in BμG@Sbase (accession number E-BUGS-
277 119; <http://bugs.sgul.ac.uk/E-BUGS-119>) and also ArrayExpress (accession number E-
278 BUGS-119).

279

280 **Electrophoretic mobility shift assays**

281 *E. coli* strains were grown overnight for 16 h at 37°C shaking at 200 rpm. Cultures were spun
282 at 4,000 rpm for 10 minutes at 4°C. The bacterial pellet was resuspended in 1 ml
283 equilibration buffer (Sigma-Aldrich). Cells were sonicated using manufacturers instructions
284 (Diagenode, Belgium), followed by centrifuged for 5 minutes at 13,000 rpm. The supernatant
285 containing lysed cell content was poured into a new 1.5 ml microcentrifuge tube. Lysed cells
286 were incubated with Ni-NTA (Qiagen) for 1 h at 4°C on a rotator. Elution was performed

287 using a His-Select spin column (Sigma-Aldrich). To demonstrate the DNA binding properties
288 of Cj1556, purified recombinant protein was hybridised to PCR amplified fragments (140 –
289 180 bp) located upstream of the translation initiation sites of the *Cj1556*, *flaA* and *flgK* genes
290 (Table 1 & 2). 2.5 µg recombinant native protein was hybridised with 20 ng of purified DNA
291 along with 2 µl hybridisation solution (20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM
292 DTT, 250 mM NaCl, 50 mM Tris-HCL (pH 7.5) and incubated at room temperature for 40
293 minutes. Samples were resuspended in Tris-Glycine native sample buffer (Invitrogen) up to
294 10 µl and analysed using a Tris-Glycine gel under non-denaturing conditions (Invitrogen)
295 followed by Western blot analysis.

296

297 **Biofilm assays**

298 *C. jejuni* were grown on blood agar plates for 24 h at 37°C under microaerobic conditions. 10
299 ml Brucella broth was preincubated in a 50 ml flask at 37°C under microaerobic conditions
300 24 h prior to inoculation, then inoculated with the bacterial cells harvested into Brucella broth
301 to an OD₆₀₀ of 0.1. 1 ml was then added to 24 well polystyrene plates (Corning) and
302 incubated under microaerobic conditions with gentle agitation for 3 days. The wells were
303 washed three times with PBS, followed by addition of 0.2% (w/v) crystal violet (Sigma-
304 Aldrich) for 10 minutes. The wells were then washed three times with PBS, followed by
305 dissolving the biofilm with 20% acetone/80% ethanol. Detection was performed using a
306 Dynex MRX II 96 well plate reader (Dynex, U.S.A) at an A₅₉₅.

307

308 ***Galleria mellonella* infection model**

309 *G. mellonella* larvae were obtained from LiveFoods Direct (U.K) and kept on wood chips at
310 16°C. Larvae were injected with a 10 µl inoculum of a 24 h *C. jejuni* culture diluted to OD₆₀₀
311 0.1 by micro-injection (Hamilton, Switzerland) in the right foremost leg, giving an infectious

312 dose of approximately 10^6 CFU (17). Injection with PBS and no injection controls were also
313 performed. Larvae were incubated 37°C with survival and percentage survival recorded at 24
314 h intervals. For each experiment, 10 *G. mellonella* larvae were infected and experiments were
315 repeated three times.

316

317 **Statistical analyses**

318 Data is presented as mean \pm SD. All experiments represent at least three biological replicates
319 performed in triplicate in each experiment. Statistical analyses were performed using Prism
320 software (GraphPad Software, USA). Variables were compared using a student's *t*-test.

321

322 **Results**

323 **Bioinformatic analysis indicates Cj1556 has a role in regulation of stress responses**

324 The 333 nucleotide predicted CDS of *Cj1556* was originally annotated as a hypothetical
325 protein in the genome sequence of *C. jejuni* NCTC11168 (29). Following re-annotation, the
326 updated product function indicated that Cj1556 is a transcriptional regulator based on the
327 identification of a new Pfam motif (PF01638), defined as an HxIR-like helix-turn-helix motif
328 (29). The HxIR-like helix-turn-helix motif is located 45 nucleotides into the CDS and
329 encompasses the remainder of the CDS. The HxIR-like helix-turn-helix motif is part of the
330 MarR family of transcriptional regulators that includes proteins that control virulence factor
331 production, bacterial responses to both antibiotics and oxidative stress and also catabolism of
332 environmental aromatics compounds (82, 85). The predicted function of Cj1556 was
333 investigated further using the Campylobacter Protein Interaction Database (60) and putative
334 interactions with Ctb (Cj0465c) were identified. Ctb is a group III truncated haemoglobin and
335 characterisation studies in *C. jejuni* have already shown Ctb to be part of the nitrosative stress
336 response regulon (49). Ctb has also been linked with moderating oxygen metabolism within
337 *C. jejuni* (49). Collectively this bioinformatic analyses suggest that Cj1556 has an important
338 role as a stress response regulator.

339

340 **Construction and characterisation of a *C. jejuni* 11168H *Cj1556* mutant**

341 To investigate the function of Cj1556, a defined isogenic 11168H *Cj1556* mutant was
342 constructed by insertion of a kanamycin resistant cassette (Km^R) using standard mutagenesis
343 techniques (35, 38) with Km^R in the same orientation as the *Cj1556* CDS to obviate potential
344 polar effects. To further confirm phenotypic changes, the *Cj1556* mutant was complemented,
345 verified by PCR / sequencing and termed *Cj1556* complement. Motility assays demonstrated

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

348

349 ***Cj1556* mutant exhibits increased sensitivity to both oxidative and heat stress**

350 Nitrosative stress assays were performed using acidified NaNO₂. However no differences
351 between the survival of the 11168H wild-type strain and the *Cj1556* mutant were observed
352 (data not shown). Oxidative stress assays were performed using H₂O₂. The *Cj1556* mutant
353 exhibited increased sensitivity to H₂O₂ compared to the 11168H wild-type strain (Fig. 1A). In
354 addition, the *Cj1556* complement restored the wild-type H₂O₂ sensitivity phenotype (Fig.
355 1A). Previous research has suggested a link between aerobic and heat stress (12, 63). In order
356 to investigate this further, a range of heat stress experiments were performed. No significant
357 differences in survival were observed at 42°C / 60 mins or 55°C / 15 mins. However the
358 *Cj1556* mutant displayed increased sensitivity compared to the wild-type strain at 60°C / 5
359 minutes and the *Cj1556* complement restored the wild-type phenotype (Fig. 1B).

360

361 ***Cj1556* mutant displays a reduced ability to interact with and invade Caco-2 intestinal** 362 **epithelial cells**

363 Interaction (adhesion and invasion) and invasion assays were performed using 11168H wild-
364 type, *Cj1556* mutant and *Cj1556* complement strains. No significant differences were
365 observed when comparing the levels of interaction at either 3 h or 6 h, however the *Cj1556*
366 mutant displayed a reduced ability to interact with Caco-2 cells after 24 h co-culture,
367 compared with the 11168H wild-type and *Cj1556* complement strains (Fig. 2A). The *Cj1556*
368 mutant also displayed a reduced ability to invade Caco-2 cells after 24 h co-culture,
369 compared with the 11168H wild-type and *Cj1556* complement strains (Fig. 2B). No
370 significant differences were observed when comparing the levels of invasion at 3 h or 6 h.

371

372 ***Cj1556* mutant exhibits reduced intracellular survival in Caco-2 intestinal epithelial**
373 **cells and in J774A.1 macrophage cells**

374 A modification of the interaction and invasion assays was used to analyse the level of
375 intracellular survival in Caco-2 intestinal epithelial cells (53), in order to investigate the
376 ability of *C. jejuni* to survive when exposed to intracellular stress conditions such as ROS.
377 There was a statistically significant reduction in the level of intracellular survival of the
378 *Cj1556* mutant compared to the 11168H wild-type and *Cj1556* complement strains (Fig. 3A).
379 Intracellular survival assays using macrophage cells were also performed to further
380 investigate the survival rates of the 11168H wild-type, *Cj1556* mutant and *Cj1556*
381 complement strains. Macrophages internalise and destroy *C. jejuni* (80) and previous studies
382 have shown that *C. jejuni* are killed within 24 h of internalisation (80). There was a
383 statistically significant reduction in the level of intracellular survival of the *Cj1556* mutant
384 compared to the 11168H wild-type strain (Fig. 3B).

385

386 ***Cj1556* mutant exhibits reduced survival in both co-culture media and in an aerobic**
387 **environment**

388 A further variation of the intracellular survival assay was used to assess the survival of *C.*
389 *jejuni* in tissue culture medium. There was a statistically significant increase in the number of
390 viable bacterial cells obtained from the supernatant after 24 h co-culture with Caco-2 cells
391 when comparing the 11168H wild-type and *Cj1556* complement strains to the *Cj1556* mutant
392 (Fig. 4A). Following the identification of significant differences between the 11168H wild-
393 type strain and *Cj1556* mutant in response to oxidative stress and intracellular survival,
394 further investigations on the ability of these strains to survive aerobic (O₂) stress were
395 performed. The difference in the level of *Cj1556* mutant survival between the interaction and

396 intracellular assays suggested that additional stresses might affect *C. jejuni* during these
397 assays. Survival assays with 11168H wild-type strain, *CjI556* mutant and *CjI556*
398 complement strains were performed under either microaerobic or aerobic conditions in either
399 Brucella broth or tissue culture media with no shaking to replicate the conditions for the co-
400 culture assays. A statistically significant reduction in the number of viable bacterial cells with
401 the *CjI556* mutant compared to the 11168H wild-type strain in both types of media was
402 observed after 24 h incubation under aerobic conditions (Fig. 4D and E) but not under
403 microaerobic conditions (Fig. 4B and C).

404

405 ***CjI556* mutant induces a reduced IL-6 response from T84 cells**

406 IL-6 and IL-8 are well-characterised markers denoting a host immune response against
407 pathogens (56). Only minimal secretion of IL-6 and IL-8 was detected when the 11168H
408 wild-type and *CjI556* mutant were co-cultured with Caco-2 cells (data not shown). However
409 using the T84 cell line, significant levels of induction of both IL-6 and IL-8 by the 11168H
410 wild-type strain and *CjI556* mutant was observed (Fig. 5). There was no significant
411 difference in the level of IL-8 induction by the *CjI556* mutant compared to that by the
412 11168H wild-type strain (Fig. 5A), however a significant reduction in the level of IL-6
413 induction by the *CjI556* mutant was observed (Fig. 5B).

414

415 **Microarray analysis indicates negative autoregulation of *CjI556* expression**

416 To analyse the gene expression profile of the *CjI556* mutant compared to the 11168H wild-
417 type strain, microarray experiments were performed using total RNA samples isolated from
418 *C. jejuni* grown to late-log phase (16 h). A total of 91 genes were differentially expressed in
419 the *CjI556* mutant compared to the 11168H wild-type with 73 genes up-regulated and 18
420 genes down-regulated, based on an ANOVA selection methodology (5, 18). Interestingly the

421 gene with the most pronounced up-regulation (10.4 fold) was *Cj1556*. Sequence analysis of
422 the *Cj1556* reporter element used on the arrays showed that this particular sequence was
423 present upstream of the Km^R cassette in the *Cj1556* mutant (data not shown). Usually the
424 mutated gene in a defined mutant would expect to appear down-regulated, however the
425 microarray data indicates that in the absence of the Cj1556 protein, *Cj1556* gene expression
426 is dramatically increased. This suggests that Cj1556 represses further expression of the
427 *Cj1556* gene, acting as a negative autoregulator. Further analysis of genes associated with
428 oxidative and aerobic (O₂) stress responses showed that many were down-regulated in the
429 *Cj1556* mutant, including *katA* (5.13 fold), *perR* (5.05 fold) and *hspR* (2.07 fold) (Table 3),
430 indicating potential reasons for the increased sensitivity of the *Cj1556* mutant to these
431 stresses.

432

433 **Electrophoretic mobility shift assays indicate binding of Cj1556 to a DNA promoter**
434 **probe upstream of the *Cj1556* gene**

435 To investigate whether Cj1556 acts as a DNA binding protein and could potentially bind to
436 the promoter region of the *Cj1556* gene to repress further expression as indicated by the
437 microarray data, electrophoretic mobility shift assays were performed. The full length Cj1556
438 protein was expressed and purified from *E. coli*. Binding of this recombinant Cj1556 protein
439 to a 170 bp DNA fragment upstream of the *Cj1556* gene was observed, indicating a
440 Protein:DNA complex (Fig. 6). Such binding of recombinant Cj1556 was not observed with
441 DNA fragments representing the promoter regions of the negative control genes *flaA* and
442 *flgK*. This data indicates that Cj1556 acts as a DNA binding protein and also supports the
443 microarray data that suggests a negative autoregulation system for the expression of Cj1556.
444 Negative autoregulation is often a feature of the MarR family of transcriptional regulators
445 (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

473 **Discussion**

474 The human intestinal pathogen *C. jejuni* must survive diverse conditions in different hosts
475 and also in the environment. The ability of *C. jejuni* to survive both oxidative and aerobic
476 (O₂) stress conditions is fundamental considering the ubiquity of this bacterial pathogen.
477 During re-annotation of the *C. jejuni* NCTC11168 genome sequence (29), Cj1556 was
478 identified as a putative transcriptional regulator. Based on motif and protein interaction data,
479 we hypothesised that Cj1556 was an important *C. jejuni* stress response regulator and
480 therefore investigated the ability of a *Cj1556* mutant to survive different stresses and further
481 explore the role of Cj1556 during host-pathogen interactions.

482 In addition to Cj1556, the *C. jejuni* NCTC11168 genome contains another CDS (Cj1546)
483 with the MarR family motif. Cj1546 was also re-annotated as a putative transcriptional
484 regulator with 43.6% identity and 58.4% similarity to Cj1556. Analysis of a comparative
485 genomics microarray dataset containing 111 *C. jejuni* strains (16) identified *Cj1546* in over
486 95% of these *C. jejuni* strains and *Cj1556* in approximately 50% of these *C. jejuni* strains.
487 One hypothesis as to the function of these MarR motif-containing proteins is that both
488 perform similar roles in relation to aerobic (O₂) and oxidative stress, however whilst all *C.*
489 *jejuni* strains contain Cj1546, strains such as *C. jejuni* NCTC11168 and 81-176 that also
490 contain Cj1556 may have a greater ability for survival within the human host due to greater
491 resistance to oxidative stresses.

492 Oxidative stress assays showed that the *Cj1556* mutant has an increased sensitivity to
493 oxidative stress compared to the 11168H wild-type strain and that the wild-type level of
494 sensitivity to oxidative stress was fully restored with complementation of the *Cj1556*
495 mutation. In fact the *Cj1556* complement demonstrated even greater resistance to H₂O₂ than
496 the 11168H wild-type strain, possibly due to the strength of the promoter as the
497 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,

523 the *Cj1556* mutant was also more susceptible to extracellular stresses when compared to the
524 11168H wild-type strain. The *Cj1556* mutant exhibits increased sensitivity to H₂O₂ *in vitro*,
525 so it is reasonable to suggest ROS released by Caco-2 cells during these experiments will
526 have an effect on *C. jejuni* survival. Standard co-culture assays will not only result in
527 exposure of *C. jejuni* to ROS released by Caco-2 cells but also to aerobic (O₂) stress as the
528 assays are performed in a CO₂ incubator. The approximate atmospheric O₂ and CO₂ levels are
529 around 21% and 0.04% respectively. During co-culture experiments, the level of CO₂ will be
530 around 5% and so the O₂ level will be around 16-18%. Based on the relative levels of
531 survival between the interaction, invasion and intracellular survival assays, we hypothesised
532 the greater level of sensitivity exhibited by the *Cj1556* mutant during the interaction assay
533 may be in part due to increased exposure of extracellular *C. jejuni* to aerobic (O₂) stress.
534 Aerobic survival assays were performed to replicate the conditions during the interaction,
535 invasion and intracellular survival assays by incubating *C. jejuni* in tissue culture media but
536 in the absence of Caco-2 cells. A reduction in survival was observed for the *Cj1556* mutant
537 compared to the 11168H wild-type strain under these aerobic (O₂) stress conditions, but not
538 under microaerobic conditions. *C. jejuni* typically loses viability within intestinal epithelial
539 cells over 24 h with no evidence of intracellular replication (41). Evidence to date suggests
540 that *C. jejuni* reside in membrane bound compartments termed *C. jejuni* containing vacuole
541 (CCV), avoiding entry into lysosomes (81). *C. jejuni* engulfed by macrophages must resist a
542 combination of unfavourable conditions such as ROS. There are contradictory reports
543 regarding the ability of *C. jejuni* to survive within macrophages, depending on the
544 macrophage cell type and *C. jejuni* strain used (20, 79). In this study, the *Cj1556* mutant
545 exhibited reduced intracellular survival within the mouse macrophage J774A.1 cell line.
546 Taken together, this data indicates that *Cj1556* plays a multi-factorial role in bacterial
547 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 CO₂ 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

573 absence of sDOC (45). Microarray analysis has shown that a number of *C. jejuni* virulence
574 factors are up-regulated in the presence of 0.1% (w/v) sDOC, including *ciaB*, *cmeABC*, *dccR*
575 and *tlyA* (45). Interestingly, *Cj1556* was also up-regulated in the presence of sDOC, with
576 transcription increased 2.8 fold (45). The transcriptional response of *E. coli* O157:H7 to bile
577 treatment has also been investigated using microarrays and has identified bile-induced
578 changes in transcription for gene encoding proteins affecting membrane structure and
579 permeability, bile resistance, adhesion and virulence potential (30). Most interestingly this
580 data indicates that bile induces expression of the *marRAB* operon, by binding to the repressor
581 protein MarR and thus preventing binding of MarR to the *marRAB* promoter site (30).
582 *Cj1556* is a member of the MarR family of transcriptional regulators and further studies will
583 be required to confirm whether bile can bind to the *Cj1556* protein and thus prevent binding
584 to the *Cj1556* promoter site, resulting in the up-regulation of *Cj1556* in the presence of bile
585 observed previously (45).

586 Microarray analysis of the *Cj1556* mutant identified *Cj1556* as the most up-regulated gene.
587 Analysis of the *Cj1556* nucleotide sequence upstream of the Km^R cassette in the *Cj1556*
588 mutant confirmed that this was the sequence printed on the oligonucleotide array, suggesting
589 that expression of *Cj1556* is controlled by a negative autoregulation feedback mechanism. In
590 the wild-type strain, basal levels of *Cj1556* would block further expression of *Cj1556* by
591 inhibiting the binding of RNA polymerase to the *Cj1556* promoter site. However in the
592 absence of *Cj1556* in the *Cj1556* mutant, expression of *Cj1556* can continue. Such negative
593 autoregulation is a feature of the MarR family of transcriptional regulators. In this study,
594 experiments confirmed the binding of recombinant *Cj1556* to a 170 bp DNA fragment
595 upstream of the *Cj1556* gene, confirming the DNA binding ability of *Cj1556*. To confirm this
596 was not a non-specific artefact, two random negative control promoter regions were selected
597 (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 (O₂) 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 (O₂) 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

623 nitrogen (9) and the complex enzymatic cascades that regulate coagulation or melanisation of
624 haemolymph (50). Cellular defence refers to haemocyte-mediated immune responses like
625 phagocytosis, nodulation and encapsulation (66, 70). Haemocytes perform many of the
626 functions of phagocytic cells in mammals, are capable of ingesting bacterial pathogens and
627 generating bactericidal compounds such as superoxide via a respiratory burst (8, 15). After
628 infection of *G. mellonella* with *Yersinia pseudotuberculosis*, bacteria accumulate in
629 haemocytes, thus suggesting that *G. mellonella* may be useful for the identification of other
630 genes associated with intracellular survival (15). Infection with the *Cj1556* mutant resulted in
631 increased survival of *G. mellonella* larvae compared to survival after infection with the
632 11168H wild-type strain. This suggests the *Cj1556* mutant is more susceptible to the host
633 immune mechanisms resulting in reduced bacterial survival within *G. mellonella*. At least six
634 types of haemocytes have been identified in insects such as *G. mellonella* with plasmatocytes
635 and granulocytes the most abundant (10). Production of ROS has also been detected in
636 haemocytes with evidence of both oxygen radicals and H₂O₂ both found in plasmatocytes of
637 *G. mellonella* (68). This data links the increased sensitivity of the *Cj1556* mutant to H₂O₂
638 stress *in vitro* with an attenuation of virulence *in vivo* using the *G. mellonella* model of
639 infection.

640 In summary, the basis of *C. jejuni* survival is dependent upon the ability to sense and respond
641 to the different environments encountered within hosts and in the environment. This study
642 has identified a novel *C. jejuni* transcriptional regulator *Cj1556* that is involved in oxidative
643 and aerobic (O₂) stress responses and is important for the survival of *C. jejuni* in the natural
644 environment and *in vivo*.

645

646 **Acknowledgements**

647 We thank Dennis Linton (University of Manchester, UK) for providing the *C. jejuni*
648 NCTC11168 complementation vector. We thank Madeleine Moule, Sofia Lourenco and
649 Meredith Stewart (all LSHTM) for technical support and guidance. Dominic Mills was
650 supported by a Bloomsbury Colleges PhD Studentship (2007-2010). We acknowledge
651 BμG@S (the Bacterial Microarray Group at St George's, University of London) for supply of
652 the microarray and advice and The Wellcome Trust for funding this multi-collaborative
653 microbial pathogen microarray facility under the Functional Genomics Resources Initiative.
654
655

656 **References**

- 657 1. **Allos, B. M.** 2001. *Campylobacter jejuni* Infections: update on emerging issues and
658 trends. Clin Infect Dis **32**:1201-6.
- 659 2. **Amabile-Cuevas, C. F., and B. Demple.** 1991. Molecular characterization of the
660 *soxRS* genes of *Escherichia coli*: two genes control a superoxide stress regulon.
661 Nucleic Acids Res **19**:4479-84.
- 662 3. **Andersen, M. T., L. Brondsted, B. M. Pearson, F. Mulholland, M. Parker, C. Pin,
663 J. M. Wells, and H. Ingmer.** 2005. Diverse roles for HspR in *Campylobacter jejuni*
664 revealed by the proteome, transcriptome and phenotypic characterization of an *hspR*
665 mutant. Microbiology **151**:905-15.
- 666 4. **Atack, J. M., and D. J. Kelly.** 2008. Contribution of the stereospecific methionine
667 sulphoxide reductases MsrA and MsrB to oxidative and nitrosative stress resistance in
668 the food-borne pathogen *Campylobacter jejuni*. Microbiology **154**:2219-30.
- 669 5. **Bacon, J., B. W. James, L. Wernisch, A. Williams, K. A. Morley, G. J. Hatch, J.
670 A. Mangan, J. Hinds, N. G. Stoker, P. D. Butcher, and P. D. Marsh.** 2004. The
671 influence of reduced oxygen availability on pathogenicity and gene expression in
672 *Mycobacterium tuberculosis*. Tuberculosis (Edinb) **84**:205-17.
- 673 6. **Baillon, M. L., A. H. van Vliet, J. M. Ketley, C. Constantinidou, and C. W. Penn.**
674 1999. An iron-regulated alkyl hydroperoxide reductase (AhpC) confers aerotolerance
675 and oxidative stress resistance to the microaerophilic pathogen *Campylobacter jejuni*.
676 J Bacteriol **181**:4798-804.
- 677 7. **Begley, M., C. G. Gahan, and C. Hill.** 2005. The interaction between bacteria and
678 bile. FEMS Microbiol Rev **29**:625-51.
- 679 8. **Bergin, D., E. P. Reeves, J. Renwick, F. B. Wientjes, and K. Kavanagh.** 2005.
680 Superoxide production in *Galleria mellonella* hemocytes: identification of proteins
681 homologous to the NADPH oxidase complex of human neutrophils. Infect Immun
682 **73**:4161-70.
- 683 9. **Bogdan, C., M. Rollinghoff, and A. Diefenbach.** 2000. Reactive oxygen and
684 reactive nitrogen intermediates in innate and specific immunity. Curr Opin Immunol
685 **12**:64-76.
- 686 10. **Boman, H. G., and D. Hultmark.** 1987. Cell-free immunity in insects. Annu Rev
687 Microbiol **41**:103-26.
- 688 11. **Boor, K. J.** 2006. Bacterial stress responses: what doesn't kill them can make them
689 stronger. PLoS Biol **4**:e23.
- 690 12. **Brondsted, L., M. T. Andersen, M. Parker, K. Jorgensen, and H. Ingmer.** 2005.
691 The HtrA protease of *Campylobacter jejuni* is required for heat and oxygen tolerance
692 and for optimal interaction with human epithelial cells. Appl Environ Microbiol
693 **71**:3205-12.
- 694 13. **Buswell, C. M., Y. M. Herlihy, L. M. Lawrence, J. T. McGuiggan, P. D. Marsh,
695 C. W. Keevil, and S. A. Leach.** 1998. Extended survival and persistence of
696 *Campylobacter* spp. in water and aquatic biofilms and their detection by
697 immunofluorescent-antibody and -rRNA staining. Appl Environ Microbiol **64**:733-
698 41.
- 699 14. **Cabiscol, E., J. Tamarit, and J. Ros.** 2000. Oxidative stress in bacteria and protein
700 damage by reactive oxygen species. Int Microbiol **3**:3-8.
- 701 15. **Champion, O. L., I. A. Cooper, S. L. James, D. Ford, A. Karlyshev, B. W. Wren,
702 M. Duffield, P. C. Oyston, and R. W. Titball.** 2009. *Galleria mellonella* as an
703 alternative infection model for *Yersinia pseudotuberculosis*. Microbiology **155**:1516-
704 22.

- 705 16. **Champion, O. L., M. W. Gaunt, O. Gundogdu, A. Elmi, A. A. Witney, J. Hinds,**
706 **N. Dorrell, and B. W. Wren.** 2005. Comparative phylogenomics of the food-borne
707 pathogen *Campylobacter jejuni* reveals genetic markers predictive of infection source.
708 Proceedings of the National Academy of Sciences of the United States of America
709 **102:16043-16048.**
- 710 17. **Champion, O. L., A. V. Karlyshev, N. J. Senior, M. Woodward, R. La Ragione,**
711 **S. L. Howard, B. W. Wren, and R. W. Titball.** 2010. Insect infection model for
712 *Campylobacter jejuni* reveals that *O*-methyl phosphoramidate has insecticidal
713 activity. *J Infect Dis* **201:776-82.**
- 714 18. **Corcionivoschi, N., M. Clyne, A. Lyons, A. Elmi, O. Gundogdu, B. W. Wren, N.**
715 **Dorrell, A. V. Karlyshev, and B. Bourke.** 2009. *Campylobacter jejuni* alters surface
716 capsular polysaccharide when co-cultured with epithelial cells. *Infect Immun.*
- 717 19. **Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M.**
718 **Lappin-Scott.** 1995. Microbial biofilms. *Annu Rev Microbiol* **49:711-45.**
- 719 20. **Day, W. A., Jr., J. L. Sajecki, T. M. Pitts, and L. A. Joens.** 2000. Role of catalase
720 in *Campylobacter jejuni* intracellular survival. *Infect Immun* **68:6337-45.**
- 721 21. **Dorrell, N., J. A. Mangan, K. G. Laing, J. Hinds, D. Linton, H. Al-Ghusein, B. G.**
722 **Barrell, J. Parkhill, N. G. Stoker, A. V. Karlyshev, P. D. Butcher, and B. W.**
723 **Wren.** 2001. Whole genome comparison of *Campylobacter jejuni* human isolates
724 using a low-cost microarray reveals extensive genetic diversity. *Genome Res*
725 **11:1706-15.**
- 726 22. **Duncan, C., H. Dougall, P. Johnston, S. Green, R. Brogan, C. Leifert, L. Smith,**
727 **M. Golden, and N. Benjamin.** 1995. Chemical generation of nitric oxide in the
728 mouth from the enterosalivary circulation of dietary nitrate. *Nat Med* **1:546-51.**
- 729 23. **Eivers, K. T., S. M. Turner, L. M. Wainwright, G. Marsden, J. Hinds, J. A. Cole,**
730 **R. K. Poole, C. W. Penn, and S. F. Park.** 2005. NssR, a member of the Crp-Fnr
731 superfamily from *Campylobacter jejuni*, regulates a nitrosative stress-responsive
732 regulon that includes both a single-domain and a truncated haemoglobin. *Mol*
733 *Microbiol* **57:735-50.**
- 734 24. **Eriksson, S., S. Lucchini, A. Thompson, M. Rhen, and J. C. Hinton.** 2003.
735 Unravelling the biology of macrophage infection by gene expression profiling of
736 intracellular *Salmonella enterica*. *Mol Microbiol* **47:103-18.**
- 737 25. **Fang, F. C.** 2004. Antimicrobial reactive oxygen and nitrogen species: concepts and
738 controversies. *Nat Rev Microbiol* **2:820-32.**
- 739 26. **Fields, J. A., and S. A. Thompson.** 2008. *Campylobacter jejuni* CsrA mediates
740 oxidative stress responses, biofilm formation, and host cell invasion. *J Bacteriol*
741 **190:3411-6.**
- 742 27. **Friis, L. M., M. Keelan, and D. E. Taylor.** 2009. *Campylobacter jejuni* drives
743 MyD88-independent interleukin-6 secretion via Toll-like receptor 2. *Infect Immun*
744 **77:1553-60.**
- 745 28. **Gaynor, E. C., D. H. Wells, J. K. MacKichan, and S. Falkow.** 2005. The
746 *Campylobacter jejuni* stringent response controls specific stress survival and
747 virulence-associated phenotypes. *Mol Microbiol* **56:8-27.**
- 748 29. **Gundogdu, O., S. D. Bentley, M. T. Holden, J. Parkhill, N. Dorrell, and B. W.**
749 **Wren.** 2007. Re-annotation and re-analysis of the *Campylobacter jejuni* NCTC11168
750 genome sequence. *BMC Genomics* **8:162.**
- 751 30. **Hamner, S., K. McInnerney, K. Williamson, M. J. Franklin, and T. E. Ford.**
752 2010. Bile salts influence the ordered expression of virulence genes in *Escherichia*
753 *coli* O157:H7. Society of General Microbiology, Abstract ED12/12.
- 754 31. **Hitchen, P., J. Brzostek, M. Panico, J. A. Butler, H. R. Morris, A. Dell, and D.**

- 755 **Linton.** 2010. Modification of the *Campylobacter jejuni* flagellin glycan by the
756 product of the Cj1295 homopolymeric tract containing gene. *Microbiology*.
- 757 32. **Hobbie, S., L. M. Chen, R. J. Davis, and J. E. Galan.** 1997. Involvement of
758 mitogen-activated protein kinase pathways in the nuclear responses and cytokine
759 production induced by *Salmonella typhimurium* in cultured intestinal epithelial cells. *J*
760 *Immunol* **159**:5550-9.
- 761 33. **Imlay, J. A., and S. Linn.** 1986. Bimodal pattern of killing of DNA-repair-defective
762 or anoxically grown *Escherichia coli* by hydrogen peroxide. *J Bacteriol* **166**:519-27.
- 763 34. **Iovine, N. M., S. Pursnani, A. Voldman, G. Wasserman, M. J. Blaser, and Y.**
764 **Weinrauch.** 2008. Reactive nitrogen species contribute to innate host defense against
765 *Campylobacter jejuni*. *Infect Immun* **76**:986-93.
- 766 35. **Jones, M. A., K. L. Marston, C. A. Woodall, D. J. Maskell, D. Linton, A. V.**
767 **Karlyshev, N. Dorrell, B. W. Wren, and P. A. Barrow.** 2004. Adaptation of
768 *Campylobacter jejuni* NCTC11168 to high-level colonization of the avian
769 gastrointestinal tract. *Infect Immun* **72**:3769-76.
- 770 36. **Joshua, G. W., C. Guthrie-Irons, A. V. Karlyshev, and B. W. Wren.** 2006.
771 Biofilm formation in *Campylobacter jejuni*. *Microbiology* **152**:387-96.
- 772 37. **Kamal, N., N. Dorrell, A. Jagannathan, S. M. Turner, C. Constantinidou, D. J.**
773 **Studholme, G. Marsden, J. Hinds, K. G. Laing, B. W. Wren, and C. W. Penn.**
774 2007. Deletion of a previously uncharacterized flagellar-hook-length control gene *fliK*
775 modulates the sigma54-dependent regulon in *Campylobacter jejuni*. *Microbiology*
776 **153**:3099-111.
- 777 38. **Karlyshev, A. V., D. Linton, N. A. Gregson, and B. W. Wren.** 2002. A novel
778 paralogous gene family involved in phase-variable flagella-mediated motility in
779 *Campylobacter jejuni*. *Microbiology* **148**:473-80.
- 780 39. **Ketley, J. M.** 1997. Pathogenesis of enteric infection by *Campylobacter*.
781 *Microbiology* **143 (Pt 1)**:5-21.
- 782 40. **Klancnik, A., N. Botteldoorn, L. Herman, and S. S. Mozina.** 2006. Survival and
783 stress induced expression of *groEL* and *rpoD* of *Campylobacter jejuni* from different
784 growth phases. *Int J Food Microbiol* **112**:200-7.
- 785 41. **Konkel, M. E., S. F. Hayes, L. A. Joens, and W. Cieplak, Jr.** 1992. Characteristics
786 of the internalization and intracellular survival of *Campylobacter jejuni* in human
787 epithelial cell cultures. *Microb Pathog* **13**:357-70.
- 788 42. **Lavine, M. D., and M. R. Strand.** 2002. Insect hemocytes and their role in
789 immunity. *Insect Biochem Mol Biol* **32**:1295-309.
- 790 43. **Linton, D., N. Dorrell, P. G. Hitchen, S. Amber, A. V. Karlyshev, H. R. Morris,**
791 **A. Dell, M. A. Valvano, M. Aebi, and B. W. Wren.** 2005. Functional analysis of the
792 *Campylobacter jejuni* N-linked protein glycosylation pathway. *Mol Microbiol*
793 **55**:1695-703.
- 794 44. **Lucchini, S., H. Liu, Q. Jin, J. C. Hinton, and J. Yu.** 2005. Transcriptional
795 adaptation of *Shigella flexneri* during infection of macrophages and epithelial cells:
796 insights into the strategies of a cytosolic bacterial pathogen. *Infect Immun* **73**:88-102.
- 797 45. **Malik-Kale, P., C. T. Parker, and M. E. Konkel.** 2008. Culture of *Campylobacter*
798 *jejuni* with sodium deoxycholate induces virulence gene expression. *J Bacteriol*
799 **190**:2286-97.
- 800 46. **McLennan, M. K., D. D. Ringoir, E. Frirdich, S. L. Svensson, D. H. Wells, H.**
801 **Jarrell, C. M. Szymanski, and E. C. Gaynor.** 2008. *Campylobacter jejuni* biofilms
802 up-regulated in the absence of the stringent response utilize a calcofluor white-
803 reactive polysaccharide. *J Bacteriol* **190**:1097-107.
- 804 47. **Meister, M., C. Hetru, and J. A. Hoffmann.** 2000. The antimicrobial host defense

- 805 of *Drosophila*. *Curr Top Microbiol Immunol* **248**:17-36.
- 806 48. **Mihaljevic, R. R., M. Sikic, A. Klancnik, G. Brumini, S. S. Mozina, and M.**
807 **Abram.** 2007. Environmental stress factors affecting survival and virulence of
808 *Campylobacter jejuni*. *Microb Pathog* **43**:120-5.
- 809 49. **Monk, C. E., B. M. Pearson, F. Mulholland, H. K. Smith, and R. K. Poole.** 2008.
810 Oxygen- and NssR-dependent globin expression and enhanced iron acquisition in the
811 response of campylobacter to nitrosative stress. *J Biol Chem* **283**:28413-25.
- 812 50. **Muta, T., and S. Iwanaga.** 1996. The role of hemolymph coagulation in innate
813 immunity. *Curr Opin Immunol* **8**:41-7.
- 814 51. **Mylonakis, E., A. Casadevall, and F. M. Ausubel.** 2007. Exploiting amoeboid and
815 non-vertebrate animal model systems to study the virulence of human pathogenic
816 fungi. *PLoS Pathog* **3**:e101.
- 817 52. **Nachamkin, I., B. M. Allos, and T. Ho.** 1998. *Campylobacter* species and Guillain-
818 Barre syndrome. *Clin Microbiol Rev* **11**:555-67.
- 819 53. **Naito, M., E. Firdich, J. A. Fields, M. Pryjma, J. Li, A. Cameron, M. Gilbert, S.**
820 **A. Thompson, and E. C. Gaynor.** 2010. Effects of sequential *Campylobacter jejuni*
821 81-176 lipooligosaccharide core truncations on biofilm formation, stress survival, and
822 pathogenesis. *J Bacteriol* **192**:2182-92.
- 823 54. **Nguyen, H. T., J. E. Corry, and C. A. Miles.** 2006. Heat resistance and mechanism
824 of heat inactivation in thermophilic campylobacters. *Appl Environ Microbiol* **72**:908-
825 13.
- 826 55. **Olson, C. K., S. Ethelberg, W. v. Pelt, and R. V. Tauxe.** 2008. Epidemiology of
827 *Campylobacter jejuni* Infections in Industrialized nations. In *Campylobacter* Third
828 Edition, I. Nachmkin, C.M. Szymanski, and M.J. Blaser, eds (ASM Press), pp.163-
829 189.
- 830 56. **Oppenheim, J. J., C. O. Zachariae, N. Mukaida, and K. Matsushima.** 1991.
831 Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu*
832 *Rev Immunol* **9**:617-48.
- 833 57. **Palyada, K., Y. Q. Sun, A. Flint, J. Butcher, H. Naikare, and A. Stintzi.** 2009.
834 Characterization of the oxidative stress stimulon and PerR regulon of *Campylobacter*
835 *jejuni*. *BMC Genomics* **10**:481.
- 836 58. **Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T.**
837 **Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev,**
838 **S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M.**
839 **Rutherford, A. H. van Vliet, S. Whitehead, and B. G. Barrell.** 2000. The genome
840 sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable
841 sequences. *Nature* **403**:665-8.
- 842 59. **Parkins, M. D., H. Ceri, and D. G. Storey.** 2001. *Pseudomonas aeruginosa* GacA, a
843 factor in multihost virulence, is also essential for biofilm formation. *Mol Microbiol*
844 **40**:1215-26.
- 845 60. **Parrish, J., J. Yu, G. Liu, J. Hines, J. Chan, B. Mangiola, H. Zhang, S. Pacifico,**
846 **F. Fotouhi, V. DiRita, T. Ideker, P. Andrews, and R. Finley.** 2007. A proteome-
847 wide protein interaction map for *Campylobacter jejuni*. *Genome Biology* **8**:R130.
- 848 61. **Pesci, E. C., D. L. Cottle, and C. L. Pickett.** 1994. Genetic, enzymatic, and
849 pathogenic studies of the iron superoxide dismutase of *Campylobacter jejuni*. *Infect*
850 *Immun* **62**:2687-94.
- 851 62. **Philpott, D. J., S. Yamaoka, A. Israel, and P. J. Sansonetti.** 2000. Invasive *Shigella*
852 *flexneri* activates NF-kappa B through a lipopolysaccharide-dependent innate
853 intracellular response and leads to IL-8 expression in epithelial cells. *J Immunol*
854 **165**:903-14.

- 855 63. **Phongsisay, V., V. N. Perera, and B. N. Fry.** 2007. Expression of the *htrB* gene is
856 essential for responsiveness of *Salmonella typhimurium* and *Campylobacter jejuni* to
857 harsh environments. *Microbiology* **153**:254-62.
- 858 64. **Pittman, M. S., K. T. Elvers, L. Lee, M. A. Jones, R. K. Poole, S. F. Park, and D.
859 J. Kelly.** 2007. Growth of *Campylobacter jejuni* on nitrate and nitrite: electron
860 transport to NapA and NrfA via NrfH and distinct roles for NrfA and the globin Cgb
861 in protection against nitrosative stress. *Mol Microbiol* **63**:575-90.
- 862 65. **Reuter, M., A. Mallett, B. M. Pearson, and A. H. van Vliet.** 2010. Biofilm
863 formation by *Campylobacter jejuni* is increased under aerobic conditions. *Appl
864 Environ Microbiol* **76**:2122-8.
- 865 66. **Schmidt, O., U. Theopold, and M. Strand.** 2001. Innate immunity and its evasion
866 and suppression by hymenopteran endoparasitoids. *Bioessays* **23**:344-51.
- 867 67. **Sikic Pogacar, M., R. Rubesa Mihaljevic, A. Klancnik, G. Brumini, M. Abram,
868 and S. Smole Mozina.** 2009. Survival of stress exposed *Campylobacter jejuni* in the
869 murine macrophage J774 cell line. *Int J Food Microbiol* **129**:68-73.
- 870 68. **Slepneva, I. A., V. V. Glupov, S. V. Sergeeva, and V. V. Khramtsov.** 1999. EPR
871 detection of reactive oxygen species in hemolymph of *Galleria mellonella* and
872 *Dendrolimus superans sibiricus* (Lepidoptera) larvae. *Biochem Biophys Res
873 Commun* **264**:212-5.
- 874 69. **Stead, D., and S. F. Park.** 2000. Roles of Fe superoxide dismutase and catalase in
875 resistance of *Campylobacter coli* to freeze-thaw stress. *Appl Environ Microbiol*
876 **66**:3110-2.
- 877 70. **Strand, M. R., and L. L. Pech.** 1995. Immunological basis for compatibility in
878 parasitoid-host relationships. *Annu Rev Entomol* **40**:31-56.
- 879 71. **Svensson, S. L., L. M. Davis, J. K. MacKichan, B. J. Allan, M. Pajaniappan, S. A.
880 Thompson, and E. C. Gaynor.** 2009. The CprS sensor kinase of the zoonotic
881 pathogen *Campylobacter jejuni* influences biofilm formation and is required for
882 optimal chick colonization. *Mol Microbiol* **71**:253-72.
- 883 72. **Svensson, S. L., E. Fridrich, and E. C. Gaynor.** 2008. Survival strategies of
884 *Campylobacter jejuni*: stress responses, the viable but nonculturable state, and
885 biofilms. In *Campylobacter* Third Edition, I. Nachmkin, C.M. Szymanski, and M.J.
886 Blaser, eds (ASM Press), pp.571-590.
- 887 73. **Trieu-Cuot, P., G. Gerbaud, T. Lambert, and P. Courvalin.** 1985. *In vivo* transfer
888 of genetic information between gram-positive and gram-negative bacteria. *Embo J*
889 **4**:3583-7.
- 890 74. **van Vliet, A. H., M. A. Baillon, C. W. Penn, and J. M. Ketley.** 2001. The iron-
891 induced ferredoxin FdxA of *Campylobacter jejuni* is involved in aerotolerance. *FEMS
892 Microbiol Lett* **196**:189-93.
- 893 75. **van Vliet, A. H., M. L. Baillon, C. W. Penn, and J. M. Ketley.** 1999.
894 *Campylobacter jejuni* contains two fur homologs: characterization of iron-responsive
895 regulation of peroxide stress defense genes by the PerR repressor. *J Bacteriol*
896 **181**:6371-6.
- 897 76. **van Vliet, A. H., K. G. Wooldridge, and J. M. Ketley.** 1998. Iron-responsive gene
898 regulation in a *Campylobacter jejuni* fur mutant. *J Bacteriol* **180**:5291-8.
- 899 77. **Wainwright, L. M., K. T. Elvers, S. F. Park, and R. K. Poole.** 2005. A truncated
900 haemoglobin implicated in oxygen metabolism by the microaerophilic food-borne
901 pathogen *Campylobacter jejuni*. *Microbiology* **151**:4079-91.
- 902 78. **Wainwright, L. M., Y. Wang, S. F. Park, S. R. Yeh, and R. K. Poole.** 2006.
903 Purification and spectroscopic characterization of Ctb, a group III truncated
904 hemoglobin implicated in oxygen metabolism in the food-borne pathogen

- 905 *Campylobacter jejuni*. Biochemistry **45**:6003-11.
- 906 79. **Wassenaar, T. M., M. Engelskirchen, S. Park, and A. Lastovica.** 1997.
- 907 Differential uptake and killing potential of *Campylobacter jejuni* by human peripheral
- 908 monocytes/macrophages. Med Microbiol Immunol (Berl) **186**:139-44.
- 909 80. **Watson, R. O., and J. E. Galan.** 2008. *Campylobacter jejuni* survives within
- 910 epithelial cells by avoiding delivery to lysosomes. PLoS Pathog **4**:e14.
- 911 81. **Watson, R. O., and J. E. Galán.** 2008. Interaction of *Campylobacter jejuni* with host
- 912 cells. In *Campylobacter* Third Edition, I. Nachmkin, C.M. Szymanski, and M.J.
- 913 Blaser, eds (ASM Press), pp.289-296.
- 914 82. **Wilkinson, S. P., and A. Grove.** 2004. HucR, a novel uric acid-responsive member
- 915 of the MarR family of transcriptional regulators from *Deinococcus radiodurans*. J
- 916 Biol Chem **279**:51442-50.
- 917 83. **Wosten, M. M., M. Boeve, W. Gaastra, and B. A. van der Zeijst.** 1998. Cloning
- 918 and characterization of the gene encoding the primary sigma-factor of *Campylobacter*
- 919 *jejuni*. FEMS Microbiol Lett **162**:97-103.
- 920 84. **Wosten, M. M., C. T. Parker, A. van Mourik, M. R. Guilhabert, L. van Dijk, and**
- 921 **J. P. van Putten.** 2006. The *Campylobacter jejuni* PhosS/PhosR operon represents a
- 922 non-classical phosphate-sensitive two-component system. Mol Microbiol **62**:278-91.
- 923 85. **Wösten, M. M. S. N., A. V. Mourik, and J. P. M. V. Putten.** 2008. Regulation of
- 924 Genes in *Campylobacter jejuni*. In *Campylobacter* Third Edition, I. Nachmkin, C.M.
- 925 Szymanski, and M.J. Blaser, eds (ASM Press), pp.611-624.
- 926 86. **Yang, I. V., E. Chen, J. P. Hasseman, W. Liang, B. C. Frank, S. Wang, V.**
- 927 **Sharov, A. I. Saeed, J. White, J. Li, N. H. Lee, T. J. Yeatman, and J.**
- 928 **Quackenbush.** 2002. Within the fold: assessing differential expression measures and
- 929 reproducibility in microarray assays. Genome Biol **3**:research0062.
- 930 87. **Zaki, M. H., T. Akuta, and T. Akaike.** 2005. Nitric oxide-induced nitrate stress
- 931 involved in microbial pathogenesis. J Pharmacol Sci **98**:117-29.
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936 **Tables**

937

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

939

Primer Name	Sequence
<i>Cj1556</i> -F	ATCATTCTCTTTGTCCTAT
<i>Cj1556</i> -R	TAAGATGGATTCTAAACTATTG
Km ^R forward-out	TGGGTTTCAAGCATTAGTCCATGCAAG
Km ^R reverse-out	GTGGTATGACATTGCCTTCTGCG
Comp- <i>Cj1556</i> -F	CCCCCATGGATAAGGATTTATAATGAAAAAATATCATTCTCT
Comp- <i>Cj1556</i> -R	CCCGCTAGCTTAAACGATATTTTTATAGCTAT
Comp- <i>Cj1556</i> -R-HIS	CCCGCTAGCTTAATGATGATGATGATGATGAACGATATTTTT ATAGCTAT
Upstream <i>Cj1556</i> - F	ATGCAATCTAGAAATTAT
Upstream <i>Cj1556</i> - R	GGACAAAGAGAATGATATT
Upstream <i>flaA</i> - F	ATCACAGCTTATATTAAAG
Upstream <i>flaA</i> - R	GTGTTAATACGAAATCCCAT
Upstream <i>flgK</i> - F	ATTTGTTCTTATTGTCAA
Upstream <i>flgK</i> - R	ATGTTCCAAAAATACCCAT

940

941

942 **Table 2. DNA fragments used as promoter probes for electrophoretic mobility shift**
943 **assays**

944

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

945

946

947 **Table 3. Changes in expression of genes linked to the *C. jejuni* oxidative and aerobic**
 948 **(O₂) stress responses in the *Cj1556* mutant compared to the 11168H wild-type strain**
 949

Gene Name	Fold Change	Product function
<i>spoT</i>	+1.26	putative guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase
<i>sodB</i>	+1.24	superoxide dismutase (Fe)
<i>htrA</i>	+1.21	serine protease (protease DO)
<i>fdxA</i>	+1.07	ferredoxin
<i>dcuA</i>	-1.17	anaerobic C4-dicarboxylate transporter
<i>ahpC</i>	-1.27	alkyl hydroperoxide reductase
<i>dps</i>	-1.36	putative bacterioferritin
<i>hspR</i>	-2.07	heat shock transcriptional regulator
<i>perR</i>	-5.05	peroxide stress regulator
<i>katA</i>	-5.13	catalase

950

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

952 analysis

953 **Figure Legends**

954

955 Fig. 1. Effect of oxidative (A) and heat stress (B) on the survival of *C. jejuni* 11168H wild-
956 type, *Cj1556* mutant and *Cj1556* complement strains (*Cj1556* comp). *C. jejuni* strains were
957 incubated with 10 mM H₂O₂ for 15 minutes at 37°C (A) or at 60°C for 5 minutes (B) and
958 bacterial survival assessed. Asterisks (*) denote a statistically significant difference ($p < 0.05$)
959 for *Cj1556* mutant compared to the 11168H wild-type strain.

960

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

968

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

976

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

984

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

990

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

996

997 Fig. 7. Biofilm assay on 11168H wild-type, *Cj1556* mutant and *Cj1556* complement strains
998 (*Cj1556* comp). *C. jejuni* biofilms were grown for 3 days, rinsed three times with PBS,
999 followed crystal violet staining. The asterisks denote a statistically significant difference ($**$
1000 $= p < 0.01$) for the *Cj1556* mutant compared to the 11168H wild-type strain.

1001

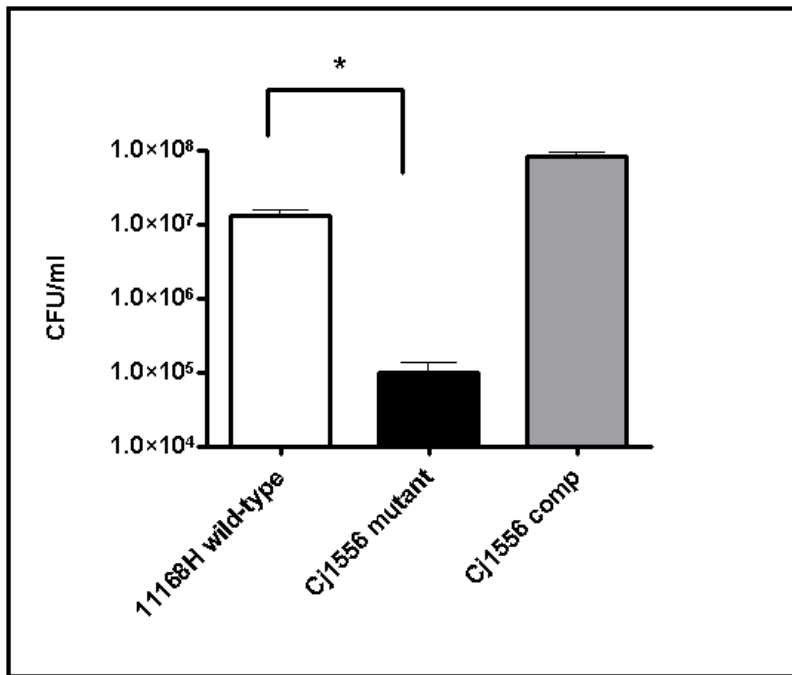
1002 Fig. 8. *G. mellonella* larvae were injected with a 10 µl inoculum of a 24 h *C. jejuni* culture
1003 diluted to OD₆₀₀ 0.1 by micro-injection in the right foremost leg, giving an infectious dose of
1004 approximately 10⁶ CFU. Larvae were incubated at 37°C with survival and appearance
1005 recorded at 24 h intervals. Brucella broth and no injection controls were used. For each
1006 experiment, 10 *G. mellonella* larvae were infected and experiments were repeated in
1007 triplicate. The asterisk denotes a statistically significant difference (* = $p < 0.05$) for *Cj1556*
1008 mutant compared to the 11168H wild-type strain.

1009

1010

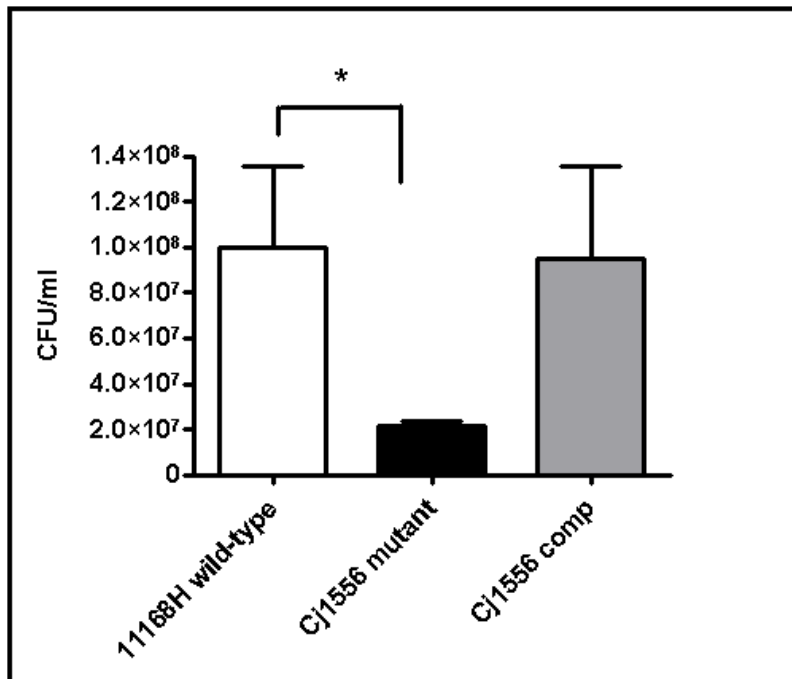
1011 **Figures**

1012 Fig. 1 (A)



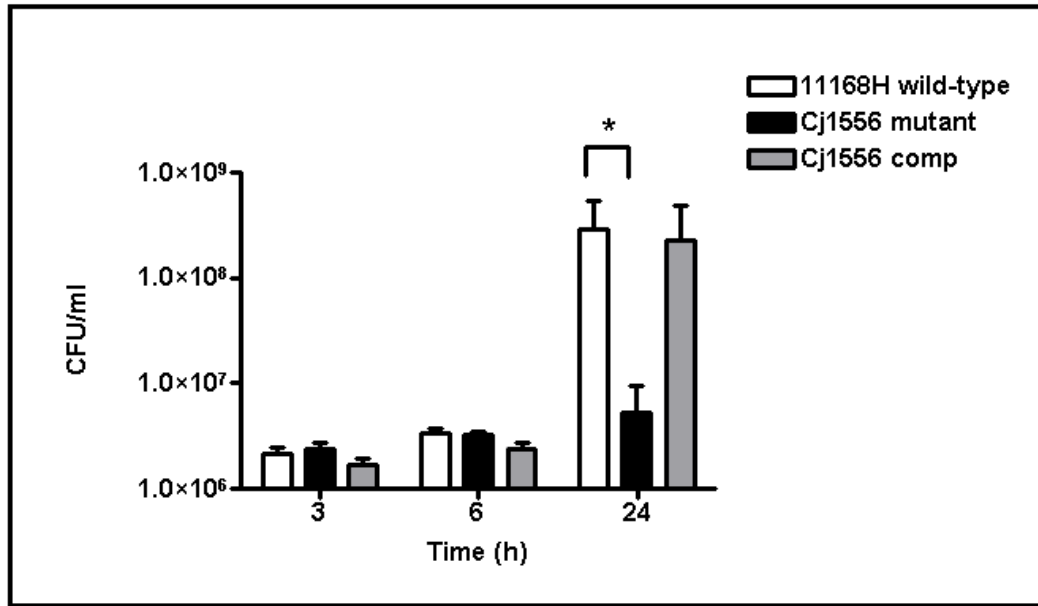
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1014 Fig. 1 (B)



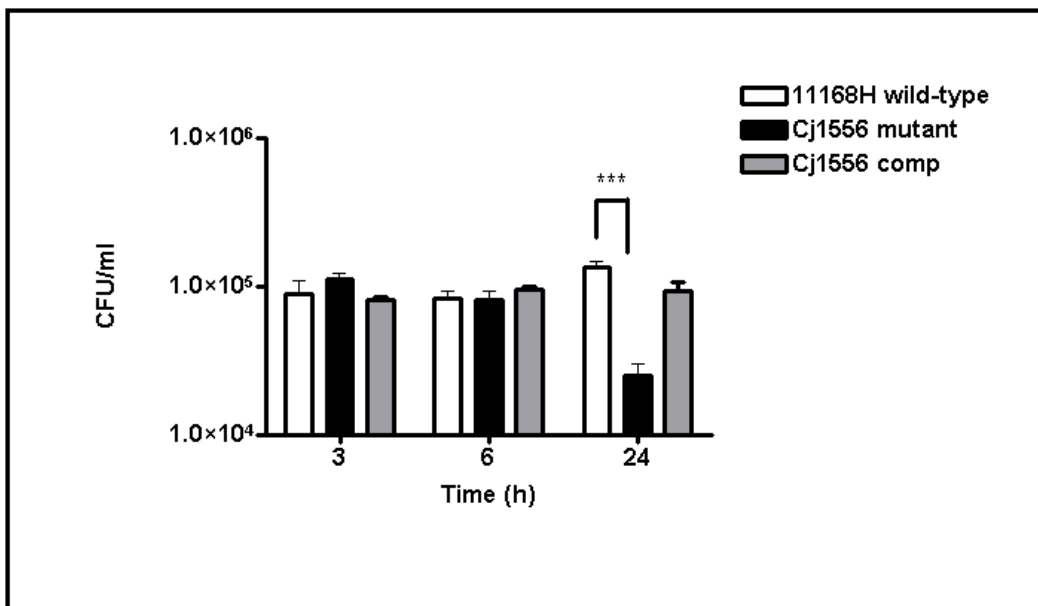
1015

1016 Fig. 2 (A)



1017

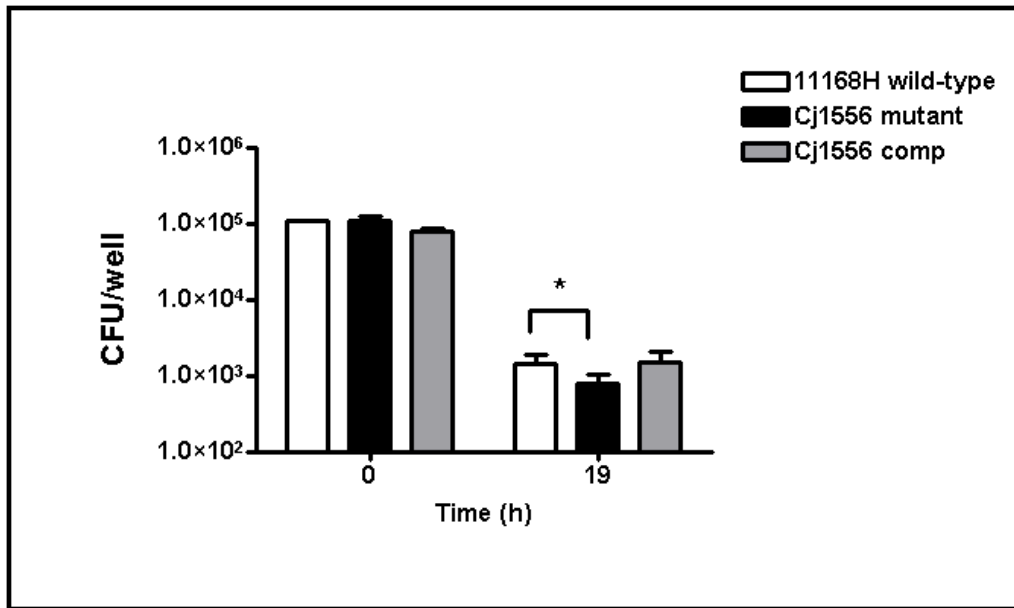
1018 Fig. 2 (B)



1019

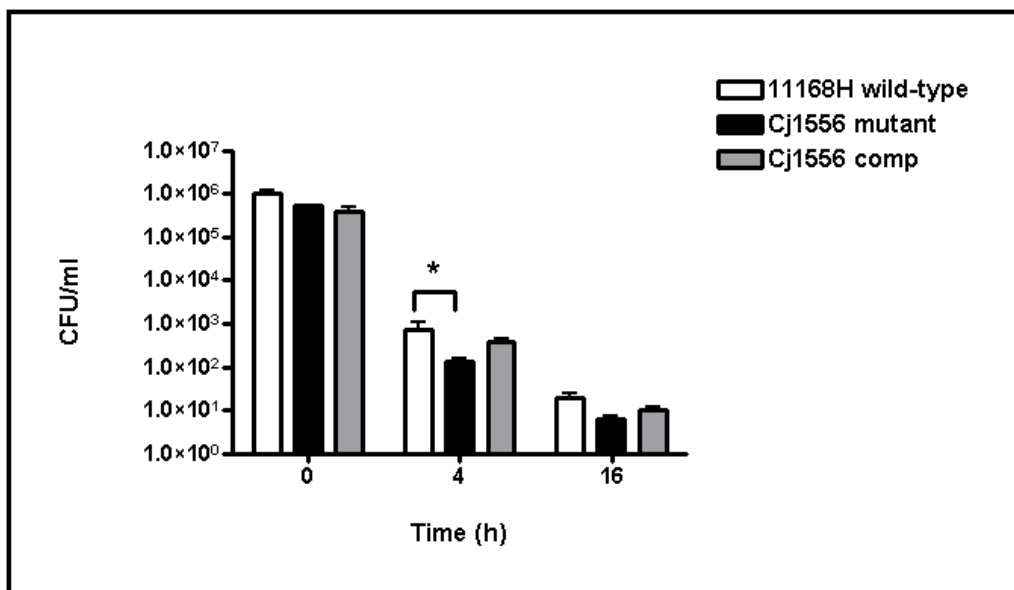
1020

1021 Fig. 3 (A)



1022

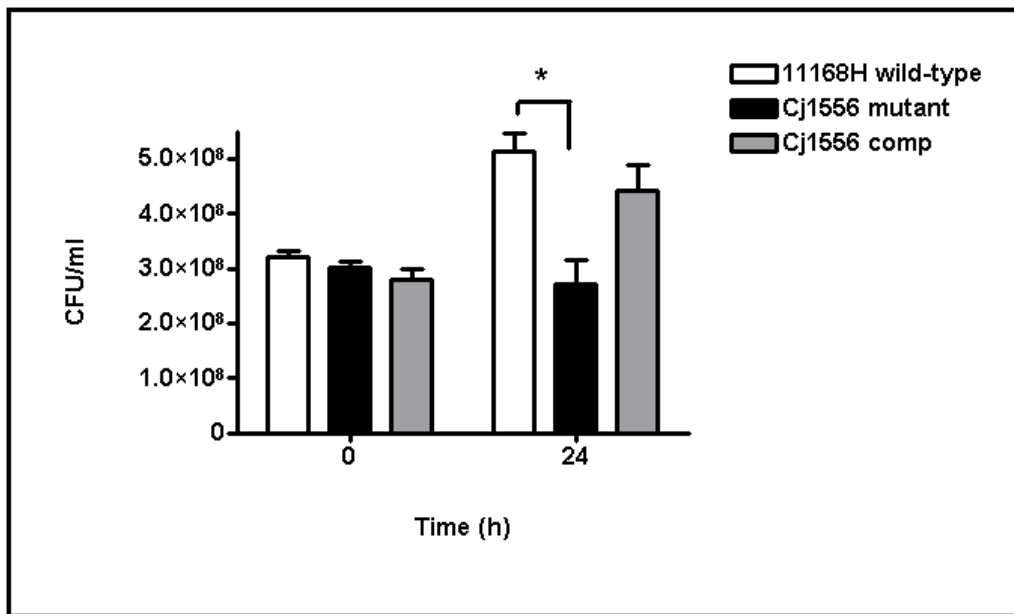
1023 Fig. 3 (B)



1024

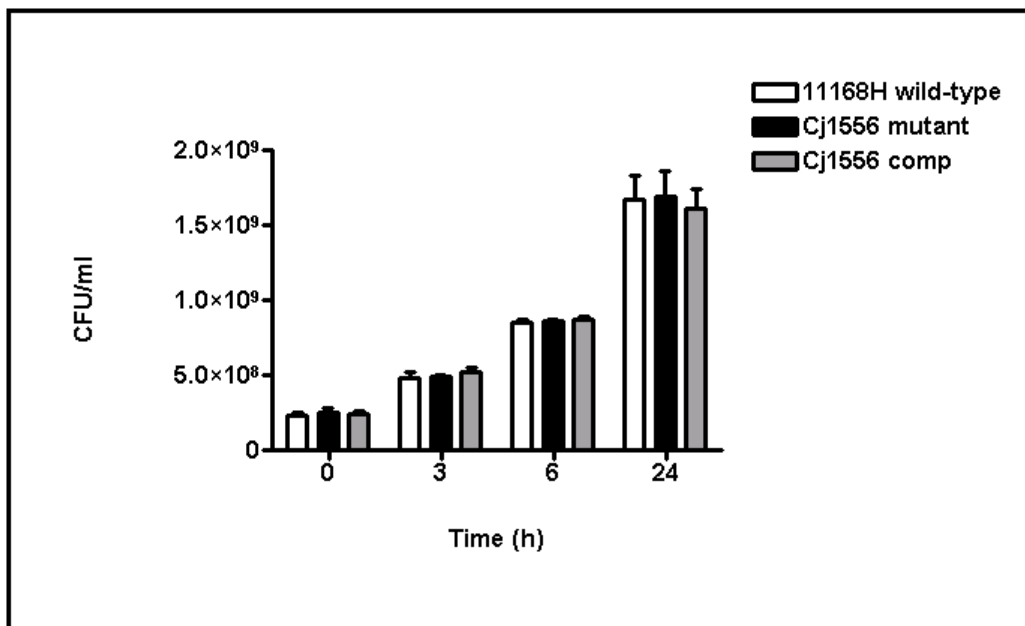
1025

1026 Fig. 4 (A)



1027

1028 Fig. 4 (B)

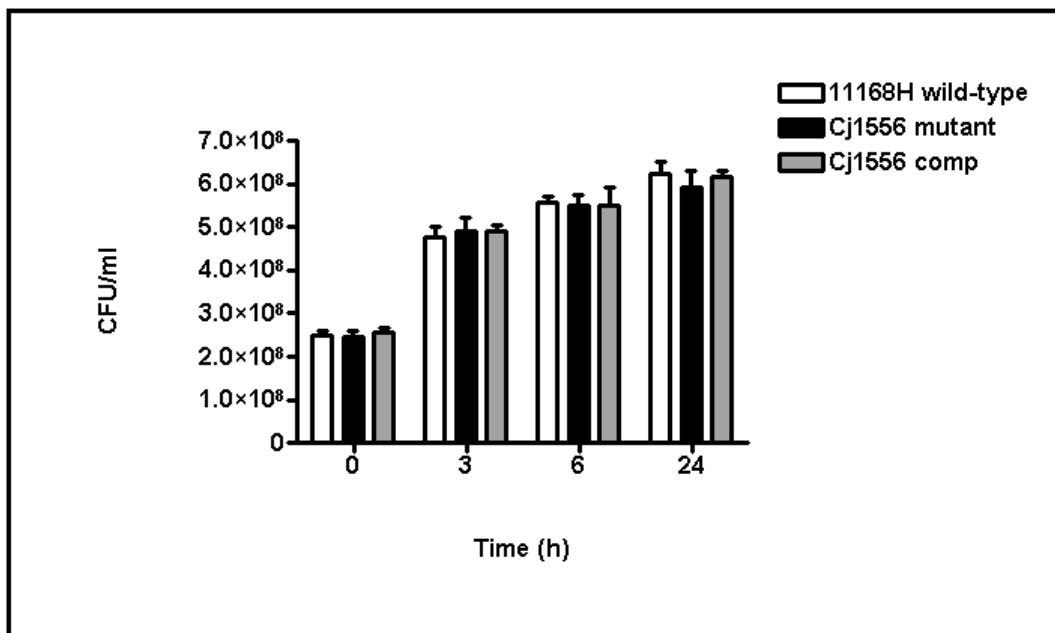


1029

1030

1031 Fig. 4 (C)

1032

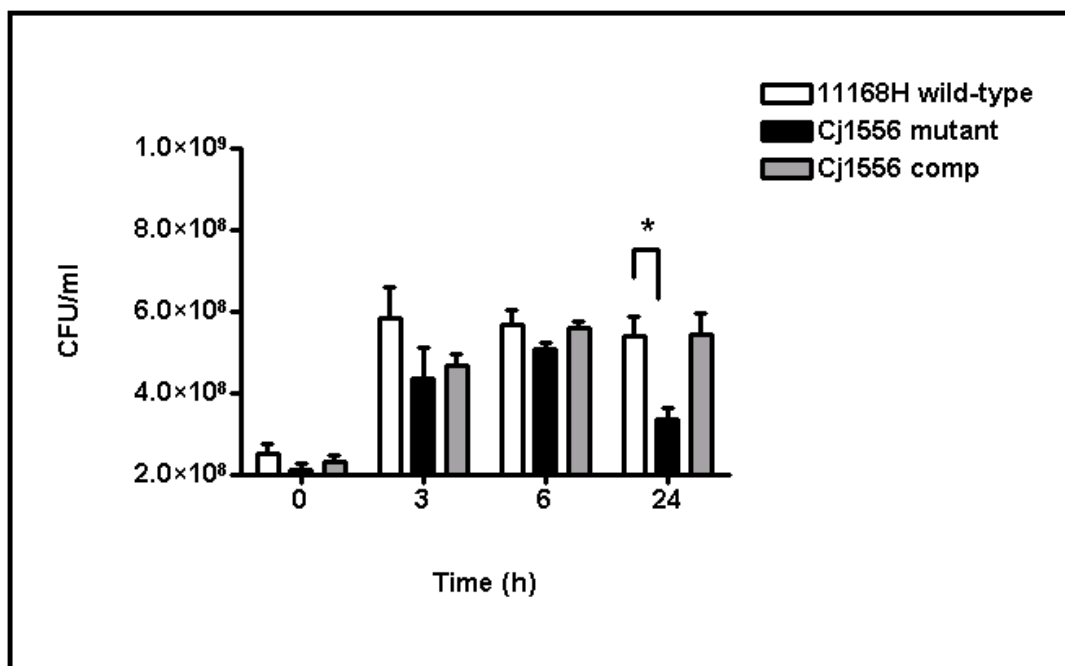


1033

1034

1035 Fig. 4 (D)

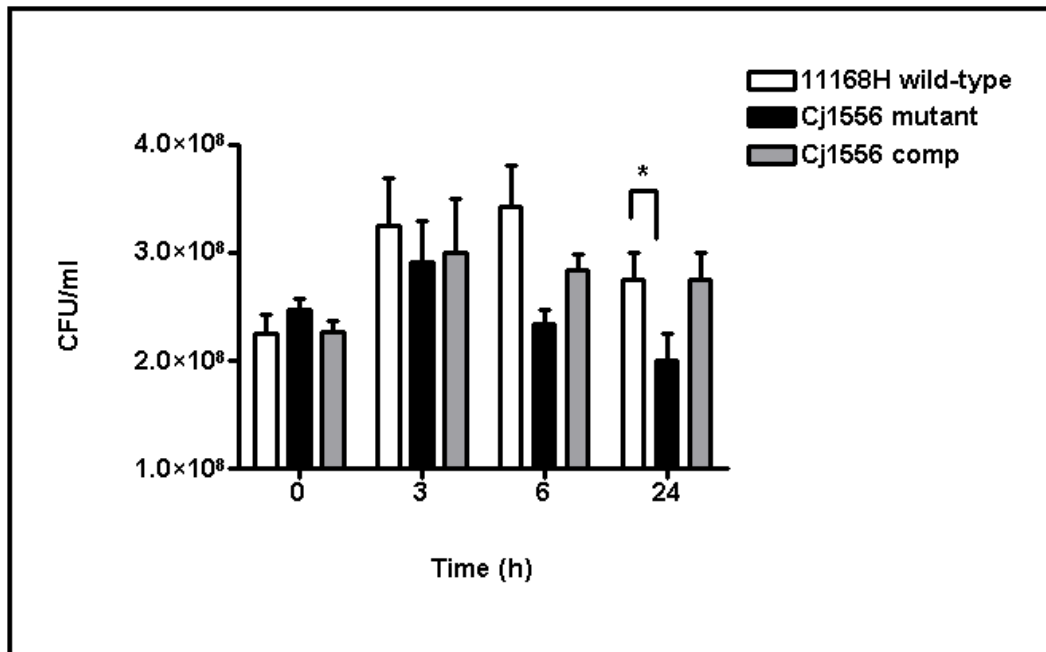
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1038

1039 Fig. 4 (E)

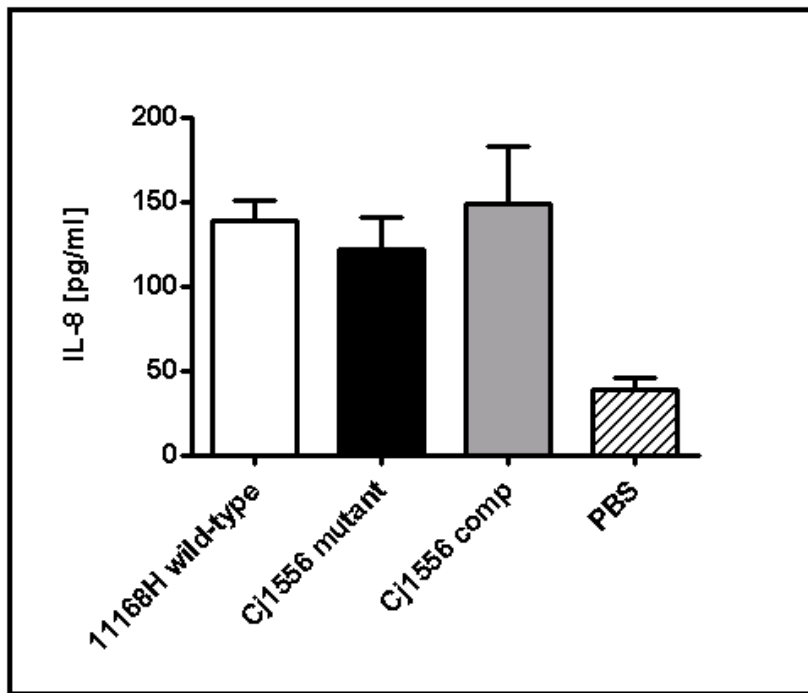


1040

1041

1042 Fig. 5 (A)

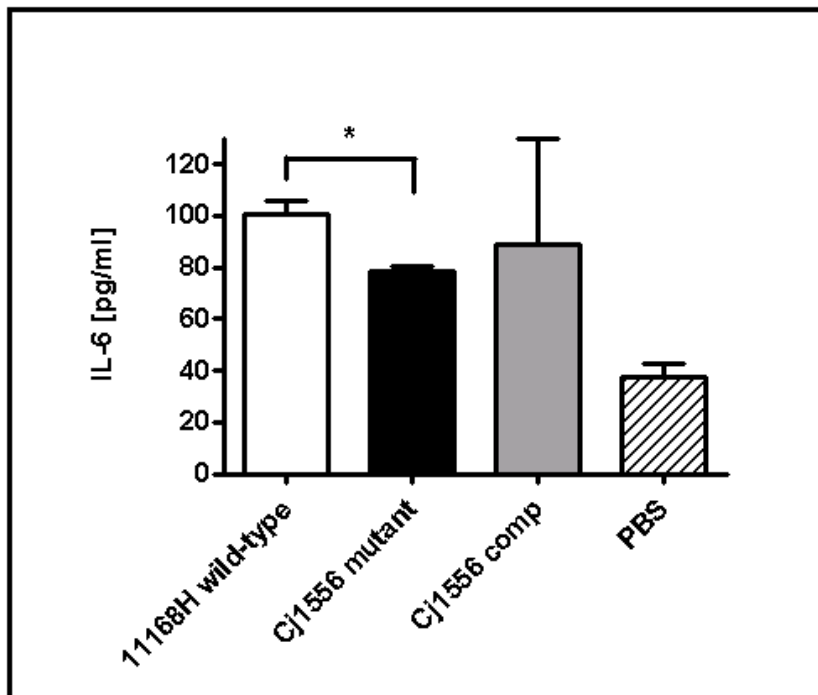
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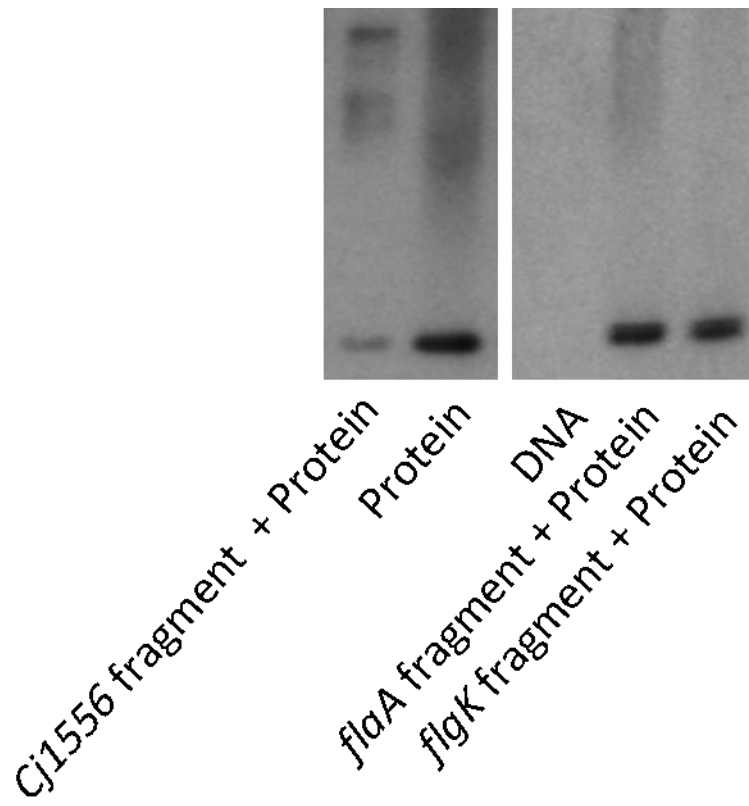
1046 Fig. 5 (B)



1047

1048

1049 Fig. 6

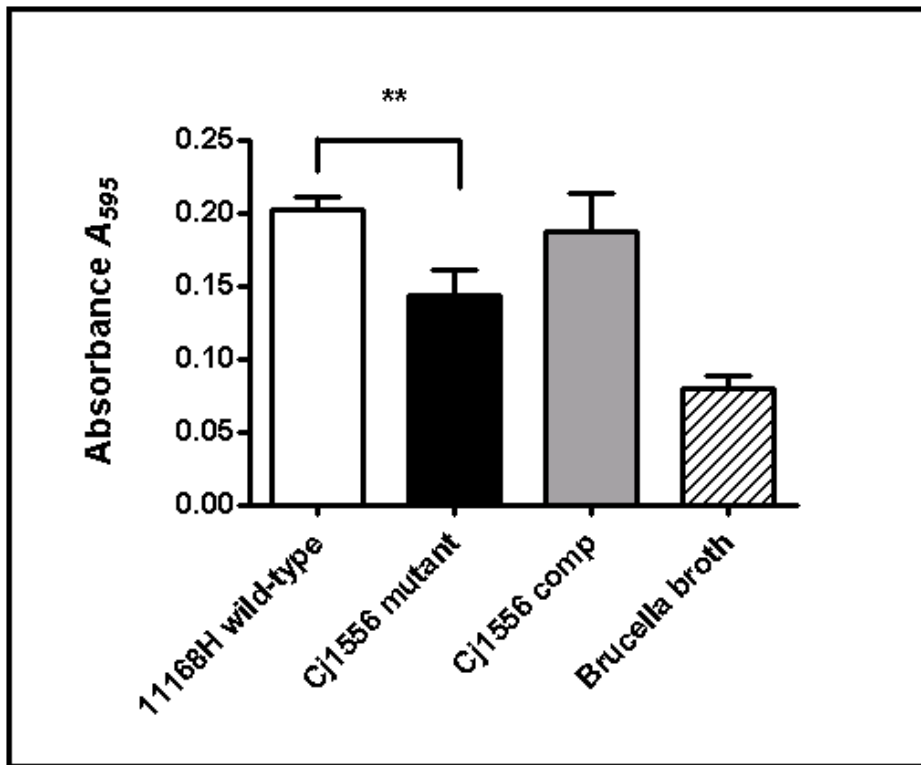


1050

1051

1052 Fig. 7

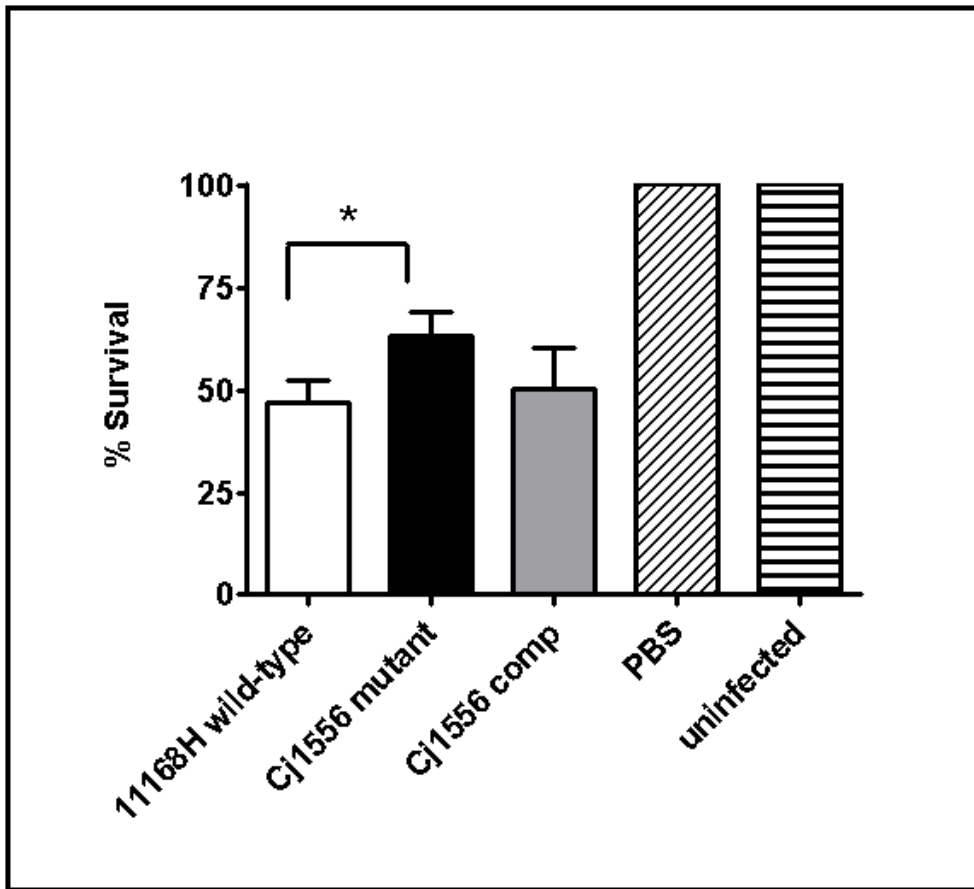
1053



1054

1055 Fig. 8

1056



1057