

REVISED MANUSCRIPT MIC-D-15-00080

PerR controls oxidative stress defence and aerotolerance, but not motility-associated phenotypes of *Campylobacter jejuni*

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Running title: *Campylobacter* PerR, oxidative stress and motility

Contents Category: Regulation

Total number of words in Summary: 248

Total number of words in Manuscript text: 6,384

Number of Figures: 5

Number of Tables: 2

Supplementary information: 1 Table, 2 Figures

Key Words: *Campylobacter jejuni*, oxidative stress defence, peroxide regulator, aerotolerance.

28 **ABSTRACT**

29 The foodborne bacterial pathogen *Campylobacter jejuni* is an obligate microaerophile, which is
30 exposed to atmospheric oxygen during transmission through the food chain. Survival under aerobic
31 conditions requires the concerted control of oxidative stress systems, which in *C. jejuni* are
32 intimately connected with iron metabolism via the PerR and Fur regulatory proteins. Here we have
33 characterised the roles of *C. jejuni* PerR in oxidative stress- and motility phenotypes, and its
34 regulon at the level of transcription, protein expression and promoter interactions. Insertional
35 inactivation of *perR* in the *C. jejuni* reference strains NCTC 11168, 81-176 and 81116 did not result
36 in any growth deficiencies, but strongly increased survival in atmospheric oxygen conditions, and
37 allowed growth around filter discs infused with up to 30% H₂O₂ (8.8 M). Expression of catalase,
38 alkyl hydroperoxide reductase, thioredoxin reductase and the Rrc desulforubrythrin were
39 increased in the *perR* mutant, and this was mediated at the transcriptional level as shown by
40 electrophoretic mobility shift assays of the *kataA*, *ahpC* and *trxB* promoters using purified PerR.
41 Differential RNA-seq analysis of a *fur perR* mutant allowed the identification of eight previously
42 unknown transcription start sites of genes controlled by either Fur and/or PerR. Finally, inactivation
43 of *perR* in *C. jejuni* did not result in reduced motility, and did not reduce killing of *Galleria*
44 *melonella* wax moth larvae. In conclusion, PerR plays an important role in controlling oxidative
45 stress resistance and aerobic survival of *C. jejuni*, but this role does not extend into control of
46 motility and associated phenotypes.

47

48

49 **INTRODUCTION**

50 The bacterial pathogen *Campylobacter jejuni* is the most common cause of bacterial
51 gastroenteritis of humans in the developed world, with an estimated annual incidence of 9.2 million
52 cases in the 27 member countries of the European Union (EFSA, 2011; Nichols *et al.*, 2012). This,
53 together with its link to neurodegenerative diseases like Guillain-Barre syndrome make it both a
54 public health and economic problem (Janssen *et al.*, 2008; McCarthy *et al.*, 2012; Poropatich *et al.*,
55 2010). *C. jejuni* requires microaerobic conditions (3-15% O₂ and 3-10% CO₂) for growth in
56 laboratory conditions, but the organism is known to survive for extended periods in non-permissive
57 atmospheric conditions, which are encountered during transmission and infection (Hazeleger *et al.*,
58 1998). When exposed to such conditions, *C. jejuni* will be under oxidative stress, and hence its
59 ability to deal with such stresses is thought to contribute significantly to its success as a bacterial
60 pathogen. *C. jejuni* expresses an array of factors combatting oxidative stresses, including several
61 peroxidases (Atack *et al.*, 2008; Baillon *et al.*, 1999; Kendall *et al.*, 2014) and an iron-cofactored
62 superoxide dismutase (Purdy *et al.*, 1999), and the absence of these factors severely affects
63 important aspects of *C. jejuni* food chain survival and transmission (Oh & Jeon, 2014; Stead &
64 Park, 2000) as well as virulence (Flint *et al.*, 2014).

65 Iron and oxidative stress are intimately connected via the capability of iron to produce reactive
66 oxygen species via the Haber-Weiss and Fenton reactions (van Vliet *et al.*, 2002). In *C. jejuni*, the
67 metalloregulatory proteins Fur and PerR control iron uptake and oxidative stress responses (Holmes
68 *et al.*, 2005; Palyada *et al.*, 2009; van Vliet *et al.*, 1999), with regulation of oxidative stress further
69 complicated by overlapping control circuitry through other regulatory proteins such as CosR and
70 Cj1556 (Dufour *et al.*, 2013; Gundogdu *et al.*, 2011; Hwang *et al.*, 2012; Svensson *et al.*, 2009).
71 Furthermore, DNA-binding by the Dps protein is activated in the presence of iron or hydrogen
72 peroxide, and protects against DNA damage by hydroxyl radicals (Huergo *et al.*, 2013).

73 The PerR regulator is found mostly in Gram-positive bacteria (Marinho *et al.*, 2014), but also in
74 the Epsilon-proteobacterial genera *Campylobacter* (van Vliet *et al.*, 1999) and *Helicobacter* (Belzer

75 *et al.*, 2011). In general, PerR proteins respond to peroxide stress through derepression of
76 expression of peroxidases and protective proteins such as Dps. In *C. jejuni*, PerR was first shown to
77 mediate iron-dependent regulation of catalase (KatA) and alkyl hydroperoxide reductase (AhpC)
78 (van Vliet *et al.*, 1999). Subsequent studies using transcriptional profiling, RNA-seq and chromatin
79 immunoprecipitation using iron, *fur* and *perR* mutants have shown that there is significant overlap
80 between the iron, Fur and PerR regulons (Butcher *et al.*, 2012; Butcher & Stintzi, 2013; Holmes *et*
81 *al.*, 2005; Palyada *et al.*, 2009). PerR has been proposed to modulate its own expression (Kim *et al.*,
82 2011), while complete derepression of catalase expression was only observed in a *fur perR* double
83 mutant (van Vliet *et al.*, 1999). Since a *fur* mutant constitutively expresses iron acquisition systems
84 (Miller *et al.*, 2009; van Vliet *et al.*, 2002), this further complicates interpretation of transcriptomic
85 and proteomic characterisation of PerR regulation in *C. jejuni*. Inactivation of *perR* results in
86 reduced colonisation in chickens, suggesting a role of oxidative stress regulation in intestinal
87 colonisation (Palyada *et al.*, 2009), although the reduced motility of the *perR* mutant reported in this
88 study makes it difficult to distinguish the roles of motility and PerR on colonisation.

89 In this study, we have used a multi-layered approach to investigate the regulatory and
90 phenotypic roles of PerR in *C. jejuni*. We show that inactivation of the *perR* gene leads to increased
91 aerotolerance and hyper-resistance to hydrogen peroxide, and have identified and validated
92 candidates for PerR regulation. Surprisingly, our data highlight that there is no direct link between
93 PerR-based oxidative stress regulation and infection when tested in an invertebrate model system.
94 Taken together, these data highlight the complex role of PerR in the biology and lifestyle of *C.*
95 *jejuni*.

96

97 **MATERIALS AND METHODS**

98

99 **Bacterial strains, plasmids and growth conditions**

100 *Campylobacter jejuni* NCTC 11168, 81-176 and 81116 (NCTC 11828) and their isogenic
101 mutants (Table 1) were routinely grown under microaerobic conditions (85% N₂, 5% O₂, 10% CO₂)
102 in a MACS-MG-100 controlled atmosphere cabinet (Don Whitley Scientific), at 37°C or 42°C.
103 Growth curves were determined by growing *C. jejuni* isolates in a FluoStar Omega controlled
104 atmosphere plate reader (BMG Labtech). For these growth curves, small volume (200 µl) *C. jejuni*
105 cultures were grown in clear, flat-bottomed, 96-well plates under microaerobic conditions (85% N₂,
106 5% O₂, 10% CO₂) at 37-42 °C, shaking at 400 rpm (double orbital), and OD₆₀₀ readings were taken
107 every 20 min. Broth cultures were carried out in Brucella (Becton, Dickinson and Company) with
108 shaking, whereas growth on plates used Brucella agar or Blood Agar Base agar No. 2 with Skirrow
109 supplements (10 µg ml⁻¹ vancomycin, 5 µg ml⁻¹ trimethoprim, 2.4 IU polymyxin B). An Innova
110 4230 incubator (New Brunswick Scientific) was used for shaking aerobic cultures at 37°C. All
111 bacterial strains and plasmids used in this study are given in Table 1.

112

113 **Construction of a *C. jejuni* *perR* mutant**

114 The region containing the *cj0322* gene and approximately 500 base pairs (bp) of flanking
115 sequence on each side was PCR amplified by Phusion DNA Polymerase (New England Biolabs)
116 using oligonucleotide primers PerRFlanksF and PerRFlanksR (Table S1). This amplified fragment
117 was purified using a commercial PCR Purification Kit (QIAGEN), digested with *EcoRI* (New
118 England Biolabs) and *PstI* (Promega) and then ligated into pNEB193 to form plasmid pOSH2. To
119 make the *perR* insertional inactivation construct, pOSH2 was used as template for inverse PCR as
120 described previously (Reuter & van Vliet, 2013), using the oligonucleotide primers PerRInverseR
121 and PerRInverseL. The kanamycin cassette from pMARKan9 and the inverse PCR product from
122 pOSH2 were digested with *BamHI* (New England Biolabs) and ligated to form plasmid pOSH3.

123 Ligated fragments were transformed into *E. coli* strain Top10 and positive transformants were
124 selected for by plating on LB agar supplemented with 30 µg ml⁻¹ kanamycin. All constructs and
125 insert orientations were confirmed by restriction digestion analysis and sequencing (The Genome
126 Analysis Centre, Norwich, UK). *C. jejuni perR* mutants were isolated after transformation with
127 pOSH3 by electroporation (Reuter & van Vliet, 2013) and subsequent selection on kanamycin-
128 containing agar. Colonies were screened by PCR using oligonucleotides PerRKOCheck FWD and
129 PerRKOCheck REV, which anneal outside of the cloned flanking regions in combination with
130 antibiotic cassette specific primers KmPrReadOut and KmReadOut (Table S1).

131

132 **Construction of *perR* complementation constructs**

133 *C. jejuni perR* mutants were complemented *in trans* by using the *perR* gene with its own
134 promoter cloned into the *cj0046* pseudogene, as described previously (Reuter & van Vliet, 2013).
135 To make the *perR* complementation construct with the native *perR* promoter, the *perR* gene plus
136 promoter region was PCR amplified using oligonucleotides PerRCompNativeFwdNcoI and
137 PerRCompRevNcoI (Table S1). The amplified fragment was digested with *NcoI* (Promega) and
138 ligated into the *Esp3I* site of pC46. This construct, known as pC46perR, was transformed into a *C.*
139 *jejuni perR* mutant using standard electroporation methods. Complementation strains were selected
140 on chloramphenicol-containing agar plates, and colonies checked by PCR using primers that anneal
141 outside of the *cj0046* flanking regions (0046Fcheck3 and 0046Rcheck 3) in combination with gene-
142 and antibiotic cassette-specific primers CatPrReadOut and PerRInternalRev (Table S1).

143

144 **Motility Assay**

145 The A₆₀₀ of an overnight *C. jejuni* culture was adjusted to 0.4 using sterile PBS. Bacterial
146 motility was assessed by spotting 10 µl of this culture onto the centre of a 0.4% Brucella agar plate
147 (Reuter & van Vliet, 2013). Plates were photographed after 24, 48, and 72 hours of incubation at
148 microaerobic conditions at 37°C, and the diameter of the halo was measured using ImageJ software

149 (version 1.41; National Institute of Health [<http://rsbweb.nih.gov/ij/>]). A *C. jejuni* NCTC 11168
150 *flaAB* non-motile mutant was included in all experiments as a negative control (Reuter *et al.*, 2010).

151

152 **Oxidative Stress and Aerotolerance Assays**

153 Resistance to oxidative stress was measured using disc inhibition assays. *C. jejuni* were grown
154 overnight on Skirrow plates at 37°C, and cells were harvested into 2 ml of Brucella broth. *C. jejuni*
155 was then added to 3 ml 1.5% Brucella agar to a final A_{600} of 1.0 and poured onto a Brucella plate.
156 After the agar had set, 6 mm diameter sterile 3M Whatman paper discs were placed on the soft agar
157 surface, and 10 μ l of hydrogen peroxide (0-30% v/v in water) or cumene hydroperoxide (0-6% v/v
158 in dimethyl sulfoxide) were applied to discs. Plates were incubated overnight in microaerobic
159 conditions at 37°C, photographed and the zone of inhibition (no growth) was measured using
160 ImageJ image analysis software (National Institute of Health). The effect of hydrogen peroxide was
161 also measured in broth culture, using overnight cultures of *C. jejuni* NCTC 11168 which were
162 adjusted to an A_{600} of 0.4 using sterile PBS buffer. Hydrogen peroxide solution was then added to a
163 final concentration of 3% (v/v). Cell survival was assessed by plating out serial dilutions after
164 incubating the broth cultures in shaking conditions for 0, 2, 5, 10, 15, 30 60,120 and 180 minutes.

165 Aerotolerance assays were adapted from (Baillon *et al.*, 1999) with some alterations. Cultures
166 (20 ml) were grown overnight in Brucella broth, and adjusted to an A_{600} of 0.4 using Brucella broth.
167 For each strain, cultures were split into two 10 ml cultures in separate flasks, with one grown
168 microaerobically (85% N₂, 5%O₂, 10% CO₂) and the other aerobically at 37°C, shaking at 200 rpm.
169 Samples of each culture were taken at three hour time intervals. Serial ten-fold dilutions were used
170 to assess cell survival; 5 μ l of each dilution was spotted onto Brucella agar plates and incubated
171 under microaerobic conditions for 2 days at 37°C.

172

173 **Galleria Infection Model**

174 The *Galleria mellonella* infection model was used to assess whether inactivation and

175 complementation of *perR* affected killing of wax moth larvae by *C. jejuni*, which has been
176 suggested to represent virulence (Champion *et al.*, 2010; Gundogdu *et al.*, 2011; van Alphen *et al.*,
177 2014). *G. mellonella* larvae were obtained from Livefoods.co.uk (United Kingdom). Larvae were
178 inoculated in the right foremost pro-leg by microinjection (Hamilton, Switzerland) with 10 μ l *C.*
179 *jejuni* overnight culture, which had been adjusted to an A_{600} of 0.1 (approximately 10^6 CFU). PBS,
180 and mock infection controls were also performed alongside each experiment. The larvae were
181 incubated at 37°C, with percentage survival scored at 24 hour intervals. For each experiment, ten *G.*
182 *mellonella* larvae were infected and a total of five independent experiments were performed.

183

184 **Two-dimensional protein gel electrophoresis and protein identification**

185 Two-dimensional protein gel electrophoresis was conducted essentially as described previously
186 (Hockin *et al.*, 2012; Shaw *et al.*, 2012). *C. jejuni* cells were grown to late log phase in Brucella
187 broth in microaerobic conditions, and were harvested from broth culture (50 ml) by centrifugation
188 at $4,000 \times g$, 10 min at room temperature. Cell pellets were resuspended in 500 μ l lysis buffer (50
189 mM Tris (pH 7.5), 0.3% sodium dodecyl sulfate (SDS), 0.2 M dithiothreitol, 3.3 mM $MgCl_2$, 16.7
190 μ g of RNase ml^{-1} , and 1.67 U of DNase ml^{-1}) and lysed (Soniprep 150 MSE; Sanyo) on ice until
191 clear. The samples were then centrifuged ($14,000 \times g$, 20 min, 4°C) to remove any unlysed cells.
192 Total cell protein was quantified using a 2D Quant kit (GE Healthcare) as per the manufacturer's
193 instructions. After two-dimensional protein gel electrophoresis, proteins of interest were removed
194 from the gel using ProPick excision robot (Genomic Solutions), and in-gel trypsin digested using a
195 ProGest Protein Digester (Genomic Solutions) (Hockin *et al.*, 2012; Shaw *et al.*, 2012). LC-MS/MS
196 analysis was performed using a LTQ-Orbitrap mass spectrometer (Thermo Electron) and a
197 nanoflow-HPLC system (nanoACQUITY: Waters).

198

199 **Expression and purification of recombinant *C. jejuni* PerR**

200 The *perR* gene was PCR amplified using primers *perRpET21aF* and *perRpET21aR* (Table S1),

201 and the amplified fragment was digested with restriction enzymes *Bam*HI and *Nde*I (New England
202 Biolabs) and ligated into pET21a, which had been digested with the same enzymes. This plasmid
203 construct (pOSH1) was subsequently checked by sequencing (TGAC, Norwich, UK) then
204 transformed into *E. coli* BL21 (DE3). *E. coli* harbouring pOSH1 were grown in LB broth, at 37°C,
205 shaking at 180 rpm to an A₆₀₀ of 1.0. The expression of recombinant PerR was induced by addition
206 of 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hours at 30°C. Bacterial cells were
207 harvested by centrifugation and resuspended in 20 mM Tris-HCl, 50 mM NaCl, pH 8.0, and then
208 lysed on ice by sonication, in six passes of 30 seconds. Soluble cell extract was isolated by
209 centrifugation at 23,000 × *g* for 30 minutes at 4°C. The soluble cell extract was filter sterilized and
210 loaded onto a HiTrap™ Heparin affinity column. *C. jejuni* PerR was eluted from the heparin
211 column across a salt gradient and further purified by gel filtration. Purified PerR protein was
212 visualised by SDS-PAGE.

213

214 **Electrophoretic Mobility Gel Shift Analysis**

215 DNA fragments (180-200 bp) located upstream of translational start sites of the *kata*, *ahpC*,
216 *trxB* and *perR* genes were amplified by PCR using 5' DIG-labelled PCR primers ('Prom' primers,
217 Table S1) and PCR products quantified by Nanodrop. The *dnaE* promoter was included as negative
218 control. Recombinant PerR protein (0-5 nM) was incubated with 0.5 nM of DIG-labelled DNA in
219 EMSA binding buffer (50 μM MnCl₂, 20 mM Tris-Borate pH 7.4, 50 mM KCl, 3 mM MgCl₂, 5%
220 glycerol, 0.1% Triton X-100), in a total volume of 20 μl. The reaction was left for 60 min at room
221 temperature. Reactions were assayed for protein-DNA binding by native PAGE, on a 12%
222 acrylamide gel at 150 V for 50 min. Labelled DNA was transferred to positively charged nylon
223 membranes (Hybond N+, Amersham Biotech) by electroblotting at 130 mA for 1 hour. DNA was
224 cross-linked to the membrane using UV radiation (1200 kJ cm²) and incubated with Anti-
225 Digoxigenin-AP fab fragments (Roche). CDP Star® (SIGMA) was used to detect DIG-labelled
226 fragments on the nylon membranes, as per the manufacturer's instructions.

227

228 **RNA-seq analysis**

229 RNA for RNA-seq analyses was extracted from *C. jejuni* NCTC 11168 wild-type (Porcelli *et*
230 *al.*, 2013) and *fur perR* mutants (this study), grown to A₆₀₀ of 0.4. RNA was isolated using hot
231 phenol (Mattatall & Sanderson, 1996) to ensure that small RNAs would not be removed by the
232 extraction procedure. The RNA was treated with DNase I to remove genomic DNA, followed by
233 optional treatment with Terminator Exonuclease (TEX, Epicentre Biotechnology) for enrichment of
234 primary RNAs, and treatment with Tobacco Acid Phosphatase (TAP, Cambio, UK) to generate 5'-P
235 ends for downstream ligation of 454 adapters (Porcelli *et al.*, 2013; Sharma *et al.*, 2010). After
236 ligation of an RNA oligonucleotide to the phosphorylated 5'-ends of RNA, and polyadenylation of
237 RNA, first strand cDNA was generated using an oligo-dT containing 454-B primer. The cDNA
238 fragments were barcoded and amplified, and used for generation of cDNA libraries for the 454 FLX
239 system at Vertis Biotech, Germany. These libraries were subsequently analysed using a Roche
240 Titanium sequencer. The same RNA-samples were also used to generate strand-inspecific RNA-seq
241 libraries for Illumina sequencing, using instructions from the manufacturer (Illumina), and further
242 analysed using an Illumina HiSeq2000 sequencer.

243 Sequencing reads were grouped based on the barcode tag, the 5' adapter was clipped, and reads
244 of >70% A were removed. The remaining reads were aligned against the *C. jejuni* NCTC 11168
245 genome sequence using Segemehl version 0.0.9.3 (Hoffmann *et al.*, 2009), and converted into
246 number of reads per nucleotide position. Graphs representing the number of mapped reads per
247 nucleotide were visualized using the Integrated Genome Browser software from Affymetrix (Nicol
248 *et al.*, 2009) and analysed as described previously (Porcelli *et al.*, 2013; Sharma *et al.*, 2010).
249 Transcript levels of individual genes were expressed as Reads Per Kilobase per Million mapped
250 reads (RPKM) values, calculated after mapping of reads using CLC Genomics Workbench v5 (CLC
251 Bio).

252

253 **Accession numbers**

254 The RNA-seq data obtained for the *C. jejuni* NCTC 11168 wild-type and *fur perR* mutant have
255 been deposited in the Gene Expression Omnibus (GEO) and Short Read Archive (SRA) databases,
256 and are available via GEO accession numbers GSE49312 (wild-type dRNA-seq), GSE49660 (*fur*
257 *perR* mutant dRNA-seq) and GSE49687 (wild-type and *fur perR* mutant RNA-seq).

258

259 **RESULTS**

260

261 **Construction and complementation of *C. jejuni perR* mutants**

262 To investigate the role of PerR in *C. jejuni* gene regulation and physiology, we constructed an
263 isogenic *perR* (*cj0322*) mutant by allelic replacement in *C. jejuni* reference strains NCTC 11168,
264 81116 (NCTC 11828) and 81-176. Approximately 100 bp of the 3' end of the *perR* gene region was
265 not removed, to avoid disruption of the *cj0323* gene promoter, for which the transcriptional start site
266 is located directly downstream of *perR* (Fig. S1) (Dugar *et al.*, 2013; Porcelli *et al.*, 2013). In
267 addition, the *perR* mutation was complemented *in trans* to ensure that phenotypic changes observed
268 were due to the *perR* mutation, and not due to secondary mutations or polar effects of the insertion
269 of the antibiotic resistance cassette. Complementation was achieved by genomic insertion of the
270 *perR* gene with its own promoter into the *cj0046* pseudogene (Reuter & van Vliet, 2013). The *C.*
271 *jejuni perR* mutants and complemented strains showed no growth defects at 37°C and 42°C in the
272 three *C. jejuni* strains investigated (NCTC 11168, 81116 and 81-176), when compared to the
273 respective wild-type strains (Fig. S2).

274

275 ***C. jejuni perR* mutants display significantly increased resistance to peroxide and oxygen stress**

276 Previous studies have shown that *C. jejuni perR* mutants are more resistant to peroxide stress
277 induced by hydrogen peroxide (H₂O₂) or cumene hydroperoxide (CHP). We quantified this using
278 plate inhibition and broth survival assays. The *perR* mutants exhibited very high levels of resistance
279 to hydrogen peroxide in both broth culture (Fig. 1A) and disc assay experiments (Fig. 1B), both in
280 microaerobic conditions. During exposure to 3% hydrogen peroxide (0.88 M) in broth culture, the
281 *perR* mutants maintained viability (1.0×10^8 cfu ml⁻¹) throughout the experiment, whereas the
282 number of viable cells in the wild-type strains declined rapidly after the addition of H₂O₂. Exposure
283 of *C. jejuni perR* mutants to hydrogen peroxide on discs revealed that the level of peroxide
284 resistance was considerably higher than previously reported, where 3% of hydrogen peroxide was

285 used (Baillon *et al.*, 1999; Flint & Stintzi, 2015; Palyada *et al.*, 2009; van Vliet *et al.*, 1999). Wild-
286 type *C. jejuni* were sensitive to 5% H₂O₂ (Fig. 1B) indicated by the inhibition of growth of the
287 bacteria around the Whatman paper disc. In contrast, the *perR* mutants in the three reference strains
288 did not show any inhibition zone at 30% H₂O₂ (8.8 M). When grown in the presence of 3-6%
289 cumene hydroperoxide (CHP), the three wild-type strains showed a reduction in growth as the
290 concentration of CHP increased (Fig. 1C). However, *C. jejuni* 81116 and 81-176 *perR* mutants were
291 able to grow better in the presence of CHP compared to the comparative wild-type strains, as is
292 evident from the reduced zones of clearance. Although there was a reduced zone of clearance with
293 CHP for the *C. jejuni* NCTC 11168 *perR* mutant, this difference was not statistically significant.
294 Overall, all three *C. jejuni* strains show a similar phenotype: inactivation of the *C. jejuni perR* gene
295 significantly increased resistance to hydrogen peroxide and cumene hydroperoxide, whereas the
296 complementation of the *perR* mutant restored a wild-type peroxide stress phenotype (Fig. 1).

297 The increase in peroxide stress resistance was associated with a general increase in survival
298 under aerobic conditions (aerotolerance). After exposure of stationary phase cells to an aerobic
299 environment, the *C. jejuni* NCTC 11168 wild-type and complemented *perR* mutants showed a rapid
300 reduction in viability, as the number of viable cells declined by two logs to 1.0×10^6 cfu ml⁻¹ after
301 six hours in an aerobic environment (Fig. 2). In contrast, the *perR* mutant showed increased
302 aerotolerance with over 1.0×10^6 cfu ml⁻¹ surviving after nine hours exposure to air, with 2-3 logs
303 more survival when compared to the wild-type and complemented strains.

304

305 ***C. jejuni* PerR controls expression of peroxidase genes at the transcriptional level**

306 To characterise the role of PerR in regulation of *C. jejuni* gene expression, the effects of the
307 *perR* mutation was assessed at the level of protein abundance (using two-dimensional gel
308 electrophoresis) and transcript levels (using RNA-sequencing). As overlaps between the Fur and
309 PerR regulatory networks were previously reported (Holmes *et al.*, 2005; Palyada *et al.*, 2009; van
310 Vliet *et al.*, 1999), we included a *fur* and *fur perR* double mutant (Table 1) in the protein expression

311 analysis. Comparison of the protein profiles of *C. jejuni* NCTC 11168 wild-type, *perR*, *fur* and *fur*
312 *perR* mutants showed that several proteins were consistently increased in abundance in the *perR*
313 mutant backgrounds (Fig. 3). Four of these proteins were further investigated by mass spectrometry
314 and identified as AhpC (Cj0334, Mascot Score 11221, sequence coverage 97%), KatA (Cj1385,
315 Mascot Score 1152, sequence coverage 56%), TrxB (Cj0146c, Mascot Score 2725, sequence
316 coverage 67%) and Rrc (Cj0012c, Mascot Score 1752, sequence coverage 67%).

317 The increased abundance of these four proteins was associated with increased levels of the
318 respective mRNAs, as shown by RNA sequencing using the wild-type strain and the *fur perR*
319 mutant (Fig. 4A). The RPKM values for the *ahpC*, *katA*, *trxB* and *rrc* genes were significantly
320 increased in the NCTC 11168 *fur perR* mutant when compared to the wild-type strain (Table 2).
321 Differential RNA-sequencing (Dugar *et al.*, 2013; Porcelli *et al.*, 2013) was used to confirm the
322 transcription start sites of the respective genes (Table 2). Each of the four PerR-regulated genes
323 showed increased transcript levels as found in the RNA-seq analysis. For each of the four genes,
324 transcription starts from a single, identical transcription start site (TSS) in both the wild-type strain
325 and *fur perR* mutant, which is preceded by a recognisable σ^{70} -10 sequence (gnTAnaaT) located 4-7
326 bp upstream, and matches the TSS described previously (Baillon *et al.*, 1999; Dugar *et al.*, 2013;
327 Hwang *et al.*, 2012; Porcelli *et al.*, 2013). This demonstrates that PerR-regulation of these genes is
328 mediated from a single promoter region, and excludes the possibility that PerR-regulation of these
329 genes is controlled from a secondary promoter. In addition, the use of the *fur perR* mutant for RNA-
330 sequencing allowed the additional identification of eight previously unknown transcription start
331 sites of Fur-repressed genes (Table 2), aiding the analysis of possible operator sequences in their
332 respective promoters. Analysis of these genes shows the presence of motifs representing potential
333 Fur/PerR-binding sites, which are rich in nAT-triplets (Baichoo & Helmann, 2002).

334

335 **PerR binds to the *C. jejuni* *katA*, *ahpC*, *trxB* and *perR* promoters**

336 Full length, untagged *C. jejuni* PerR protein was heterologously expressed in *E. coli* purified to

337 approximately 90% purity, as assessed by SDS-PAGE analysis. This was subsequently used in
338 electrophoretic mobility shift assays (EMSAs) to assess whether PerR binds specifically to the
339 *ahpC*, *katA*, *trxB* and *rrc* promoter regions identified by differential RNA-seq. The DNA fragments
340 used included the transcriptional start sites shown in Table 2. Binding of PerR was observed for the
341 *ahpC*, *katA*, and *trxB* promoters, confirming the direct role of PerR in their regulation (Fig. 4B).
342 The *perR* promoter was used as positive control as autoregulation of *perR* was previously described
343 (Kim *et al.*, 2011), and indeed this also resulted in a mobility shift (Fig. 4B). No mobility shift was
344 observed with the *dnaE* (*cj0718*) promoter, which was included to control for non-specific binding
345 of *C. jejuni* PerR protein to *C. jejuni* promoter DNA (Fig. 4B). We were unable to detect binding of
346 PerR to the promoter region of *rrc* (data not shown).

347

348 **Inactivation of PerR does not affect *C. jejuni* motility-associated phenotypes**

349 It was previously reported that inactivation of *perR* led to a reduction in motility in *C. jejuni*
350 NCTC 11168, and this was associated with reduced colonisation of the chicken intestine (Flint *et*
351 *al.*, 2014; Palyada *et al.*, 2009). Hence we compared the swarming motility of the wild-type, *perR*
352 mutant and complemented *perR* mutant strains in semi-solid (0.4 %) agar, compared to a non-motile
353 control strain (*flaAB* mutant). There was no loss or reduction of motility in the *C. jejuni perR*
354 mutants, when compared to the corresponding wild-type strain, in any of the three *C. jejuni* strains
355 investigated (Fig. 5A). We also assessed whether the inactivation of the *perR* gene affected the
356 ability of *C. jejuni* to kill *G. mellonella* larvae (Champion *et al.*, 2010; Senior *et al.*, 2011).
357 Inoculation with the wild-type NCTC 11168 strain resulted in a median killing of 80% of larvae.
358 Similarly, inoculation of *G. mellonella* with the *C. jejuni perR* mutant or complemented *perR*
359 mutant did not result in increased *G. mellonella* survival when compared to the wild-type, when
360 measured 24 or 48 hours after inoculation (Fig. 5B).

361

362 **DISCUSSION**

363 The microaerophilic lifestyle of bacteria like *Campylobacter jejuni* continuously poses a
364 problem for the cell: there is a need for some oxygen for metabolism (i.e. as preferred terminal
365 electron acceptor), but there is also exquisite sensitivity to the possible deleterious effects of
366 reactive oxygen species formed during metabolism, or exposure to external sources of oxygen. This
367 is especially a problem for pathogenic bacteria which require transmission from host to host (often
368 fecal-oral), during which they may need to survive long-term exposure to atmospheric oxygen
369 conditions. These stresses necessitate a tight control of oxidative stress defence systems, and hence
370 it is not surprising that many of the characterised *C. jejuni* regulatory systems directly or indirectly
371 affect oxidative stress (Gundogdu *et al.*, 2011; Hwang *et al.*, 2012; Palyada *et al.*, 2009; Svensson
372 *et al.*, 2009; van der Stel *et al.*, 2014; van Vliet *et al.*, 2002). In this study we have focused on the
373 PerR regulator, a member of the Fur family of metalloregulatory proteins, which was initially
374 identified as controlling expression of peroxidases (van Vliet *et al.*, 1999) and subsequently
375 suggested to affect colonisation properties of *C. jejuni*, probably via motility (Palyada *et al.*, 2009).
376 To understand how PerR mediates oxidative stress responses in *C. jejuni*, we have characterised the
377 phenotypes of *C. jejuni perR* mutants in response to multiple sources of oxidative stress. Our results
378 support the role of PerR as a regulator of oxidative-stress mediated genes (Kim *et al.*, 2015; Kim *et*
379 *al.*, 2011; Palyada *et al.*, 2009; van Vliet *et al.*, 1999) and as mediator of very high levels of
380 peroxide stress resistance, but inactivation of *perR* did not affect the ability of *C. jejuni* to kill wax
381 moth larvae. Interestingly, the increased resistance to peroxide stress of a *perR* mutant is matched
382 by a significantly increased aerobic survival (aerotolerance) of *C. jejuni*.

383 Although some targets of PerR were described previously (Hwang *et al.*, 2012; Kim *et al.*,
384 2015; Kim *et al.*, 2011; Palyada *et al.*, 2009), the reported phenotypes of *perR* mutants of *C. jejuni*
385 have varied, especially with regard to its role in motility and intestinal colonisation. In this study, *C.*
386 *jejuni perR* mutants displayed growth similar to wild-type (Fig. S2), did not show lowered motility
387 in three well-characterised *C. jejuni* reference strains (Fig. 5A) and did not show reduced ability to

388 kill wax moth larvae (Fig. 5B). This differs from the phenotypes described previously (Palyada *et*
389 *al.*, 2009) where inactivation of *perR* in strain NCTC 11168 resulted in decreased motility and
390 colonisation of the chicken intestine, while complementation with *perR* restored motility. We
391 currently do not have an explanation for this difference in phenotypes between the different *perR*
392 mutants and have insufficient information for anything more than speculation. Flagellar biosynthesis
393 and motility requires the coordinated expression of >50 genes, and it is well established that the
394 inactivation or absence of expression of a single factor (by mutation or phase variation) can
395 significantly affect motility (Carrillo *et al.*, 2004; Hendrixson, 2006). All this does highlight the
396 complexity of regulation of oxidative stress responses and motility, which in *C. jejuni* are affected
397 by several regulatory systems (Carrillo *et al.*, 2004; Gundogdu *et al.*, 2011; Hendrixson, 2006;
398 Holmes *et al.*, 2005; Hwang *et al.*, 2011a; Hwang *et al.*, 2011c; Hwang *et al.*, 2012; Palyada *et al.*,
399 2009; van der Stel *et al.*, 2014; van Vliet *et al.*, 1999).

400 It was previously shown that inactivation of *perR* in strain NCTC 11168 results in high level
401 production of the peroxidases catalase and alkyl hydroperoxide reductase (van Vliet *et al.*, 1999).
402 We have confirmed this phenotype in two other commonly used reference strains of *C. jejuni*
403 (81116 and 81-176). More importantly, the high levels of especially catalase resulted in a
404 significant increase in resistance against hydrogen peroxide, with the *perR* mutant able to grow
405 close to discs infused with 30% (8.8 M) hydrogen peroxide, which is the highest commercially
406 available concentration of hydrogen peroxide. This concentration is much higher than previously
407 tested, where either 3% hydrogen peroxide was used (Baillon *et al.*, 1999; Flint & Stintzi, 2015;
408 Palyada *et al.*, 2009; van Vliet *et al.*, 1999), or concentrations ranging from 1-5 mM (Huergo *et al.*,
409 2013; Hwang *et al.*, 2011a; Hwang *et al.*, 2011c), and highlights the very high levels of catalase
410 expression in the *perR* mutant. A similar phenotype (albeit not as striking) was observed for cumene
411 hydroperoxide (Fig. 1C), which is a substrate for AhpC (Baillon *et al.*, 1999). The difference
412 between these phenotypes may be explained by the difference in expression levels in the wild-type
413 strain; while expression of catalase is strongly repressed in the iron-sufficient conditions of the

414 Brucella medium (Fig. 3) (van Vliet *et al.*, 1999), there is constitutive (but inducible) expression of
415 AhpC in the wild-type strain, thus giving a much higher level of protection independent of the *perR*
416 mutation.

417 Next to the clear increase in resistance to peroxides, we also observed a significant increase in
418 aerotolerance in the *perR* mutant (Fig. 2). Aerobic tolerance plays a key role in the infection route
419 of *C. jejuni*, as the organism needs to survive aerobic exposure in order to spread and proliferate.
420 The increased hydrogen peroxide and cumene hydroperoxide resistance of the *perR* mutant (Fig.
421 1A-C) were associated with a clear increase in aerobic survival (Fig. 2) of a *C. jejuni* NCTC 11168
422 *perR* mutant compared to the wild-type strain, with a 3-4 log higher survival than the wild-type
423 strain and complemented mutant. Although the exact mechanism underlying the increased
424 aerotolerance was outside the remit of this study, it is likely to be due to the increased expression of
425 peroxidases, since inactivation of the *ahpC* gene was previously shown to reduce aerotolerance
426 (Baillon *et al.*, 1999). Aerobic stress will be abundant during the transmission of *C. jejuni* to food
427 preparation surfaces and hence aerotolerance may be a key factor in aiding the spread and survival
428 of *C. jejuni* in the environment. Taken at face value, this means that PerR reduces the capability of
429 *C. jejuni* to survive outside the avian or animal host, and hence it would be counter-intuitive that
430 this system is found in all *C. jejuni* and *C. coli* genomes, and also in several related *Helicobacter*
431 species (Belzer *et al.*, 2011). One possible explanation for this is that uncontrolled production of
432 oxidative stress response systems may be deleterious during infection or transmission.

433 Proteomic analysis of the *C. jejuni* NCTC 11168 *perR* mutant revealed a significant increase in
434 abundance of Rrc (Cj0012c) (Pinto *et al.*, 2011; Yamasaki *et al.*, 2004). The role of Rrc in oxidative
435 stress defences in *C. jejuni* has not yet fully been elucidated, yet it has been shown to be regulated
436 by several oxidative stress regulators including PerR, Fur, CosR and CprSR (Holmes *et al.*, 2005;
437 Hwang *et al.*, 2012; Palyada *et al.*, 2009; Svensson *et al.*, 2009). The protein itself has been
438 characterised as having a significant NADH-linked hydrogen peroxide reductase activity, and hence
439 may contribute to the hydrogen peroxide resistance of *C. jejuni perR* mutants (Fig. 1A and 1B)

440 (Pinto *et al.*, 2011).

441 The increased aerotolerance of the *perR* mutant may be linked to the joint derepression of alkyl
442 hydroperoxide reductase (*ahpC*) and thioredoxin reductase (*trxB*) genes. although we cannot
443 exclude other mechanisms. Inactivation of *ahpC* in *C. jejuni* resulted in reduced aerobic survival,
444 therefore it seems likely that an increased expression of *ahpC* may confer increased resistance and
445 survival in aerobic environments although further investigation is required (Baillon *et al.*, 1999). An
446 alternative hypothesis is that AhpC has a dual function of peroxidase and chaperone, as described
447 for the *Helicobacter pylori* AhpC protein (Chuang *et al.*, 2006), which could protect proteins from
448 oxidative damage. The co-regulation of the *trxB* and *aphC* genes by PerR also gives clues about the
449 role of TrxB in *C. jejuni*. In *C. jejuni*, AhpC catalyses the reduction of organic peroxides to their
450 corresponding alcohols, a process in which AhpC becomes oxidised. *C. jejuni* lacks a homologue
451 for the known AhpC recycling partner AhpF, which is found in *E. coli* (Poole, 1996). There has
452 been some speculation about potential candidates for AhpC recycling partners in *C. jejuni*,
453 including ferredoxin (Baillon *et al.*, 1999; van Vliet *et al.*, 2001). The joint regulation of *trxB*
454 alongside *ahpC* suggests that this role may be performed by *C. jejuni* TrxB, although this requires
455 experimental validation.

456 The advances in DNA sequencing technologies now allow identification of transcript levels by
457 RNA-sequencing, and in this study we have utilised differential RNA-sequencing to identify the
458 transcription start sites and promoter location of PerR- and Fur-regulated genes in *C. jejuni*,
459 including eight transcription start sites lacking in prior RNA-seq analyses (Dugar *et al.*, 2013;
460 Porcelli *et al.*, 2013). An analysis for conserved motifs by MEME (Bailey *et al.*, 2009) of 17
461 Fur/PerR-regulated promoter regions (Table 2) showed the presence of sequences resembling Fur
462 boxes (Baichoo & Helmann, 2002) in each promoter, but no specific motif distinguishing the PerR-
463 dependent promoters and the Fur-dependent promoters (Table 2). This is not unique to *C. jejuni*, as
464 a single amino acid mutation in the *B. subtilis* PerR is sufficient for it to bind to Fur recognition
465 sites (Caux-Thang *et al.*, 2014). In addition, it was previously shown that Fur and PerR can work

466 synergistically in *C. jejuni* (van Vliet *et al.*, 1999), and the subtle differences between the *C. jejuni*
467 PerR-box and Fur-box may not be easily distinguished by bioinformatic means.

468 In conclusion, we show here different roles for PerR and have further characterised parts of its
469 regulon. This work supports the role of PerR as a regulator of oxidative stress, but not as a regulator
470 of motility-associated phenotypes. The finding that PerR reduces aerotolerance of *C. jejuni* is
471 somewhat counter-intuitive in view of the importance of aerotolerance in the foodborne
472 transmission of this important bacterial pathogen, but may be best viewed as an example of the
473 complicated regulatory network governing expression of oxidative stress defence genes in *C. jejuni*.
474 Hence, future studies are required to further unravel the respective roles of, and crosstalk between,
475 regulatory systems.

476

477 **ACKNOWLEDGMENTS**

478 This work was supported by a Norwich Research Park PhD studentship to R.H., and the
479 Biotechnology and Biological Sciences Research Council (BBSRC) via the BBSRC Institute
480 Strategic Programme Grant BB/J004529/1, responsive mode grant BB/F00978X/1, and The
481 Genome Analysis Centre Capacity and Capability Challenge programme (project number CCC-1-
482 18). We thank Vertis Biotech for dRNA-seq cDNA library generation, Alain Stintzi for *C. jejuni*
483 NCTC 11168 *fur* and *perR* mutants, and EMBL Hamburg (PEPC7) for protein purification advice
484 and training. We also acknowledge the members of the IFR *Campylobacter* group, and Jason Crack
485 and Nick Cull (University of East Anglia) for experimental support and suggestions.

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710

711

712 **LEGENDS TO FIGURES**

713

714 **Figure 1: Inactivation of the *C. jejuni perR* gene results in increased resistance to oxidative**
715 **stress.** [A] Incubation of *C. jejuni* wild-type (filled circles), *perR* mutant (grey triangles) and the
716 complemented *perR* mutant (open squares) in Brucella broth supplemented with 3% hydrogen
717 peroxide, results in a rapid loss in viability of the wild-type strain and complemented *perR* mutant,
718 but no loss of viability of the *perR* mutant [B] The *C. jejuni perR* mutant is not sensitive to
719 hydrogen peroxide up to 30% (~ 8.8 M) in disc inhibition assays, whereas the wild-type and
720 complemented mutant show inhibition zones at hydrogen peroxide concentrations of 5%. Hydrogen
721 peroxide concentrations used are 0%, 5%, 10%, 15%, 20% and 30%. Asterisks indicate the absence
722 of an inhibition zone. [C] A *C. jejuni perR* mutant is more resistant to 3% (black bars) and 6%
723 (white bars) cumene hydroperoxide than the wild-type strain. Error bars indicate standard error of
724 the mean calculated over three independent experiments. Asterisks indicate a significant difference
725 with the wild-type strain (t-test), the # symbol indicates the absence of an inhibition zone.

726

727 **Figure 2: Inactivation of *perR* increases survival of *C. jejuni* in aerobic conditions.** Stationary-
728 phase cells of *C. jejuni* NCTC 11168 (black bars), *perR* mutant (white bars) and complemented
729 *perR* mutant (grey bars) were exposed to atmospheric oxygen conditions at 37°C. Aerobic survival
730 is expressed as the number of viable cells remaining in a culture exposed to air as determined by 10
731 fold serial dilution and spot plates. Statistically significant differences are indicated (*) as
732 determined by a 1-way ANOVA test.

733

734 **Figure 3: Inactivation of *perR* results in increased levels of peroxidases and related proteins.**
735 Two-dimensional gel analysis of *C. jejuni* NCTC 11168 wild-type, *perR*, *fur* and *fur perR* mutant
736 grown in Brucella broth. Boxes show enlarged regions to highlight differential protein abundance
737 between the wild-type strain and mutants. Circle A indicates multiple protein spots for catalase

738 (Cj1385: KatA), Circle B indicates thioredoxin reductase (Cj0146c: TrxB), Circle C indicates
739 protein spots for the rubreythin/rubredoxin-like protein of *C. jejuni* (Cj0012c: Rrc) and Circle D
740 indicates alkyl hydroperoxide reductase (Cj0334: AhpC).

741

742 **Figure 4: PerR-mediated regulation of peroxidase expression is mediated at the**
743 **transcriptional level, via binding of PerR to promoter regions.** [A] Illumina-based RNA-
744 sequencing of *C. jejuni* NCTC 11168 wild-type and an isogenic *fur perR* double mutant identifies
745 differentially regulated genes, as shown by RPKM values for Illumina RNA sequencing (Chaudhuri
746 *et al.*, 2011). Examples of deregulated genes are indicated. [B] Electrophoretic mobility gel shift
747 assays (EMSAs) using purified PerR protein demonstrate that PerR binds directly to regulated
748 promoter regions of the *ahpC*, *kataA*, and *trxB* genes. The auto-regulated *perR* promoter is included
749 as comparison (Kim *et al.*, 2011), and the *dnaE* promoter as negative control.

750

751 **Figure 5: Inactivation of *perR* does not affect *C. jejuni* motility or ability of *C. jejuni* to kill**
752 **wax moth larvae.** [A] Swarming motility of *C. jejuni* strains NCTC 11168, 81-176 and 81116
753 (NCTC 11828) is not affected by the inactivation of the *perR* gene, as shown using soft agar (0.4%).
754 Pictures shown are representative data from each of the three reference strains, for the wild-type
755 strain, the *perR* mutant and the complemented *perR::perR⁺* mutant, and compared to a non-motile
756 control (D) (*flaAB* mutant). [B] *G. mellonella* larvae were inoculated with 10 µl of an overnight *C.*
757 *jejuni* NCTC 11168 culture, diluted to an OD₆₀₀ of 0.1, by injection into the right foremost proleg,
758 which corresponds to an infectious dose of approximately 10⁶ CFU. The larvae were incubated at
759 37°C, with survival recorded at 24 and 48 hours post injection. Phosphate buffer saline and mock-
760 injection controls were included for comparison. For each experiment, ten *G. mellonella* larvae
761 were used per group, and five independent experiments were performed. There was no statistically
762 significant difference in survival between the wild-type strain, *perR* mutant or complemented strain.

1 **Table 1:** Bacterial strains and vectors used in this study.

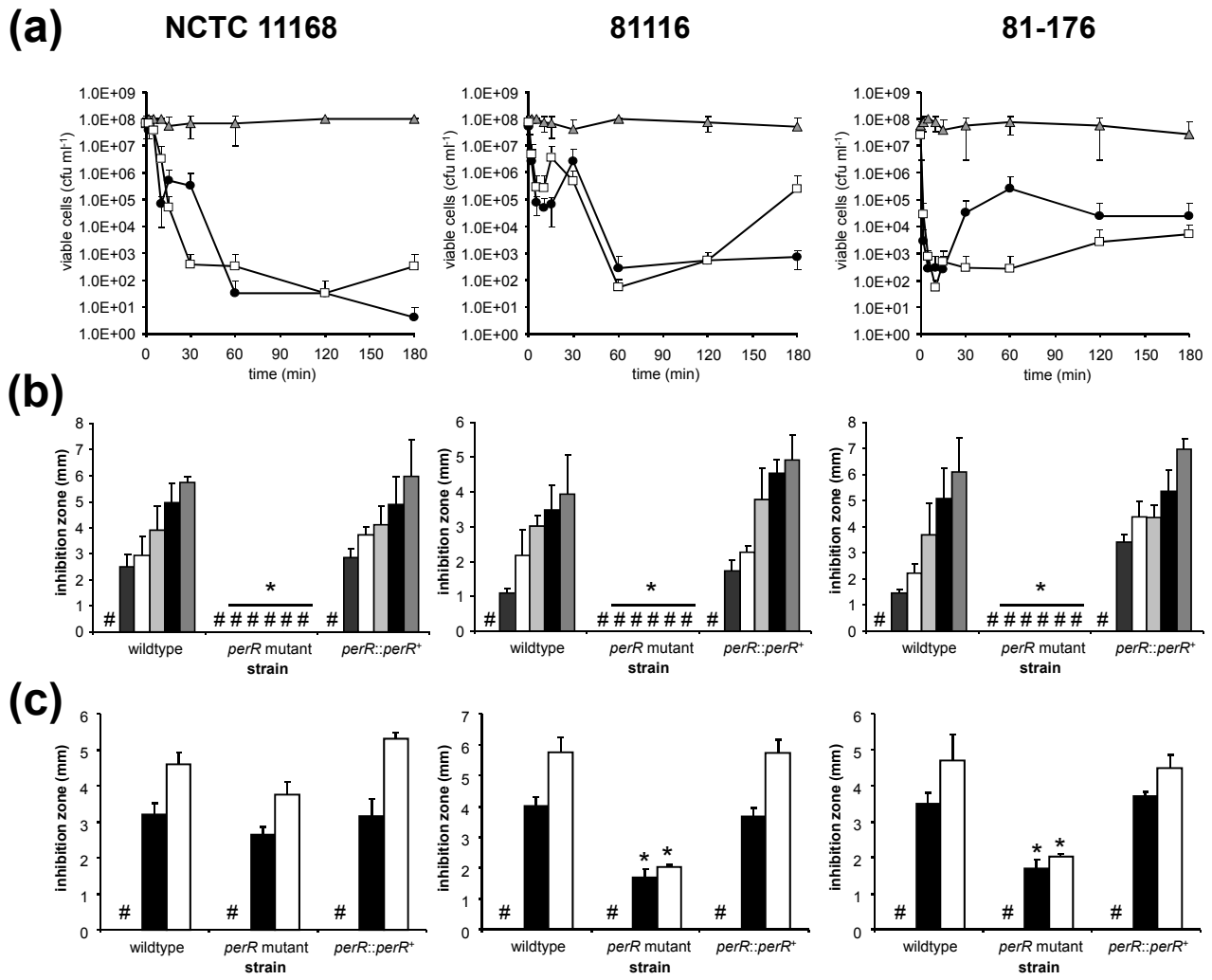
Strains	Description *	Source
<i>E. coli</i>		
BL21 (DE3)	F ⁻ ompT hsdSB (r _B ⁻ m _B ⁻) gal dcm (DE3)	Novagen
Top10	General cloning strain	Invitrogen
<i>C. jejuni</i>		
NCTC 11168	Wild-type <i>C. jejuni</i>	(Parkhill <i>et al.</i> , 2000)
11168 <i>perR</i>	NCTC 11168 <i>perR</i> ::kan ^R	This study
11168 <i>perR</i> :: <i>perR</i> ⁺	NCTC 11168 <i>perR</i> :: kan ^R <i>cj0046</i> :: <i>perR</i> ^{native} *cat ^R	This study
11168 <i>fur</i>	NCTC 11168 <i>perR</i> ::kan ^R	This study
11168 <i>fur perR</i>	NCTC 11168 <i>perR</i> ::kan ^R <i>fur</i> ::cat ^R	This study
11168 <i>fur perR</i>	11168 <i>fur perR</i>	(Palyada <i>et al.</i> , 2009)
11168 <i>flaAB</i>	NCTC 11168 (<i>cj1338-39c</i>)::kan ^R	(Reuter & van Vliet, 2013)
81116	Wild-type <i>C. jejuni</i> (NCTC 11828)	NCTC
81116 <i>perR</i>	NCTC 11828 <i>perR</i> ::kan ^R	This study
81116 <i>perR</i> :: <i>perR</i> ⁺	NCTC 11828 <i>perR</i> :: kan ^R <i>cj0046</i> :: <i>perR</i> ^{native} *cat ^R	This study
81-176	Wild-type <i>C. jejuni</i>	ATCC
81-176 <i>perR</i>	81-176 <i>perR</i> ::kan ^R	This study
81-176 <i>perR</i> :: <i>perR</i> ⁺	81-176 <i>perR</i> :: kan ^R <i>cj0046</i> :: <i>perR</i> ^{native} *cat ^R	This study
<u>Vectors</u>		
pNEB193	General cloning vector	New England Biolabs
pC46	Complementation plasmid containing <i>cj0046</i> flanks, cat ^R (for native promoter complementation)	(Reuter & van Vliet, 2013)
pET21a	General Cloning Vector	Invitrogen
pMARKan9	Source of kanamycin resistance cassette	(Reuter & van Vliet, 2013)
pAV35	Source of chloramphenicol resistance cassette	(van Vliet <i>et al.</i> , 1998)
pOSH1	PerR over-expression plasmid, pET21a, amp ^R	This study
pOSH2	<i>perR</i> plus flanking regions in pNEB193	This study
pOSH3	<i>perR</i> disruption plasmid, kan ^R	This study
pC46 <i>perR</i>	<i>perR</i> complementation plasmid, native promoter; cat ^R .	This study

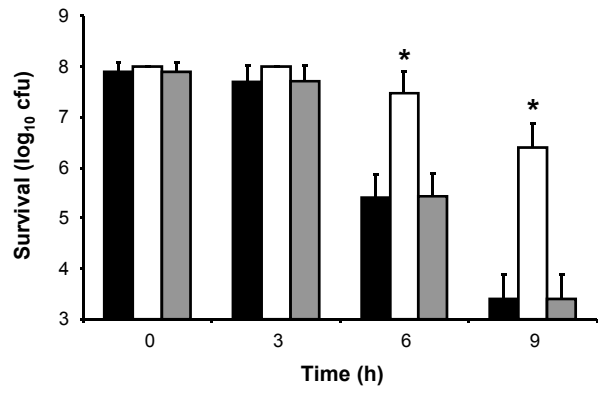
2 * kan^R indicates the presence of a kanamycin resistance gene, cat^R a chloramphenicol resistance
3 gene, amp^R an ampicillin resistance gene.

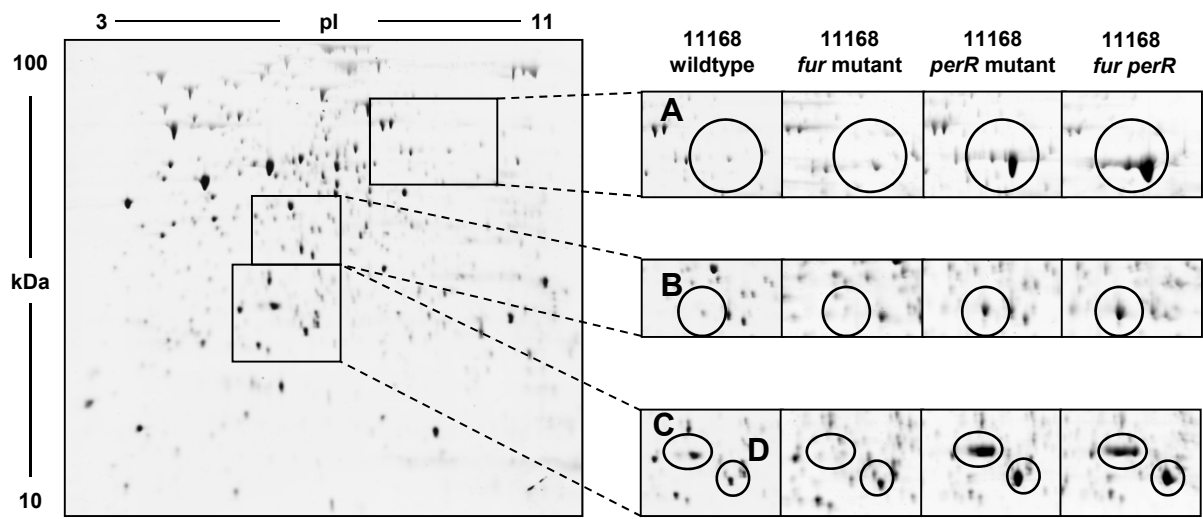
1 **Table 2.** *C. jejuni* promoter regions derepressed in a *C. jejuni fur perR* mutant

Gene *	TSS †	promoter + 5' UTR ‡ (putative PerR/Fur operator)	Ratio §
<i>rrc</i> (Cj0012c)	17426	ttat ttt gatatacatt ttt cataaatgataaaaattattaattgacaaaaat at ttt tttataaaTACAATact ttt TgataatcaaaaaAGGAGtcaaaATG	3.70
<i>trxB</i> (Cj0146c)	151607	taataat ttt tattaactactattaagaataat ttt taTATCATtatt A ata ataaaaattcaatataaaAGGAaatataaaATG	9.85
<i>cj0176c</i> (Cj0176c)	172717	aataaaaatgaaaat ttt atcagttataaaaattattaagttactttatattt act ttt taaatatTAATAT ttt ctt A tcaaaaatctctaaaAGGAattaATG	2.22
<i>chaN</i> (Cj0177)	172881	ataa ttt tgataataattataaaat ttt taaat ttt taataaaatatttaaat agt ttt tttatataataaaat ttt TAATAT tt caat A tcaaaaaataaccactaa GAAAGtaacaataataATG	100.63
<i>ahpC</i> (Cj0334)	302353	at tt ac ct ttat tt tgataataattataact ttt ttta ttt ta ttt ta ttt atattatct tagat ttt taaaaaaaataaagatt tt aa cg aTATAAT tt cg tt A tcaata aa ttt tatt ttt AGGAGaaaatATG	3.08
<i>cfrB</i> (Cj0444)	412299	at ttt tatt ttt tataaattg tt atcaaaa tt aa g tt tt aa ag tt tt ct tt tttTATACT tt caaaa A t g att tt aa at atcaaaa tt at ttt tt tt aaAGGAa ttaatTTG	28.26
<i>cfrA</i> (Cj0755)	705419	aa ttt tt tt aa tt aa ct aa tt atcaca at tgatacaaaa ttt atct tt aa tt aa tt aa tt tattatcaTAATAT ttt gat T t ct aa tt caat tt ta ca aaaAGGA GaaaaATG	29.20
<i>ceuB</i> (Cj1352)	1283976	cttactaaaat at c ttt ttatag ttt ttatcatt tt ct tt aa ca aa ttt catTA CAAT tt cat T tt tt gataa tt tatt tt actataAGGAaagatTTG	7.55
<i>cj1384c</i> (Cj1384c)	1322394	tgcat ttt tattgataa tt aa ttt caaaa tt aa ttt ag ttt tt tt taTAT TT ATa ataata A ttatcaaaa tt at ttt taaaa tt caaaaAGGAGa ttt ATG	239.28
<i>katA</i> (Cj1385)	1322495	taaa ttt at ttt tgaaa tt tattatcaat tt aa tt gcaattattcag tt aa ttt ta ttt tt tt aa tt agaTATAAT tt tacta A tt tt aa tt aa tt tatt ttt AGGAGa aa ca ATG	439.02
<i>chuZ</i> (Cj1613c)	1540646	atata ttt taagtg ctt tt tt aa ttt gataa tt gaTACCAT tt tt ttt A at tt ttt ttt taaaa tt caagaaAGGAtaaaaATG	11.01
<i>chuA</i> (Cj1614)	1540788	gataa ttt tatt tt ctaa tt tattatgatataaa tt TATCAT tt tattat T ttat ttaAGGAGtaaaa tt TTG	294.17
<i>exbB2</i> (Cj1628)	1555995	ga ttt ct tt aa tt aa tt atgataa tt gatatta tt taaa ttt at ttt tt tt at acagTATAAT ctt tt tt a A a ttt tataag tt at ttt GGAGAAAA tt ATG	30.83
<i>cj1658</i> (Cj1658)	1580782	tata ttt taataaaaa tt at tt atcaacataaaa g ctt ttt tt tt ag tt ct tt tt tt ag ttt tt tt agTATTAT ct ag cc A tt ttt tt tt gataa tt aa tt at ttt ttatcaaaa tt caAGGAtaatgataATG	6.61

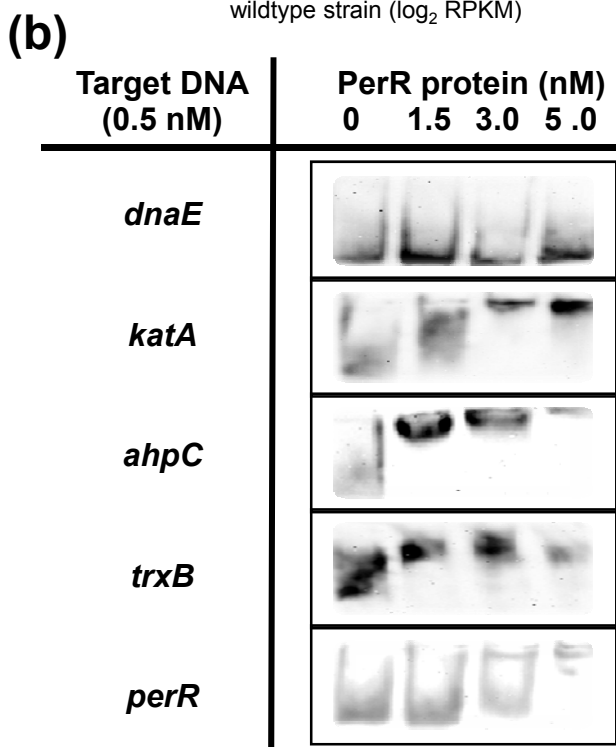
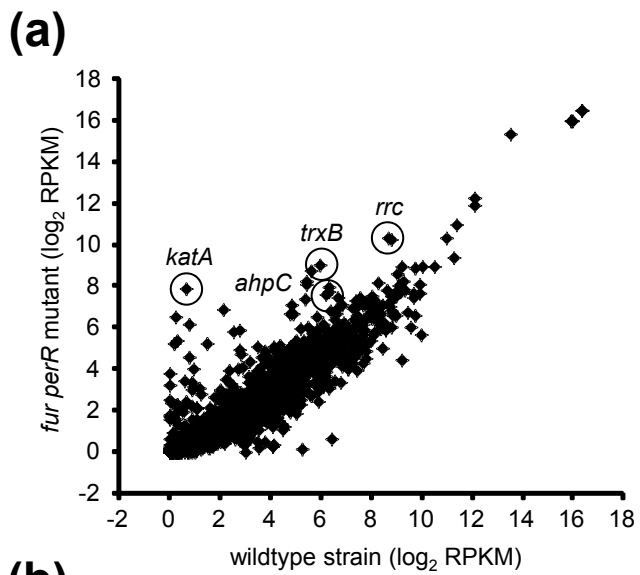
2 * Gene number from *C. jejuni* NCTC 11168 (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000)3 † Position of the transcription start site (TSS) on the *C. jejuni* genome, as determined by
4 differential RNA-sequencing (Porcelli *et al.*, 2013).5 ‡ The TSS is indicated in bold typeface and underlined. Capital letters indicate the ribosome
6 binding site (consensus sequence AGGAg), ATG or TTG startcodon, σ^{70} -dependent -10
7 region (TAnaAT), and indicated in grey are potential operator sequences.8 § Ratio of RPKM values obtained for the *fur perR* mutant divided by the RPKM value obtained
9 for wild-type *C. jejuni* NCTC 11168, using RNA-sequencing on the Illumina HiSeq 2000.







Handley et al, Figure 3



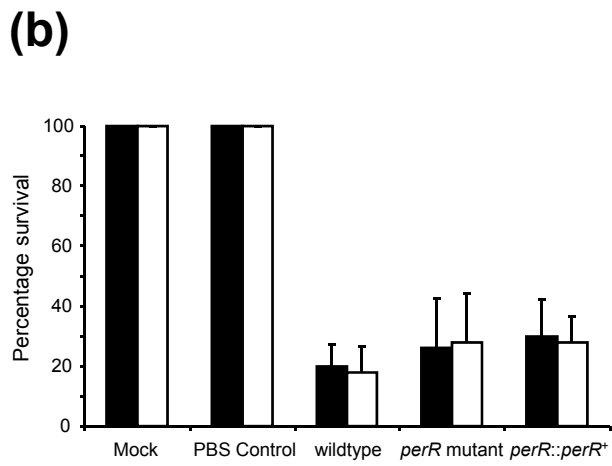
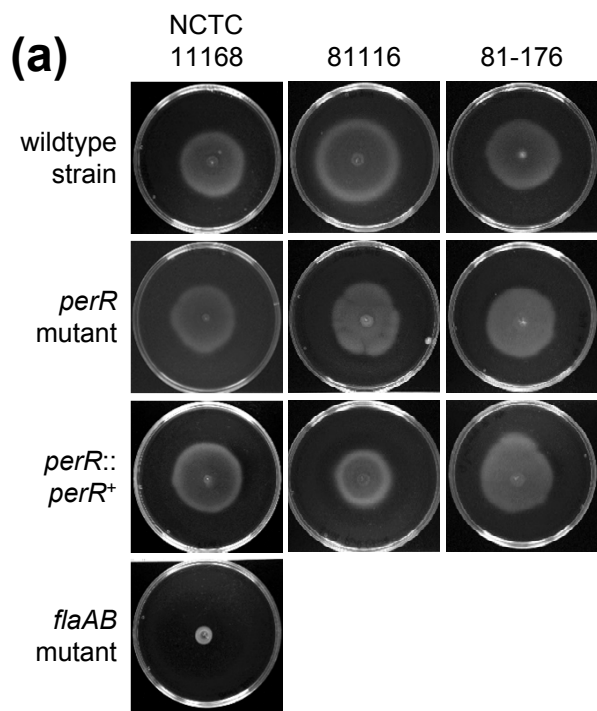


Table S1: Oligonucleotides used in this study.

Primer Designation	Sequence (5' > 3')
PerRflanksF	GGACCTATTGAATTCCGTTATCCTAG
PerRflanksR	GGAAACTGCAGGCTAAAATTCTG
PerRInverseR	GATCGGATCCGCTAAGCTTTATGAATATCAAGAGC
PerRInverseL	GATCGGATCCCACATAGTCTTTGCGGAGTAGC
PerRKOChek FWD	GCCCCAAGAGATGAACAAATGATGC
PerRKOChek REV	GCTTATCTTTTCTCTCTAAAGATAAAGTCC
KmPrReadOut	GCGATATCTTCTATATAAGCGTACCG
KmReadOut	CGGGGAAGAACAGTATGTCGAGC
PerRCompNativeFwdNcoI	GGAAAACCATGGAGAAAAAGATG
PerRCompRevNcoI	GGGTTATTTTAACCATGGTTTGC
0046Fcheck3	GCAGAGCACTTGATTTTAGTGTGTGC
0046Rcheck 3	CCTGGAGAAGTATTAGATAGTAGCGG
CatPrReadOut	GTCGAAATACTCTTTTCGTGTCC
PerRInternalRev	CACAAACAGACAGATGATTGACG
<i>perRp</i> ET21aF	GGAATAAATCATATGGAATTACTACAAATG
<i>perRp</i> ET21aR	CCCTTAAAATATATGGATCCTTTTAACC
KatA Prom Fwd	[DIG]-CCAATAACCAAAACTGACATAAATTCTCC
KatA Prom Rev	CGTTAGTCAATTTTTTCATTGTTTTCTCC
DnaE Prom Fwd	[DIG]-GCAATTTTACTTTCATCATTTTCATCC
DnaE Prom Rev	GCAAGTGTGTAAATTGACTCATTTTCTTCC
AhpC Prom Fwd	[DIG]-GCAATACATATATCTGTAATTTTACAGC
AhpC Prom Rev	GCTTTTTTAGTAACTATCATATTTTCTCC
PerR Prom Fwd	[DIG]-GGAAAAACAAGTGAGGTGG
PerR Prom Rev	GCTTTTAACTCATGTTTTTTAAGC
TrxB Prom Fwd	[DIG]-GGTGAAGTTGTAGATCAACTCGTTGGTGC
TrxB Prom Rev	CCAGCAGGACCTCCACCTATGATTGC
RNA adaptor	AUAUGC GCGAAUCCUGUAGAACGAACACUAG AAGAAA

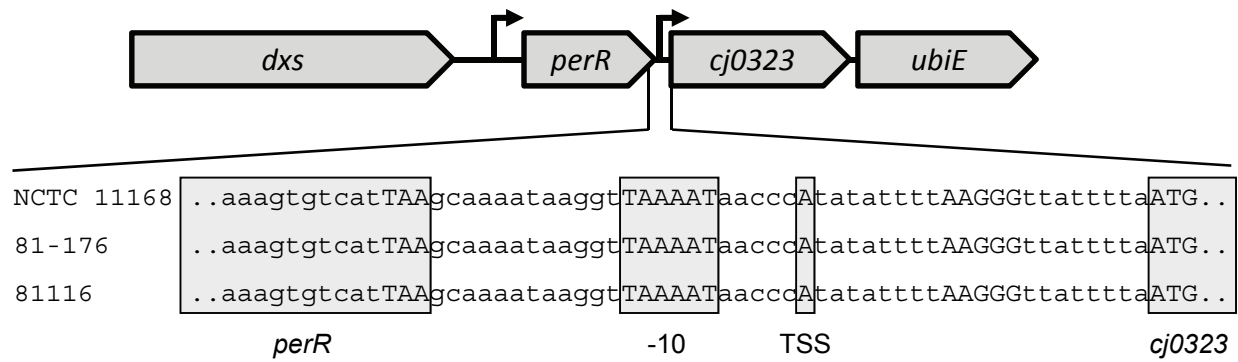
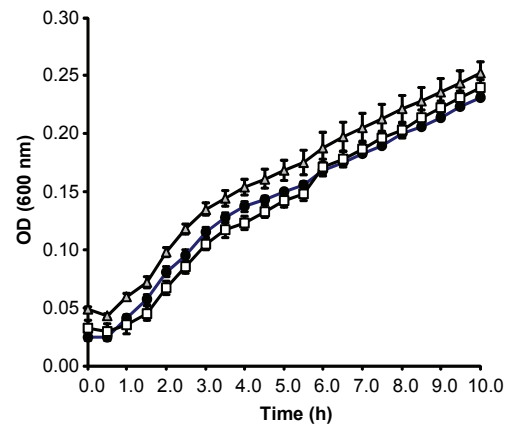
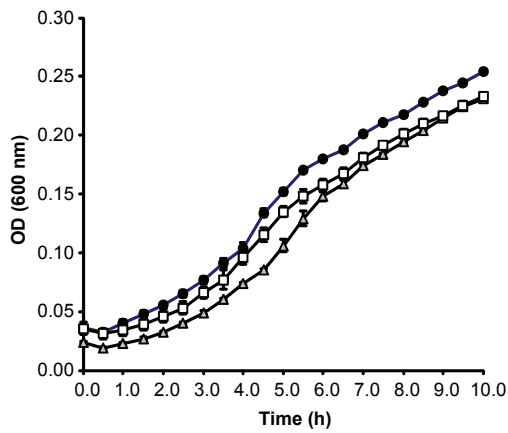
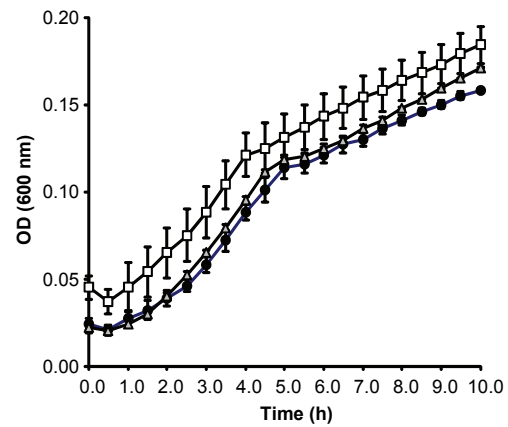
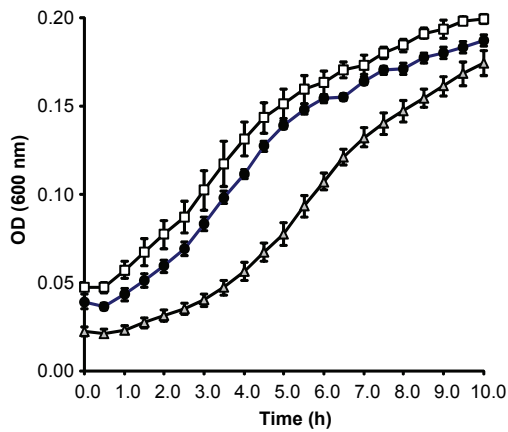


Figure S1: The *perR* genomic region is conserved across *C. jejuni* reference strains NCTC 11168, 81116 and 81-176, including a promoter just downstream of *perR* driving transcription of *cj0323* (Dugar *et al*, 2013; Porcelli *et al*, 2013). The final 54 nt of the 3' end of *perR* were not deleted in the *perR* mutants, to avoid disruption of the *cj0323* promoter. Promoters are indicated by arrows, with -10 sequences, transcription start site and the respective genes highlighted.

C. jejuni NCTC 11168



C. jejuni 81116



C. jejuni 81-176

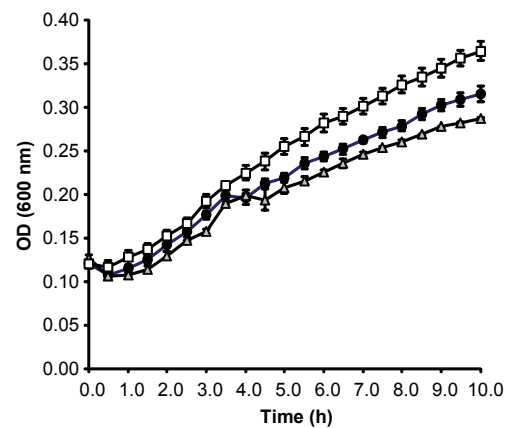
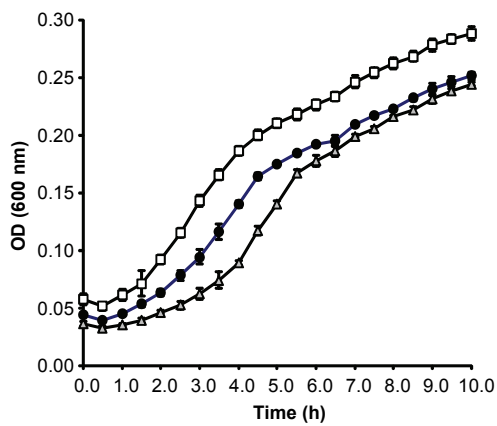


Figure S2: The *C. jejuni* *perR* mutants and complemented strains do not show any growth defects when compared to the wildtype strain when grown in Brucella media at 37°C and 42°C, in either of the three *C. jejuni* strains investigated (NCTC 11168, 81116 and 81-176). Black dots represent the wildtype strain, grey triangles the *perR* mutant, white squares the complemented *perR::perR+* strain. Error bars denote standard deviation of three independent experiments.