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

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

**INTRODUCTION**

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

Iron and oxidative stress are intimately connected via the capability of iron to produce reactive oxygen species via the Haber–Weiss and Fenton reactions (van Vliet et al., 2002). In C. jejuni, the metalloregulatory proteins Fur and PerR control iron uptake and oxidative stress responses (Holmes et al., 2005; Palyada et al., 2009; van Vliet et al., 2009), with regulation of oxidative stress further complicated by overlapping control circuitry through other regulatory proteins such as CosR and Gj1556 (Dufour et al., 2013; Gundogdu et al., 2011; Hwang et al., 2012; Svensson et al., 2009). Furthermore, DNA–binding by the Dps protein is activated in the presence of iron or hydrogen peroxide, and protects against DNA damage by hydroxyl radicals (Huerdo et al., 2013).

The PerR regulator is found mostly in Gram-positive bacteria (Marinho et al., 2014), but also in the Epsilonproteobacterial genera Campylobacter (van Vliet et al., 2009) and Helicobacter (Belzer et al., 2011). In general, PerR proteins respond to peroxide stress through derepression of expression of peroxidases and protective proteins such as Dps. In C. jejuni, PerR was first shown to mediate iron-dependent regulation of catalase (KatA) and alkyl hydroperoxide reductase (AhpC) (van Vliet et al., 1999). Subsequent studies using transcriptional profiling, RNA sequencing (RNA-seq) and chromatin immunoprecipitation using iron, fur and perR mutants have shown that there is significant overlap between the iron, Fur and PerR regulons (Butcher et al., 2012; Butcher & Stintzi, 2013; Holmes et al., 2005; Palyada et al., 2009). PerR has been proposed to modulate its own expression (Kim et al., 2011), while complete derepression of catalase expression was only observed in a fur perR double mutant (van Vliet et al., 1999). Since a fur mutant constitutively expresses iron acquisition systems (Miller et al., 2009; van Vliet et al., 2002), this further complicates interpretation of transcriptomic and proteomic characterization of PerR regulation in C. jejuni. Inactivation of perR results in reduced colonization in chickens, suggesting a role of oxidative stress regulation in intestinal colonization (Palyada et al., 2009), although the reduced motility of the perR mutant reported in this study makes it difficult to distinguish the roles of motility and PerR on colonization.

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

**METHODS**

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

**Construction of a C. jejuni perR mutant.** The region containing the cjo322 gene and approximately 500 bp of flanking sequence on each side was PCR amplified by Phusion DNA polymerase (New England Biolabs) using oligonucleotide primers PerRFlnksF and PerRFlnksR (Table S1, available in the online Supplementary Material). This amplified fragment was purified using a commercial PCR purification kit (Qiagen), digested with EcoRI (New England Biolabs) and PstI (Promega), and then ligated into pNBE193 to form plasmid pOSH2. To make the perR insertional inactivation construct, pOSH2 was used as template for inverse PCR as described previously (Reuter & van Vliet, 2013), using the oligonucleotide primers PerRInvRe and PerRInvFor. The kanamycin cassette from pMARKan9 and the inverse PCR product from pOSH2 were digested with BamHI (New England Biolabs) and ligated to form plasmid pOSH3. Ligated fragments were used to transform Escherichia coli strain Top10 and positive transformants were selected by plating on LB agar supplemented with 30 µg kanamycin ml⁻¹. All constructs and insert orientations were confirmed by restriction digestion analysis and sequencing (The Genome Analysis Centre, Norwich, UK). C. jejuni perR mutants were isolated after transformation with pOSH3 by electroporation (Reuter & van Vliet, 2013) and subsequent selection on kanamycin-containing agar. Colonies were screened by PCR using oligonucleotides PerRKOChek FWD and PerRKOChek REV, which anneal outside of the cloned flanking regions in combination with antibiotic-cassette-specific primers KmPrReadOut and KmReadOut (Table S1).

**Construction of perR complementation constructs.** C. jejuni perR mutants were complemented in trans by using the perR gene with its own promoter cloned into the cjo046 pseudogene, as described previously (Reuter & van Vliet, 2013). To make the perR complementation construct with the native perR promoter, the perR gene plus promoter region was PCR amplified using oligonucleotides PerRCompNatFwdNcoI and PerRCompRevNcoI (Table S1). The amplified fragment was digested with NcoI (Promega) and ligated into the Esp31 site of pC46. This construct, known as pC46perR, was used to transform a C. jejuni perR mutant using standard electroporation methods. Complementation strains were selected on chloramphenicol-containing agar plates, and colonies
checked by PCR using primers that anneal outside of the cj0046 flanking regions (0046Fcheck3 and 0046Rcheck 3) in combination with gene- and antibiotic-cassette-specific primers CatPrReadOut and PerRInternalRev (Table S1).

Motility assay. The OD₆₅₀ of an overnight C. jejuni culture was adjusted to 0.4 using sterile PBS. Bacterial motility was assessed by spotting 10 μl culture onto the centre of a 0.4 % Brucella agar plate (Reuter & van Vliet, 2013). Plates were photographed after 24, 48 and 72 h of incubation under microaerobic conditions at 37 °C, and the diameter of the halo was measured using ImageJ software [version 1.41; National Institute of Health (http://rsbweb.nih.gov/ij/)]. A C. jejuni NCTC 11168 flaAB non-motile mutant was included in all experiments as a negative control (Reuter et al., 2010).

Oxidative stress and aerotolerance assays. Resistance to oxidative stress was measured using disc inhibition assays. C. jejuni were grown overnight on Skirrow plates at 37 °C, and cells were harvested into 2 ml Brucella broth. C. jejuni was then added to 3 ml 1.5 % Brucella agar to a final OD₆₅₀ of 1.0 and poured onto a Brucella plate. After the agar had set, 6 mm diameter sterile 3M Whatman paper discs were placed on the soft agar surface, and 10 μl hydrogen peroxide (0–30 %, v/v, in water) or cumene hydroperoxide (0–6 %, v/v, in DMSO) was applied to the discs. Discs were incubated overnight in microaerobic conditions at 37 °C, photographed and the zone of inhibition (no growth) was measured using ImageJ image analysis software. The effect of hydrogen peroxide was also measured in broth culture, using overnight cultures of C. jejuni NCTC 11168 which were adjusted to an OD₆₅₀ of 0.4 using sterile PBS buffer. Hydrogen peroxide solution was then added to a final concentration of 3 % (v/v). Cell survival was assessed by plating out serial dilutions after incubating the broth cultures in shaking conditions for 0, 2, 5, 10, 15, 30, 60, 120 and 180 min.

Aerotolerance assays were adapted from Baillon et al. (1999) with some alterations. Cultures (20 ml) were grown overnight in Brucella broth, and adjusted to an OD₆₅₀ of 0.4 using Brucella broth. For each strain, cultures were split into two 10 ml cultures in separate flasks, with one grown microaerobically (85 % N₂, 5 % O₂, 10 % CO₂) and the other aerobically at 37 °C, shaking at 200 r.p.m. Samples of each culture were taken at 3 h time intervals. Serial tenfold dilutions were used to assess cell survival; 5 μl of each dilution was spotted onto Brucella agar plates and incubated under microaerobic conditions for 2 days at 37 °C.

Galleria infection model. The Galleria mellonella infection model was used to assess whether inactivation and complementation of perR affected killing of wax moth larvae by C. jejuni, which has been suggested to represent virulence (Champion et al., 2011; van Alphen et al., 2014). G. mellonella larvae were obtained from Livefood UK. Larvae were inoculated in the right foremost pro-leg by microinjection (Hamilton) with 10 μl C. jejuni overnight culture, which had been adjusted to an OD₆₅₀ of 0.1 (approximately 10⁶ c.f.u.). PBS and mock infection controls were also

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<th>Strain/vector</th>
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performed alongside each experiment. The larvae were incubated at 37 °C, with percentage survival scored at 24 h intervals. For each experiment, ten G. mellonella larvae were infected and a total of five independent experiments were performed.

2D protein gel electrophoresis and protein identification. 2D protein gel electrophoresis was conducted essentially as described previously (Hockin et al., 2012; Shaw et al., 2012). C. jejuni cells were grown to late log phase in Brucella broth under microaerobic conditions, and were harvested from broth culture (50 ml) by centrifugation at 4000 g for 10 min at room temperature. Cell pellets were resuspended in 300 μl lysis buffer (50 mM Tris pH 7.5, 0.3 % SDS, 0.2 M DTT, 3.3 mM MgCl₂, 16.7 μg RNase ml⁻¹ and 1.67 U DNase ml⁻¹) and lysed (Soniprep 150 MSE; Sanyo) on ice until clear. The samples were then centrifuged (14 000 g, 20 min, 4 °C) to remove any unlysed cells. Total cell protein was quantified using a 2D Quant kit (GE Healthcare) as per the manufacturer’s instructions. After 2D protein gel electrophoresis, proteins of interest were visualized using a ProToshot gel excision robot (Genomic Solutions), and in-gel trypsin digested using a ProGrast protein digester (Genomic Solutions) (Hockin et al., 2012; Shaw et al., 2012). HPLC-MS-MS analysis was performed using a LTQ-Orbitrap mass spectrometer (Thermo Electron) and a nanoflow-HPLC system (nanoACQUITY; Waters).

Expression and purification of recombinant C. jejuni PerR. The perR gene was PCR amplified using primers perRpet21aF and perRpet21aR (Table S1), and the amplified fragment was digested with restriction enzymes BamHI and NdeI (New England Biolabs) and ligated into pET21a, which had been digested with the same enzymes. This plasmid construct (pOSh) was subsequently checked by sequencing (The Genome Analysis Centre, Norwich, UK) then used to transform E. coli BL21(DE3). E. coli harbouring pOSh was grown in LB broth, at 37 °C, with shaking at 180 r.p.m. to an OD₆₀₀ of 1.0. The expression of recombinant PerR was induced by addition of 1.0 mM IPTG for 4 h at 30 °C. Bacterial cells were harvested by centrifugation and resuspended in 20 mM Tris-HCl, 50 mM NaCl, pH 8.0, and then lysed on ice by sonication. The samples were then centrifuged at 23 000 g for 30 min at 4 °C. The soluble cell extract was treated with DNase I (New England Biolabs) and RNase A (Promega) and the DNA was purified using hot phenol (Mattatall & Sanderson, 1996) to ensure that small RNAs would not be removed by the extraction procedure. The RNA was treated with DNase I to remove genomic DNA, followed by optional treatment with Terminator exonuclease (Epicentre Biotechnologies) for enrichment of primary RNAs, and treatment with tobacco acid phosphorylase (Cambio) to generate 5’-P ends for downstream ligation of 454 fords (Porcelli et al., 2013; Sharma et al., 2010). After ligation of an RNA oligonucleotide to the phosphorylated 5’-ends of RNA, and polyadenylation of RNA, first strand cDNA was generated using an oligo-dT containing 454-B primer. The cDNA fragments were barcoded and amplified, and used for generation of cDNA libraries for the 454 FLX system at Vertis Biotech (Germany). These libraries were subsequently analysed using a Roche Titanium sequencer. The same RNA samples were also used to generate strand-unspecific RNA-seq libraries for Illumina sequencing, using instructions from the manufacturer (Illumina), and further analysed using an Illumina HiSeq2000 sequencer.

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

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

RESULTS

Construction and complementation of C. jejuni perR mutants

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

Previous studies have shown that C. jejuni perR mutants are more resistant to peroxide stress induced by hydrogen peroxide (H₂O₂) or cumene hydroperoxide (CHP). We quantified this using plate inhibition and broth survival assays. The perR mutants exhibited very high levels of resistance to hydrogen peroxide in both broth culture (Fig. 1a) and disc assay experiments (Fig. 1b), both under micro-aerobic conditions. During exposure to 3 % hydrogen peroxide (0.88 M) in broth culture, the perR mutants maintained viability (1.0 × 10⁸ c.f.u. ml⁻¹) throughout the experiment, whereas the number of viable cells in the wild-type strains declined rapidly after the addition of H₂O₂. Exposure of C. jejuni perR mutants to hydrogen peroxide on discs revealed that the level of peroxide resistance was considerably higher than previously reported, where 3 % hydrogen peroxide was used (Baillon et al., 1999; Flint & Stintzi, 2015; Palyada et al., 2009; van Vliet et al., 1999). Wild-type C. jejuni were sensitive to 5 % H₂O₂ (Fig. 1b), as indicated by the inhibition of growth of the bacteria around the Whatman paper disc. In contrast, the perR mutants in the three reference strains did not show any inhibition zone at 30 % H₂O₂ (8.8 M). When grown in the presence of 3–6 % CHP, the three wild-type strains showed a reduction in growth as the concentration of CHP increased (Fig. 1c). However, C. jejuni 81116 and 81-176 perR mutants were able to grow better in the presence of CHP compared to the comparative wild-type strains, as is evident from the reduced zones of clearance. Although there was a reduced zone of clearance with CHP for the C. jejuni NCTC 11168 perR mutant, this difference was not statistically significant. Overall, all three C. jejuni strains show a similar phenotype, inactivation of the C. jejuni perR gene significantly increased resistance to hydrogen peroxide and cumene hydroperoxide, whereas the complementation of the perR mutant restored a wild-type peroxide stress phenotype (Fig. 1).

The increase in peroxide stress resistance was associated with a general increase in survival under aerobic conditions (aerotolerance). After exposure of stationary phase cells to an aerobic environment, the C. jejuni NCTC 11168 wild-type and complemented perR mutants showed a rapid reduction in viability, as the number of viable cells declined by two logs to 1.0 × 10⁶ c.f.u. ml⁻¹ after 6 h in an aerobic environment (Fig. 2). In contrast, the perR mutant showed increased aerotolerance with over 1.0 × 10⁶ c.f.u. ml⁻¹ surviving after 9 h exposure to air, with 2–3 logs more survival when compared to the wild-type and complemented strains.

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

To characterize the role of PerR in regulation of C. jejuni gene expression, the effects of the perR mutation were assessed at the level of protein abundance (using 2D gel electrophoresis) and transcript levels (using RNA-seq). As overlaps between the Fur and PerR regulatory networks have been reported (Holmes et al., 2005; Palyada et al., 2009; van Vliet et al., 1999), we included a fur and fur perR double mutant (Table 1) in the protein expression analysis. Comparison of the protein profiles of C. jejuni NCTC 11168 wild-type, perR, fur and fur perR mutants showed that several proteins were consistently increased in abundance in the perR mutant backgrounds (Fig. 3). Four of these proteins were further investigated by MS and identified as AhpC (Cj0334, Mascot score 11221, sequence coverage 97 %), KatA (Cj1385, Mascot score 1152, sequence coverage 56 %), TrxB (Cj0146c, Mascot score 2725, sequence coverage 67 %) and Rcr (Cj0012c, Mascot score 1752, sequence coverage 67 %).

The increased abundance of these four proteins was associated with increased levels of the respective mRNAs, as shown by RNA sequencing using the wild-type strain and the fur perR mutant (Fig. 4a). The RPKM values for the ahpC, katA, trxB and rrc genes were significantly increased in the NCTC 11168 fur perR mutant when compared to the wild-type strain (Table 2). Differential RNA-seq (Dugar et al., 2013; Porcelli et al., 2013) was used to confirm the TSSs of the respective genes (Table 2). Each of the four PerR-regulated genes showed increased transcript levels as found in the RNA-seq analysis. For each of the four genes, transcription starts from a single, identical TSS in both the wild-type strain and fur perR mutant, which is preceded by a recognizable σ⁷₀–10 sequence (gntTAnaaT) located 4–7 bp upstream, and matches the TSS described elsewhere (Baillon et al., 1999; Dugar et al., 2013; Hwang et al., 2012; Porcelli et al., 2013). This demonstrates that PerR regulation of these genes is mediated from a single promoter region, and excludes the possibility that this regulation is controlled from a secondary promoter. In addition, the use of the fur perR mutant for RNA-seq allowed the additional identification of eight previously unknown TSSs of Fur-repressed genes (Table 2), aiding the analysis of possible operator sequences in their respective promoters. Analysis of these genes shows the presence of motifs representing potential Fur/PerR-binding sites, which are rich in nAT-triplets (Baichoo & Helmann, 2002).

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

Full length, untagged C. jejuni PerR protein was heterologously expressed in E. coli purified to approximately 90 % purity, as assessed by SDS-PAGE analysis. This was subsequently used in EMSAs to assess whether PerR binds specifically to the ahpC, katA, trxB and rrc promoter regions identified by differential RNA-seq. The DNA fragments used included the TSSs shown in Table 2. Binding of PerR was observed for the ahpC, katA and trxB promoters, confirming the direct role of PerR in their regulation.
(Fig. 4b). The perR promoter was used as positive control as autoregulation of perR was described elsewhere (Kim et al., 2011), and indeed this also resulted in a mobility shift (Fig. 4b). No mobility shift was observed with the dnaE (cj0718) promoter, which was included to control for non-specific binding of C. jejuni PerR protein to C. jejuni promoter DNA (Fig. 4b). We were unable to detect binding of PerR to the promoter region of rrc (data not shown).

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

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

**DISCUSSION**

The microaerophilic lifestyle of bacteria like C. jejuni continuously poses a problem for the cell: there is a need for some oxygen for metabolism (i.e. as preferred terminal electron acceptor), but there is also exquisite sensitivity to the possible deleterious effects of reactive oxygen species formed during metabolism, or exposure to external sources of oxygen. This is especially a problem for pathogenic bacteria which require transmission from host to host (often faecal–oral), during which they may need to survive long-term exposure to atmospheric oxygen conditions. These stresses necessitate a tight control of oxidative stress defence systems; hence, it is not surprising that many of the characterized C. jejuni regulatory systems directly or indirectly affect oxidative stress (Gundogdu et al., 2011; Hwang et al., 2012; Palyada et al., 2009; Svensson et al., 2009; van der Stel et al., 2015; van Vliet et al., 2002). In this study, we have focused on the PerR regulator, a member of the Fur family of metalloregulatory proteins, which was initially
stress responses in *C. jejuni*, we have characterised the phenotypes of *C. jejuni* perR mutants in response to multiple sources of oxidative stress. Our results support the role of PerR as a regulator of oxidative–stress–mediated genes (Kim et al., 2011, 2015; Palyada et al., 2009; van Vliet et al., 1999) and as mediator of very high levels of peroxide stress resistance, but inactivation of perR did not affect the ability of *C. jejuni* to kill wax moth larvae. Interestingly, the increased resistance to peroxide stress of a perR mutant is matched by a significantly increased aerobic survival (aerotolerance) of *C. jejuni*.

Although some targets of PerR have been described elsewhere (Hwang et al., 2012; Kim et al., 2011, 2015; Palyada et al., 2009), the reported phenotypes of perR mutants of *C. jejuni* have varied, especially with regard to the role of PerR in motility and intestinal colonization. In this study, *C. jejuni* perR mutants displayed growth similar to wild-type (Fig. S2), did not show lowered motility in three well-characterized *C. jejuni* reference strains (Fig. 5a) and did not show reduced ability to kill wax moth larvae (Fig. 5b). This differs from the phenotypes described elsewhere (Palyada et al., 2009), where inactivation of perR in strain NCTC 11168 resulted in decreased motility and colonization of the chicken intestine, while complementation with *perR* restored motility. We currently do not have an explanation for this difference in phenotypes between the different *perR* mutants and have insufficient information for anything more that speculation. Flagellar biosynthesis and motility requires the coordinated expression of >50 genes, and it is well established that the inactivation or absence of expression of a single factor (by mutation or phase variation) can significantly affect motility (Carrillo et al., 2004; Hendrixson, 2006). All this does highlight the complexity of regulation of oxidative stress responses and motility, which in *C. jejuni* are affected by several regulatory systems (Carrillo et al., 2004; Gundogdu et al., 2011; Hendrixson, 2006; Holmes et al., 2005; Hwang et al., 2011a, b, 2012; Palyada et al., 2009; van der Stel et al., 2015; van Vliet et al., 1999).

It was previously shown that inactivation of perR in strain NCTC 11168 results in high-level production of the peroxidases catalase and alkyl hydroperoxide reductase (van Vliet et al., 1999). We have confirmed this phenotype in two other commonly used reference strains of *C. jejuni* (81116 and 81-176). More importantly, the high levels of, especially, catalase resulted in a significant increase in resistance against hydrogen peroxide, with the perR mutant able to grow close to discs infused with 30 % (8.8 M) hydrogen peroxide, which is the highest commercially available concentration of hydrogen peroxide. This concentration is much higher than previously tested, where either 3 % hydrogen peroxide was used (Baillon et al., 1999; Flint & Stintzi, 2015; Palyada et al., 2009; van Vliet et al., 1999), or concentrations ranging from 1–5 mM (Huergo et al., 2013; Hwang et al., 2011a, b), and highlights the very high levels of catalase expression in the perR mutant. A similar phenotype (albeit not as striking) was observed

![Fig. 4. PerR-mediated regulation of peroxidase expression is mediated at the transcriptional level, via binding of PerR to promoter regions. (a) Illumina-based RNA-seq of *C. jejuni* NCTC 11168 wild-type and an isogenic fur perR double mutant identified differentially regulated genes, as shown by RPKM values for Illumina RNA-seq (Chaudhuri et al., 2011). Examples of deregulated genes are indicated. (b) EMSAs using purified PerR protein demonstrate that PerR binds directly to regulated promoter regions of the ahpC, katA and trxB genes. The auto-regulated perR promoter is included as a comparison (Kim et al., 2011), and the dnaE promoter as a negative control.](http://mic.sgmjournals.org)
for cumene hydroperoxide (Fig. 1c), which is a substrate for AhpC (Baillon et al., 1999). The difference between these phenotypes may be explained by the difference in expression levels in the wild-type strain; while expression of catalase is strongly repressed in the iron-sufficient conditions of the Brucella medium (Fig. 3) (van Vliet et al., 1999), there is constitutive (but inducible) expression of AhpC in the wild-type strain, thus giving a much higher level of protection independent of the perR mutation.

In addition to the clear increase in resistance to peroxides, we also observed a significant increase in aerotolerance in the perR mutant (Fig. 2). Aerobic stress will be abundant during the transmission of C. jejuni to food preparation surfaces; hence aerotolerance may be a key factor in the infection route of C. jejuni, as the organism needs to survive aerobic exposure in order to spread and proliferate. The increased hydrogen peroxide and cumene hydroperoxide resistance of the perR mutant (Fig. 1a–c) was associated with a clear increase in aerobic survival (Fig. 2) of a C. jejuni NCTC 11168 perR mutant compared to the wild-type strain, with a 3–4 log higher survival than the wild-type strain and complemented mutant. Although the exact mechanism underlying the increased aerotolerance was outside the remit of this study, it is likely to be due to the increased expression of peroxidases, since activation of the ahpC gene was previously shown to reduce aerotolerance (Baillon et al., 1999). Aerobic stress will be abundant during the transmission of C. jejuni to food preparation surfaces; hence aerotolerance may be a key factor in aiding the spread and survival of C. jejuni in the environment. Taken at face value, this means that PerR reduces the capability of C. jejuni to survive outside the avian or animal host; hence, it would be counter-intuitive that this system is found in all C. jejuni and Campylobacter

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

<table>
<thead>
<tr>
<th>Gene (accession number)</th>
<th>TSS position</th>
<th>Promoter + 5' untranslated region</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>rrC (cj0012c)</td>
<td>17 426</td>
<td>TATAATGATACACAAATTTATTTTTTTTA</td>
<td>3.70</td>
</tr>
<tr>
<td>trxB (cj0146c)</td>
<td>151 607</td>
<td>TATAATGATACACAAATTTATTTTTTTTA</td>
<td>9.85</td>
</tr>
<tr>
<td>cj0176c (cj0176c)</td>
<td>172 717</td>
<td>TATAATGATACACAAATTTATTTTTTTTA</td>
<td>2.22</td>
</tr>
<tr>
<td>chaN (cj0177)</td>
<td>172 881</td>
<td>TATAATGATACACAAATTTATTTTTTTTA</td>
<td>100.63</td>
</tr>
<tr>
<td>ahpC (cj0334)</td>
<td>302 353</td>
<td>TATAATGATACACAAATTTATTTTTTTTA</td>
<td>3.08</td>
</tr>
<tr>
<td>cfrB (cj0444)</td>
<td>412 299</td>
<td>TATAATGATACACAAATTTATTTTTTTTA</td>
<td>28.26</td>
</tr>
<tr>
<td>cfrA (cj0755)</td>
<td>705 419</td>
<td>TATAATGATACACAAATTTATTTTTTTTA</td>
<td>29.20</td>
</tr>
<tr>
<td>ccrB (cj1352)</td>
<td>1 283 976</td>
<td>TATAATGATACACAAATTTATTTTTTTTA</td>
<td>7.55</td>
</tr>
<tr>
<td>cfrB (cj1384c)</td>
<td>1 322 394</td>
<td>TATAATGATACACAAATTTATTTTTTTTA</td>
<td>239.28</td>
</tr>
<tr>
<td>katA (cj1385)</td>
<td>1 322 495</td>
<td>TATAATGATACACAAATTTATTTTTTTTA</td>
<td>439.02</td>
</tr>
<tr>
<td>chuZ (cj1613c)</td>
<td>1 540 646</td>
<td>TATAATGATACACAAATTTATTTTTTTTA</td>
<td>11.01</td>
</tr>
<tr>
<td>chuA (cj1614)</td>
<td>1 540 788</td>
<td>TATAATGATACACAAATTTATTTTTTTTA</td>
<td>294.17</td>
</tr>
<tr>
<td>exbB2 (cj1628)</td>
<td>1 555 995</td>
<td>TATAATGATACACAAATTTATTTTTTTTA</td>
<td>30.83</td>
</tr>
<tr>
<td>chuA (cj1658)</td>
<td>1 580 782</td>
<td>TATAATGATACACAAATTTATTTTTTTTA</td>
<td>6.61</td>
</tr>
</tbody>
</table>

*Gene number from C. jejuni NCTC 11168 (Gundogdu et al., 2007; Parkhill et al., 2000).
†Position of the TSS on the C. jejuni genome, as determined by differential RNA-seq (Porcelli et al., 2013).
‡The TSS is indicated in bold typeface and underlined. Capital letters indicate the ribosome–binding site (consensus sequence AGGAg), ATG or TTG start codon and σ70-dependent –10 region (TAnaAT), and highlighted in grey are the potential operator sequences.
§Ratio of RPKM values obtained for the fur perR mutant divided by the RPKM value obtained for wild-type C. jejuni NCTC 11168, using RNA-seq on the Illumina HiSeq 2000.
coli genomes, and also in several related *Helicobacter* species (Belzer *et al.*, 2011). One possible explanation for this is that uncontrolled production of oxidative stress response systems may be deleterious during infection or transmission.

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

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

The advances in DNA–sequencing technologies now allow identification of transcript levels by RNA-seq, and in this study we have utilized differential RNA-seq to identify the TSSs and promoter location of PerR- and Fur-regulated genes in *C. jejuni*, including eight TSSs lacking in prior RNA-seq analyses (Dugar *et al.*, 2013; Porcelli *et al.*, 2013). An analysis for conserved motifs by MEME (Bailey *et al.*, 2009) of 17 Fur/PerR-regulated promoter regions (Table 2) showed the presence of sequences resembling Fur–boxes (Baichoo & Helmann, 2002) in each promoter, but no specific motif distinguishing the PerR-dependent promoters and the Fur-dependent promoters (Table 2). This is not unique to *C. jejuni*, as a single amino acid mutation in the *Bacillus subtilis* PerR is sufficient for it to bind to Fur
recognition sites (Caux-Thang et al., 2015). In addition, it was previously shown that Fur and PerR can work synergistically in C. jejuni (van Vliet et al., 1999), and the subtle differences between the C. jejuni PerR-box and Fur-box may not be easily distinguished by bioinformatic means.

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

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