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

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

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ABSTRACT

The foodborne bacterial pathogen *Campylobacter jejuni* is an obligate microaerophile, which 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 characterised 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% H_2O_2 (8.8 M). Expression of catalase, alkyl hydroperoxide reductase, thioredoxin reductase and the Rrc desulforubrerythrin were 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-seq analysis of a *fur perR* mutant allowed the identification of eight previously unknown transcription start sites of genes controlled by either Fur and/or PerR. Finally, inactivation of *perR* in *C. jejuni* did not result in reduced motility, and did not reduce killing of *Galleria melonella* 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, 2011; Nichols *et al.*, 2012). This, together with its link to neurodegenerative diseases like Guillain-Barre syndrome make 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% O_2 and 3-10% CO_2) 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 (Hazeleger *et al.*, 1998). When exposed to such conditions, *C. jejuni* will be under oxidative stress, and 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.*, 1999), with regulation of oxidative stress further complicated by overlapping control circuitry through other regulatory proteins such as CosR and Cj1556 (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 (Huergo *et al.*, 2013).

The PerR regulator is found mostly in Gram-positive bacteria (Marinho *et al.*, 2014), but also in the Epsilon-proteobacterial genera *Campylobacter* (van Vliet *et al.*, 1999) 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-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 characterisation of PerR regulation in *C. jejuni*. Inactivation of *perR* results in reduced colonisation in chickens, suggesting a role of oxidative stress regulation in intestinal colonisation (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 colonisation.

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*.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Campylobacter jejuni NCTC 11168, 81-176 and 81116 (NCTC 11828) and their isogenic mutants (Table 1) were routinely grown under microaerobic conditions (85% N_2, 5% O_2, 10% CO_2) 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 FluoStar 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% N_2, 5% O_2, 10% CO_2) at 37-42 °C, shaking at 400 rpm (double orbital), and OD_{600} readings were taken every 20 min. Broth cultures were carried out in Brucella (Becton, Dickinson and Company) with shaking, whereas growth on plates used Brucella agar or Blood Agar Base agar No. 2 with Skirrow supplements (10 µg ml^{-1} vancomycin, 5 µg ml^{-1} trimethoprim, 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 *cj0322* gene and approximately 500 base pairs (bp) of flanking sequence on each side was PCR amplified by Phusion DNA Polymerase (New England Biolabs) using oligonucleotide primers PerRFlanksF and PerRFlanksR (Table S1). This amplified fragment was purified using a commercial PCR Purification Kit (QIAgen), digested with *Eco*RI (New England Biolabs) and *Pst*I (Promega) and then ligated into pNEB193 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 PerRInverseR and PerRInverseL. The kanamycin cassette from pMARKan9 and the inverse PCR product from pOSH2 were digested with *Bam*HI (New England Biolabs) and ligated to form plasmid pOSH3.

Ligated fragments were transformed into *E. coli* strain Top10 and positive transformants were selected for by plating on LB agar supplemented with 30 µg ml^{-1} kanamycin. 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 PerRKOCheck FWD and PerRKOCheck 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 *cj0046* 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 PerRCompNativeFwdNcoI and PerRCompRevNcoI (Table S1). The amplified fragment was digested with *Nco*I (Promega) and ligated into the *Esp*3I site of pC46. This construct, known as pC46perR, was transformed into 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 A_{600} of an overnight *C. jejuni* culture was adjusted to 0.4 using sterile PBS. Bacterial motility was assessed by spotting 10 µl of this culture onto the centre of a 0.4% Brucella agar plate (Reuter & van Vliet, 2013). Plates were photographed after 24, 48, and 72 hours of incubation at 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 37ºC, and cells were harvested into 2 ml of Brucella broth. *C. jejuni* was then added to 3 ml 1.5% Brucella agar to a final A_{600} 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 of hydrogen peroxide (0-30% v/v in water) or cumene hydroperoxide (0-6% v/v in dimethyl sulfoxide) were applied to discs. Plates were incubated overnight in microaerobic conditions at 37°C, photographed and the zone of inhibition (no growth) was measured using ImageJ image analysis software (National Institute of Health). The effect of hydrogen peroxide was also measured in broth culture, using overnight cultures of *C. jejuni* NCTC 11168 which were adjusted to an A_{600} 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 minutes.

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 A_{600} 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% N_2, 5%O_2, 10% CO_2) and the other aerobically at 37ºC, shaking at 200 rpm. Samples of each culture were taken at three hour time intervals. Serial ten-fold 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.*, 2010; Gundogdu *et al.*, 2011; van Alphen *et al.*, 2014). *G. mellonella* larvae were obtained from Livefoods.co.uk (United Kingdom). Larvae were inoculated in the right foremost pro-leg by microinjection (Hamilton, Switzerland) with 10 µl *C. jejuni* overnight culture, which had been adjusted to an A_{600} of 0.1 (approximately 10^6 CFU). PBS, and mock infection controls were also performed alongside each experiment. The larvae were incubated at 37°C, with percentage survival scored at 24 hour intervals. For each experiment, ten *G. mellonella* larvae were infected and a total of five independent experiments were performed.

Two-dimensional protein gel electrophoresis and protein identification

Two-dimensional 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 in microaerobic conditions, and were harvested from broth culture (50 ml) by centrifugation at 4,000 × *g*, 10 min at room temperature. Cell pellets were resuspended in 500 µl lysis buffer (50 mM Tris (pH 7.5), 0.3% sodium dodecyl sulfate (SDS), 0.2 M dithiothreitol, 3.3 mM $MgCl_2$, 16.7 µg of RNase ml^{-1}, and 1.67 U of 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 two-dimensional protein gel electrophoresis, proteins of interest were removed from the gel using ProPick excision robot (Genomic Solutions), and in-gel trypsin digested using a ProGest Protein Digester (Genomic Solutions) (Hockin *et al.*, 2012; Shaw *et al.*, 2012). LC-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 *perR*pET21aF and *perR*pET21aR (Table S1),

and the amplified fragment was digested with restriction enzymes *Bam*HI and *Nde*I (New England Biolabs) and ligated into pET21a, which had been digested with the same enzymes. This plasmid construct (pOSH1) was subsequently checked by sequencing (TGAC, Norwich, UK) then transformed into *E. coli* BL21 (DE3). *E. coli* harbouring pOSH1 were grown in LB broth, at 37°C, shaking at 180 rpm to an A_{600} of 1.0. The expression of recombinant PerR was induced by addition of 1.0 mM isopropyl-β-$_D$-thiogalactopyranoside (IPTG) for 4 hours 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, in six passes of 30 seconds. Soluble cell extract was isolated by centrifugation at 23,000 × *g* for 30 minutes at 4°C. The soluble cell extract was filter sterilized and loaded onto a HiTrap™ Heparin affinity column. *C. jejuni* PerR was eluted from the heparin column across a salt gradient and further purified by gel filtration. Purified PerR protein was visualised by SDS-PAGE.

Electrophoretic Mobility Gel Shift Analysis

DNA fragments (180-200 bp) located upstream of translational start sites of the *katA*, *ahpC*, *trxB* and *perR* genes were amplified by PCR using 5' DIG-labelled PCR primers ('Prom' primers, Table S1) and PCR products quantified by Nanodrop. The *dnaE* promoter was included as negative control. Recombinant PerR protein (0-5 nM) was incubated with 0.5 nM of DIG-labelled DNA in EMSA binding buffer (50 µM $MnCl_2$, 20 mM Tris-Borate pH 7.4, 50 mM KCl, 3 mM $MgCl_2$, 5% glycerol, 0.1% Triton X-100), in a total volume of 20 µl. The reaction was left for 60 min at room temperature. Reactions were assayed for protein-DNA binding by native PAGE, on a 12% acrylamide gel at 150 V for 50 min. Labelled DNA was transferred to positively charged nylon membranes (Hybond N+, Amersham Biotech) by electroblotting at 130 mA for 1 hour. DNA was cross-linked to the membrane using UV radiation (1200 kJ cm^2) and incubated with Anti-Digoxigenin-AP fab fragments (Roche). CDP Star ® (SIGMA) was used to detect DIG-labelled 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 A_{600} 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

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 11828) 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 transcriptional start site 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°C 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 (H_2O_2) 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 in microaerobic conditions. During exposure to 3% hydrogen peroxide (0.88 M) in broth culture, the *perR* mutants maintained viability (1.0×10^8 cfu ml^{-1}) throughout the experiment, whereas the number of viable cells in the wild-type strains declined rapidly after the addition of H_2O_2. 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% of 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% H_2O_2 (Fig. 1B) 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% H_2O_2 (8.8 M). When grown in the presence of 3-6% cumene hydroperoxide (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 1.0×10^6 cfu ml^{-1} after six hours in an aerobic environment (Fig. 2). In contrast, the *perR* mutant showed increased aerotolerance with over 1.0×10^6 cfu ml^{-1} surviving after nine hours 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 characterise the role of PerR in regulation of *C. jejuni* gene expression, the effects of the *perR* mutation was assessed at the level of protein abundance (using two-dimensional gel electrophoresis) and transcript levels (using RNA-sequencing). As overlaps between the Fur and PerR regulatory networks were previously 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 mass spectrometry 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 Rrc (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-sequencing (Dugar *et al.*, 2013; Porcelli *et al.*, 2013) was used to confirm the transcription start sites 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 transcription start site (TSS) in both the wild-type strain and *fur perR* mutant, which is preceded by a recognisable σ^{70} -10 sequence (gnTAnaaT) located 4-7 bp upstream, and matches the TSS described previously (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 PerR-regulation of these genes is controlled from a secondary promoter. In addition, the use of the *fur perR* mutant for RNA-sequencing allowed the additional identification of eight previously unknown transcription start sites 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 electrophoretic mobility shift assays (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 transcriptional start sites 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 previously described (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 colonisation 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. melonella* 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 hours after inoculation (Fig. 5B).

DISCUSSION

The microaerophilic lifestyle of bacteria like *Campylobacter 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 fecal-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, and hence it is not surprising that many of the characterised *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.*, 2014; 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 identified as controlling expression of peroxidases (van Vliet *et al.*, 1999) and subsequently suggested to affect colonisation properties of *C. jejuni*, probably via motility (Palyada *et al.*, 2009). To understand how PerR mediates oxidative 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.*, 2015; Kim *et al.*, 2011; 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 were described previously (Hwang *et al.*, 2012; Kim *et al.*, 2015; Kim *et al.*, 2011; Palyada *et al.*, 2009), the reported phenotypes of *perR* mutants of *C. jejuni* have varied, especially with regard to its role in motility and intestinal colonisation. In this study, *C. jejuni perR* mutants displayed growth similar to wild-type (Fig. S2), did not show lowered motility in three well-characterised *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 previously (Palyada *et al.*, 2009) where inactivation of *perR* in strain NCTC 11168 resulted in decreased motility and colonisation 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; Hwang *et al.*, 2011c; Hwang *et al.*, 2012; Palyada *et al.*, 2009; van der Stel *et al.*, 2014; 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; Hwang *et al.*, 2011c), and highlights the very high levels of catalase expression in the *perR* mutant. A similar phenotype (albeit not as striking) was observed 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.

Next to the clear increase in resistance to peroxides, we also observed a significant increase in aerotolerance in the *perR* mutant (Fig. 2). Aerobic tolerance plays a key role 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) were 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 inactivation 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 and 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, and hence it would be counter-intuitive that this system is found in all *C. jejuni* and *C. 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 characterised 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 and 1B)

(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 *aphC* 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 oxidised. *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-sequencing, and in this study we have utilised differential RNA-sequencing to identify the transcription start sites and promoter location of PerR- and Fur-regulated genes in *C. jejuni*, including eight transcription start sites 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 *B. subtilis* PerR is sufficient for it to bind to Fur recognition 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

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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

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

Figure 4: PerR-mediated regulation of peroxidase expression is mediated at the transcriptional level, via binding of PerR to promoter regions. [A] Illumina-based RNA-sequencing of *C. jejuni* NCTC 11168 wild-type and an isogenic *fur perR* double mutant identifies differentially regulated genes, as shown by RPKM values for Illumina RNA sequencing (Chaudhuri *et al.*, 2011). Examples of deregulated genes are indicated. [B] Electrophoretic mobility gel shift assays (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 comparison (Kim *et al.*, 2011), and the *dnaE* promoter as negative control.

Figure 5: Inactivation of *perR* does not affect *C. jejuni* motility or ability of *C. jejuni* to kill wax moth larvae. [A] Swarming motility of *C. jejuni* strains NCTC 11168, 81-176 and 81116 (NCTC 11828) is not affected by the inactivation of the *perR* gene, as shown using soft agar (0.4%). Pictures shown are representative data from each of the three reference strains, for the wild-type strain, the *perR* mutant and the complemented *perR::perR*$^{+}$ mutant, and compared to a non-motile control (D) (*flaAB* mutant). [B] *G. mellonella* larvae were inoculated with 10 µl of an overnight *C. jejuni* NCTC 11168 culture, diluted to an OD_{600} of 0.1, by injection into the right foremost proleg, which corresponds to an infectious dose of approximately 10^6 CFU. The larvae were incubated at 37°C, with survival recorded at 24 and 48 hours post injection. Phosphate buffer saline and mock-injection controls were included for comparison. For each experiment, ten *G. mellonella* larvae were used per group, and five independent experiments were performed. There was no statistically significant difference in survival between the wild-type strain, *perR* mutant or complemented strain.

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

Strains	Description *	Source
E. coli		
BL21 (DE3)	F^- ompT hsdSB (r_B^- m_B^-) gal dcm (DE3)	Novagen
Top10	General cloning strain	Invitrogen
C. jejuni		
NCTC 11168	Wild-type *C. jejuni*	(Parkhill *et al.*, 2000)
11168 *perR*	NCTC 11168 *perR*::kan^R	This study
11168 *perR*::*perR*$^+$	NCTC 11168 *perR*:: kan^R *cj0046*::*perR*^{native}*cat^R	This study
11168 *fur*	NCTC 11168 *perR*::kan^R	This study
11168 *fur perR*	NCTC 11168 *perR*::kan^R *fur*::cat^R	This study
11168 *fur perR*	11168 *fur perR*	(Palyada *et al.*, 2009)
11168 *flaAB*	NCTC 11168 (*cj1338-39c*)::kan^R	(Reuter & van Vliet, 2013)
81116	Wild-type *C. jejuni* (NCTC 11828)	NCTC
81116 *perR*	NCTC 11828 *perR*::kan^R	This study
81116 *perR*::*perR*$^+$	NCTC 11828 *perR*:: kan^R *cj0046*::*perR*^{native}*cat^R	This study
81-176	Wild-type *C. jejuni*	ATCC
81-176 *perR*	81-176 *perR*::kan^R	This study
81-176 *perR*::*perR*$^+$	81-176 *perR*:: kan^R *cj0046*::*perR*^{native}*cat^R	This study
Vectors		
pNEB193	General cloning vector	New England Biolabs
pC46	Complementation plasmid containing *cj0046* 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 *et al.*, 1998)
pOSH1	PerR over-expression plasmid, pET21a, amp^R	This study
pOSH2	*perR* plus flanking regions in pNEB193	This study
pOSH3	*perR* disruption plasmid, kan^R	This study
pC46*perR*	*perR* complementation plasmid, native promoter; cat^R.	This study

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

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

Gene *	TSS †	promoter + 5' UTR ‡ (putative PerR/Fur operator)	Ratio §
rrc (Cj0012c)	17426	ttatttgatatacattttcataaatgataaaaattattaattgacaaaaat attttttataaTACAATactttt**T**gataatcaaaaaAGGAGtcaaaATG	3.70
trxB (Cj0146c)	151607	taataatttttattaactactattaagaataattttaTATCATtatt**A**ata ataaaattcaatataaAGGAaatataaATG	9.85
cj0176c (Cj0176c)	172717	aataaaatgaaaatatttatcagttataaaattattaagttactttatattt actttttaatatTAATATttctt**A**tcaaaatatctaaaAGGAattaATG	2.22
chaN (Cj0177)	172881	ataaattttgataataattataaataattaaattttaatataaatatttaat agtatttttatatataaaatatTAATATtcaat**A**tcaaaaataaaccactaa GAAAGtaacaataataATG	100.63
ahpC (Cj0334)	302353	atttaccttatttgataataattataacttttttaaatttttaatattatct tagatatttaaaaaaaaataaagatttaaacgaTATAATttcgtt**A**tcaata aaatttattatttAGGAGaaaatATG	3.08
cfrB (Cj0444)	412299	attttattatttataattgttatcaaaattaagttaattaaaagtattcttt tttTATACTccaaaa**A**tgattttaaatatcaaaataatttttttttaaAGGAa ttaatTTG	28.26
cfrA (Cj0755)	705419	aatttttaattaatactaattatcacaattgatacaaaatttatcttaaatt aataattattatcaTAATATtttgat**T**tctaaatcaatatttaacaaaAGGA GaaaaATG	29.20
ceuB (Cj1352)	1283976	cttactaaaatatcctttatagttttatcatttcttaaaacaaatttcatTA CAATttcat**T**tttgataattattattactataAGGAaagatTTG	7.55
cj1384c (Cj1384c)	1322394	tgcattttattgataataaatttcaaaataaatttagttttttttaTATTATa ataata**A**ttatcaaaatatattttaaaaatcacaaAGGAGaatttATG	239.28
katA (Cj1385)	1322495	taaatttattttgaaatttattatcaataaaatgcaattattcagttaattt taatttttaatagaTATAATttacta**A**ttaataaaatttattatttAGGAGa aaacaATG	439.02
chuZ (Cj1613c)	1540646	atatattttaagtgctttttaatttgataattgaTACCATttttttt**A**atat tttatttttaaaatcaagaaAGGAtaaaaATG	11.01
chuA (Cj1614)	1540788	gataaatttatttctaaattattatgatataaatTATCATtatttat**T**ttat ttaAGGAGtaaaatTTG	294.17
exbB2 (Cj1628)	1555995	gaatttcttaaaaattatgataattgatattaattataaatttatttttttat acagTATAATctttta**A**aatttataagtatatttttGGAGAaaaatATG	30.83
cj1658 (Cj1658)	1580782	tataatttaataaaaaaatattatcaacataaaagcttatttttttagttcta tttaagattttttagTATTATactagcc**A**ttatttattttgataataaatatt attttttatcaaaaatcaAGGAtaatgataATG	6.61

* Gene number from *C. jejuni* NCTC 11168 (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000)

† Position of the transcription start site (TSS) on the *C. jejuni* genome, as determined by differential RNA-sequencing (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 startcodon, σ^{70}-dependent -10 region (TAnaAT), and indicated in grey are 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-sequencing on the Illumina HiSeq 2000.

Figure 1 (Powerpoint)

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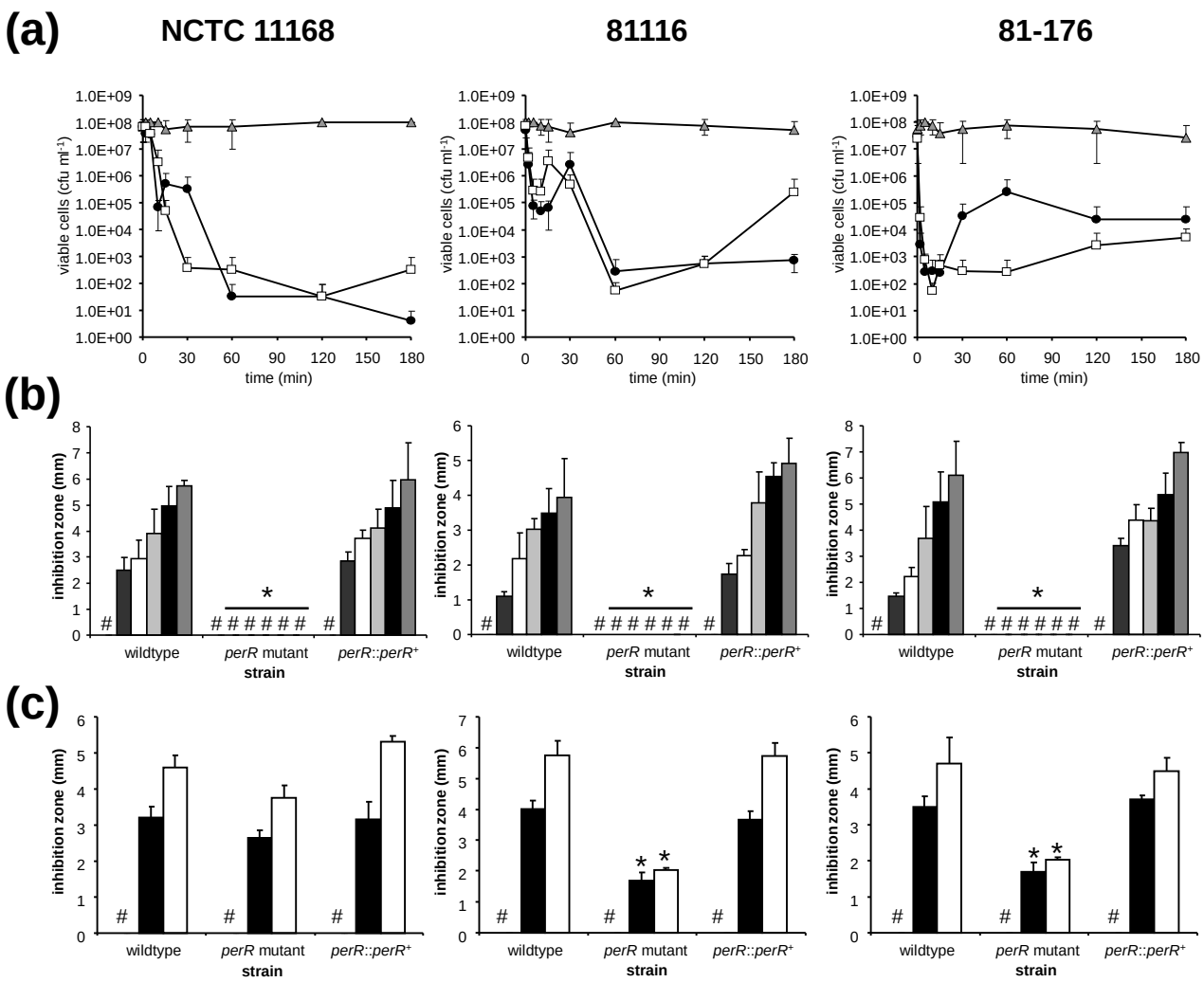

Figure 2 (Powerpoint)
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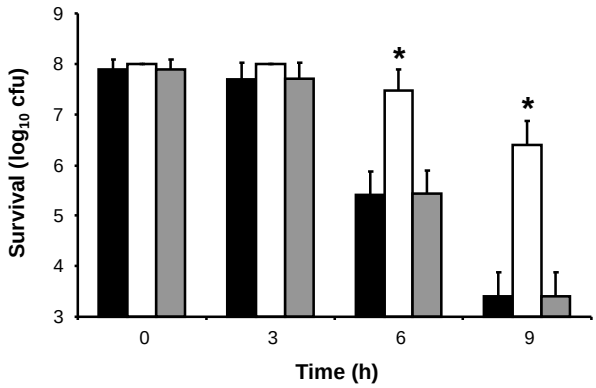

Figure 3 (Powerpoint)
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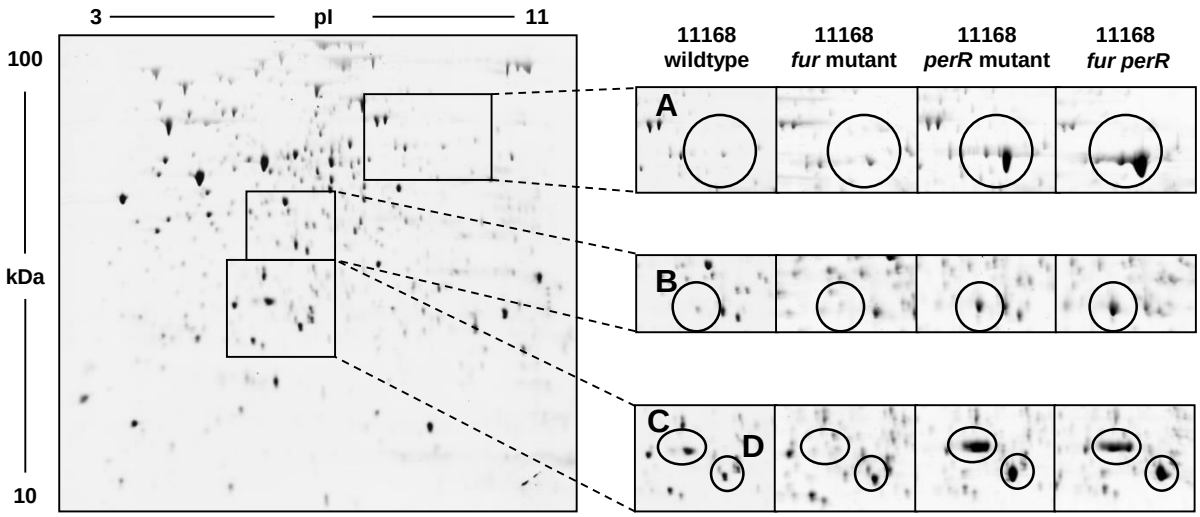

Figure 4 (Powerpoint)
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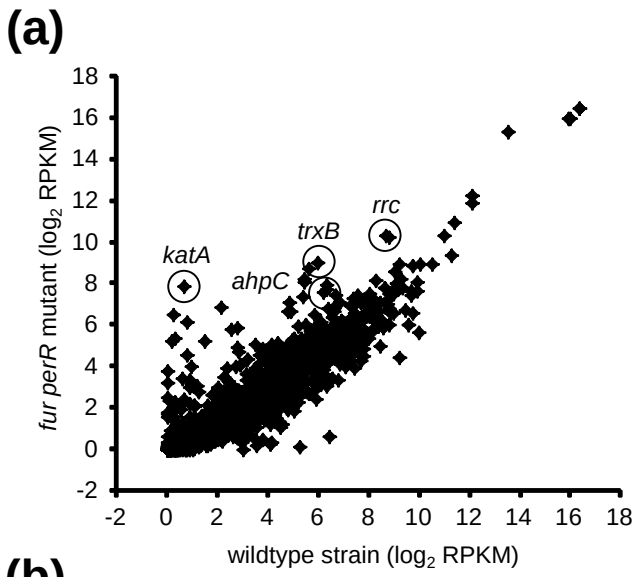

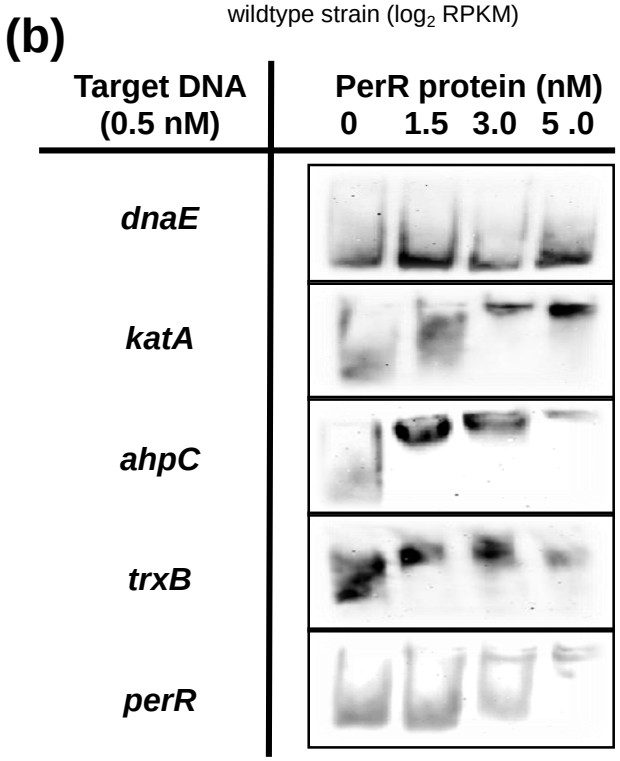

Figure 5 (Powerpoint)
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