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The *Campylobacter jejuni* Oxidative Stress Regulator RrpB Is Associated with a Genomic Hypervariable Region and Altered Oxidative Stress Resistance

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INTRODUCTION

*Campylobacter jejuni* is the leading cause of bacterial foodborne diarrheal disease worldwide. Despite the microaerophilic nature of the bacterium, *C. jejuni* can survive the atmospheric oxygen conditions in the environment. Bacteria that can survive either within a host or in the environment like *C. jejuni* require variable responses to survive the stresses associated with exposure to different levels of reactive oxygen species. The MarR-type transcriptional regulators RrpA and RrpB have recently been shown to play a role in controlling both the *C. jejuni* oxidative and aerobic stress responses. Analysis of 3,746 *C. jejuni* and 486 *C. coli* genome sequences showed that whilst *rrpA* is present in over 99% of *C. jejuni* strains, the presence of *rrpB* is restricted and appears to correlate with specific MLST clonal complexes (predominantly ST-21 and ST-61). *C. coli* strains in contrast lack both *rrpA* and *rrpB*. In *C. jejuni* *rrpB*+ strains, the *rrpB* gene is located within a variable genomic region containing the IF subtype of the type I Restriction-Modification (hsd) system, whilst this variable genomic region in *C. jejuni* *rrpB*− strains contains the IAB subtype hsd system and not the *rrpB* gene. *C. jejuni* *rrpB*− strains exhibit greater resistance to peroxide and aerobic stress than *C. jejuni* *rrpB*+ strains. Inactivation of *rrpA* resulted in increased sensitivity to peroxide stress in *rrpB*+ strains, but not in *rrpB*− strains. Mutation of *rrpA* resulted in reduced killing of *Galleria mellonella* larvae and enhanced biofilm formation independent of *rrpB* status. The oxidative and aerobic stress responses of *rrpB*− and *rrpB*+ strains suggest adaptation of *C. jejuni* within different hosts and niches that can be linked to specific MLST clonal complexes.

Keywords: *Campylobacter jejuni*, oxidative stress response regulation, aerobic stress response regulation, transcription factors, restriction modification system

INTRODUCTION

*Campylobacter jejuni* is the leading cause of bacterial foodborne diarrheal disease worldwide with an estimated 400 million human infections occurring each year (Ruiz-Palacios, 2007). The predominance of *C. jejuni* can be attributed to the ability to survive in the environment as well as within avian and mammalian hosts despite the microaerophilic nature of this bacterium
C. jejuni has evolved specific adaptation mechanisms to survive under atmospheric oxygen conditions (Kim et al., 2015). In addition to aerobic stress such as the exposure to increased levels of oxygen under atmospheric conditions, C. jejuni can also encounter stress conditions within the host, specifically oxidative stress in the form of reactive oxygen species (ROS) during in vivo survival (Fang, 2004; Palayda et al., 2009). ROS is a collective term that describes the chemical species generated upon incomplete reduction of oxygen (Imlay, 2003) with examples including the superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and the hydroxyl radical (•OH) (D’Autreaux and Toledano, 2007). The accumulation of ROS in the bacterial cytoplasm and periplasm leads to damage of nucleic acids, proteins and membrane structures (Atack and Kelly, 2009).

Bacteria that can survive either within a host or in the environment like C. jejuni require variable responses to survive the stresses associated with exposure to different levels of ROS (Kim et al., 2015). Therefore it is not surprising that C. jejuni contains a number of regulatory proteins involved in the oxidative stress response such as PerR (Handley et al., 2015), Fur (van Vliet et al., 2000), and CosR (Hwang et al., 2011). The C. jejuni NCTC 11168 genome also contains two MarR-type transcriptional regulators that have previously been designated as RrpA and RrpB (Gundogdu et al., 2011, 2015). Using C. jejuni 11168H [a hypermotive derivative of the original sequenced strain NCTC 11168 that shows higher levels of cecal colonization in a chick colonization model (Karlyshev et al., 2002; Jones et al., 2004)] we have shown that both RrpA and RrpB play a role in oxidative and aerobic stress responses with auto-regulatory activity typical of MarR-type transcriptional regulators (Gundogdu et al., 2011, 2015). In addition, RrpA has also been shown to bind upstream of katA suggesting that RrpA directly influences the expression of catalase (KatA). Both 11168H $rrpA$ and $rrpB$ mutants exhibited reduced KatA activity. However, a 11168H $rrpAB$ double mutant exhibited higher levels of resistance to hydrogen peroxide oxidative stress, but similar levels of KatA activity compared to the wild-type strain. Neither the 11168H $rrpA$ mutant nor the 11168H $rrpB$ mutant exhibited any significant difference in sensitivity to either cumene hydroperoxide or menadione oxidative stresses, but both mutants exhibited reduced cytotoxicity in the Galliera mellonella model of infection and enhanced biofilm formation. However, the 11168H $rrpAB$ double mutant exhibited wild-type levels of both cytotoxicity in the G. mellonella model of infection and biofilm formation. Together these data indicate a role for both RrpA and RrpB in the C. jejuni oxidative and aerobic stress responses, enhancing bacterial survival both within a host and in the environment, but also prompted further investigations in order to understand the specific roles of RrpA and RrpB.

Traditional typing methods have failed to identify C. jejuni strains from different sources that cause disease in humans (Champion et al., 2005). Human infections are largely attributed to undercooking of poultry products or poor food hygiene practices involving handling of such produce (Sheppard et al., 2009). C. jejuni can survive in many different niches with the organism isolated from avian, animal, human, and environmental sources (Young et al., 2007). However, whole genome phylogenetic analysis of C. jejuni strains using microarrays has identified different clades and subclades linked to the source of the isolate (Champion et al., 2005; Stabler et al., 2013). Previously we identified differences in the distribution of $rrpA$ and $rrpB$ regulators amongst 111 C. jejuni strains with $rrpA$ present in over 95% and $rrpB$ in approximately only 50% of these strains (Gundogdu et al., 2011). A more recent analysis of 270 C. jejuni strains identified nine clusters (C1–C9) based on genotype and multilocus sequence typing (MLST) data and indicated that clusters C1–C6 were dominated by livestock-associated clonal complexes, whilst clusters C7–C9 contained the majority of the water and wildlife associated clonal complexes (Stabler et al., 2013). Using the original data from this study, $rrpA$ was identified in 129/133 (96.99%) strains within the C1–C6 subclades and in 130/137 (94.89%) strains within the C7–C9 subclades. In contrast $rrpB$ was identified in 102/133 (76.67%) strains within the C1–C6 subclades and only in 19/137 (13.87%) strains within the C7–C9 subclades. In total $rrpA$ was identified in 259/270 (95.92%) strains whilst $rrpB$ was identified in only 121/270 (44.81%) strains (Supplementary Figure 1; Supplementary Table 1).

The discovery of the varied distribution for $rrpA$ and $rrpB$ has led us to further explore the potential reasons as to why certain strains have one or both regulators. To try and understand the reasons for this, we have investigated the presence or absence of $rrpA$ and $rrpB$ in 4,232 C. jejuni and C. coli genome sequences. Analysis of 4,232 Campylobacter genomes showed that whilst $rrpA$ is present in over 99% of C. jejuni strains, the presence of $rrpB$ is restricted and appears to correlate with livestock-associated MLST clonal complexes. Further analysis showed that the presence of $rrpB$ is linked to a hypervariable region containing the Iδ subtype of the type I Restriction-Modification (hsd) system, whereas $rrpB^−$ strains contain the IAB subtype hsd system. Further investigation of the phenotypes of different C. jejuni $rrpB^−$ and $rrpB^+$ strains identified a link between the presence of the MarR-type transcriptional regulator RrpB with the ability of C. jejuni to adapt and survive in different environmental niches. The oxidative and aerobic stress response of $rrpB^−$ and $rrpB^+$ strains suggests adaptation of C. jejuni within different hosts and niches that can be linked with specific MLST clonal complexes.

**MATERIALS AND METHODS**

**Comparative Genomics of $rrpA$ and $rrpB$ within Campylobacter Genomes**

A total of 4,232 complete and draft Campylobacter genome sequences (3,746 C. jejuni and 486 C. coli) were obtained from public collections (Jolley and Maiden, 2010; Cody et al., 2013) and are listed in Supplementary Table 2 with accession numbers/pubMLST IDs and assembly status. These genomes were previously used for identification of DNase-genes (Brown et al., 2015), CRISPR repeats and cas genes (Pearson et al., 2015) and the fucose utilization operon (Dwivedi et al., 2016). Genomes...
Bacterial Strains and Growth Conditions

*Campylobacter jejuni* strains (Table 1) were grown at 37°C in a microaerobic chamber (Don Whitley Scientific, United Kingdom) containing 85% N₂, 10% CO₂ and 5% O₂ either on blood agar (BA) plates containing Columbia agar base (Oxoid, United Kingdom) supplemented with 7% (v/v) horse blood (TCS Microbiology, United Kingdom) and *Campylobacter* Selective Supplement (Oxoid) or in Brucella broth (Oxoid) with shaking at 75 rpm. *C. jejuni* strains were grown on BA plates for 24 h prior to use in all assays unless otherwise stated. *Escherichia coli* XL-2 Blue MRF’ competent cells (Stratagene, United Kingdom) were used for cloning experiments and were grown at 37°C in aerobic conditions either on Luria-Bertani (LB) agar plates (Oxoid) or in LB broth (Oxoid) with shaking at 200 rpm. Antibiotics were added at the following concentrations: ampicillin (100 µg/ml), kanamycin (50 µg/ml), and chloramphenicol (50 µg/ml for *E. coli* studies or 10 µg/ml for *C. jejuni* studies). All reagents were obtained from Fisher Scientific (United Kingdom) unless otherwise stated.

Construction of *C. jejuni* Mutants

*Campylobacter jejuni* mutants were constructed as described previously (Gundogdu et al., 2011). Briefly, genes or gene fragments were amplified from *C. jejuni* genomic DNA using the appropriate gene specific primers (Table 2). PCR products were ligated with pGEM-T Easy vector (Promega, United Kingdom) and then transformed into XL-2 Blue MRF’ cells. If required, inverse PCR mutagenesis (IPCRM) was performed to introduce a unique BglII site into the cloned gene. A kanamycin cassette (KanR) was then ligated into the unique BglII site within the cloned gene (Trieu-Cuot et al., 1985; van Vliet et al., 1998). These constructs were electroporated into competent *C. jejuni* cells and putative clones were confirmed by PCR and sequencing as described previously (Gundogdu et al., 2011).

Oxidative Stress and Aerobic Growth Assays

Oxidative stress and aerobic growth assays were performed as described previously (Gundogdu et al., 2011, 2015). Briefly, bacterial cells were harvested into 1 ml PBS and diluted to an OD₆₀₀ of 1. For oxidative stress assays, bacterial cells were exposed to H₂O₂ at final concentrations of 25, 50, or 100 mM for 15 min, menadione at a final concentration of 100 mM for 60 min and cumene hydroperoxide at 0.05% (w/v) for 15 min, all at 37°C under microaerobic conditions. Serial dilutions were prepared and 10 µl of the 10⁻¹⁻¹⁶ dilutions spotted onto BA plates, incubated for 48 h and colonies counted. For growth curves, 10 ml Brucella broth was pre-incubated in a 30 ml flask at 37°C under microaerobic conditions for 24 h. Bacterial cells grown on BA plates for 24 h were used to inoculate pre-incubated Brucella broth at an OD₆₀₀ of 0.1 and grown for up to 24 h at 37°C under microaerobic and aerobic conditions. OD₆₀₀ readings were performed at selected time points. In addition bacterial colony forming units (CFUs) were assessed at time point 16 h under microaerobic and aerobic conditions.

Galleria mellonella Infection Model

*Galleria mellonella* infection assays were performed as described previously (Champion et al., 2010; Gundogdu et al., 2015). Briefly, *G. mellonella* larvae (LiveFoods Direct, United Kingdom) were stored at 16°C on wood chips. Ten larvae for each experiment were infected with a 10 µl inoculum of a 24 h *C. jejuni* culture diluted to OD₆₀₀ 0.1 by micro-injection (Hamilton, Switzerland) in the right foremost leg, giving an infectious dose of approximately 10⁶ CFU (Champion et al., 2010). Controls were injection with PBS and no injection. Larvae were inoculated at 37°C with survival recorded at 24 h.

Biofilm Assays

Biofilm assays were performed as described previously (Gundogdu et al., 2015). Briefly, bacterial cells were harvested into Mueller Hinton broth, then inoculated to an OD₆₀₀ of 0.1 into 10 ml Mueller Hinton broth pre-incubated in a 25 ml flask at 37°C under microaerobic conditions for 24 h prior to inoculation, then grown for 5 h at 37°C under microaerobic conditions with shaking at 75 rpm. The OD₆₀₀ was readjusted to 0.1, then 1 ml of culture was added to a 24 well polystyrene plate (Corning, U.S.A) and incubated at 37°C under either aerobic or microaerobic conditions stationary for 72 h. The wells were washed twice with PBS, dried for 20 min at 37°C followed by addition of 1% (w/v) crystal violet (Sigma–Aldrich) for 15 min. The wells were washed three times with PBS, then destained with 10% (v/v) acetic acid / 30% (v/v) methanol. Absorbance (A₅₉₅) was measured using a SpectraMax M3 microplate reader (Molecular Devices, USA).

Statistical Analyses

The data is presented as mean ± SD. All experiments were performed with at least three biological replicates. Each biological replicate was performed in three technical replicates. Statistical analyses were performed using Prism software (GraphPad...
TABLE 1 | Campylobacter jejuni strains used in this study.

| Strain                  | Description                                                                 | Reference |
|-------------------------|-----------------------------------------------------------------------------|-----------|
| 11168H                  | A hypermotile derivative of the original sequence strain NCTC 11168 that shows higher levels of caecal colonization in a chick colonization model. C3 clade. MLST clonal complex ST-21. | Karlyshev et al., 2002; Jones et al., 2004 |
| 81-176                  | Highly virulent and widely studied laboratory strain of C. jejuni. MLST clonal complex ST-42. | Korlath et al., 1985 |
| 81116                   | Genetically stable strain which remains infective in avian models. C9ii clade. MLST clonal complex ST-283. | Wassenaar et al., 1991 |
| M1                      | M1 (laboratory designation 99/308) is a rarely documented case of direct transmission of C. jejuni from chicken to a person, resulting in enteritis. C9ii clade. MLST clonal complex ST-45. | Fris et al., 2010 |
| 11168H rrpB mutant      | Isogenic 11168H rrpB mutant with insertion of a 1.4 kb KanR cassette         | Gundogdu et al., 2011 |
| 11168H rrpB complement  | rrpB complement constructed by the insertion of the rrpB CDS into the Cj0233 pseudogene in the 11168H rrpB mutant (pDENNIS complementation vector used) | Gundogdu et al., 2011 |
| 81-176 rrpB mutant      | Isogenic 81-176 rrpB mutant with insertion of a 1.4 kb KanR cassette         | This study |
| 81-176 rrpB complement  | rrpB complement constructed by the insertion of the rrpB CDS into a rRNA gene in the 81-176 rrpB mutant (pRRC complementation vector used) | This study |
| 11168H rrpA mutant      | Isogenic 11168H rrpA mutant with insertion of a 1.4 kb KanR cassette         | Gundogdu et al., 2015 |
| 11168H rrpA complement  | rrpA complement constructed by the insertion of the rrpA CDS into a rRNA gene in the 11168H rrpA mutant (pRRC complementation vector used). | Gundogdu et al., 2015 |
| 81-176 rrpA mutant      | Isogenic 81-176 Cj1546 mutant with insertion of a 1.4 kb KanR cassette       | This study |
| 81-176 rrpA complement  | rrpA complement constructed by the insertion of the rrpA CDS into a rRNA gene in the 81-176 rrpA mutant (pRRC complementation vector used) | This study |
| 81116 rrpA mutant       | Isogenic 81116 rrpA mutant with insertion of a 1.4 kb KanR cassette         | This study |
| 81116 rrpA complement   | rrpA complement constructed by the insertion of the rrpA CDS into a rRNA gene in the 81116 rrpA mutant (pRRC complementation vector used) | This study |
| M1 rrpA mutant          | Isogenic M1 rrpA mutant with insertion of a 1.4 kb KanR cassette            | This study |
| 40917                   | Clinical bloody diarrhea isolate                                           | Campylobacter Reference Lab, UK |
|                         | HS type 21                                                                  |           |
|                         | Water and wildlife associated strain. C9ii clade. MLST clonal complex ST-45. |           |
| 12241                   | Ovine isolate                                                              | Campylobacter Reference Lab, UK |
|                         | HS type 50                                                                  |           |
|                         | Water and wildlife associated strain. C9ii clade. MLST clonal complex ST-206. |           |
| 64555                   | Clinical bloody diarrhea isolate                                           | Campylobacter Reference Lab, UK |
|                         | HS type 31                                                                  |           |
|                         | Water and wildlife associated strain. C8 clade.                             |           |
| 47693                   | Isolated from chicken isolate                                              | Campylobacter Reference Lab, UK |
|                         | HS type 27                                                                  |           |
|                         | Water and wildlife associated strain. C8 clade.                             |           |
| 62914                   | Clinical vomiting isolate                                                   | Campylobacter Reference Lab, UK |
|                         | HS type – untypeable                                                       |           |
|                         | Water and wildlife associated strain. C7 clade.                             |           |
| 44119                   | Clinical septicaemia isolate                                                | Campylobacter Reference Lab, UK |
|                         | HS type – 18                                                                |           |
|                         | Water and wildlife associated strain. C7 clade.                             |           |
| 32787                   | Clinical asymptomatic isolate                                               | Campylobacter Reference Lab, UK |
|                         | HS type – 18                                                                |           |
|                         | Water and wildlife associated strain. C9ii clade.                           |           |
| Hl80614                 | Human isolate                                                              | Campylobacter Reference Lab, UK |
|                         | Water and wildlife associated strain. C9ii clade.                           |           |
| Hl41100305              | Human isolate                                                              | Campylobacter Reference Lab, UK |
|                         | Water and wildlife associated strain. C9ii clade.                           |           |
| 31481                   | Clinical asymptomatic isolate                                               | Campylobacter Reference Lab, UK |
|                         | HS type – 37                                                                |           |
|                         | Water and wildlife associated strain. C8 clade.                             |           |
| 47886                   | Clinical septicaemia isolate                                                | Campylobacter Reference Lab, UK |
|                         | HS type – untypeable                                                       |           |
|                         | Livestock associated strain.                                                |           |
|                         | C8 clade.                                                                  |           |

(Continued)
TABLE 1 | Continued

| Strain          | Description                                      | Reference                                      |
|-----------------|--------------------------------------------------|------------------------------------------------|
| 30280           | Clinical diarrhea isolate                        | Campylobacter Reference Lab, UK               |
|                 | HS type – 16                                      |                                                 |
|                 | Livestock associated strain. C2 clade.           |                                                 |
| 13713           | Ox liver portion isolate                         | Campylobacter Reference Lab, UK               |
|                 | HS type – 2                                       |                                                 |
|                 | Livestock associated strain. C1 clade.           |                                                 |
| 11973           | Chicken isolate                                  | Campylobacter Reference Lab, UK               |
|                 | HS type – 2                                       |                                                 |
|                 | Livestock associated strain. C1 clade.           |                                                 |
| G1              | Clinical GBS isolate                             | Guy's Hospital, UK                             |
|                 | HS type – 1                                       |                                                 |
|                 | Livestock associated strain. C5 clade.           |                                                 |
|                 | MLST clonal complex ST-21.                       |                                                 |
| Bovine27        | Bovine isolate                                   | University of Bristol, UK                      |
|                 | Livestock associated strain. C2 clade.           |                                                 |
| 12912           | Ox liver portion isolate                          | Campylobacter Reference Lab, UK               |
|                 | HS type – 50                                      |                                                 |
|                 | Livestock associated strain. C4 clade.           |                                                 |
| 13040           | Chicken isolate                                  | Campylobacter Reference Lab, UK               |
|                 | HS type – 50                                      |                                                 |
|                 | Livestock associated strain. C6 clade.           |                                                 |
| 40209           | Chicken isolate                                  | Campylobacter Reference Lab, UK               |
|                 | HS type – 5                                       |                                                 |
|                 | Livestock associated strain. C4 clade.           |                                                 |
| 91B1            | Chicken isolate                                  | Oxford University, UK                          |
|                 | Livestock associated strain. C5 clade.           |                                                 |
| Hi41380304      | Human isolate                                    | Campylobacter Reference Lab, UK               |
|                 | Livestock associated strain. C3 clade.           |                                                 |
| 11168H perR mutant | Obtained from Campylobacter mutant bank      | LSHTM mutant bank                             |
|                 | http://crf.lshtm.ac.uk/wren_mutants.htm          |                                                 |
| 11168H sodB mutant | Obtained from Campylobacter mutant bank      | LSHTM mutant bank                             |
|                 | http://crf.lshtm.ac.uk/wren_mutants.htm          |                                                 |
| 11168H ahpC mutant | Obtained from Campylobacter mutant bank      | LSHTM mutant bank                             |
|                 | http://crf.lshtm.ac.uk/wren_mutants.htm          |                                                 |
| 11168H katA mutant | Obtained from Campylobacter mutant bank      | LSHTM mutant bank                             |
|                 | http://crf.lshtm.ac.uk/wren_mutants.htm          |                                                 |

Software). All statistical analyses were performed comparing two data sets directly assuming a normal distribution using a two-way student’s t-test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).

RESULTS

Presence of rrpA and rrpB in a Collection of 4,232 C. jejuni and C. coli

To assess the distribution of rrpA and rrpB in C. jejuni and C. coli we utilized a collection of 4,232 Campylobacter genome sequences (3,746 C. jejuni and 486 C. coli) from public databases (Cody et al., 2013; Brown et al., 2015), which were phylogenetically clustered using FFPry feature frequency profiling (Van Vliet and Kusters, 2015; Dwivedi et al., 2016) and further analyzed for MLST sequence type and clonal complex (Pearson et al., 2015). The vast majority of C. jejuni strains contain rrpA whilst the presence of rrpB is more restricted (Figure 1; Supplementary Table 2 – Sheet 2). The presence or absence of rrpB appears to correlate with MLST clonal complex, especially ST-21 and ST-61. Most C. coli strains contain neither rrpA nor rrpB, with only 12 C. coli genomes encoding an RrpA ortholog and only a single C. coli genome encoding an RrpB ortholog (Supplementary Table 2). Variation in RrpA and RrpB was observed with isolates from C. jejuni MLST clonal complex ST-607 having a shorter RrpA protein lacking the N-terminal 27 amino acids, while a proportion of C. jejuni ST-353 isolates have a RrpB protein either lacking the N-terminal 29 amino acids, the C-terminal 29 amino acids or a combination of 29 N-terminal amino acids and 9 C-terminal amino acids (Supplementary Figure 2). There was no apparent link between the truncated versions and source of the isolates (Supplementary Table 2), suggesting these are different forms of RrpA and RrpB circulating within clonal complexes.

Further analysis of the C. jejuni and C. coli genome sequences investigated the distribution of genes flanking rrpA and rrpB...
TABLE 2 | Oligonucleotide primers used in this study.

| Primer Name | Sequence |
|-------------|----------|
| 11168H and 81-176 Cj155-F | ATCATTCTTTGTCCTAT |
| 11168H and 81-176 Cj155-R | TAAGATGQATCTAAACTATT |
| 11168H and 81-176 Cj154-F | CCCCCATGQATAAAGGTTTATAATAGAAAAATATCATT |
| 11168H and 81-176 Cj154-R | CCGQCTGQCTAAACGATATTATAGTCTAT |
| 81-176 Comp-Q1556-F | CCGTCTAGATGAAATGACTAAAGAGAATTCTCAG |
| 81-176 Comp-Q1556-R | CCGTCTAGATGAAATGACTAAAGAGAATTCTCAG |
| 11168H and 81-176 Cj154 F | ATGAGTTTGATCTACTTCG |
| 11168H and 81-176 Cj154 R | AGATATTAAATCTCACTGCT |
| 81116 Cj154-F | GGGAGATCTCTCTTAAGGTATTGGTTA |
| 81116 Cj154-R | GGGAGATCTCTCTTAAGGTATTGGTTA |
| 81116 Cj154-F checking primers | ATGACTAAAGGAATGTTAAATGACTAAAGAGAATTCTCAG |
| 81116 Cj154-R checking primers | ATGACTAAAGGAATGTTAAATGACTAAAGAGAATTCTCAG |
| KanR forward-out | TGGGTTTCAAGCATTAGTCCATGCAAG |
| KanR reverse-out | GTGGTATGACATTGCCTTCTGCG |
| CatR forward-out | CGATTGATGACATTGTCG |
| CatR reverse-out | TGTCCTGQCAATTTGTA |

FIGURE 1 | Prevalence of the rrpA and rrpB genes among 3,746 Campylobacter jejuni and 486 C. coli genomes. Genomes were phylogenetically clustered using FFPry feature frequency profiling with \( L = 18 \) (Van Vliet and Kusters, 2015). The first two rows labeled ‘rrpA’ and ‘rrpB’ indicate genomes possessing the respective genes, which are shown in red, while those lacking the pathway are shown in black. The third bar shows the primary combinations of MLST clonal complexes for C. jejuni and C. coli, with red-labeled clonal complexes representing livestock-associated lineages, blue-labeled clonal complexes representing water and wildlife-associated lineages (Stabler et al., 2013), with the exception of the ST-61 clonal complex, which has been described as cattle (livestock)-associated (Kwan et al., 2008; Rotariu et al., 2009). The asterisks at ST-42 and ST-353 indicate that these clonal complexes may have a proportion of livestock-associated isolates. The association of some clonal complexes such as ST-464 was not reported previously and these are in black font. The lowercase letters indicate the approximate position of reference strains 81116 and M1 (a), 81-176 (b), RM1221 (c) and NCTC 11168 (d).
and identified two conserved flanking regions with a more variable central region (Figure 2). The upstream flanking region contains rrpA whilst the downstream flanking region contains an arsenic resistance operon, an MCP-gene and a paralyzed flagella gene pflA. The hypervariable central region contains a type I Restriction-Modification (hsd) system. The NCTC 11168 version of this hypervariable central region contains rrpB whilst the 81116 version does not (Supplementary Table 3). The 81116 version of this hypervariable central region is representative of all the other C. jejuni ST strains analyzed in this study. Analysis of the C. coli genome sequences (which have primarily a structure very similar to 81116) has versions mostly without rrpA and rrpB.

**Campylobacter jejuni rrpB− Wild-Type Strains Exhibit Increased Resistance to Oxidative Stress Compared to C. jejuni rrpB+ Wild-Type Strains**

Characterization of the role of RrpA and RrpB in the C. jejuni 11168H wild-type strain has shown that both rrpA and rrpB mutants exhibit increased sensitivity to H$_2$O$_2$ when compared to the wild-type strain (Gundogdu et al., 2011, 2015). Based on the observed variation of rrpA and rrpB amongst C. jejuni isolates (Gundogdu et al., 2011), we utilized the Stabler et al. (2013) study to select 25 wild-type strains (Table 1) (13 rrpB− and 12 rrpB+ strains) for testing sensitivity toward H$_2$O$_2$ (Figure 3). rrpB− strains (Figure 3B) displayed significantly greater resistance to H$_2$O$_2$ when compared to rrpB+ strains (Figure 3A). The variation in the presence of rrpA and rrpB identified from the microarray data (Champion et al., 2005) for these 25 strains was also confirmed using PCR (data not shown).

**Mutation of rrpA Results in Increased Sensitivity to Peroxide Stress in Strains 11168H and 81-176 (rrpB+), But Not in Strains 81116 and M1 (rrpB−)**

To further investigate the link between oxidative stress resistance and the varied distribution of rrpA and rrpB, rrpA and rrpB single mutants were constructed in the 11168H and 81-176 wild-type strains (rrpB+) and rrpA mutants were also constructed in the 81116 and M1 wild-type strains (rrpB−). The mutants obtained did not have any observable deficiency in motility or growth rate under standard conditions (data not shown). Compared to the respective wild-type strains, both the 11168H and 81-176 rrpA mutants exhibited an increased sensitivity to 25 mM H$_2$O$_2$ however, the 81116 and M1 rrpA mutants exhibited wild-type levels of survival (Figure 4). Neither the 11168H and 81-176 wild-type strains nor the respective rrpA mutants survived exposure to higher concentrations of H$_2$O$_2$ (50 or 100 mM), however, the 81116 and M1 wild-type strains and the respective rrpA mutants all exhibited only slightly increased sensitivity to these higher concentrations of H$_2$O$_2$ (Figure 4). 11168H and 81-176 rrpB mutants exhibited the same increased sensitivity to 25 mM H$_2$O$_2$, however, the 81116 and M1 rrpA mutants exhibited wild-type levels of survival (Figure 4). Neither the 11168H and 81-176 rrpB mutants exhibited only slightly increased sensitivity to 25 mM H$_2$O$_2$, however, the 81116 and M1 rrpA mutants exhibited wild-type levels of survival (Figure 4). Cumene hydroperoxide and menadione stress assays were also performed on the 11168H, 81-176, 81116, M1 wild-type strains and the respective rrpA and rrpB mutants (Supplementary Figure 3). There were no significant differences in sensitivity to either cumene hydroperoxide or menadione between any of the wild-type strains and respective mutants, with the exception that the 81116 rrpA mutant appeared to be more resistant to cumene hydroperoxide stress than the 81116 wild-type strain.
FIGURE 3 | Effect of oxidative stress on the survival of *C. jejuni* rrpB+ (A) and rrpB− (B) wild-type strains. rrpB+ strains are 11168H, 81-176, 47886, 30280, 13713, 11973, G1, Bovine27, 12912, 13040, 40209, 91B1, and Hi4130304. rrpB− strains are M1, 81116, 40917, 12241, 64555, 47693, 62914, 44119, 32787, Hi80614, Hi441100305, and 31481. These strains were selected from the Stabler et al. (2013) study (Table 1). *C. jejuni* strains were incubated with 25 mM, 50 or 100 mM H$_2$O$_2$ for 15 min at 37°C under microaerobic conditions. * For 81-176 denotes that this strain was not included in Stabler et al. (2013), but was selected here as a common laboratory strain. Bacterial survival was subsequently assessed. # Symbol indicates lack of growth. Asterisks denote a statistically significant difference (* = p < 0.05, ** = p < 0.01, *** = p < 0.001) between strains.
Mutation of *rrpA* Reduces Growth under Aerobic Stress Conditions in *rrpB*+ Strains, But Not in *rrpB*− Strains

In *C. jejuni* 11168H, *rrpA* and *rrpB* mutants exhibit reduced growth under aerobic stress conditions (Gundogdu et al., 2011, 2015). The ability of *rrpA* and *rrpB* mutants to grow under aerobic stress conditions was compared to the respective four wild-type strains. No differences in bacterial growth kinetics (Supplementary Figure 4A) or in CFU counts after 16 h (Figure 6A) was observed under microaerobic conditions. However, differences were observed under aerobic conditions. All four *rrpA* mutants displayed significantly reduced growth kinetics during the logarithmic phase under aerobic conditions (Supplementary Figure 4B). However, compared to the respective wild-type strains, only the 11168H and 81-176 *rrpA* mutants exhibited a reduction in CFU counts after 16 h, whilst the 81116 and M1 *rrpA* mutants did not (Figure 6B).

Mutation of *rrpA* Results in Decreased Cytotoxicity in the *Galleria mellonella* Larvae Model of Infection

*Galleria mellonella* larvae have been used as a model to study infection with many different enteric pathogens including *C. jejuni* (Champion et al., 2009). Insect larvae possess specialized phagocytic cells, termed haemocytes (Bergin et al., 2005; Mylonakis et al., 2007). Haemocytes mimic the functions of phagocytic cells in mammals and are able to degrade bacterial pathogens as well as generate bactericidal compounds such as superoxide via a respiratory burst (Lavine and Strand, 2002; Bergin et al., 2005). Both 11168H *rrpA* and *rrpB* mutants have been shown to exhibit reduced cytotoxicity in this model of infection compared to the wild-type strain (Gundogdu
et al., 2011, 2015). The cytotoxicity of these mutants in *G. mellonella* larvae compared to the respective wild-type strains was investigated. Infection with either the 11168H or 81-176 *rrpA* mutants resulted in a statistically significant decrease in cytotoxicity to *G. mellonella* larvae compared to infection with the respective wild-type strains (Figure 7). Infection with the 81116 or M1 *rrpA* mutants also resulted in a decrease in cytotoxicity to *G. mellonella* larvae compared to infection with the respective wild-type strains, although this decrease was not statistically significant (Figure 7).

**DISCUSSION**

*Campylobacter jejuni* will be exposed to ROS both during colonization or infection of a host and in the environment as well as during the course of normal bacterial metabolism. It remains a conundrum as to how this microaerophilic pathogen is so widely dispersed, highly prevalent and able to survive...
in the ambient environment. *C. jejuni* contains a number of different mechanisms for counteracting the effects of oxidative stress and the control of the *C. jejuni* oxidative stress response is complex involving multiple inter-linked levels of regulation (Atack and Kelly, 2009). The re-annotation of the *C. jejuni* NCTC 11168 genome sequence (Gundogdu et al., 2007) identified both RrpA and RrpB as putative MarR-type transcriptional regulators which were subsequently shown to be involved in the *C. jejuni* peroxide and aerobic stress response (Gundogdu et al., 2011, 2015). All the other regulators of the *C. jejuni* oxidative stress response, such as PerR, Fur, CosR, CsrA, CprRS, and RacRS, are all conserved (> 99.8%) amongst all the *C. jejuni* and *C. coli* wild-type strains in this study (van Vliet et al., 1999; Atack and Kelly, 2009; Palyada et al., 2009; Hwang et al., 2012). Analysis of the distribution of *rrpA* and *rrpB* in 3,746 *C. jejuni* and 486 *C. coli* genomes from public databases confirmed that the vast majority of *C. jejuni* strains contain *rrpA*, whilst the presence of *rrpB* is more restricted, with the distribution linked to MLST clonal complex. The majority of *C. jejuni* strains that contain both *rrpA* and *rrpB* are from ST-21 and ST-61. ST-21 strains are often associated with human infections (Sheppard et al., 2009) and ST-61 strains with infections of livestock (Kwan et al., 2008; Rotariu et al., 2009).

Analysis of the distribution of *rrpA* and *rrpB* from the microarray data study of 270 *C. jejuni* strains (Stabler et al., 2013) identified water and wildlife strains predominantly as *rrpB*+, whereas livestock-associated strains were predominantly *rrpB*− (Supplementary Figure 1; Supplementary Table 1). This distribution of *rrpA* and *rrpB* was also reflected in the analysis of the whole genome sequence data (Figure 1) where red-labeled clonal complexes represent livestock-associated lineages and blue-labeled clonal complexes represent water and wildlife-associated lineages. It is, however, not always clear whether each clonal complex is associated with a single host as clonal complexes are regularly isolated from multiple animal species and thus attribution to a single host reservoir is difficult using MLST data alone (Dearlove et al., 2016). One example is the ST-48 complex that has been isolated from humans, cattle and sand from beaches (Dingle et al., 2002). Another example is ST-257 and ST-61 that have been isolated from chicken and ruminants, yet ST-257 is designated as a water and wildlife-associated lineage, whereas ST-61 is denoted as a livestock-associated lineage (Sheppard et al., 2011). These livestock and water and wildlife descriptions may be somewhat generalized and investigating the properties of individual STs will be more important to understand the source. ST-21, ST-206, and ST-48 may form a “complex group” of related genotypes that are widely distributed, perhaps reflecting the “generalist” ability to colonize a wide range of hosts (Dingle et al., 2002; Sheppard et al., 2011).

The location of *rrpA* and *rrpB* close to a type I Restriction-Modification (hsd) system suggests an explanation as to why *rrpB* is present in almost all *C. jejuni* strains, whilst the presence of *rrpB* is restricted and appears to correlate with MLST clonal complexes. Restriction-modification (R-M) systems are ubiquitous in the bacterial world and provide a defense against foreign DNA and bacteriophages (Wilson and Murray, 1991). Foreign DNA is cleaved by endonucleases but host DNA avoids damage due to methylation. R-M systems have been classified into four distinct groups, type I, type II, type III, and type IV. A type I R-M locus was identified in the genome sequence of NCTC 11168 (*Cj1549–Cj1553*) (Parkhill et al., 2000). The type I enzyme is a bi-functional, multi-subunit complex consisting of HsdR, HsdM, and HsdS. Further analysis of the type I R-M system from 73 *C. jejuni* strains (including NCTC 11168, 81-176, and 81116) assigned some *hsd* systems to the classical type IC family but also identified two additional type I R-M families, termed type IAB and type IF (Miller et al., 2005). 81116 contains a type IAB *hsd* system, whilst both NCTC 11168 and 81-176 contain a type IF *hsd* system. This study also found evidence for

![FIGURE 8 | Biofilm assay on *C. jejuni* 11168H, 81-176, 81116, and M1 wild-type strains and respective *rrpA* and *rrpB* mutants. *C. jejuni* 11168H wild-type strain and mutants were grown for 72 h under aerobic (A) and microaerobic (B) growth conditions at 37°C without shaking, rinsed three times with PBS, followed by crystal violet staining. Asterisks denote a statistically significant difference (* = p < 0.05, ** = p < 0.01, *** = p < 0.001) between strains.](image-url)
extensive rearrangements within the *C. jejuni hsd* loci of the same family but suggested that the low sequence similarity between the IC, IAB, and IF families would make inter-family recombination events unlikely. The NCTC 11168 version of the hypervariable central region identified in this study, which contains the type IF *hsd* system and *rrpB*, represents the majority of ST-21, ST-61, ST-42, and ST-353 strains. 81-176 also contains the type IF *hsd* system and *rrpB*. However, the 81116 version of this hypervariable central region, which represents the majority of all the other *C. jejuni* ST strains analyzed in this study, contains a type IAB *hsd* system and does not contain *rrpB*. The lack of recombination between *C. jejuni* strains containing the type IF *hsd* system and *rrpB* with other *C. jejuni* strains containing a different family of type I *hsd* system could be the explanation for the restricted distribution of *rrpB* amongst *C. jejuni* strains. This region of the *C. jejuni* genome has previously been identified as hypervariable region 14, one of 16 hypervariable regions (Taboada et al., 2004). The presence of intervening ORFs between *hsdR* and *hsdS* (referred to as *rlo* genes for R-linked ORF) and also between *hsdS* and *hsdM* (referred to as *mlo* genes for M-linked ORFs) are a distinct feature of the *C. jejuni* hsd loci (Miller et al., 2005). *H. pylori* ModH from the type III R-M system has been shown to have a regulatory role (Srikhanta et al., 2011) suggesting that R-M systems may also have a role in the regulation of virulence gene expression (Vasu and Nagaraja, 2013). Recently the same hypervariable region in *C. coli* has been shown to contain a novel streptomycin resistance gene (Olkkola et al., 2016). The genomics-based screening also showed that there are different alleles of RrpA and RrpB (Supplementary Figure 2) leading to a N-terminal truncation, a C-terminal truncation or both. The truncated version of RrpB was predominantly observed in isolates of the ST-353 clonal complex, while truncated versions of RrpA were rare in most clonal complexes, but dominant in ST-607 isolates (Supplementary Table 2). Future studies could utilize these truncated versions to test for the role of the N-terminal and C-terminal regions in functionality of RrpA and RrpB.

*Campylobacter jejuni* rrpB+ strains exhibit a pattern of greater resistance to peroxide stress when compared to rrpB− strains. This pattern was the same when comparing the 11168H, 81-176, 81116, and M1 rrpA mutants against the respective wild-type strain where rrpA mutants in rrpB+ strains were more resistant to peroxide stress compared to rrpA mutants in rrpB− strains. In addition, when comparing bacterial growth of 11168H, 81-176, 81116, and M1 rrpA mutants against the respective wild-type strain under aerobic conditions, all four rrpA mutants displayed a reduced growth rate during the logarithmic phase. Both the 11168H and 81-176 rrpA mutants exhibited lower CFUs at 16 h compared to the respective wild-type strains. Even though both 81116 and M1 rrpA mutants also displayed slower growth rates compared to the respective wild-type strains when comparing respective CFU counts at 16 h, these differences were not statistically significant. The general pattern that rrpB+ strains exhibit increased sensitivity to peroxide and aerobic stresses is rather counterintuitive. Possibly having only a single Rrp regulator is more efficient in responding to such stresses. Certainly rrpA is the most conserved amongst *C. jejuni* strains. Even though the oxidative stress assays were performed on a relatively small number of selected strains, it is interesting to speculate why livestock-associated strains such as NCTC 11168 (ST-21) would be more sensitive to peroxide and aerobic stress, whilst water and wildlife-associated strains such as 81116 (ST-283) and M1 (ST-45) would be more resistant to such stresses is difficult to explain. One possible hypothesis is that the ability to survive aerobic stress is more important for the latter strains. The variation in the presence of rrpA and rrpB between different wild-type strains may play an important role in the ability of *C. jejuni* to adapt and survive in different environmental niches.

Infection with rrpA mutants from both rrpB+ and rrpB− strains resulted in an increase in survival of *G. mellonella* larvae compared to infection with the respective wild-type strains. We have previously demonstrated that infection of *G. mellonella* with the 11168H rrpA mutant leads to increased survival of the *G. mellonella* larvae (Gundogdu et al., 2015). Here we demonstrate that the 81-176 rrpA mutant (rrpB+) also exhibits a similar statistically significant reduction in larval cytotoxicity. Both 81116 and M1 rrpA mutants (rrpB−) exhibit reduced larval cytotoxicity, but not to a significant degree. This suggests the rrpA mutants (like the rrpB mutants) are more susceptible to the host immune mechanisms resulting in reduced bacterial survival within *G. mellonella*. This also suggests that RrpA may in fact play a role in the oxidative stress response of rrpB− strains such as 81116 and M1, but at a different level compared to rrpB+ strains, such as 11168H and 81-176. Certainly the enhanced resistance of the 81116 and M1 rrpA mutants to peroxide stress compared to the 11168H and 81-176 rrpA mutants is not reflected by increased virulence in the *G. mellonella* larvae infection model.

*Campylobacter jejuni* forms biofilms (Joshua et al., 2006; Gundogdu et al., 2011) and this may be an important factor in the survival of *C. jejuni* both within hosts and in the environment. Though our understanding of the specific mechanisms underlying biofilm formation in *C. jejuni* is still limited (Svensson et al., 2008), *C. jejuni* lacks the classical two component regulatory systems involved in biofilm formation that are present in other bacteria such as GacSA in *Pseudomonas aeruginosa* (Parkins et al., 2001). Biofilm formation has been linked to responses to oxidative and aerobic stress as *C. jejuni* biofilm formation is increased under aerobic conditions (Reuter et al., 2010) and mutation of genes encoding oxidative stress response proteins results in changes in biofilm formation (Oh and Jeon, 2014; Gundogdu et al., 2015). Analysis of biofilm formation demonstrated that rrpA mutants from both rrpB+ and rrpB− strains exhibited an increased biofilm phenotype compared to the respective wild-type strain under both microaerobic and aerobic conditions. Again the enhanced resistance of the 81116 and M1 rrpA mutants to peroxide stress compared to the 11168H and 81-176 rrpA mutants is not reflected by a decrease in biofilm formation.

The basis of *C. jejuni* survival is dependent upon the ability to sense and respond to the different environments encountered within hosts and in the environment. In this study we identified bioinformatically that over 99% of *C. jejuni* strains contain rrpA, whilst rrpB is restricted and appears to correlate with livestock-associated MLST clonal complexes. There exists a conserved genetic structure for rrpA, whilst rrpB seems to be...
part of a transferable hypervariable region linked with variation in the type I R-M (hsd) system, giving an explanation for the more restricted distribution of rrpB amongst C. jejuni strains. rrpB+ strains possess an increased level of resistance to peroxide and aerobic stress compared to rrpB− strains. So whilst all the other oxidative stress response regulators such as PerR, Fur, CosR, CsrA, CprRS, RacRS, and RrpA appear to be conserved in C. jejuni, variation in the presence of RrpB between different wild-type strains may play an important role for altered oxidative stress responses through the concerted actions of these multiple regulators in this microaerophilic pathogen. This highlights the potential of genetic variation in the natural population in the adaptation to different environmental niches.

AUTHOR CONTRIBUTIONS
OG, DdS, BW, and ND designed this study. OG, DdS, BM, and AE performed all the lab-based experimental work. OG, DdS, BW, and ND designed this study. OG, DdS, BM, and AE performed all the lab-based experimental work. OG, DdS, BW, and ND performed all bioinformatic analysis. OG, DdS, BW, AvV, and ND wrote the manuscript.

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SUPPLEMENTARY MATERIAL
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