Introduction

The relative paucity of genetic techniques available for the manipulation of *Campylobacter jejuni* has historically been a limiting factor in the study and molecular biology of the leading cause of bacterial gastroenteritis in the developed world [1]. *C. jejuni* is a member of a large genus of microaerophilic Gram-negative $\varepsilon$-proteobacteria and is carried harmlessly by many animals, especially poultry, but is an endemic cause of a range of diarrheal illnesses and medical complications in humans. Many laboratories are actively studying the bacterium to understand the genetic determinants and physiological features that contribute to *C. jejuni*’s virulence and prevalence as a food-borne enteric pathogen. Today, research in the area continues to benefit from and depends on a small arsenal of molecular tools, such as gene deletion strategies and plasmids for genetic complementation. Since the 1980’s, only selection for kanamycin and chloramphenicol resistance has been widely adopted for the genetic manipulation of *Campylobacter*. 

The development of the first genetic tools for *C. jejuni* was precipitated after the demonstration of gene transfer from *Escherichia coli* to *C. jejuni* via plasmids carrying kanamycin resistance in 1987 [2]. This led to the development, in 1988, of a kanamycin resistance cassette for use in gene disruption experiments [3]. Cloning and expression of a chloramphenicol resistance gene from *Campylobacter coli* in 1990 [4] was followed by development of replicative cloning vectors and mutational constructs marked with chloramphenicol resistance in 1995 [5]. Approximately a decade later, three groups successfully mutagenized *C. jejuni* with transposons carrying kanamycin or chloramphenicol resistance genes [6-8]. The finite number of resistance markers has limited genetic analyses to single-gene or single-operon studies, and has prevented complementation of double-deletion strains. As our understanding of *C. jejuni* grows, so does the need for new markers to rapidly delete and restore complex multi-gene systems, and/or to simultaneously express a reporter such as green fluorescent protein (GFP), arylsulfatase, or luciferase in mutant and/or complemented strains. To address this need, we adapted current *C. jejuni* genetic technologies to harbor resistance genes against the antibiotics hygromycin B and apramycin.

Hygromycin B is an aminoglycoside antibiotic produced by *Streptomyces hygroscopicus* that inhibits protein synthesis in both prokaryotes and eukaryotes [9]. Apramycin is another aminoglycoside, an aminocyclitol synthesized by *Streptomyces tenuebrarius* [10]. Like other aminoglycosides, such as kanamycin, both hygromycin
B and apramycin prevent ribosome translation during translation elongation by binding to the 30 s rRNA proximal to the ribosomal E, P and A sites [11]. Hygromycin B is not used clinically, but is sometimes a component of poultry feed where it has antimicrobial activity against nematode parasites of chickens [12]. Apramycin is also used as a veterinary antibiotic [12,13]. The hygromycin B (HygB) resistance marker used in this study confers resistance by the activity of a specific aminoglycoside phosphotransferase encoded by the 999 bp aph(3’') gene, encoding hygromycin B 7’’O-kinase or simply hygromycin phosphotransferase [14]. The specific modification of hygromycin B is a phosphorylation at the 7’’-OH of the desamino acid ring [15]. Resistance to apramycin (Apr’’’’) is conferred by the 777 bp aac(3’IV) aminoglycoside 3-‘’-acetyltransferase gene [13]. Specifically, the enzyme catalyzes the 3-amino group of apramycin’s deoxystreptamine ring [16]. Neither aph(3’’) nor aac(3’IV) confers resistance to the other’s respective antibiotic, nor do they confer resistance to kanamycin. Vice versa, the C. jejuni kanamycin resistance gene aphA-3 does not bestow resistance to either hygromycin B or apramycin (data not shown).

In this study, we modified existing C. jejuni gene deletion/ mutagenesis and insertion strategies and plasmids to encode either HygB or Apr’’’’. We based our construction of a non-polar mutagenesis construct on the approach devised by Ménard, Sansonetti and Parsot [17], in which the resistance gene is promoterless, does not harbor a terminator, and transcription is driven from the promoter of the operon into which the gene is introduced. We also modified Karlyshev and Wren’s pRRC C. jejuni genome-insertional gene delivery and expression system [18], replacing the cat chloramphenicol acetyltransferase cassette with either aph(3’’) or aac(3’IV). The expression of aph(3’’) and aac(3’IV) was not detrimental to C. jejuni under common laboratory conditions. Furthermore, to demonstrate the potential of these new markers and plasmids, we deleted and then complemented the C. jejuni arylsulphate sulfatotransferase astA, since the product of astA cleaves a chromogenic substance that can be used to report transcriptional activity [19,20]. With the addition of hygromycin B and apramycin resistance markers, we have provided several new, but relatively familiar, well defined and easy-to-use tools to aid other Campylobacter researchers in a variety of genetic approaches.

Materials and Methods

Bacterial Strains and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table 1. E. coli strains used for plasmid construction were grown at 37°C in Luria-Bertani (LB, Sigma) broth or on 1.7% (w/v) agar plates supplemented with ampicillin (100 μg/mL, Ap’’), chloramphenicol (15 μg/mL, Cm’’), kanamycin (50 μg/mL, Kan’’), hygromycin B (100 μg/mL’’) or apramycin (50 μg/mL’’), as necessary. C. jejuni strains were grown at 37°C or 42°C in Mueller-Hinton (MH, Oxoid) broth or agar supplemented with vancomycin (10 μg/mL’’’’) and trimethoprim (5 μg/mL’’’’). C. jejuni were grown under standard growth conditions (6% O2, 12% CO2) using the Oxoid CampyGen system for shaking broth cultures, or in a Sanyo tri-gas incubator for plates. MH was supplemented with chloramphenicol (15 μg/mL’’’’), kanamycin (50 μg/mL’’’’), hygromycin B (250 μg/mL’’’’) or apramycin (50 μg/mL’’’’) where appropriate.

Construction of Plasmids pAC1H and pAC1A, pRRH and pRRA

Oligonucleotide primers used in this study are listed in Table 2 and were synthesized by Integrated DNA Technologies. The design of pAC1H and pAC1A plasmids containing the non-polar aph(3’’) or aac(3’’’’) markers is described in Results. The aph(3’’) or aac(3’’’’) sequence was amplified from pMV261.hyg or p261com-p.apra with primers pMV261.hyg or p261com-p.apra with primer set 5631 and 5632, or 5633 and 5634, respectively. The polymerase chain reaction (PCR) was carried out with iProof high-fidelity DNA polymerase (Bio-Rad). A-ends were incorporated on the purified products by incubation with Taq DNA polymerase (Invitrogen) and dATP. The purified products were then introduced to linearized pGEM-T Easy (Novagen), ligated overnight with T4 DNA ligase (NEB), and transformed into E. coli DH5α (Invitrogen). Transformants were selected for on LB media supplemented with ampicillin and either hygromycin B or apramycin.

Sequencing verified that the fragment containing aac(3’IV) was correctly inserted into pGEM-T and this plasmid was then designated pAC1A. Sequencing of pGEM-T containing aph(3’’’) indicated that the restriction sites flanking aph(3’’’) and aph(3’’’’) sequence itself were incorrect. The initial aph(3’’’) PCR product was instead digested with MfI and SpI (NEB), purified, and ligated to low-copy pBAD24 digested with EcoRI and SpI. The ligation was transformed into E. coli DH5α and sequencing of the transformants indicated the correct aph(3’’’) sequence was incorporated. The resulting plasmid with the aph(3’’’) non-polar marker inserted in pBAD24 was designated pAC1H.

The design of the pRRH and pRRA gene delivery and expression plasmids is also described in the text. Inverse PCR amplification of pRRC was carried out using iProof with primers 5705 and 5706. The resulting PCR product was purified and digested with KmI and XhoI, and ligated to gel-purified aph(3’’’) or aac(3’’’’) markers from similarly-digested pAC1H and pAC1A. Transformants were selected on LB supplemented with hygromycin B or apramycin, and the resulting plasmids were named pRRH or pRRA respectively. C. jejuni were transformed with 15 μg of plasmid DNA from pRRH, pRRA, pRRK and pRRR as per established procedure [21] to create antibiotic resistant strains, and verified by PCR against the corresponding resistance gene.

Growth Analyses and Competition Assays

For standard growth curve analyses, 10 mL overnight broth cultures of C. jejuni 81–176 integrated with pRRH (HygB’’), pRRA (Apr’’’’), pRRK (Kan’’) and pRRR (Cm’’’’) were inoculated from growth on agar plates containing the appropriate antibiotic. The next day, at the zero time point, strains were standardized to OD600 0.005 in 10 mL of pre-warmed MH (no antibiotics) and grown for 48 hours shaking at 200 rpm at either 37°C or 42°C. Colony forming units (CFU) were assessed over time by plating 10-fold dilutions of aliquots on MH agar plates. Plates were incubated for 48 hours and colonies counted. To assess relative fitness of each antibiotic resistant strain, a co-culture competition was set up. Cultures were inoculated as above, but at the zero time point, 2.5 mL from each of the 10 mL OD600 0.005 cultures were mixed to create a 10 mL culture containing the 4 marked strains. These were grown at 37°C alongside a wild-type control, and CFU were assessed by plating 10-fold dilutions on MH only, or MH containing one of the four antibiotics. Colonies were counted after 48 hours incubation. Three biological replicates, each with 2 technical replicates, were carried out for each assay.

Deletion and Complementation of astA and Assay for Enzymatic Activity

For mutagenesis, the astA gene was PCR-amplified from wild-type 81–176 genomic DNA with primers 5707 and 5708 using iProof DNA polymerase. The PCR product was purified, A-tailed and ligated to pGEM-T to make pGEM-T+astA, which was
transformed into *E. coli* DH5α and selected for with ampicillin. Inverse PCR was performed on the resulting plasmid with primers 5707 and 5708 which deleted all 1,863 bp of *astA* and introduced *KpnI* and *XbaI* sites. The inverse PCR product was digested with *KpnI* and *XbaI*, ligated to similarly-digested *aph* (7') or *aac(3)IV* non-polar markers from pAC1H or pAC1A, and transformed into *E. coli* DH5α. The resulting constructs, pGEM-T-*astA::aph* and pGEM-T-*astA::hyg*, were purified, verified and then transformed into *C. jejuni* 81–176 to create ΔastA::hyg* and ΔastA::aph*. For complementation, iProof PCR was used to amplify *astA* with primer sets 0688 and 0689 [promoterless *astA*], 0690 and 0691 [promoter and *astA*], and 0692 and 0693 [promoter and *astA* in reverse]. This introduced *XbaI* and *MfeI* restriction sites to each of the 3 products. The PCR products, and pRRH and pRRK, were digested with *XbaI* and *MfeI*, and the plasmids were dephosphorylated with Antarctic Phosphatase (NEB). Following clean-up, each *astA* gene was ligated to each plasmid and transformed into DH5α. Colonies were screened by PCR for the inserts, sequenced, and the resulting plasmids were introduced into *E. coli* DH5α for genome integration at rRNA loci; *AprR*, *HygR*, and 1 of 5-bromo-4-chloro-3-indolyl sulfonate potassium salt (XS, Sigma). For quantification, the liquid bacterial culture was spotted on MH agar supplemented with 100 μg mL−1 of 5-bromo-4-chloro-3-indolyl sulfate potassium salt (XS, Sigma). For quantification, the liquid arylsulfatase assay was carried out as described [19,21], with the exception that strains were incubated in AB3 buffer for 2 h instead of 18 h.

### Table 1. Bacterial strains or plasmids used in this study.

| Strain or plasmid | Genotype or description | Source |
|-------------------|-------------------------|--------|
| **E. coli strains** |                         |        |
| DH5α              | F−, ΔgalE, ΔlacZΔM15, ΔendA1, recA1, hsdR17(k-mcK-mcsB) supE44 thi-1 gyrA96 relA1 Δ(lacZYA-argF) U169 | Invitrogen |
| **C. jejuni strains** |                         |        |
| 81–176            | Wild type isolated from a diarrheic patient | [28] |
| 81–176 pRRH       | Strain 81–176 with genome-integrated pRRH; HygR | This study |
| 81–176 pRRA       | Strain 81–176 with genome-integrated pRRA; AprR | This study |
| 81–176 pRRC       | Strain 81–176 with genome-integrated pRRC; CmR | This study |
| 81–176 pRRK       | Strain 81–176 with genome-integrated pRRK; KanR | This study |
| 81–176 ΔastA::hygR | Deletion of *astA* with *aph*(7'); HygR | This study |
| 81–176 ΔastA::aprR | Deletion of *astA* with *aac(3)IV*; AprR | This study |
| DRH461            | Strain 81–176 with an unmarked deletion of *astA* | [19] |
| DRH461 pRRH+astA  | DRH461 with integrated pRRH and polycistronic promoterless *astA*; HygR | This study |
| DRH461 pRRA+astA  | DRH461 with integrated pRRA and polycistronic promoterless *astA*; AprR | This study |
| DRH461 pRRH+astA (reverse) | DRH461 with integrated pRRH and reverse orientation promoterless *astA*; HygR | This study |
| DRH461 pRRA+astA (reverse) | DRH461 with integrated pRRA and reverse orientation promoterless *astA*; AprR | This study |
| DRH461 pRRH+(p)astA (reverse) | DRH461 with integrated pRRH and reverse orientation endogenous promoter and *astA*; HygR | This study |
| DRH461 pRRH+(p)astA (reverse) | DRH461 with integrated pRRH and reverse orientation endogenous promoter and *astA*; AprR | This study |

**Plasmids**

| Plasmid | Source |
|---------|--------|
| pMV261.hyg | Source of *aph*(7'); HygR | [29,30] |
| p261comp.apra | Source of *aac(3)IV*; AprR | [30] |
| pGEM-T | Linearized cloning vector, blue-white screening; ApR | Novagen |
| pBAD24 | Low-copy arabinose-inducible expression vector; ApR | [31] |
| pAC1H | pBAD24 ligated to *aph*(7') amplified with 5631 and 5632; HygR, ApR | This study |
| pAC1A | pGEM-T ligated to *aac(3)IV* amplified with 5633 and 5634; ApR, ApR | This study |
| pRRC | *C. jejuni* vector for genome integration at rRNA loci; CmR | [18] |
| pRRK | *C. jejuni* vector for genome integration at rRNA loci; KanR | J. Ketley |
| pRRH | *C. jejuni* vector for genome integration at rRNA loci; HygR | This study |
| pRRA | *C. jejuni* vector for genome integration at rRNA loci; AprR | This study |
| pGEM-T+astA | pGEM-T ligated to *astA* amplified with 5707 and 5708; ApR | This study |
| pGEM-T+astA::hygR | pGEM-T with *astA* interrupted with *aph*(7') from pAC1H; HygR, ApR | This study |
| pGEM-T+astA::aprR | pGEM-T with *astA* interrupted with *aac(3)IV* from pAC1A; AprR, ApR | This study |
| pRRH+astA | pRRH ligated to *astA* amplified with 0688 and 0689; HygR | This study |
| pRR+astA | pRR ligated to *astA* amplified with 0690 and 0691; HygR | This study |
| pRR+astA (reverse) | pRR ligated to *astA* amplified with 0690 and 0691; HygR | This study |
| pRRH+(p)astA (reverse) | pRRH ligated to *astA* amplified with 0692 and 0691; HygR | This study |
| pRR+astA (reverse) | pRR ligated to *astA* amplified with 0692 and 0691; HygR | This study |
of 1 h. Two biological replicates, each with two technical replicates, were carried out.

Results

Creation of Hygromycin and Apramycin Resistance Markers for C. jejuni Gene Replacement/Deletion

To construct Hyg<sup>R</sup> and Apr<sup>R</sup> cassettes that could be used for mutagenesis, we synthesized PCR ultramers to aph(7)<sup>™</sup> or aac(3)IV, which included the restriction enzyme cut sites and features depicted in Fig. 1A based on the non-polar Kan<sup>R</sup> cassette described by Menard, Sansonetti and Parsot [17]. This construct contains neither a promoter nor a transcription terminator, with the resistance genes preceded at the 5’-end by translational stop codons in all reading frames and also including a Shine-Dalgarno sequence or ribosome binding site (RBS). The 3’-end is followed by another RBS, multiple restriction sites for cloning, and a start codon upstream of and in-frame with the Smal and BamHI cut sites. This start codon is designed to overcome translational coupling of genes in polycistrons if the Smal or BamHI cut sites are employed. The Apr<sup>R</sup> construct was subsequently introduced into high-copy pGEM for clonal amplification (pAC1<sub>A</sub>, Fig. 1B).

However, unwanted recombination and loss of restriction cut sites flanking the Hyg<sup>R</sup> gene necessitated introducing the Hyg<sup>R</sup> construct into the low-copy pBAD24 vector instead (pAC1<sub>H</sub>, Fig. 1D). Via restriction analyses, we confirmed that all introduced restriction sites can be effectively used to excise the resistance cassettes (Fig. 1 C, E). When harbored by E. coli, expression of the resistance markers is driven by lac or ara inducible promoters in pGEM and pBAD respectively; however, induction was not required for E. coli growth in the presence of the corresponding antibiotic. Each antibiotic resistance cassette, lacking a transcriptional terminator, was subsequently introduced into an E. coli cloning vector convenient for non-polar insertion of genes. When harbored by C. jejuni, expression of the resistance markers is driven by the natural promoter of the cat gene encoded on the endogenous cat gene necessitated introducing the Hyg<sup>R</sup> construct into an E. coli cloning vector convenient for non-polar insertion of genes. When harbored by C. jejuni, expression of the resistance markers is driven by the natural promoter of the cat gene encoded on the endogenous cat gene.

Modification of the pRRC Genome-insertional Gene Delivery Vector to Carry aph(7)<sup>™</sup> or aac(3)IV

The chloramphenicol resistance marker (cat, Cm<sup>R</sup>) encoded on pRRC (Fig. 2A) [18] was exchanged with Hyg<sup>R</sup> or Apr<sup>R</sup>. The endogenous aph(7)<sup>™</sup> or aac(3)IV promoters were non-functional in C. jejuni (data not shown), so the pRRC cat promoter was retained for integrated expression.

Table 2. Oligonucleotides used in this study (with restriction sites underlined).

| Primer | Sequence 5’-3’ | Target, sense and description | Restriction sites |
|--------|----------------|-------------------------------|------------------|
| 5631   | ACACCAATTGGTTACCCGGGTTACATACTAGAGGAGATAATGACACAAGAATCCTAGTTTAC | aph(7)<sup>™</sup>, sense, start codon changed to ATG. | MfeI, KpnI, Smal |
| 5632   | GTGGCATGCTGCTGACATATGCTAGAGGATCCGGGATCATATCCCT-CCAGGATCTACGAGCGCCGGGGGGCCGGGTGTC | aph(7)<sup>™</sup>, antisense. | Smal, BamHI, XbaI, NdeI, PstI, SpI |
| 5633   | ACACCAATTGGTTACCCGGGTTACATACTAGAGGAGATAATGACACAAGAATCCTAGTTTAC | aac(3)IV, sense, start codon changed to ATG. | MfeI, KpnI, Smal |
| 5634   | GTGGCATGCTGCTGACATATGCTAGAGGATCCGGGATCATATCCCT-CCAGGATCTACGAGCGCCGGGGGGCCGGGTGTC | aac(3)IV, antisense. | Smal, BamHI, -XbaI, NdeI, PstI, SpI |
| 5705   | ACACCAATTGGTTACCCGGGTTACATACTAGAGGAGATAATGACACAAGAATCCTAGTTTAC | pRRC cat, sense. | KpnI |
| 5706   | TGATGGTACATGACTGCTG | pRRC cat, sense. | |
| 5707   | TATAGCCGAAACAAAAATCC | Flanking astA, sense. | |
| 5708   | AAATGAAATTTGGAAAGCTTCTC | Flanking astA, antisense. | |
| 5709   | ACACCAATTGGTTACCCGGGTTACATACTAGAGGAGATAATGACACAAGAATCCTAGTTTAC | 5’-internal astA, antisense. | KpnI |
| 5710   | ACACCAATTGGTTACCCGGGTTACATACTAGAGGAGATAATGACACAAGAATCCTAGTTTAC | 3’-internal astA, sense. | XbaI |
| 0688   | ACACCAATTGGTTACCCGGGTTACATACTAGAGGAGATAATGACACAAGAATCCTAGTTTAC | Promoterless astA, sense. For polycistronic expression. | XbaI |
| 0689   | ACACCAATTGGTTACCCGGGTTACATACTAGAGGAGATAATGACACAAGAATCCTAGTTTAC | astA, antisense. For polycistronic expression. | MfeI |
| 0690   | ACACCAATTGGTTACCCGGGTTACATACTAGAGGAGATAATGACACAAGAATCCTAGTTTAC | Promoterless astA, sense. For reverse expression. | MfeI |
| 0691   | ACACCAATTGGTTACCCGGGTTACATACTAGAGGAGATAATGACACAAGAATCCTAGTTTAC | astA, antisense. For reverse expression. | XbaI |
| 0692   | ACACCAATTGGTTACCCGGGTTACATACTAGAGGAGATAATGACACAAGAATCCTAGTTTAC | Upstream astA, sense. For reverse expression. | MfeI |

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Figure 1. Synthesis of plasmids containing *aph*(7') or *aac*(3)IV as non-polar hygromycin B and apramycin resistance markers. (A) Schematic of ultramers designed to amplify *aph*(7') or *aac*(3)IV. The 5' ultramers 5631 and 5633, for *aph*(7') or *aac*(3)IV respectively, include *Mfe*I, *Kpn*I and *Sma*I restriction sites, stop codons in all three reading frames, and a ribosome binding site. The 3' ultramers 5632 and 5634 include a ribosome binding site, a start codon in-frame with restriction sites for *Sma*I and *Bam*HI, and restriction sites for *Xba*I, *Nde*I, *Pst*I and *Sph*I. (B) The amplified *aac*(3)IV was introduced by TA cloning into linearized pGEM-T, conserving the restriction sites in the pGEM-T multiple cloning site (MCS). The resulting plasmid is pAC1A. The pGEM sites may also be used for the sub-cloning of the apramycin resistance marker (*Apr*R). (C) All introduced sites in pAC1A were tested by restriction digest. (D) The *Mfe*I- and *Sph*I-digested *aph*(7) amplification product was cloned into pBAD24 digested with *Eco*RI (*Mfe*I-compatible) and *Sph*I. The *Mfe*I site was lost in the resulting plasmid, pAC1H. (E) All restriction sites introduced to pAC1H were tested by digest.

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to ensure expression. Exchange of cat was achieved using an inverse PCR methodology, in which a KpnI site was introduced to the 5'-end of the antisense primer (Fig. 2B). The antisense primer was targeted to the DNA immediately upstream of cat, allowing conservation of the cat promoter. The inverse PCR product was next digested with KpnI and XbaI and ligated to similarly digested aph(7') or aac(3)IV from pAC1H or pAC1A to create pRRH and pRRA respectively (only pRRH is shown). Restriction digest analysis confirmed the function of all introduced sites. The resistance markers from pRRK, pRRC, pRRH and pRRA were inserted into the C. jejuni 81–176 genome, and each resulting strain was analyzed for microaerobic growth and survival in shaken Mueller-Hinton (MH) broth by counting CFU over 48 hours at both 42 °C (left panel) and 37 °C (right panel). To determine if the introduction of either marker contributed any fitness cost that could affect competitiveness against wild-type or the other marked strains, a competition assay was performed. Equal numbers of wild-type marked with hygromycin, apramycin, chloramphenicol and kanamycin resistance markers were co-cultured with unmarked wild-type in shaking MH broth at 37 °C under microaerobic conditions. CFU were assessed by plating a dilution series on MH agar. CFU were further assessed from the co-culture by plating on MH only (the total CFU, same data as in F) or MH supplemented with each antibiotic, representing the number of bacteria resistant to each antibiotic.

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The Presence of aph(7') or aac(3)IV in C. jejuni is not Detrimental to Growth

To test if the hygromycin B or apramycin resistance genes affected C. jejuni growth and survival, pRRH and pRRA were integrated into the genome of C. jejuni 81–176 to create HygR or AprR wild-type strains. In the absence of their respective antibiotics, we assessed the time-course growth profile of these strains and compared CFU recovered to those of wild-type and wild-type marked with aphA-3 from pRRK and cat from pRRC. Neither the HygR or AprR strains were defective for growth in MH broth under microaerobic conditions in at 37 °C or 42 °C, the optimal temperature range of the bacterium (Fig. 2E). However, because differences in fitness cost of the antibiotic markers may not have been detected in the first experiment, we also carried out a competitive index-style assay. Broth cultures were inoculated with equal numbers of each of the 4 resistant strains, and the mixed cultures were grown at 37 °C alongside a wild-type only control (Fig. 2F). At each time point, CFU were assessed by plating dilutions on MH or MH containing hygromycin B, apramycin, kanamycin or chloramphenicol. The total CFU were represented on the MH-only plate, while the number of resistant bacteria were determined on each antibiotic-containing plate. No fitness cost was observed for either HygR or AprR strains between 0–24 hours (Fig. 2G). At 48 hours there was a modest decrease in CFU.
recovered for HygR strains; however, this was less pronounced than the defect observed for strains carrying the well-established KanR marker. It should be noted that at the 48 hour timepoint, each culture exhibited an overall decrease in the relative CFU recovered under antibiotic selection, suggesting that older cultures are generally more sensitive to antibiotic pressure.

Deletion and Complementation of Arylsulfatase astA with HygR or AprR Constructs

In C. jejuni, expression of arylsulfatase (astA) can be monitored via colorimetric plate and broth assays [19,21]. To further test the usability of pAC1H, pAC1A, pRRH and pRRA, we mutagenized astA with the non-polar HygR or AprR markers from pAC1H and pAC1A and also restored a copy of astA to the genome of a ΔastA strain using pRRH or pRRA. First, astA (Fig. 3A) was completely deleted and replaced with the non-polar HygR or AprR markers from pAC1H and pAC1A, respectively (Fig. 3B). Next, a promoterless astA was cloned into pRRH or pRRA in the transcriptional direction of, and thus expressed by, the cat promoter (Fig. 3C), and these constructs were integrated into DRH461, an unmarked ΔastA strain [19]. The astA gene was also cloned without (Fig. 3D) and with (Fig. 3E) its native promoter into pRRH or pRRA in the reverse orientation to the cat promoter. These latter constructs test expression only from the endogenous astA promoter and were likewise integrated into DRH461. Each strain was spotted onto MH solid media containing the chromogenic substrate XS, which is cleaved by arylsulfatase [20], and grown for 72 hours. No arylsulfatase activity was observed in any deletion strain. Partial complementation was observed for astA expressed from the cat promoter, full complementation was observed for astA expressed from its native promoter, and no complementation was observed for the promoterless astA cloned in the reverse orientation to the cat promoter (Fig. 3F). A quantitative liquid spectrophotometric assay confirmed the plate readouts (Fig. 3G).

Figure 3. Mutagenesis of the arylsulfatase gene astA with aph(7′) or aac(3)IV non-polar markers and complementation of ΔastA via genomic insertion with pRRH or pRRA. (A) Loci arrangement of astA single-gene operon in C. jejuni 81–176. (B) Deletion of astA with either aph(7′) or aac(3)IV from pAC1H or pAC1H. (C) Introduction of promoterless astA into pRRH or pRRA in the same orientation as the cat promoter created pRRH+astA or pRRA+astA and resulted in polycistronic expression of astA with aph(7′) or aac(3)IV. (D) Promoterless astA inserted in the opposite orientation to the cat promoter (designated pRRH+astA (reverse) or pRRA+astA (reverse) (E) Insertion of the endogenous astA promoter and astA in the opposite orientation to the cat promoter in pRRH and pRRA created pRRH+(p)astA (reverse) and pRRA+(p)astA (reverse). Only HygR plasmids/strains are depicted in B–E, but both HygR and AprR plasmids represented with HygR in D, C and E were integrated into the genome of the ΔastA strain, DRH461. (F) Arylsulfatase activity of the deletion and complementation strains was assessed by spotting 10 μL of OD-standardized cultures onto MH agar plates supplemented with the chromogenic XS cleaved by arylsulfatase. A blue-green color indicates activity, and the spots correspond to labels on the bar graph below. (G) Quantification of arylsulfatase activity from broth cultures to assess transcription of astA.

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Discussion

With the introduction of hygromycin B and apramycin resistance markers, we have provided researchers in the C. jejuni field with additional genetic tools, essentially doubling the number of broadly usable markers for this organism. We envision that these markers will be especially useful for deletion of multiple genes, complementation, and/or the addition of various promoter-reporter constructs. Although it is possible to construct a potentially unlimited number of unmarked deletion mutants in C. jejuni [7], a disadvantage of unmarked mutations is that they cannot be easily transferred from one genetic background to another. This is especially critical for an organism like C. jejuni with high rates of phenotypic variation associated with phase-variable, highly mutable contingency loci [1,22,23]. This variation is often unpredictable and may result in phenotypes unlinked to the intended mutation, and it is often pertinent to test several mutant clones or re-introduce a marked mutation to a wild-type background to ensure the veracity of any phenotype.

The non-polar markers harbored by gene disruption cassettes in pAC1H and pAC1A have both advantages and disadvantages when compared to markers that also introduce a promoter, such as the C. jejuni cat cassette in pRY109 [5]. Primarily, the main advantage of a promoterless disruption construct is that the introduction of a non-endogenous promoter affects transcription of any co-transcribed gene at the 3′-end of the deleted gene in an operon. Vice versa, one disadvantage of the promoterless marker is that it is reliant on transcription from the promoter of the gene into which it has inserted. Therefore, if the target gene is not highly expressed, then the same will be true for the resistance gene, and antibiotic resistance will not be conferred to the bacterium. Our laboratory has experienced this problem, and if a mutant cannot be made with a non-polar marker, then it can be attempted using the cat cassette from pRY109. Another advantageous feature of the non-polar markers in pAC1H and pAC1A is the start codon at the 3′-end of the resistance gene; specifically, use of the 3′ SmaI or BamHI restriction sites allows placement of the start codon in the reading frame of the stop codon of the mutated gene. This configuration allows for translation of a remainder of the gene into which the marker is inserted, preventing polar effects on the downstream gene due to translational coupling. Translational coupling is the interdependence of translation efficiency of co-transcribed genes on a polycistronic mRNA [17,24]. We do not routinely design our deletion/replacement strategies to take translational coupling into account for the initial study of a gene; however, translational coupling was recently observed for the C. jejuni astA gene. For cloning in pAC1H and pAC1A, the promoter is considerably lower than from p261comp.apra and helpful advice. We are also grateful to Dmitry Apel and Emilisa Friedich for technical assistance and manuscript critique, and Jenny Vermeulen for 81–176 genome-integrated pRRK and pRRC.

In summary, we have adapted a set of well-established plasmids to encode hygromycin B and apramycin resistance for gene deletion, replacement, and expression in C. jejuni. We also established the optimal concentrations of both hygromycin B and apramycin for the purposes of selection. In addition, we determined that introduction of aphB(7) or aac(3)IV was not detrimental and that there was no appreciable fitness cost to C. jejuni when compared to chloramphenicol or kanamycin resistance markers. These constructs were validated using the astA reporter, and are currently being utilized in our laboratory for exploratory studies of uncharacterized genes. These new molecular tools will provide a broader range of possible experiments, will assist in the mechanistic study of C. jejuni, and contribute to a better understanding of the microorganism’s lifecycle and pathogenicity.

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Author Contributions

Conceived and designed the experiments: AC ECG. Performed the experiments: AC. Analyzed the data: AC ECG. Contributed reagents/materials/analysis tools: ECG. Wrote the paper: AC ECG.

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