INTRODUCTION

Campylobacter jejuni is the leading bacterial cause of foodborne gastroenteritis worldwide. The impact of C. jejuni infections is significant due to their high incidence, duration of infection and possible post-infection sequelae (Ruiz-Palacios, 2007). C. jejuni has a broad range of environmental reservoirs including water, birds and other domestic animals, with chickens representing the largest source of human infection (Young et al., 2007). Although C. jejuni is considered as commensal in chickens, it has a significant impact on animal welfare in certain breeds of bird (Humphrey et al., 2014). The genome of C. jejuni is subject to considerable genetic variation, which is thought to play an important role in the survival of the species within host organisms and the environment, especially by altering surface exposed structures such as flagella, lipooligosaccharide and capsular polysaccharide (Balaban et al., 2009; Jerome et al., 2011; Joslin & Hendrixson, 2009; Mohawk et al., 2014; Thomas et al., 2014). Genetic heterogeneity arises in the C. jejuni population as a consequence of mutations due to replication errors, phase variation of homopolymeric or repetitive heteropolymeric tracts either in coding sequences or in promoter regions and to a lesser extent, recombination (Thomas et al., 2014; Wassenaar, 2011; Wilson et al., 2009). It has been suggested that the lack of a functional mismatch repair system enhances the frequency of phase variation and random mutations, and thus the overall heterogeneity of the C. jejuni population (Gaasbeek et al., 2009).

Flagellar-mediated motility plays a central role in commensal and pathogenic behaviours of C. jejuni (Guerry, 2007; Lertsetthakarn et al., 2011). The flagellar system is subject to genetic variation at multiple levels, which often leads to alteration of motility (Gao et al., 2014; Guerry, 2007; Hendrixson & DiRita, 2004). C. jejuni possesses polar flagella at one or both ends of the bacterial cell which consist of a membrane-embedded basal body, a hook structure and
Second-site mutation causing motility defects

Electroporation and natural transformation of C. jejuni. Electroporation of C. jejuni was performed as described previously (Holt et al., 2012) with minor adjustments. C. jejuni M1 was cultured on BHI-TrM blood agar plates for ~48 h then replated onto fresh BHI-TrM blood agar plates and grown for ~16 h. Bacteria were harvested from plates in BHI broth, washed four times in ice-cold wash buffer [272 mM sucrose and 15 % (v/v) glycerol] and then resuspended in wash buffer. Of this suspension, 100 μl was added per 0.2 cm pre-chilled electroporation MicroPulser cuvette (Bio-Rad) and mixed with DNA (~3 μg). Electroporation was performed using a GenePulser Xcell system (Bio-Rad) using the following settings: 2.5 kV, 200 Ω and 25 μF. After a single pulse, 200 μl of pre-warmed SOC medium was added and the suspension was transferred onto 2 ml BHI-TrM blood agar in a Universal tube (Greiner). After 5 h incubation under microaerophilic conditions, 1 ml of pre-warmed BHI medium was added to resuspend the bacteria, which were plated onto selective agar plates and grown under microaerophilic conditions for 2 to 3 days.

Natural transformation of C. jejuni was performed using an adapted biphasic method (Holt et al., 2012; van Vliet et al., 1998). C. jejuni was grown on BHI-TrM blood agar plates for ~48 h and replated onto fresh BHI-TrM blood agar plates and grown for ~16 h. Bacteria were harvested from plates in BHI broth and the OD600 was adjusted to ~0.5. Of this suspension, 0.5 ml was pipetted onto 2 ml BHI-TrM blood agar in a Universal tube (Greiner) and incubated for 3 h under microaerophilic conditions. Thereafter, DNA was added and incubated for 3–5 h before bacteria were harvested and then plated on selective agar plates and grown for 2 to 3 days under microaerophilic conditions.

Construction of defined gene deletion mutants. C. jejuni defined gene deletion mutants were generated by allelic replacement of the gene with a chloramphenicol resistance cassette (cat). The cat cassette was amplified by PCR from the plasmid pCC027 (Coward et al., 2008) and the 5’ (L1 and L2 primers) and 3’ (R1 and R2 primers) flanking regions of the target gene were amplified by PCR from C. jejuni M1 genomic DNA. PCRs were performed using Q5 DNA polymerase (New England Biolabs) and purified using the QIAquick PCR purification kit (Qiagen). The gene flanking region PCR primers L2 (reverse primer 5’ flank) and R1 (forward primer 3’ flank) contain extensions that are complementary to the primers used for amplification of the cat cassette. The cat cassette (640 ng) and gene flanking regions (200 ng each) were joined in an overlap PCR without primers, resulting in the incorporation of the cat cassette in between the 5’ and 3’ flanking regions. Two microlitres of the product of this overlap PCR were then used as a template in a second round of amplification, this time using the primers L1 (forward primer 5’ flank) and R2 (reverse primer 3’ flank), which flank the original gene sequence and thus amplify the whole fragment; a total of four 50 μl PCRs were performed for each mutant, and subsequently pooled and purified. Of this, ~3 μg of the overlap PCR product was used for electroporation of C. jejuni M1 to generate first generation defined gene deletion mutants, which were selected on BHI-TrM-Cm blood agar. Next, first generation mutant DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen) and checked by PCR for recombination at the desired genomic location using the L1 forward primer (5’ gene flanking region) and a control reverse primer located in the gene flanking) and R1 (forward primer 3’ flank) contain extensions that are complementary to the primers used for amplification of the cat cassette. The cat cassette (640 ng) and gene flanking regions (200 ng each) were joined in an overlap PCR without primers, resulting in the incorporation of the cat cassette in between the 5’ and 3’ flanking regions. Two microlitres of the product of this overlap PCR were then used as a template in a second round of amplification, this time using the primers L1 (forward primer 5’ flank) and R2 (reverse primer 3’ flank), which flank the original gene sequence and thus amplify the whole fragment; a total of four 50 μl PCRs were performed for each mutant, and subsequently pooled and purified. Of this, ~3 μg of the overlap PCR product was used for electroporation of C. jejuni M1 to generate first generation defined gene deletion mutants, which were selected on BHI-TrM-Cm blood agar. Next, first generation mutant DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen) and checked by PCR for recombination at the desired genomic location using the L1 forward primer (5’ gene flanking region) and a control reverse primer located in the gene (‘A’ primer, which should not generate a product) and a cat cassette primer (CC069, which should generate a product of predicted size). First generation gene deletion mutant DNA (50 ng) was used for natural transformation of C. jejuni M1 WT; the gene replacement event was selected and confirmed by PCR. For flaB deletion mutant clones, correct integration of the cat cassette was also analysed by DNA sequencing. In addition, the WT was processed in parallel through the transformation procedure without any added mutagenic DNA to obtain a ‘coupled’ WT strain. This was done to reduce the genetic variation between the WT and defined mutant strains. Primer sequences are given in Table S1.

METHODS

Bacterial strains and growth conditions. All WT strains, defined mutants, genetically complemented mutants and plasmids are summarized in Table 1. C. jejuni strain M1 was originally isolated from a researcher after visiting a poultry processing plant (Friis et al., 2010) and is fully motile and spiral-shaped. C. jejuni M1 was routinely cultured on brain heart infusion (BHI, Oxoid) agar plates supplemented with 5 % defibrinated horse blood (Oxoid) and 5 μg trimethoprim ml⁻¹ (TrM). Gene deletion mutants were cultured in the presence of 10 μg chloramphenicol ml⁻¹ and genetically complemented mutant strains were also supplemented with 50 μg kanamycin ml⁻¹. C. jejuni was cultured under microaerophilic conditions (5 % O₂, 10 % CO₂ and 85 % N₂) in a MACS VA500 Variable Atmosphere Work Station (Don Whitley). Escherichia coli NEB 5α or 10-β (New England Biolabs) was used for cloning and was cultured in LB medium at 37 °C, supplemented as appropriate with antibiotics.

a protruding polymeric filament that is composed of the major flagellin subunit FlaA and the minor subunit FlaB (Gao et al., 2014; Guerry et al., 1991). In C. jejuni strain 81116, inactivation of flaA led to a non-flagellated and non-motile phenotype, whereas flagella and motility were retained after the inactivation of flaB (Nuijten et al., 1990; Wassenaar et al., 1991). The motor subunits at the flagellar base, encoded by motA and motB, are responsible for flagellar rotation. Mohawk et al. (2014) reported that non-motile variants of C. jejuni 81-176 carried distinct mutations [i.e. single nucleotide polymorphisms (SNPs) and insertions and deletions (INDELs)] in the motA gene. Flagellar gene expression is tightly controlled and requires the alternative δ²⁸ and δ²⁴ factors, the FlgSR two-component system, the FlhF GTPase and the flagellar secretion system (Balaban et al., 2009; Hendrixson & DiRita, 2003; Joslin & Hendrixson, 2009). Phase variation mechanisms acting on the FlgSR system that affect motility in C. jejuni 81-176 include reversible phase variation in (i) homopolymeric poly-A and poly-T tracts within the flgR response regulator (Hendrixson, 2006) and (ii) poly-A tracts and heteropolymeric repeats located in the flgS sensor histidine kinase (Hendrixson, 2008). It is noteworthy that the C. jejuni flagellar filament is extensively glycosylated, predominantly with pseudaminic acid and legionaminic acid and derivatives thereof. Recombination within the flagellar glycosylation gene locus and homopolymeric poly-G tract phase variation are mechanisms of variation (Howard et al., 2009; Karlyshev et al., 2002; van Alphen et al., 2008).

Here we show that the propensity for stochastic mutation events in C. jejuni has clear implications for gene function analysis in an experimental setting. Analysis of C. jejuni M1 flaB mutant clones revealed inconsistent motility phenotypes. Using genome resequencing and in-depth genotypic and phenotypic characterization, we identified second-site mutations and confirmed that these were responsible for the observed inconsistent motility amongst flaB mutant clones. We also describe a new versatile and user-friendly system for genetic complementation in C. jejuni.
Table 1. Bacterial strains and plasmids used in this study

| Strain or plasmid * | Relevant genotype† or description | Source/reference |
|---------------------|----------------------------------|------------------|
| **C. jejuni**       |                                  |                  |
| M1                  | WT                               | Friis et al. (2010) |
| M1 coupled to ΔflaB | WT                               | This study       |
| M1 coupled to ΔflaB*, ΔflaA, ΔflaAB | WT | This study       |
| M1 coupled to ΔflaB+empty GC | WT; Km' | This study       |
| M1 coupled to ΔfliD, ΔfliA | WT | This study       |
| M1 coupled to ΔfliW | WT | This study       |
| M1 coupled to ΔfliW+empty GC | WT; Km' | This study       |
| M1 ΔfliB clones A, B, C, D, E | ΔCJM1_1294; Cm' | This study       |
| M1 ΔfliA clones A, B, C | ΔCJM1_1296; Cm' | This study       |
| M1 ΔfliAB clones A, B, C | ΔCJM1_1295-1294/CJM1_1296; Cm' | This study       |
| M1 ΔfliD clones A, B, C | ΔCJM1_0851; Cm' | This study       |
| M1 ΔfliA clones A, B, C | ΔCJM1_1501; Cm' | This study       |
| M1 ΔfliW clone A, B, C | ΔCJM1_1052; Cm' | This study       |
| M1 ΔfliW clone A+empty GC | ΔCJM1_1052; Cm' | This study       |
| M1 ΔfliW clone A+ΔfliW GC | ΔCJM1_1052+ΔCJM1_1052; Cm' | This study       |
| M1 ΔfliB clone D-empty GC | ΔCJM1_1294; Cm' | This study       |
| M1 ΔfliB clone D+ΔfliW GC | ΔCJM1_1294+ΔCJM1_1295; Cm' | This study       |
| M1 ΔfliB clone E-empty GC | ΔCJM1_1294; Cm' | This study       |
| M1 ΔfliB clone E+ΔfliW GC | ΔCJM1_1294+ΔCJM1_1052; Cm' | This study       |
| **E. coli strains** |                                  |                  |
| NEB 10–β or 5–x   | Host strains for pSV009 and derivatives thereof | New England Biolabs |
| Plasmids           |                                  |                  |
| pCC027             | Cm' donor plasmid                 | Coward et al. (2008) |
| pMiniT             | E. coli PCR cloning plasmid       | New England Biolabs |
| pUC19              | E. coli cloning plasmid           | New England Biolabs; Yanisch-Perron et al. (1985) |
| pSV009             | C. jejuni genetic complementation plasmid, pUC19 backbone; donor for empty GC region | This study       |
| pSV012             | pSV009 containing fliW; donor for fliW GC region | This study       |

*empty GC, Empty genetic complementation region amplified from pSV009; fliW GC, genetic complementation region containing the fliW promoter and coding sequence amplified from pSV012.

†Cm', chloramphenicol resistance; Km', kanamycin resistance.

**Motility assay.** *C. jejuni* was grown on BHI blood agar plates for ~48 h then replated onto fresh BHI blood agar plates and grown for ~16 h. BHI suspensions of OD600 ~0.5 were made and stabbed, using a 20 μl pipette tip, into the middle of a 9 cm petri dish containing 25 ml of 0.4 % select agar (Sigma Aldrich) BHI medium and grown overnight under microaerophilic conditions. The diameter of the ring of bacterial growth was measured using a ruler (n=3).

**Genetic complementation.** For genetic complementation of defined gene deletion mutants, the target gene was inserted into a pseudogene region between CJM1_0055 and CJM1_0057 (Coward et al., 2008). For this, a new genetic complementation system was designed in *silico* and synthesized *de novo* from oligonucleotides (GeneArt Strings, Life Technologies). The genetic complementation system consists of the cat promoter region from pRY011 (Coward et al., 2008; Yao et al., 1993), a partial pUC19 multiple cloning site and the kanamycin resistance cassette including its promoter region from pRY107 (Yao et al., 1993), all of which is flanked by 400 bp of 5’ and 3’ sequence of the CJM1_0055–0057 pseudogene region (98–100 % identity with sequences in *C. jejuni* 11168, 81116 and 81-176), see Fig. 5(a) for a schematic overview. The *de novo* synthesized genetic complementation region was first cloned into pMiniT using the NEB PCR cloning kit (New England Biolabs) and subsequently subcloned into pUC19 using the HindIII and EcoRI restriction enzyme sites, yielding the plasmid pSV009. Of note, the 5’ pseudogene region (CJM1_0055–0057) sequenced originally harboured a HindIII site, which was eliminated by introducing a synonymous mutation during the *in silico* design of the genetic complementation insert. The fliW coding sequence including its upstream sequence was amplified by PCR and subcloned into pSV009 using the Xhol and BamHI sites, yielding pSV012-fliW. Sequences of pSV009 and pSV012-fliW were confirmed using DNA sequencing. The fliW complementation region and the empty genetic complementation region were amplified by PCR from pSV012 or pSV009, respectively, using the primers pSV009_GCampl_FW1/RV1, which amplify the complete genetic complementation region. The fliW complementation or the empty genetic complementation region was subsequently introduced into *C. jejuni* by electroporation. For a schematic overview of the genetic complementation strategy, see Fig. 5(a). Primer sequences are given in Table S1.
Scanning and transmission electron microscopy (EM) analysis. C. jejuni M1 WT and defined gene deletion mutants were cultured for \( \sim 48 \) h, harvested and washed four times in water. For scanning EM, bacteria were fixed in 2 \% glutaraldehyde in 0.05 M sodium cacodylate buffer at pH 7.4 for 48 h, allowed to settle onto 10 mm poly-l-lysine-coated glass coverslips, rinsed twice in deionized water and quench frozen in propane cooled in liquid nitrogen. Samples were freeze-dried overnight from \(-95\) to \(+30 \)°C in a K775X freeze dryer (Quorum Etmitech). The coverslips were mounted on Cambridge stubs with silver DAG and coated with 4 nm of gold in a K575X sputter coater (Quorum Etmitech), then imaged using a Verios 460 (FEI) scanning electron microscope operated at 2 or 5 kV. For transmission EM, bacteria were fixed in 2 \% glutaraldehyde in 0.05 M sodium cacodylate buffer at pH 7.4 for 6–12 h, and adsorbed onto glow discharged 400 mesh copper grids with a carbon film attached for 30 s. They were rinsed twice with deionized water and stained for 30 s with 1 \% aqueous uranyl acetate. Grids were allowed to dry for 20 min and viewed in a Tecnai G2 (FEI) transmission electron microscope operated at 200 keV. Images were captured with an AMT XR60B camera running Deben software.

Illumina sequencing. Genomic DNA was isolated using Genomic-tip 20/G columns (Qiagen) or the DNeasy Blood and Tissue kit (Qiagen) according to manufacturer’s instructions. Libraries for Illumina sequencing were prepared using the NEBNext Ultra DNA library prep kit (New England Biolabs) or the Nextera XT DNA kit (Illumina). For the NEBNext Ultra DNA kit, the DNA was first sheared to \( \sim 300 \) or 400 bp fragments in microTUBE screw-cap tubes in an M220 focused-ultrasonicator (Covaris). Following NEBNext Ultra DNA library preparation, the library size was determined with a Bioanalyzer 2100 (Agilent) and the DNA concentration was measured with the Qubit dsDNA BR kit (Life Technologies) and pooled at equimolar amounts. Nextera XT library preparation was performed according to the manufacturer’s instructions. The libraries for pflA (CJM1_1501), flaD (CJM1_0851), flaB (CJM1_1294) gene deletion mutant clones and their coupled WT strains were sequenced using 76 bp paired-end sequencing, whereas the \( fliW \) gene deletion mutant clone A and its coupled WT were sequenced on the Illumina MiSeq platform using 301 bp paired-end sequencing (v3 chemistry).

Illumina sequence data analysis. Raw sequence reads for pflA (CJM1_1501), flaD (CJM1_0851), flaB (CJM1_1294) mutant clones and their coupled WTs were mapped to the M1 reference genome sequence [GenBank accession no. CP001900 (Friis et al., 2010)] using the CLC Genomics Workbench version 7.5.1. Variants were called only if covered by >20 reads, a variant count of >5 reads and detected in both forward and reverse sequence reads. Hierarchical clustering based on SNPs and INDELs presence/absence was performed using CLC Main Workbench (v7); variants present in all sequenced mutants and WTs were excluded from the clustering analysis.

Variant analysis of \( fliW \) mutant clone A and its coupled WT was performed as follows: (1) Illumina sequence reads were mapped to the M1cam reference genome (accession no. CP012149, see details below) using Stampy (Lunter & Goodson, 2011), (2) Samtools was used to identify variants (Li et al., 2009); the minimum quality for variant calling was set to 100. The variant effect at the protein level was predicted using SnpEff (Cingolani et al., 2012).

Genome resequencing and annotation of C. jejuni M1 Cambridge. The M1 WT strain used in our laboratory and ‘coupled’ WT strains (i.e. the M1 WT processed through the natural transformation procedure) obtained during the generation of flaB (one WT), pflA and flaD (two WTs) gene deletion mutants were Illumina sequenced and their sequence reads mapped to the M1 reference sequence [GenBank accession no. CP001900 (Friis et al., 2010)]. SNPs and INDELs detected in all four sequenced M1 WTs were collected and incorporated into the C. jejuni M1 reference genome sequence using a custom Python script, yielding an in-house C. jejuni M1 genome sequence (designated CJM1cam). The effect of SNPs and INDELs at the protein level was predicted using SnpEff (Cingolani et al., 2012). Sequence variants detected in this study by Sanger DNA sequencing were also incorporated into the CJM1cam genome sequence. An overview of the SNPs and INDELs relative to the published M1 reference genome can be found in Table S2. The obtained CJM1cam genome sequence was annotated with Prokka (Seemann, 2014) and assigned locus tags starting with CJM1cam; where possible the locus tag numbering of the original M1 reference genome was retained. The annotation of the flagellin locus CJM1_1295-1294 (\( fliB \) and CJM1_1296 (\( fliA \)) was manually corrected. Automated ORF calling using Prokka resulted in the convergence of 33 partial coding sequences (CDSs) into 16 CDSs relative to the published M1 genome (Friis et al., 2010). The CJM1cam proteins were assigned Cluster of Orthologous Genes (Tatusov et al., 1997) functional categories using a custom R script. The obtained CJM1cam genome sequence and annotation are available through GenBank (accession no. CP012149). Statistical analyses. Statistical analyses were performed in GraphPad Prism 6.0 (GraphPad software) with \( P<0.05 \) considered to be significant.

Sequencing data. The CJM1cam genome sequence is deposited at GenBank (CP012149) and the sequence of the pSV009 genetic complementation plasmid is deposited KT373982. The Illumina sequence data are deposited at the European Nucleotide Archive under study accession number PRJEB10223 (http://www.ebi.ac.uk/ena).

RESULTS AND DISCUSSION

Differential motility of defined gene deletion mutant clones

For our studies into the molecular mechanisms of C. jejuni pathogenesis, e.g. invasion of gut epithelial cells (to be reported elsewhere), three defined deletion mutants were generated in C. jejuni M1 flagella genes using an overlap PCR method. Gene deletions were constructed in flaD, also referred to as flgL (CJM1_0851; hook filament junction protein) (Neal-McKinney & Konkel, 2012), pflA (CJM1_1501; paralysed flagellum protein) (Yao et al., 1994) and CJM1_1294, predicted to encode the major flagellin subunit FlaA (CJM1_1294) in the published C. jejuni M1 reference genome (Friis et al., 2010). We anticipated that deletion of these flagella genes would result in non-motile phenotypes (Neal-McKinney & Konkel, 2012; Wassenaar et al., 1991; Yao et al., 1994). As expected (Neal-McKinney & Konkel, 2012; Yao et al., 1994) three independent mutant clones for each of flaD (CJM1_0851) and pflA (CJM1_1501) were found to be non-motile (Fig. 1). Scanning EM confirmed the previously reported non-flagellated and flagellated phenotype of flaD and pflA gene deletion mutants, respectively (data not shown) (Neal-McKinney & Konkel, 2012; Yao et al., 1994). In contrast, five independent flaA (CJM1_1294) mutant clones were tested for motility with inconsistent results: mutant clones A and B displayed WT motility, clone C showed intermediate motility and clones D and E had severely attenuated motility (Fig. 1).
To unravel the basis of the inconsistent motility phenotypes of \textit{flaA} (CJM1\_1294) mutant clones, we first confirmed the correct insertion of the chloramphenicol (\textit{cat}) resistance cassette in \textit{flaA} (CJM1\_1294) by Sanger sequencing. Importantly, this revealed the insertion of three A nucleotides in an 11 bp stretch at the 3' end of CJM1\_1295 [annotated as 'hypothetical protein' (Friis et al., 2010)], which was also independently confirmed by Sanger sequencing of the complete CJM1\_1295–1294 region. Incorporation of the A insertions into the original M1 sequence (Friis et al., 2010) led to CJM1\_1295 and CJM1\_1294 being merged into a larger ORF of 1731 bp (Fig. 2a). The difference between the published \textit{C. jejuni} M1 reference genome (Friis et al., 2010) and the sequence of our M1 strain may reflect genotypic variation or sequencing or assembly errors in the original M1 genome sequence.

In-depth analysis of the altered flagellin locus in \textit{C. jejuni} M1 revealed that: (1) the merged CJM1\_1295–1294 gene actually represents the full-length \textit{flaB} gene as it was identical to the published \textit{flaB} sequence of \textit{C. jejuni} 81116 (Nuijten et al., 1990; Pearson et al., 2007) and (2) CJM1\_1296 was incorrectly annotated as \textit{flaB}; it is identical to \textit{flaA} of \textit{C. jejuni} 81116 (Friis et al., 2010), see Fig. 2(a). Therefore, our CJM\_1294 gene deletion mutant, described above, is not a \textit{flaA} mutant but the \textit{cat} cassette is replacing a region ranging from position 822 to 1671 bp of the \textit{flaB} gene. Consequently, the motile phenotype of \textit{flaB} (CJM1\_1294) mutant clones (Fig. 1) confirmed the findings by Wassenaar et al., (1991), reporting a motile phenotype after the inactivation of \textit{flaB} in \textit{C. jejuni} 81116. Deletion of the full-length \textit{flaB} gene (CJM1\_1295–1294, referred to as the \textit{flaB}\textsuperscript{*} mutant) also resulted in WT motility levels for mutant clones B and C, although the motility of \textit{flaB}\textsuperscript{*} (CJM1\_1295–1294) mutant clone A was slightly lower compared to the WT (Fig. 2b). In line with previous findings of Wassenaar et al. (1991) in \textit{C. jejuni} 81116, deletion of \textit{flaA} (CJM1\_1296) and the complete flagellin locus (\textit{flaAB}; CJM1\_1296 and CJM1\_1295–1294) led to abolished motility (Fig. 2b). Analysis of the combined motility data for the \textit{flaA} (CJM1\_1296) and \textit{flaAB} mutant clones showed that deletion of the complete flagellin locus resulted in more severely attenuated motility compared to deletion of \textit{flaA} alone (P<0.001), suggesting that \textit{flaB} plays a minor role in motility, at least in \textit{C. jejuni} strain M1.

Based on our findings, we hypothesized that the attenuated motility in \textit{flaB} (CJM1\_1294) mutant clones C, D and E might be caused by loss-of-function mutations in other motility-associated genes or as previously reported, by a

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**Fig. 1.** Motility of \textit{C. jejuni} M1 flagella defined gene deletion mutants. Motility was analysed on BHI plates with 0.4 \% (w/v) agar after overnight incubation. Three \textit{flaD} (CJM1\_0851) and \textit{pflA} (CJM1\_1501) deletion mutant clones were assayed along with their coupled WT strain and five \textit{flaB} [CJM1\_1294, previously annotated to encode FlaA (Friis et al., 2010)] mutant clones were tested with two coupled WT strains (A and B), with the average motility shown for the latter. All three \textit{flaD} (CJM1\_0851) and \textit{pflA} (CJM1\_1501) mutant clones were non-motile; however, motility of \textit{flaB} (CJM1\_1294) mutant clones was inconsistent: clones A and B showed WT motility, clone C displayed intermediate motility, whereas the motility of clones D and E was severely attenuated. Differential motility of mutant clones was tested against the coupled WT strain(s) with a Mann–Whitney test (n=3). Data shown are the mean and SEM, with ***P<0.001.
compensatory mechanism, for example, by increased *flaB* expression or by genome rearrangements, which have been shown to (partially) restore motility (Nuijten *et al.*, 2000; Wassenaar *et al.*, 1994, 1995).

**Genotypic and phenotypic analysis of motility defects**

A genome resequencing approach was used to assess whether second-site mutations might be responsible for the attenuated motility of *flaB* (CJM1_1294) mutant clones C, D and E. Three *pflA*, three *flaD* and five *flaB* mutant clones and their ‘coupled’ M1 WT strains were genome sequenced. Eighty-one variants, i.e. SNPs and INDELs, were detected relative to the *C. jejuni* M1 reference genome [GenBank CP001900 (Friis *et al.*, 2010)]. Of these, 34 SNPs and INDELs were present in all sequenced gene deletion mutant clones and their ‘coupled’ WTs, whereas 47 SNPs and INDELs were only detected in a subselection of them (Fig. 3). Although the *flaB* mutant
clones subclustered, limited mutant-specific hierarchical clustering was detected, indicating that variation may be introduced throughout all growth steps during the construction of the gene deletion mutants. The SNPs and INDELs nucleotide position numbers correspond to the position in the original C. jejuni M1 reference genome sequence (Friis et al., 2010). Interestingly, the genome resequencing approach revealed the presence of SNPs and INDELs unique to the severely attenuated motility flaB (CJM1_1294) mutant clones D and E. These were an insertion of a G at position 689 014 within CJM1_0719 (encoding a putative periplasmic protein) and deletion of a TA-dinucleotide at position 1 021 140 within CJM1cam_0792 (encoding a putative periplasmic protein) and deletion of a TA-dinucleotide at position 1 021 140 within CJM1_0051 (flaB) which suggests that they did not originate from a single clone. D and E differed at two variant positions (Fig. 3), previously it was shown that deletion of flaB in C. jejuni 81-176 resulted in a phenotype consisting of severely attenuated motility with the presence of a probable flagellar hook structure but without flagellin filaments (Barrero-Tobon & Hendrixson, 2014). Scanning EM analysis revealed that the majority of the flaB (CJM1_1294) mutant clones D and E also lacked flagellar filaments and instead possessed hook-like structures, but with very few bacteria displaying a possible filament structure (Fig. 4d, e). In contrast, the majority of the bacteria imaged for the flaB (CJM1_1294) coupled WT (Fig. 4f, g) and flaB (CJM1_1294) mutant clones A, B and C harboured a flagellar filament at one or both poles (Fig. 4a–c). Notably, the flaB (CJM1_1294) mutant clones D and E differed at two variant positions (Fig. 3), which suggests that they did not originate from a single transformation event. Further, the intermediate motile flaB (CJM1_1294) mutant clone C possessed a unique deletion of an A at position 69 410 in CJM1_0051 (flgD), encoding a flagellar hook assembly protein. This deletion was predicted to result in a severely truncated flgD ORF, encoding a protein of 55 aa instead of 294 aa. In C. jejuni 81-176 a flgD transposon mutant was previously reported to be non-motile (Hendrixson et al., 2001).

The genome sequencing data obtained in this study for the M1 WT were used to generate a reference genome sequence (designated CJM1cam) for the C. jejuni M1 strain used in our laboratory. The CJM1cam genome sequence and annotation are available via GenBank accession no. CP012149; for more information see Methods.

Second-site mutations in fliW responsible for motility defects in flaB mutants

We hypothesized that the predicted C-terminal truncation of the FliW protein by 3 aa may be responsible for the observed attenuated motility of flaB (CJM1_1294) mutant clones D and E. To confirm that fliW is also required for motility in C. jejuni M1, a flaB gene deletion mutant was generated, which showed abolished motility (Fig. 5b). To exclude that this was a clone-specific phenotype, we assayed two other flaB gene deletion mutant clones; both clones were found to be non-motile (Fig. S1). Genome sequencing of fliW mutant clone A and its coupled WT showed that 14 SNPs and INDELs were shared between them, five variants were unique to the WT and two were only found in the fliW mutant clone A (Table S3). Unique variants to the fliW mutant clone A were an intergenic A insertion between CJM1cam_0120 (putative metalloprotease) and CJM1cam_0121 (hypothetical protein) and a synonymous SNP (A to G) in CJM1cam_0791 (hypothetical protein), indicating that the non-motile phenotype is not caused by second-site mutations. Electron micrographs of the C. jejuni M1 fliW mutant were similar to those reported previously of a C. jejuni 81-176 fliW mutant, i.e. lack of flagellar filaments and presence of a hook-like structure (Barrero-Tobon & Hendrixson, 2014) (Fig. 4h). The motility of the fliW mutant was slightly less than that of the flaB (CJM1_1294) mutant clones D and E, although this difference was not statistically significant. It is possible that the predicted 3 aa truncated FliW protein in flaB (CJM1_1294) mutant clones D and E could still possess limited functional activity.

To confirm that the TA-deletion in fliW was responsible for the severely attenuated motility of flaB (CJM1_1294) mutant clones D and E, the WT fliW gene including its native promoter was inserted into the CJM1_0055–0057 pseudogene region. For this, the new genetic complementation plasmid pSV009 was developed, which enables: (1) flexible clonal options through a whole range of restriction sites, (2) the possibility to drive the expression from the cat promoter or replace this with the native promoter of the gene of interest and (3) pseudogene region insertion in well-studied C. jejuni strains, e.g. M1, 81-176, 81116 and 11168, via homologous recombination regions with >98 % identity (Fig. 5a). Using the newly developed complementation system, insertion of fliW into the CJM1_0055–0057 pseudogene region in the fliW mutant as well as in flaB (CJM1_1294) mutant clones D and E was shown to restore motility to WT levels, suggesting that the flagella are restored, although this was not confirmed by EM. Insertion of the empty genetic complementation backbone did not restore motility (Fig. 5b). These results confirm that the TA-deletion in fliW was responsible for the attenuated motility phenotype of flaB (CJM1_1294) mutant clones D and E.

Concluding remarks

Many studies aimed at investigating the role of genes in C. jejuni make use of targeted deletion of genes by replacing them with an antibiotic resistance cassette. Here we have demonstrated that it is of critical importance to assess the phenotype of
Fig. 3. Genome sequencing revealed second-site mutations associated with motility defects. Genetic variation, i.e. SNPs and INDELs, in C. jejuni M1 defined gene deletion mutant clones and corresponding WT strains (Table 1) were analysed by illumina sequencing; their presence is indicated with black boxes or with an asterisk when either found to be unique to individual mutant clones, WT strains, or associated with differential motility. Motility phenotypes are indicated as severely attenuated motility in Δ-2-B (JM1_294) mutant clones C, D and E are indicated with an arrow on the right side of the panel. SNPs/INDELs that were detected in all sequenced M1 WTs and mutant strains compared to the M1 reference (Fris et al., 2010) were excluded from the analysis (Table S2). Hierarchical clustering was performed based on the presence/absence of SNPs and INDELs.

| Position | Type     | Reference | Variant | Gene or intergenic region | Motility |
|----------|----------|-----------|---------|---------------------------|----------|
| 961924   | Insertion | T CJM1   | T       | CJM1_0649                 | ++       |
| 904406   | SNP A    | T CJM1   | 0934 (nt33) |                         | ++       |
| 501191   | Insertion | T        | A       | Intergenic CJM1_0226 and CJM1_0227 (nt63) | ++       |
| 69618    | Insertion | T        | A       | CJM1_0696 (nt56)         | ++       |
| 605642   | Deletion  | C        | T CJM1  | 0659                      | ++       |
| 524299   | Insertion | T        | A       | CJM1_0649 (nt33)         | ++       |
| 725709   | Insertion | T        | A       | CJM1_0286                 | ++       |
| 275074   | SNP A    | T CJM1   | 0280 (nt63) |                         | ++       |
| 166431   | Deletion  | T        | - CJM1 | 0159                      | ++       |
| 154543   | Insertion | T        | T Intergenic CJM1_1548 and CJM1_1547 (nt56) | ++       |
| 114341   | Insertion | T        | T Intergenic CJM1_1180 (nt56) and CJM1_1181 (nt56) | ++       |
| 1008767  | SNP C    | A CJM1  | T       | CJM1_1081                 | ++       |
| 577459   | Insertion | T        | G       | CJM1_1009                 | ++       |
| 998911   | Insertion | T        | A       | CJM1_1007 (nt56) and CJM1_1008 (nt56) | ++       |
| 919486   | Insertion | T        | A       | CJM1_0941                 | ++       |
| 88367    | SNP T    | A CJM1  | C       | CJM1_0072                 | ++       |
| 88836    | SNP G    | T CJM1  | G       | CJM1_0072                 | ++       |
| 57743    | Insertion | T        | A       | Intergenic CJM1_0071 and CJM1_0072 (nt33) | ++       |
| 817022   | Insertion | T        | - CJM1 | 0844                      | ++       |
| 78442    | Insertion | T        | - CJM1 | 0661 (nt7)                | ++       |
| 779491   | Insertion | T        | - A    | Intergenic CJM1_0801 (nt4) and CJM1_0802 (nt4) | ++       |
| 744768   | SNP C    | T CJM1  | T       | Intergenic CJM1_0769 (nt56) and CJM1_0770 (nt56) | ++       |
| 73660    | Insertion | T        | - C    | CJM1_0054                 | ++       |
| 73929    | Insertion | T        | A       | Intergenic CJM1_0753 (nt63) and CJM1_0754 (nt63) | ++       |
| 99410    | Deletion  | A        | T CJM1 | 0051 (nt56)               | ++       |
| 699014   | Insertion | T        | - G    | CJM1_0719                 | ++       |
| 89273    | Insertion | T        | A       | CJM1_0049                 | ++       |
| 835644   | Deletion  | C        | - CJM1 | 0659                      | ++       |
| 641968   | Insertion | T        | A       | Intergenic CJM1_0604 and CJM1_0605 (nt63) | ++       |
| 505496   | Insertion | G        | - T    | Intergenic CJM1_0539 and CJM1_0540 (nt63) | ++       |
| 239042   | Deletion  | T        | T CJM1 | 0238                      | ++       |
| 1618648  | Deletion  | T        | A       | Intergenic CJM1_1680 (nt46) and CJM1_0001 (nt46) | ++       |
| 154069   | Insertion | T        | A       | CJM1_1879 (nt46)          | ++       |
| 144016   | SNP A    | G CJM1  | T       | CJM1_1491 (nt56)          | ++       |
| 144016   | SNP A    | G CJM1  | T       | Intergenic CJM1_1480 (nt56) | ++       |
| 128820   | Deletion  | T        | C       | CJM1_1299                 | ++       |
| 126370   | Insertion | T        | T Intergenic CJM1_1296 (nt63) and CJM1_1297 (nt66) | ++       |
| 126095   | Deletion  | A        | - CJM1 | 1294 (nt63)               | ++       |
| 128380   | SNP A    | T CJM1  | T       | CJM1_1293 (nt66)          | ++       |
| 126859   | SNP T    | C CJM1  | T       | CJM1_1277                 | ++       |
| 114230   | Insertion | T        | G       | CJM1_1277                 | ++       |
| 114245   | Deletion  | T        | T Intergenic CJM1_1222 and CJM1_1223 (nt63) | ++       |
| 111516   | SNP G    | T CJM1  | T       | Intergenic CJM1_1052 (nt56) and CJM1_1053 (nt56) | ++       |
| 111516   | SNP T    | T CJM1  | A       | Intergenic CJM1_1052 (nt56) and CJM1_1053 (nt56) | ++       |
| 1087510  | Deletion  | T        | T CJM1  | 1123                      | ++       |
| 1087146  | Deletion  | T        | T CJM1  | 1052 (nt66)               | ++       |

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multiple mutant clones and/or genetically complement the gene deletion. Ideally, genome sequencing may be used to screen for second-site mutations that may interfere with the pathways/mechanism under study, especially when mutant clones show inconsistent behaviour or when genetic complementation fails. As shown in this study, genome sequencing can be used as a troubleshooting tool and may also yield novel genes associated with the phenotypes under study.

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Fig. 5. Genetic complementation of \( \text{fliW} \) in severely attenuated motility \( \text{flaB} \) (CJM1_1294) mutant clones and a \( \text{fliW} \) (CJM1_1052) mutant restores motility. (a) Overview of the genetic complementation strategy using plasmid pSV009. The \( \text{fliW} \) gene including its upstream sequence was cloned into the \( \text{BamHI} \) site located in the multiple cloning site (MCS) and \( \text{XhoI} \) site of pSV009, yielding pSV012-\( \text{fliW} \). This replaced the pSV009 cat promoter and enabled \( \text{fliW} \) expression from its native promoter. The genetic complementation region is flanked by regions for homologous recombination to facilitate insertion into the CJM1_0055–0057 pseudogene region, which is predicted to be compatible with other commonly used \( \text{C. jejuni} \) isolates (e.g. 11168, 81116 and 81-176). For insertion of \( \text{fliW} \) (CJM1_1052) into the pseudogene region, the complementation region was amplified by PCR using the pSV009_GCamplf primers and subsequently introduced by electroporation. (b) As expected, insertion of \( \text{fliW} \) (CJM1_1052) in the CJM1_0055–0057 pseudogene region in a \( \text{fliW} \) (CJM1_1052) mutant restored motility. Insertion of \( \text{fliW} \) (CJM1_1052) in the pseudogene region also restored motility in the severely attenuated \( \text{flaB} \) (CJM1_1294) mutant clones D and E, whereas insertion of the empty genetic complementation (‘empty GC’) region did not restore motility. The white bars indicate the coupled WT strains for \( \text{flaB} \) (CJM1_1294) and \( \text{fliW} \) (CJM1_1052) gene deletion mutants. Differential motility of mutant clones was tested against coupled WT isolates using a Mann–Whitney test (\( n=3 \)). Data shown are the mean and SEM, with * \( p<0.05 \) and ** \( p<0.01 \).

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