A novel paralogous gene family involved in phase-variable flagella-mediated motility in *Campylobacter jejuni*

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Flagella-mediated motility is recognized to be one of the major factors contributing to virulence in *Campylobacter jejuni*. Motility of this bacterium is known to be phase variable, although the mechanism of such variation remains unknown. *C. jejuni* genome sequencing revealed a number of genes prone to phase variation via a slipped-strand mispairing mechanism. Many of these genes are hypothetical and are clustered in the regions involved in formation of three major cell surface structures: capsular polysaccharide, lipooligosaccharide and flagella. Among the genes of unknown function, the flagellar biosynthesis and modification region contains seven hypothetical paralogous genes designated as the motility accessory factor (*maf*) family. Remarkably, two of these genes (*maf1* and *maf4*) were found to be identical and both contain homopolymeric G tracts. Using insertional mutagenesis it was demonstrated that one of the genes, *maf5*, is involved in formation of flagella. Phase variation of the *maf1* gene via slipped-strand mispairing partially restored motility of the *maf5* mutant. The *maf* family represents a new class of bacterial genes related to flagellar biosynthesis and phase variation. Reversible expression of flagella may be advantageous for the adaptation of *C. jejuni* to the varied *in vivo* and *ex vivo* environments encountered during its life cycle, as well in evasion of the host immune response.

Keywords: *C. jejuni*, flagellar biosynthesis, phase variation, slipped-strand mispairing, genetic instability

INTRODUCTION

The flagellum is an essential virulence factor of *Campylobacter jejuni* and is involved in colonization of the gastro-intestinal tract (Nuijten *et al.*, 1992). Flagella-associated motility of *C. jejuni* has been found to be variable (Nuijten *et al.*, 1995), and reversible non-motile variants have been reported after extended incubation (Caldwell *et al.*, 1985). Different mechanisms for high-frequency phase variation in other bacteria, including those mediated by the slipped-strand mispairing mechanism, are well documented (Henderson *et al.*, 1999). In *Neisseria gonorrhoeae*, phase variation of expression of pili is attributed to homopolymeric tracts present in a gene involved in pilin biosynthesis (Jonsson *et al.*, 1991). Among other examples are the control of *Neisseria*

meningitidis pilin modification by a phase-variable gene (Jennings *et al.*, 1998) and phase variation of a gene encoding a galactosyltransferase of *C. jejuni* (Linton *et al.*, 2000a). Recently, in the closely related pathogen, *Campylobacter coli*, phase-variable flagellar expression due to reversible frameshift mutation in a short homopolymeric tract of T residues in the *flhA* gene has been reported (Park *et al.*, 2000).

The flagellar biogenesis apparatus in bacteria is complex and, despite extensive studies, functions of some flagella-related genes remain unknown (Macnab, 1996). Analysis of the *C. jejuni* genome sequence (Parkhill *et al.*, 2000) revealed a number of putative flagella-related genes similar to genes of *Escherichia coli* and other bacteria. In addition, genes involved in *Campylobacter* flagellin

Fig. 1. Structural and possible functional relationship among Maf proteins and their homologues in other species. The genes are indicated as arrows with fill patterns according to their predicted function and similarity: vertical hatching, *maf* family or related genes; horizontal hatching, genes related to sugar biosynthesis, transport and processing of glycoproteins; dots, genes involved in flagellin glycosylation; solid black, other genes related to flagellar biosynthesis. The putative functions of all the proteins were taken from a comprehensive genomic database available at <http://wit.IntegratedGenomics.com/WIT2/>, except for *Clostridium acetobutylicum*, for which the information was taken from <http://mystic.genomecorp.com/genesequences/clostridium>. A, *C. jejuni*; B, *C. acetobutylicum*; C, *Bacillus stearothermophilus*; D, *H. pylori*. E, a fragment of multiple alignment of amino acid sequences of Maf proteins and their orthologues in *H. pylori* (HP0114), *C. acetobutylicum* (CAC2168) and *B. stearothermophilus* (BE0797). Positions of invariant and conserved amino acid residues are labelled by asterisks and semicolons respectively. Positions of identical residues present in more than a half of the sequences are shown in bold. The consensus sequence for the region of highest similarity is shown below.

glycosylation were found (Guerry *et al.*, 1996; Linton *et al.*, 2000b). The genes involved in flagellin glycosylation are part of a large cluster also containing genes thought to be involved in sugar biosynthesis and transport, as well as seven closely related genes of unknown function (Parkhill *et al.*, 2000). We have termed these genes (Fig. 1) the *motility accessory factor* (*maf*) family of flagellin-associated proteins. Two *maf* genes (*maf1* and *maf4*) appear to be identical, both containing homopolymeric G tracts. In this study we demonstrate that the genes of the *maf* family are involved in the variation of motility via the slipped-strand mispairing mechanism.

METHODS

Bacterial strains, plasmids and growth conditions. *C. jejuni* NCTC 11168 was received from J. Ketley (University of Leicester, UK). *C. jejuni* strains were grown at 37 °C on 7 % blood agar or Mueller–Hinton agar plates in a microaerobic atmosphere (CampyGen, Oxoid) for 2 d. *E. coli* XL2 Blue MRF' (Stratagene), used in cloning experiments, was grown overnight at 37 °C on LB agar plates. Unless stated otherwise, 0.3 % Mueller–Hinton agar swarm plates were used in motility experiments and the plates were incubated at 37 °C in microaerobic conditions for either 2 d (motility studies) or 5 d (for isolation of spontaneous derivatives with increased

Fig. 2. Characterization of motility mutants of strain NCTC 11168. (A) Organization of the two putative operons containing paralogous genes *maf1* and *maf2-5* (shown in open arrows) and location of the PCR and sequencing primers. No gaps are shown between genes separated by less than 30 bp. Two other genes of the *maf1* family (*maf6* and *maf7*), which are located next to *flaA* and *flaB*, are not shown. Homopolymeric tracts are shown as grey areas. Positions and orientation of *kan^r* and *cm^r* cassettes are indicated. Short arrows indicate positions of PCR primers. Effect of the variation in poly(G) tract on the translation of *maf1* and *maf4* genes is shown in a box. (B) Motility and the genes affected in the derivatives of strain NCTC 11168. 1, wild-type; 2, *maf5::kan^r*; 3, *maf5::kan^r rev*; 4, *maf5::kan^r rev maf1::cm^r*.

motility). Where necessary the agar plates were supplemented with kanamycin at 50 µg ml⁻¹, chloramphenicol at 10 µg ml⁻¹ and ampicillin at 100 µg ml⁻¹.

Construction of mutants. In order to mutate the *maf5* gene, a 2 kb fragment of this gene was amplified using primers AK100 (GCT TTA AGC GGT TTT GAG TAT AAC AAC TTA CGC) and AK101 (CGT TCG TGT GCA TAA ACC CAA GC) (Fig. 2) and cloned into the pGEM-T vector. A unique *Bsa*BI site in the cloned fragment was used for insertion of blunt-ended *Bam*HI *kan^r* cassette (Trieu-Cuot *et al.*, 1985). The size and origin of the inserts were confirmed using PCR and restriction mapping techniques (data not shown). The construct was used for transformation of *C. jejuni* via electroporation (Wassenaar *et al.*, 1993). Mutants with direct orientation of the cassette were selected using PCR with primers DL3 (*kan^r* cassette specific, ACC CAG CGA ACC ATT TGA GG) and DL38 (*flaB* specific, GTC AGG CTA ATG CAG TGC AG) (Fig. 2).

Double mutants were constructed using a chloramphenicol-resistance (*cm^r*) cassette (Wang & Taylor, 1990) isolated from the pAV35 delivery plasmid (van Vliet *et al.*, 1998) and used as a selection marker. A recombinant plasmid cam11f9 with a 1.4 kb insert containing fragments of both *Cj1317* and *Cj1318* genes was selected from the random library used in the *C. jejuni* genome sequencing project (<http://www.sanger.ac.uk/Projects/C-jejuni/>). A *Sma*I fragment containing the *cm^r*

cassette was inserted into the *Bsa*BI site of plasmid cam11f9 and chloramphenicol-resistant colonies were selected after transformation of the revertant strain *Cj1337::kan^r rev*. The size and origin of the inserts were confirmed using PCR and restriction mapping (data not shown).

Sequencing of the homopolymeric tracts. The poly(G)-tract-containing regions of each gene were amplified from chromosomal DNA using primer DL8 (CAT AAT AAT GAC TCA TCA GTT CTA) in combination with a primer derived from an adjacent gene (*neuB3* primer DL2, GCA AGA AGC TTA TAT GCA AGT AAG G, for the *maf1* gene and *maf3* primer AK93, GCT ATT TAT TTT CAT AAC GAA TGC G, for the *maf4* gene) and the PCR products were sequenced directly in both directions (Fig. 2).

PCR conditions. Crude cell lysates were amplified using Gibco-BRL *Taq* polymerase in 20 µl volumes containing 0.1 µg primers and DNA: 94 °C for 1 min, 25 cycles of 94 °C 45 s, 50 °C 45 s, 72 °C 2 min, followed by 7 min extension at 72 °C. The PCR products were purified using S300 microspin columns (Bio-Rad) and/or analysed on agarose gels.

Western blotting. Polyacrylamide gels were Western blotted onto PVDF membrane (Millipore) using a semi-dry electroblotting apparatus (Hoefer Scientific Instruments). Blots were blocked overnight at 4 °C in Tris-buffered saline containing

0.01% Tween 20 (TBST) and 3% skimmed milk. After blocking, blots were incubated for 1 h with anti-flagellin serum at a dilution of 1:100 in TBST containing 1% bovine serum albumin (TBST-BSA) and washed three times in TBST, followed by incubation with peroxidase-labelled anti-rabbit IgG (Sigma) diluted 1:1000 in TBST-BSA for a further 1 h. Following washing as above, blots were developed using the DAB staining kit with nickel enhancement according to the manufacturer's instructions (Vector Laboratories).

Electron microscopy. *C. jejuni* colonies from a 2 d culture grown on Columbia agar containing 7% horse blood were gently resuspended in phosphate-buffered saline. Copper grids were placed Formvar-coated side down onto a drop of bacterial suspension for 1 min and then onto a drop of 3% ammonium molybdate for a further 1 min. Grids were left to dry and viewed by transmission electron microscopy.

RESULTS

Characterization of *maf* genes and their putative products

The *maf* genes are located in a large (37 kb) gene cluster, consisting of two adjacent regions, *Cj1317* (*neuB3*)–*maf5* and *maf6*–*Cj1347*, and belong to a family of seven paralogous genes, *maf1* (*Cj1318*), *maf2* (*Cj1333*), *maf3* (*Cj1334*), *maf4* (*Cj1335/6*), *maf5* (*Cj1337*), *maf6* (*Cj1341*) and *maf7* (*Cj1342*) (Fig. 1A). A similarity matrix between the genes and their products is presented in Table 1; two of the genes (*maf1* and *maf4*) are identical. Although the presence of homopolymeric tracts in both *maf1* and *maf4* implied that the genes might be involved in a slipped-strand mispairing mechanism of phase variation, no clues to their function could be found via database similarity searches. As such they were designated 'function unknown genes' in the annotation of the genome sequence (http://www.sanger.ac.uk/Projects/C_jejuni/).

Using ProSite (<http://www.expasy.ch/tools/scnpsit1.html>), most of the *maf* gene products are predicted to be cytoplasmic, with *maf3* having a potential transmembrane region. Proteins from this region with similarities in the database include flagellar modification proteins (*Cj1331* and *Cj1332*), sugar modification/biosynthesis proteins (*Cj1319* and *Cj1328*), sugar-, amino- or nucleotidyl-transferases (*Cj1321*, *Cj1329* and *Cj1331*) and an aminotransferase (*Cj1320*). Some genes in this region, such as *neuB3*

Fig. 3. Variable motility in single-colony isolates of strain NCTC 11168 (wild-type). Hypermotile (H), medium-motile (M1 and M2), low-motile (L) and non-motile (N1 and N2) isolates are shown.

(*Cj1317*) and *ptmA* (*Cj1332*), are also known to be involved in flagellar biosynthesis and modification (Guerry *et al.*, 1996; Linton *et al.*, 2000b). Since the stop codons of these genes partially overlap with N-terminal regions of some of the *maf* genes (Parkhill *et al.*, 2000), we hypothesized that *maf* genes might also be involved in flagella biosynthesis/modification.

Closer inspection of the *maf* genes revealed orthologues in three other species: *Helicobacter pylori*, *Clostridium acetobutylicum* and *Bacillus stearothermophilus* (Fig. 1B–D). In all cases the *maf*-like genes are linked to either flagellar biosynthesis genes or/and genes involved in sugar biosynthesis and transport. For example, in *H. pylori* an orthologue HP0114 is present in the same operon and immediately downstream from the *flaB* gene encoding flagellin (Fig. 1D). Although the genes present in the operon containing paralogous gene HP0465 are hypothetical, the product of one of them (HP0466) is predicted to interact with FlgB and may be a part of the flagellar biosynthesis apparatus (Fig. 1D). Four paralogous genes similar to the *maf* family (CAC2168, CAC2196, CAC2200 and CAC2202) are present in *Cl. acetobutylicum* and are associated with the genes related

Table 1. Percentage identity between *maf* genes and (in parentheses) their respective products

	<i>maf1</i>	<i>maf2</i>	<i>maf3</i>	<i>maf4</i>	<i>maf5</i>	<i>maf6</i>	<i>maf7</i>
<i>maf1</i>		82 (74)	60 (42)	100 (100)	62 (49)	52 (32)	53 (32)
<i>maf2</i>			59 (43)	82 (74)	62 (49)	52 (33)	52 (32)
<i>maf3</i>				60 (42)	61 (45)	53 (35)	55 (35)
<i>maf4</i>					62 (49)	52 (32)	53 (32)
<i>maf5</i>						54 (34)	54 (32)
<i>maf6</i>							87 (83)

Fig. 4. Determination of recombination site in a spontaneous non-motile variant. (A) The black areas in the genes (shown as arrows) represent regions of highest similarity. (B) Alignment between the *maf4* and *maf5* sequences corresponding to dark areas in (A). The hybrid gene contains a region including the 5'-terminal part of the *maf4* gene (top sequence, underlined) and a region including the 3'-terminal part of the *maf5* gene (lower sequence, underlined). These areas are separated by a region identical in both genes (in bold) where the recombination has occurred.

to both sugar and flagellar biosynthesis. Similarly, both flagellar and sugar/biosynthesis-related genes are associated with a single *maf* orthologue present in *B. stearothermophilus* (Fig. 1C). All Maf-like proteins share extensive local regions of high similarity, particularly in the N-terminal and central regions. One such region is presented in Fig. 1(E). Despite the different organization of the regions containing *maf*-like genes, the striking similarity is that they are always associated with genes involved in biosynthesis/transport of sugars and flagella.

Subclonal variation in motility of *C. jejuni* NCTC 11168

We found that motility of the original strain NCTC 11168 was significantly lower than that of fresh clinical isolates (data not shown). However, a small fraction of the clonal population of this strain revealed variable motility. Variants with motility ranging from almost non-motile to hypermotile, occurring with a frequency of approximately 10^{-3} , can be isolated from the wild-type strain (Fig. 3). A hypermotile variant (11168H) was selected for mutagenesis along with the wild-type strain NCTC 11168 (see below).

In some cases such variation could be a result of reversible changes in a number of genes involved in

chemotaxis, motility and flagellar biosynthesis. Due to the presence of homopolymeric G tracts, genes *maf1* and *maf4* could potentially be involved in such variation. However, sequencing of the regions containing homopolymeric tracts in these genes revealed that both genes were out of frame in all variants, and therefore not involved in this type of motility variation.

Gene *maf5* is involved in motility

During analysis of naturally occurring non-motile mutants we also investigated the integrity of other *maf* genes and found that some of these mutants contained deletions, resulting from recombination between homologous regions of the adjacent paralogous genes. In one such case the region was PCR amplified and sequenced, confirming recombination between adjoining *maf4* and *maf5* genes (Fig. 4). These preliminary studies suggested that some genes of the *maf* family might be involved in motility. However, as these changes may be coincidental with independent mutations in other genes, defined mutants were required. We selected one of the genes, *maf5*, for further studies.

In order to establish that *maf5* is involved in motility, non-polar site-directed insertional mutants were constructed. Insertion of the *kan^r* cassette in the *maf5* gene of strain 11168H (see Methods) resulted in the inability

Fig. 5. Effect of inactivation of the *maf5* gene on motility of strain 11168H. The plate (0.45 % agar) was incubated overnight.

Fig. 6. Electron micrographs of the wild-type strain of *C. jejuni* 11168H (A) and its *maf5::kanr* derivative (B). The arrows indicate full-sized flagella in the wild-type strain and a hook-like structure in the mutant. Bars, 0.5 μ m.

of the bacteria to swarm, confirming the hypothesis that this gene is indispensable for full motility (Figs 2B and 5).

Several independent clones resulting from the mutagenesis of the *maf5* gene in strain 11168H were non-motile, confirming that the phenotype was not coincidental with possible simultaneous genetic variation of another locus. To further prove that *maf5* is essential for motility we extracted chromosomal DNA from the 11168H *maf5::kanr* mutant and transformed the wild-type strain NCTC 11168. All transformants in this strain were also non-motile, giving additional confirmation that gene *maf5* is associated with motility.

The *maf5::kanr* mutation affects flagellar biosynthesis

Electron microscopy revealed the complete lack of the flagella in the *maf5::kanr* mutant, whereas the hook organelle could be seen (Fig. 6B). Analysis of cell lysates demonstrated reduced intensity of a band corresponding to flagellin in the *maf5::kanr* mutant both on the

Fig. 7. Effect of flagellin production in the 11168H *maf5::kanr* mutant. (A) Coomassie-stained gel; (B) blotting with anti-flagellin antibodies. Lane 1, molecular mass standards; lane 2, 11168H; lane 3, 11168H *maf5::kanr* mutant. The samples contained similar total amount of proteins as judged by the intensity of common bands in different samples in (A). The position of flagellin is indicated by an arrow.

Fig. 8. Partial restoration of motility of the *maf5::kan^r* mutant (2) in the pseudorevertant *maf5::kan^r rev* (3). Two variants of double mutants, *maf5::kan^r rev maf1::cm^r-5* (1) and 11168H *maf5::kan^r rev maf1::cm^r-4* (4), are also shown. The plate (0.34% agar) was incubated for 4 d.

polyacrylamide gel (Fig. 7A) and on a Western blot with anti-flagellin antibodies (Fig. 7B).

Motility in the *maf5::kan^r* mutant can be partially restored by the *maf1* gene product

Prolonged incubation of the 11168H *maf5::kan^r* mutant resulted in the formation of outgrowths of motile cells from the non-motile inoculum in motility plates of reduced agar concentration (Fig. 8, 3). Analysis of two such pseudorevertants obtained in two independent experiments revealed that in both cases the reading frame of the *maf1* gene was restored due to the presence of an extra G (G10→G11) in the homopolymeric tract. The *maf4* frame was unchanged (inactive, 10 Gs). Motility of the pseudorevertants was significantly lower than that of the parent 11168H strain.

In order to exclude the possibility of other coincidental mutations that could contribute to the partial restoration of motility, we constructed double mutants in which the restored copy (*maf1*) of a pseudorevertant was knocked out by insertion of a *cm^r* cassette (see Methods). Due to the identical sequences of genes *maf1* and *maf4*, the *cm^r* cassette was integrated in either the *maf1* or the *maf4* gene, resulting in two classes of double mutants. As expected, motility of those containing inserts in the inactive *maf4* gene was unaffected (Fig. 8, 4). However, the mutants containing inserts into the restored copy (*maf1*) were non-motile (Fig. 8, 1). These results confirm that partial restoration of motility in the 11168H *maf5::kan^r* was the result of activation of the *maf1* gene via a presumed slipped-strand mispairing mechanism.

DISCUSSION

In this report we describe a new class of genes involved in *C. jejuni* flagellar biosynthesis and phase variation. The genes appear to represent a unique class of flagella-related genes. A recent study has also suggested that a member of the *maf* gene family is involved in motility of *C. jejuni* 81116 (Bleumink-Pluym *et al.*, 1999). The closest orthologue to the *maf* family of proteins is a hypothetical protein of *H. pylori*, HP0114. As in *C.*

jejuni, the *H. pylori* orthologue is positioned close to the *flaB* gene, which encodes the major structural subunit of the *H. pylori* flagellar filament. Similarly to the *C. jejuni* *maf* genes, no significant similarity to any known protein with experimentally determined function could be found for the putative products encoded by orthologous genes present in other bacteria. However, comparative analysis of the genetic organization of the respective loci demonstrates that the *maf*-like genes in other species are also associated with the genes involved in flagellar biosynthesis and might be related in function.

Over 40 genes are involved in flagellar biogenesis of *E. coli* and *Salmonella typhimurium* (Macnab, 1996). Since Maf proteins do not have similarity in the non-redundant amino acid sequence database, their exact role in flagellar biogenesis remains an enigma. However, based on their position in the *C. jejuni* genome, and, more importantly, partial overlap with sugar biosynthesis (*neuB* for *maf1*) or flagellar modification (*ptmA* for *maf2*) genes, one can hypothesize that the *maf* genes may also be involved in flagellin modification. In *C. coli* VC167, motility is modulated by flagellin modification (Guerry *et al.*, 1992). We found that, similarly to NCTC 11168, in a number of other *C. jejuni* strains the representatives of the *maf* family are linked to either the *neuB3* gene or the *ptmA* gene (data not shown). Both *neuB3* and *ptmA* are known to be involved in flagellin modification (Doig *et al.*, 1996; Linton *et al.*, 2000b). Flagellin modification is thought to occur in the cytoplasm (Doig *et al.*, 1996). ProSite analysis of the putative *maf* gene products suggests that they are likely to be cytoplasmic or inner membrane bound. Our Western blot results demonstrate decreased intensity of the flagellin band in the *maf5::kan^r* mutant. It seems likely that the *maf* genes are involved in post-translational processing of the flagellin and/or assembly of the flagella. The lack of modification or transport may result in proteolytic degradation of the flagellin.

Reversible expression of flagella in *C. coli* resulting from a slipped-strand mispairing mechanism of phase variation in *flhA* gene has recently been demonstrated (Park *et al.*, 2000). In contrast to *C. coli*, *C. jejuni* does not have a homopolymeric tract in this gene. The results presented here indicate the presence of alternative mechanisms of variable flagellar expression in *C. jejuni*.

One mechanism involves slipped-strand mispairing involving the contingency genes *maf1* and *maf4*, whereas the other involves recombination between homologous genes. As demonstrated in this report, homologous recombination between adjacent genes can also be involved in variation. Further evidence of homologous recombination between *maf* genes is suggested by the absence of genes between *maf1* and *maf5* in *C. jejuni* strains 81116 and 81-176 (unpublished observation). The presence in strain NCTC 11168 of four *maf* genes, organized in a tandem fashion, indicates a potential for gene deletion/duplication.

The formation of non-motile deletion derivatives in the natural *C. jejuni* population may confer flexibility upon bacteria in adapting to changing environmental conditions when flagellar expression and motility are undesirable. The presence of the remaining homologous gene copies might potentially allow restoration of the missing copy through gene duplication. The need for flexibility in the expression of the flagellum could be explained by its possible dual function. As an adhesin, the glycosylated flagellum might be required for initial attachment to the host cells (Nuijten *et al.*, 1992). Once colonization is established, flagella may not be required and their formation may be switched off. When the nutrients at the infection site become limiting, a fraction of bacteria expressing flagella may acquire an advantage, as they can move towards a new colonization site. In addition, reversible expression of flagella may be beneficial in evading the host immune response (Nuijten *et al.*, 1995). For an organism with a relatively small genome size (1.64 Mb for *C. jejuni* NCTC 11168), the availability of such a large number of genes dedicated to flagellar biosynthesis, modification and phase variability seems extraordinary. The presence of a complex mechanism for flagellar expression may provide *C. jejuni* with a selective advantage in the ecological niches it occupies.

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