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Significance of Duplicated Flagellin Genes in Campylobacter

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The complex flagellum of Campylobacter coli VC167 contains two highly related (98%) flagellin subunit proteins which are produced from two 92% homologous, tandemly orientated genes, flaA and flaB. Mutants expressing only flaA form a full-length flagellar filament that confers slightly less than wild-type motility to the bacterium. However, flagellin mutants expressing only flaB produce extremely short, truncated filaments, and are only slightly motile. We have shown that the presence of two essentially identical genes is advantageous, in that flaAflaB mutants become highly motile upon passage by an event which allows the production of a full length simple flagellar filament containing a single Flaa-FlaB chimeric flagellin protein. Furthermore, we have demonstrated that the reassortment of DNA that results in this chimeric protein can occur by two mechanisms: intragenomic recombination and transformation-mediated intergenomic recombination.

Keywords: flagella; chimeric flagellin; Campylobacter; recombination; motility

Duplicated genes have been identified in a variety of eubacteria and archaeabacteria. Such gene duplications may in fact be a mechanism by which bacteria can amplify particular functions, thus adapting to stressful or unfavourable conditions (Sonti & Roth, 1989). Indeed, multiple copies of genes for important virulence factors have been found in the genomes of pathogenic bacteria either in expressed or silent loci (Haas & Meyer, 1986; Mekalanos, 1983; Swanson et al., 1986).

The thermophilic spiral organisms Campylobacter coli and Campylobacter jejuni are among the most frequently isolated enteric pathogens, causing severe diarrhea in humans (Butzler & Skirrow, 1979; Skirrow, 1977; Walker et al., 1986). The polar flagellum of the Campylobacter cell imparts high motility to the bacterium and plays an important role in colonization of the viscous mucous lining of the gastrointestinal tract, and is regarded as an important virulence determinant (Black et al., 1988; Caldwell et al., 1985; Morooka et al., 1985; Pavlovskis et al., 1991). The flagellar filament of C. coli is complex. In the case of C. coli VC167, the flagellar filament is composed of two highly homologous 572 amino acid residue flagellin subunit proteins. These subunit flagellins are encoded by two tandemly orientated genes, flaA and flaB which display 92% identity within their coding regions (Guerry et al., 1992). A second flagellin gene has been reported in C. jejuni strains 81-116 and IX1 (Fischer & Nachamkin, 1991; Nuijten et al., 1990), and evidence now exists that all Campylobacter isolates carry duplicated flagellin genes, with significant homology (R. A. Alm. P. Guerry & T. J. Trust, unpublished results). Mutants that only express the flaA gene produce flagellar filaments that are indistinguishable in size from the wild-type strain and are composed solely of Flaa flagellin. Mutants that only express the flaB gene product produce a severely truncated filament that imparts only partial motility to the cell (Guerry et al., 1991).

The conservation and stability of the tandemly duplicated flagellin genes in Campylobacter suggests that this property must endow these bacteria with a significant strategic advantage that outweighs the genetic instability of such an arrangement because tandem gene duplications are usually rapidly eliminated in bacteria by intragenomic recombination (Anderson & Roth, 1981; Tlsty et al., 1984). Using the previously described flaAflaB+ mutant KX15 (Guerry et al., 1991), we demonstrate here that the extra genetic information of a second highly homologous fla gene carried by Campylobacter strains can...
be used as a reserve copy to assure the organism of site. A distance of 72 bp. The point of recombination
created the chimeric coding sequences for each of the mutants was determined. (B) PCR analysis of the wild-type
VC167-T2 (lane 1), and representative chimeric mutants RAl (lane 2), RAI2 (lane 3) and RAl3 (lane 4), and a no
DNA control (lane 5). Lane 1 shows a 3771 kb fragment representing 2 intact flagellin genes, whereas a 1889 kb
fragment is seen for the chimeric mutants in lanes 2,3 and 4. Lambda (4) size markers are bacteriophage lambda
DNA digested with HindIII.

The flaA flaB+ mutant KX15 was constructed by insertion of a kanamycin (Kan') cassette into the unique EcoRV site of flaA (Fig. 1A; Guerry et al., 1991). We observed, after plating KX15 on Mueller-Hinton medium (BBL, Cockeysville, MD) containing 0.4% Bacto-agar (Difco), that highly motile bacteria irregularly swarmed out from the zone of growth after 24 to 36 hours' incubation at 37°C. All of the 58 independently isolated motile colonies tested were Kan'. The flagellins from four of these motile cells were purified and subjected to -2 hours. With the need to maintain the
culture from ated using the RAI2 RAA7 primer pair from these
mutants with BglII. or HaeII, both of which are
specific for the flaA gene, or HaeII and SepI which are
unique in the flaB sequence (Fig. 2). followed by
separation on a 12% acrylamide gel according to
the method described by Sambrook et al. (1989), we
were able to determine the areas in which recombi-
nation had occurred to produce the chimeric coding sequence (Fig. 1A). The recombinational
crossover in mutant RAI was localized to the area
between the 3' end of primer RAI2 and the BglII site, a distance of 72 bp. The point of recombination
in mutants RAI2 and RAI3 lay between the HaeII
and EcoRV restriction sites, a distance of 97 bp.
The high homology between the two fla genes does not enable the determination of the exact position of the recombinational event.

However, when the selective pressure for Kan'
(100 μg/ml) was kept during growth of the flaA flaB+ mutant KX15 on the motility agar, spikes of the swarming growth caused by highly motile bacteria were still apparent. Although the time for this growth to occur was increased to 60 to
72 hours. With the need to maintain the Kan' cartridge, the genetic rearrangements encountered
were not always as simple as those described above.
PCR and Southern analysis indicated that duplica-
tions of flagellin information had occurred, with
extra partial copies containing fla gene information
comprising the 3' end of the flaA gene (data not shown). When the flaA flaB+ mutant KX15 was fed
to rabbits, the strain consistently failed to colonize
and was cleared by 24 hours. Providing further evidence that full motility is a crucial colonization
determinant. No Kan' revertants were isolated from
the rabbit, and the few Kan' colonies that were
recovered were shown to display poor motility similar to the original flaA flaB+ mutant. However,
after only two days' incubation on motility agar,
one isolate displayed a phenotype of irregular motility identical to the Kan' mutants producing the
chimeric flagellin, yet retained Kan'. Purification
and N-terminal sequencing of the flagellin produced
by this isolate demonstrated that it possessed the first 27 N-terminal residues identical to FlaA. PCR
analysis of this mutant using primer pairs that

† Abbreviations used: Km, kanamycin; bp, base-pairs;
PMT, polymerase chain reaction.
could specifically amplify either the flaA (RAA12; RAA11: 5'-TGCATCGAAAAGATTAACAGAAC-3') or the flaB (RAA7 RAA10:5'-TCTTGTCTTTATCTTTCGATGCA-3') region demonstrated that the KmR cartridge was now located in the flaB gene, and the flaA gene was functional and intact (Fig. 3).

The rearrangement of flagellin information resulting in the restoration of full motility could be occurring by two distinct mechanisms, involving either intergenicomic or intragenomic recombinational events. When the selective pressure for KmR is removed, a simple recombination could occur between the two highly homologous flagellin genes. However, an event which would maintain KmR would likely involve DNA inserted by cells that had undergone autolysis, and taken up via natural transformation. The requirement for cell death may be one explanation as to why a longer incubation time is required to observe KmR motile revertants. To test this hypothesis, motility plates containing DNAse I (40 μg/ml) with and without Km were used. When the flaA flaB+ mutant was grown on motility plates containing DNAse without the selective pressure of Km, all 48 colonies were motile after 24 to 36 hours' growth, and were KmR. Flagella were purified from three independent colonies, and in all cases upon X-terminal protein sequencing, the flagellins displayed the first 27 X-terminal residues of FlaA. PCR analysis in conjunction with restriction endonuclease digestion demonstrated that the gene encoding this flagellin was composed of both flaA and flaB sequences. However, when both Km and DNAse were added to the motility plate, the cells remained non-motile after 5 days incubation at 37°C. This confirms that all processes of natural transformation combined with intergenicomic recombinational were crucial for the rearrangement of flagellin information when the pressure of kanamycin was maintained. Although a mechanism similar to that described for the V. cholerae RsI element duplication is theoretically possible (Goldberg & Mekalanos, 1986; Mekalanos, 1983), we were unable to isolate any motile revertants in the presence of both DNAse and Km, indicating that natural transformation mediated recombination is the predominant mechanism.

One would assume that there exists a functional reason why two flagellin genes are stably maintained in wild-type Campylobacter strains. The two fla genes are controlled by two different classes of promoters. The flaA gene is regulated by a σ28 promoter, similar to the flagella genes of Escherichia coli, Salmonella typhiurium and Bacillus subtilis (Guerry et al., 1991; Helmann & Chamberlain, 1987), whereas the flaB gene is controlled by a σ54 promoter similar to the flagellin and hook genes of Caulobacter (Minnich & Newton, 1987; Mullen & Newton, 1989). FlaB is environmentally regulated and differentially expressed at different stages during the Campylobacter life cycle which allows the cell to modulate its motility (Aim. Guerry & Trust, unpublished results). The fact that the two fla genes are under the control of distinct classes of promoters, and are expressed at vastly different levels under standard growth conditions (Guerry et al., 1991; Guerry et al., 1990) may be vital to the stable maintenance of the tandem fla genes.
Moreover, the amino and carboxy terminal domains, normally highly conserved among eubacterial flagellins (Joys, 1985, Trachtenberg & DeRosier, 1988; Wei & Joys, 1985) are important for flagellin export, polymerization into a flagellum and filament stability (Fedorov et al., 1988; Vonderviszt et al., 1989, 1991). However, it is in these regions that 70% of the amino acid differences between the FlaA and FlaB flagellins of *C. coli* VC107 occur. These sequence differences may contribute to different structural roles for the two flagellins in the assembled filament, and these functional differences could also contribute to the stable maintenance of the duplicated genes in wild-type cells. Interestingly, we never isolated a mutant capable of producing a full length FlaB filament. The fact that all the full length flagellins producing FlaA-FlaB chimeric flagellins we have isolated always contain the FlaA amino terminal domain suggests that if any structural constraint contributes to the inability of FlaB to produce a full length filament, this constraint must reside in the sequence differences carried in the N-terminal domain.

In summary, this study has provided additional evidence of the biological importance of tandemly duplicated flagelin genes in *Campylobacter*. Motility appears to be crucial to the ability of *Campylobacter* to colonize its intestinal niche, and the presence of a second flagelin gene can, under certain circumstances such as any possible spontaneous mutations which may impair motility, provide this organism with a backup or reserve gene copy, allowing the organism to maintain close to full motility. Moreover, two distinct and independent mechanisms can be utilized to accomplish this rearrangement of its genetic information, emphasizing the importance of motility to *Campylobacter*. Therefore, in addition to any structural and/or regulatory advantages in maintaining two highly homologous tandem fla genes (Guerry et al., 1991; Alm, Guerry & Trust, unpublished results), the presence of duplicated flagelin genes and their ability to ensure motility appears to endow this pathogenic bacterium with a significant biological advantage.

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