Concerted evolution of duplicate *fla* genes in *Campylobacter*

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Campylobacters have two similar copies (*flaA* and *flaB*) of their flagellin gene. It has been hypothesized that the two copies can serve for antigenic phase variation. Analysis of polymorphisms within aligned multiple DNA sequences of the *Campylobacter* flagellin genes revealed high pairwise homoplasy indexes between *flaB/flaB* pairs that were not observed between any *flaA/flaA* pairings or *flaA/flaB* pairings. Thus it seems there are constraints on the sequence of *flaB* that distinguish it from *flaA*. Nevertheless, segments of the two genes that are highly variable between strains are conserved between the *flaA* and *flaB* copies of the genes within a strain. The patterns of synonymous and non-synonymous differences suggest that one segment of the flagellin sequence is under selective pressure at the amino acid sequence level. Another segment of the protein is maintained within a strain by conversion or recombination. Comparisons of strict consensus amino acid sequences did not reveal any motifs that are uniquely FlaA or FlaB, but there are differences between FlaA and FlaB in those amino acids available for post-translational modification. The observed pattern of concerted evolution of portions of a structural gene is an unusual finding in bacteria and should be searched for with other duplicated genes. Concerted evolution was unexpected for genes involved in phase variation since it minimizes the antigenic repertoire that can be expressed by a single clone in the face of the host immune response.

Keywords: *Campylobacter*, flagellin, concerted evolution, DNA multiple-sequence analyses

INTRODUCTION

Gene duplication followed by specialization of each copy is thought to be a major mechanism of the evolution of genes (Jensen & Gu, 1996). Gene duplication initially results in the production of paralogues with a high degree of similarity. In time, each gene accumulates differences or mutations independently of the other copy. Consequently, the genes diverge in their sequences. Duplicate genes with a high degree of similarity presumably either arose recently, or are subjected to selective pressures which maintain the similarity. Concerted evolution is observed when paralogous members of a gene family within a species diverge at a rate slower than the homologous genes in other species. Concerted evolution has been detected in several eukaryotic multicopy gene families (Li, 1997). This indicates that there are mechanisms to homogenize within a species the sequence of genes that are undergoing concerted evolution. Examples of concerted evolution in bacteria include complete operons such as the

rRNA operons (Mattatall *et al.*, 1996), entire genes such as *tufA* and *tufB* in *Salmonella typhimurium* (Abdulkarim & Hughes, 1996) or *catIJF* and *pcaIJF* in *Acinetobacter calcoaceticus* (Kowalchuk *et al.*, 1995), and tandem repeats within genes, such as those seen in alpha C protein of group B streptococci (Madoff *et al.*, 1996).

The genes encoding flagellin in *Campylobacter* (*flaA* and *flaB*) serve as an opportunity to further study gene duplication. The sequences for both *flaA* and *flaB* have been published for two isolates of *Campylobacter jejuni* (Khawaja *et al.*, 1992; Nuijten *et al.*, 1990) and one isolate of *Campylobacter coli* (Logan *et al.*, 1989). The coding regions for the *flaA* and *flaB* sequences were 1722–1731 bases in length and the *flaB* sequence is separated from *flaA* by approximately 180 bases. In each pair of genes from a single isolate, *flaA* differed from the *flaB* by about 5%. Variation of each of the two genes from one strain to another was as much as 30%, with most of the variation concentrated in a central region

about a third the length of the gene (Khawaja *et al.*, 1992; Logan *et al.*, 1989; Nuijten *et al.*, 1990). We have recently completed DNA sequence analyses of *flaA* from 15 *Campylobacter* isolates and verified the presence of a major hypervariable region from approximately base 700 to base 1450, and a short variable region between bases 450 to 600 (Meinersmann *et al.*, 1997). In *Salmonella*, nanostructure analyses of the flagellin demonstrated that similar variable regions are located within domains of the protein that are surface-exposed (Yamashita *et al.*, 1995). We expect that the flagellins from *Campylobacter* have a similar conformational structure. It has been hypothesized that the second copy of *fla* serves as a potential donor for reassortment and recombination of the DNA as a mechanism for creating new antigenic variants for immune avoidance (Alm *et al.*, 1993). Wassenaar *et al.* (1993) supported this hypothesis by selecting a variant in which *flaB* apparently replaced a defective *flaA*.

A σ^{28} -like promoter site has been identified for *flaA* and a σ^{54} -like site has been identified for *flaB* (Guerry *et al.*, 1990; Nuijten *et al.*, 1990). An inverted repeat suggestive of a transcription terminator was found downstream from the *flaA* and upstream of the *flaB* promoter (Khawaja *et al.*, 1992; Logan *et al.*, 1989; Nuijten *et al.*, 1990). Different environmental factors appear to affect the separate promoters and, in culture-grown cells, *flaB* is expressed at a rate lower than that of *flaA* (Guerry *et al.*, 1990) or not at all (Nuijten *et al.*, 1990). Insertional mutagenesis of *flaA* yields cells with a truncated flagellum, greatly reduced motility, and reduced ability to colonize animals (Nachamkin *et al.*, 1993; Wassenaar *et al.*, 1991, 1993). Insertional mutagenesis of *flaB* yields cells that have flagella with apparently normal morphology and colonization ability and, perhaps, slightly decreased motility.

The high similarity between *flaA* and *flaB* in individual strains suggests that duplication of the gene is a relatively recent event. However, given the strain-to-strain variation of the *fla* genes, this conclusion is not clear. This study was undertaken to determine if patterns of evolution could be determined to explain the maintenance of a pair of highly similar *fla* genes within each strain of *Campylobacter*. We expected that the hypothesis that the duplicates provided variants for phase variation was correct and we wanted to determine the extent of variation that mechanism would create. However, the results indicated that the evolution of the *flaA* and *flaB* genes in *Campylobacter* is coordinated. The residues that are predicted to be exposed are conserved between the *flaA* and *flaB* of a particular isolate while the same region has a high variability between strains. At the same time, there are specific portions of the sequence that distinguish *flaA* from *flaB* and are conserved between strains.

METHODS

DNA sequences. DNA sequences for *flaA* and *flaB* for strains VC167, TGH9011 and 81116, and the *flaA* sequence for strain IN1, were obtained from the GenBank database (accession

nos M26945, Z29327, J05635 and X57173, respectively). Sequences for *flaA* and *flaB* of strain NCTC 11186 were obtained by BLAST search of the genomic database at the Sanger Centre (<http://www.sanger.ac.uk/Projects/Cjejuni/>). The sequence generation for *flaA* of the remaining strains has been described previously (Meinersmann *et al.*, 1997). The GenBank accession numbers are: strain A74/O, AF050184; strain D772, AF050185; strain D935, AF050186 (*flaA*) and AF050187 (*flaB*); strain D2290, AF050188 (*flaA*) and AF050189 (*flaB*); strain D2640, AF050190; strain D2677, AF050191; strain L17, AF050192 (*flaA*) and AF050193 (*flaB*); strain L19, AF050194; strain SSU9894, AF050195; strain SSU9896, AF050196; strain D1118, AF050197.

To obtain the DNA sequences for *flaB*, sequences upstream of *flaB* were first determined as described below in order to produce additional information on the non-coding intergenic region. Template DNA was generated by PCR using primers that annealed to a conserved portion of the 3' end of *flaA* paired with a downstream primer that annealed to a conserved portion at the 5' end of the known *flaB* sequences. The products of these PCR reactions were ligated into a T/A cloning vector and sequenced as described by Meinersmann *et al.* (1997). An upstream primer was then designed based on a conserved portion of the intergenic sequence upstream of the *flaB* start codon. This was paired with a primer that was designed to complement conserved sequence 3' distal to the *flaB* coding region of aligned sequences for strains VC167, TGH9011 and 81116. These primers were used in a PCR to amplify the entire *flaB* and a small amount of flanking sequence at both ends under permissive conditions (i.e. with an annealing temperature 5 °C lower than expected optimum), and the products were sequenced as described by Meinersmann *et al.* (1997) utilizing the same primers as were used for *flaA* sequencing. The sequences of this final PCR product were used for the *flaB* data in the analyses described below.

Computational analyses. Alignments were made of the DNA sequence of *flaA* from 16 strains or *flaB* from seven strains using CLUSTALX (Thompson *et al.*, 1994) and the alignments were manually edited to move gaps inserted by the alignment program so that they did not interrupt the codon reading frame. Alignment scores were either unchanged or improved by such editing. The aligned sequences were analysed with PAUP version 4.0b2a (Swofford, 1999) to reconstruct possible phylogenetic relationships and produce homoplasy indexes [homoplasy index = 1 minus (the minimum possible number of steps between two individuals on a tree divided by the number of steps observed in the reconstructed tree between those two individuals); see Discussion for further details on homoplasy]. The aligned sequences were subjected to Sawyer's runs test (Sawyer, 1989), which looks for substitution patterns that are statistically consistent with recombination events, implemented in the program GENECONV version 1.02 (distributed by S. Sawyer at <http://www.math.wustl.edu/~sawyer/>). This program performs the Bonferroni correction of Karlin-Altschul *P* values, which is the most conservative method of determining the *P* values.

Alignments were analysed in the MULTICOMP program (Reeves *et al.*, 1994) using the algorithm developed by Li (1993) for determining synonymous and non-synonymous substitutions. K_s is the synonymous substitution rate and K_a is the non-synonymous substitution rate expressed as the corrected number of substitutions per site. Rates greater than 1.00 are not real, but are due to corrections for possible multiple substitutions at a single site and remain proportional to the rate of neighbouring substitutions. The analyses were performed with a window size of 30 bases (10 codons). Use of

Table 1. Pairwise distance and homoplasmy matrix for *Campylobacter flaA* and *flaB* DNA sequences

Data above-right of the diagonal are the numbers of different nucleotide bases between paired sequences. Data below-left of the diagonal are the homoplasmy indexes for each pair. Data in the upper-right quadrant and lower-left quadrant are *flaA*–*flaB* comparisons. Upper left data are *flaB*–*flaB* and lower right are *flaA*–*flaA* comparisons. Boxed data are within-strain *flaA*–*flaB* comparisons. Data for strains with only *flaA* data are omitted from the table.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. D2290 <i>flaB</i>		2	2	78	81	248	271	97	100	87	147	151	293	315
2. L17 <i>flaB</i>	0		2	78	81	248	271	97	100	87	147	151	293	315
3. 11168 <i>flaB</i>	0	0		76	81	246	271	95	98	87	145	151	291	315
4. TGH9011 <i>flaB</i>	134	134	134		103	242	259	145	144	139	85	163	289	307
5. D935 <i>flaB</i>	114	114	112	110		259	268	150	153	150	174	78	300	314
6. 81116 <i>flaB</i>	164	164	164	130	154		177	297	296	297	297	301	89	235
7. VC167 <i>flaB</i>	162	162	160	134	166	86		326	325	318	316	316	240	116
8. D2290 <i>flaA</i>	18	18	18	32	10	80	72		3	10	76	82	248	276
9. L17 <i>flaA</i>	18	18	18	36	10	84	76	0		13	75	81	247	275
10. 11168 <i>flaA</i>	30	30	28	40	12	82	82	0	0		74	86	248	272
11. TGH9011 <i>flaA</i>	30	30	30	0	4	40	42	66	70	70		104	248	266
12. D935 <i>flaA</i>	8	8	6	14	0	76	82	42	46	40	38		252	266
13. 81116 <i>flaA</i>	116	116	116	80	110	4	20	126	130	128	86	122		198
14. VC167 <i>flaA</i>	92	92	90	60	94	2	0	96	100	102	66	106	36

Fig. 1. Gene conversion map. The fragments of possible gene-conversion events identified by the Sawyer's runs test are indicated by cross-hatched bars located along lines representing the DNA sequences of *flaA* and *flaB*. Intrastrain *flaA* to *flaB* comparisons are indicated for each strain having both sequences and the bottom line is a composite of all the possible interstrain *flaA* to *flaA* conversion events. See text for explanation of block position (I–V).

longer windows gave less saturated results but the resolution was less than desired. To analyse synonymous and non-synonymous substitutions from *flaA* to *flaB* within strains, the sequences for seven strains were aligned pairwise for each strain, and each pair was analysed with the SITES program (distributed by Jody Hey, Rutgers University) to show the sites of synonymous and non-synonymous substitutions for each sequence pair. Aligned sequences were analysed with PAUP* 4.0b2a (Swofford, 1999) to construct the most parsimonious relationships and the homoplasmy indexes were also determined with PAUP*.

Peptide sequences. The DNA sequences were translated to peptide sequences that were then aligned. One hundred per cent consensus peptide sequences were generated for the aligned *flaA* sequences and the aligned *flaB* sequences. Positions in which a consensus was not achieved were marked

with an X, unless all the residues were in the same pam250S group of similar amino acids (Dayhoff *et al.*, 1978), in which case the position was marked 0 for Ala, Gly, Pro, Ser, Thr; 1 for Asp, Glu, Asn, Gln, Asx, Glx; 2 for His, Lys, Arg; 3 for Ile, Leu, Met, Val; 4 for Phe, Trp, Tyr; or 5 for Cys. The two consensus sequences were aligned and a new consensus was generated.

RESULTS

The DNA sequences of *flaA* for 16 isolates of *Campylobacter* (15 isolates of *C. jejuni* and one isolate of *C. coli*) were aligned along with the DNA nucleotide sequences of *flaB* from seven of the same isolates (six isolates of *C. jejuni* and one isolate of *C. coli*). The homoplasmy index

Fig. 2. Site analyses of synonymous (K_S) and non-synonymous (K_A) base substitutions in (a) *flaA* for multiple strain sequence alignments, (b) *flaB* for multiple strain sequence alignments and (c) *flaA* to *flaB* within individual strains. The dashed lines represent the density of the synonymous base substitutions and the solid lines represent the density of non-synonymous substitutions. Note that the abscissae indicate the centre of the window site.

was determined for every pair of all the sequences. It was found that the homoplasy indexes for paired *flaB* genes were greater than for *flaA/flaA* or *flaA/flaB* pairs, both interstrain and intrastrain, except in pairs of the three *flaB* sequences that were highly similar (Table 1). D2290, L17 and NCTC 1168 differed by only 2 bp each and are thus expected to have low homoplasy indexes with each other.

Analyses were performed to determine the segments of the sequences that contributed to the homoplasy. The alignment of *flaA* DNA sequences from 16 strains and the *flaB* DNA sequences from seven of the same strains was subjected to the Sawyer's runs test (Sawyer, 1989). Using the most conservative method of determining P values, 104 possible gene conversion events were identified with $P < 0.05$. Sixteen of these were between the *flaA* and *flaB* of the same strain and the sites of these events are mapped in Fig. 1. The majority of the postulated recombination events were found in the

segment of the flagellin genes that we later identify as Block IV (see below). Possible *flaA* to *flaA* interstrain conversion events are also shown and reflect those seen by Harrington *et al.* (1997).

Patterns of synonymous versus non-synonymous base changes may indicate the nature of selective forces on structural genes. Window analyses of synonymous and non-synonymous base substitutions (Li, 1993; Reeves *et al.*, 1994) for *flaA* and *flaB* were scored separately and are presented in Fig. 2(a, b). The areas of sequence variability between isolates of *Campylobacter* are very similar for *flaA* and *flaB*. The sites of intrastrain synonymous and non-synonymous substitutions in the *flaA* and *flaB* sequences for each isolate for which data were available for both genes (seven pairs) were analysed and the results are shown in Fig. 2(c). The SITES program (distributed by Jody Hey, Rutgers University) gives a precise location of base substitutions and was used to delimit the blocks that are indicated in Fig. 2. The

Table 2. Summary of synonymous substitution rate (K_s), non-synonymous substitution rate (K_A) and K_s to K_A ratio for blocks of aligned flagellin sequence

Pairwise K_s and K_A were determined by the algorithm developed by Li (1993) and averaged for all the interstrain *flaA* comparisons, all the interstrain *flaB* comparisons and all the intrastrain *flaA/flaB* comparisons. The K_s and K_A are window-size-dependent, especially for small windows such as Block III. Comparisons should be made across the rows, not down the columns. However, the K_s/K_A ratio should not be affected by window size.

Block*	<i>flaA</i>			<i>flaB</i>			<i>flaA-flaB</i>		
	K_s	K_A	K_s/K_A	K_s	K_A	K_s/K_A	K_s	K_A	K_s/K_A
All (1–end)	0.476	0.106	4.49	0.454	0.100	4.54	0.159	0.047	3.47
I (1–243)	0.249	0.032	7.78	0.149	0.012	12.42	0.608	0.151	4.03
II (244–735)	0.423	0.071	5.96	0.383	0.065	5.89	0.040	0.010	4.24
III (736–831)	8.104	0.662	12.24	17.357	1.266	13.71	0.351	0.109	3.21
IV (832–1515)	0.650	0.199	3.27	0.671	0.197	3.41	0.008	0.006	1.38
V (1516–end)	0.372	0.025	14.88	0.461	0.013	35.46	0.955	0.148	6.43

* Block equals the segment of the aligned sequence for which data were analysed (base pair positions shown in parentheses).

Consensus	MGFRINTN.0	OLN020N0.3	N0..LD.SL0	RLSSGLRINS	AAD.ASGMAI	150
<i>flaA</i>	X0	A..AK0.0DL	.020..X..0		...X.....	150
<i>flaB</i>	IG	0..0HA.SVV	.0XE..K..S		...D.....	150
Consensus	.D0LRSQ0.T	LQQA1.NGND	A3GILQTADK	AMDEQLKILD	TIKTATQAA	300
<i>flaA</i>	X.0....0X.	S....	.3.....			300
<i>flaB</i>	A.S....AA.	N....	.I.....			300
Consensus	1DGQSLKTRT	MLQA1IN2LM	EELDNIAINTT	SF.GKQLLSG	.F.NQEFQIG	450
<i>flaA</i>	1.....	1..2..		..X.....	X.X.....	450
<i>flaB</i>	Q.....	D..R..		..N.....	X.X.....	450
Consensus	0SSNQ3K00	IGATQSSKIG	3TR.1.....	...G.V..T3	KNYNGILD.F	600
<i>flaA</i>	0.....3.00		3..X1XXXXX	XXX.X.XX.3	1..X	600
<i>flaB</i>	0.....3.A0		3..FETGXXX	XX0.X.XX.3	1..X	600
Consensus	F1.VVISTSV	GTGLGALA1E	IN2.AD.TG3	RAT4.3...G	..A32.G.TS	750
<i>flaA</i>	.1X.....	1..	..2X..K..3	..4X3XXX.	XX.32X.X..	750
<i>flaB</i>	.1X.....	E..	..2X..X..3	..4XVX0X.	XX.32X.X..	750
Consensus	1.F0INGV.I	G.3.Y.DGD.	NG.L30AIN0	VKDTTGV1AS	.D.NG.L3LT	900
<i>flaA</i>	1X.0....X.	.X3X.X...X	.X.30...0	1..	X.X..X.3..	900
<i>flaB</i>	1X.A....X.	.Q3X.X...X	.X.30...0	1..	3.X..X.3..	900
Consensus	S.1GRGIKI.	G.IG.G0.I.	...ENYGR	SLV.KNDG21	I.ISG0..0.	1050
<i>flaA</i>	.X1.....X	.X..X.0X.X	XXXX.....	...X....2D	.X...0XX0X	1050
<i>flaB</i>	.X1.....X	.X..X.0X.X	X1XX.....	...-....21	.X...0XLSX	1047
Consensus	.G.G....S	Q.SVSLRESK	G...A..ADA	MGF.0...G.		1200
<i>flaA</i>	X.X.XXXXX.	.X.....	..XX.XX...	..X0XXX.X	XXXXX3XXXXX	1200
<i>flaB</i>	X.X.001XI.	.X.....	..XX.XX...	..X0XXX.X	XXXXX3XXXXX	1197
Consensus	S030.4MS..	.SGFS.GSG.	SVGSGK..S.	S	0.0.3S..Y.	1350
<i>flaA</i>	.030X4..XX	.X...X...4	XX.X	XX0XXXXXX.	0X0X3.XX.X	1350
<i>flaB</i>	.S30X4..XX	G...X...X	XX.X	XXXXXXX3.	0A0X3.XX.X	1347
Consensus	3S.GSGFS0.	S..SQF0.32	.0.....	DETAGVTTLK	GAMA.MDIAE	1500
<i>flaA</i>	3.X.....0X	.XX...0X32	X0XXXXXXX		...X....	1500
<i>flaB</i>	V.X.....0G	.XX...A032	X00XXXXXXX		...V....	1497
Consensus	T..TN.DQIR	ADIG03QNQ3	..TINNITVT	QVNVK0AES.	IRDVDF00ES	1650
<i>flaA</i>	.AX..L....	S3...3	TS.....	0...Q	00..	1650
<i>flaB</i>	.XX..X....	0V...L	QV.....	A...T	A0..	1647
Consensus	AN4SK.NILA	QSGS.0MOQ.	N0..QNVL2L	LQ		1746
<i>flaA</i>	..Y..A....	X0..A.A	.SXX....R.			1746
<i>flaB</i>	..F..Y....	YA.S.X	.AVQ....K.			1743

Fig. 3. Comparison of the consensus sequence of translated amino acid sequence of *flaA* with the consensus sequence of translated *flaB*. The sequence indicated 'Consensus' shows the residues in common between the *flaA* and *flaB* consensus. Residues indicated in bold are sites that are highly conserved in both sequences but differ between *flaA* and *flaB*. Numbering on the right is the nucleotide position of the corresponding DNA sequence. Numbers substituted for the amino acid designation are the pam250S scores: 0 for Ala, Gly, Pro, Ser, Thr; 1 for Asp, Glu, Asn, Gln, Asx, Glx; 2 for His, Lys, Arg; 3 for Ile, Leu, Met, Val; 4 for Phe, Trp, Tyr; or 5 for Cys.

greatest variability of *flaA* compared to *flaB* of the same isolate occurs in three regions, one close to the 5' terminus of the DNA sequence, a short segment at the centre of the sequence, and another region at the 3' end of the sequence. Five blocks with differing patterns of base substitutions in the flagellin gene sequence were identified as follows: Block I, base positions 1–243; Block II, base positions 244–735; Block III, base positions 736–831; Block IV, base positions 832–1515;

Block V, base positions 1516 to end. The mean synonymous substitution rate (K_s), non-synonymous substitution rate (K_A) and the K_s to K_A ratio were calculated for pairwise comparisons (120 interstrain *flaA* to *flaA* comparisons, 21 *flaB* to *flaB* interstrain comparisons and seven intrastrain *flaA* to *flaB* comparisons) for each block and are presented in Table 2. Because K_s and K_A calculations become sensitive to window size as the window size gets small (compare

data in Table 2 with scales for Fig. 2, which are for windows of 30 bp), the numbers should be compared across the table and not up and down the columns.

Consensus sequences for the predicted amino acid sequences of *flaA* and *flaB* were constructed. Any residue position that did not give a 100% consensus was scored using the pam250S score for conserved amino acid side chain structure (Dayhoff *et al.*, 1978). Residues that did not match a conservation group were scored as not having a consensus. The scored consensus amino acid sequences for FlaA and FlaB were then aligned (Fig. 3). Only 12 strictly conserved differences were observed, i.e. a specific amino acid was found in all the FlaAs and a different specific amino acid was found in all the FlaBs. Six of these 12 differences were pairs of amino acids that were in the same pam250S groups. Four of the 12 differences were within 76 amino acid residues of the amino-terminal end of the protein. The remaining differences were within 62 residues of the carboxy-terminus.

DISCUSSION

Homoplasy is considered a departure from random mutations, and this departure may be due to parallel or convergent evolution or evolutionary reversals (Swofford *et al.*, 1996; Stewart, 1993). The homoplasy index is derived from phylogenetic trees to indicate the frequency of matching character states that are not found in putative ancestors. Phylogenetic trees are not usually considered valid lineage reconstructions when paralogous genes are included in the analysis (Swofford *et al.*, 1996). Nevertheless, our analyses showed that the homoplasy index was a useful tool in this study. The *flaA* and *flaB* sequences from the same isolates clustered together in dendrograms (not shown). This is an indication of concerted evolution (Li, 1997). However, the homoplasy index also revealed associations of *flaB* between strains. Pairwise comparisons of *flaA* and *flaB* sequences within isolates then revealed regions (at least Block IV, defined above) of apparent concerted evolution. The Sawyer's runs test supported this hypothesis by showing that Block IV appeared as a possible recombinant fragment between *flaA* and *flaB* in all the strains for which both sequences were available.

The extremely low number of intrastrain *flaA* to *flaB* synonymous differences in the region of DNA sequence bases 832 and 1515 between the *flaA* and *flaB* of individual strains implies editing or gene conversion with selection at the DNA level (Endo *et al.*, 1996). The higher level of intrastrain *flaA* to *flaB* synonymous differences between bases 244 and 735 suggests selection for conservation at the protein or functional level without gene conversion (Endo *et al.*, 1996). However, the observed rates for this region could be within the range that might occur in parts of the *Campylobacter* genome that do not have selection for diversity. No studies of multiple-sequence analyses of *Campylobacter* housekeeping genes have yet been published that will allow this comparison to be made. From the K_S/K_A

ratios in Table 2 it appears that there is greatest functional constraint on sequence in Block V and the lowest functional constraint in Block II and Block IV. Block IV is the segment that has almost no differences between the *flaA* and *flaB* within individual strains. However, even with the degree of variability seen, one constraint that was maintained was the G + C content. The G + C content of the *fla* sequences from *C. jejuni* strains used in this study was from 35.0 to 37.2 mol% (mean 36.4 mol%).

Recombinant shuffling of the variable regions of *flaA* and *flaB* within a strain would not itself generate interstrain variability with intrastrain conservation. In fact, the rate of concerted evolution is expected to be higher when there are structural constraints (Li, 1997). Recombination errors that may increase variability should occur at the recombination junctions. If this was occurring with *fla*, then it is expected that the interstrain variability would be concentrated at the ends of the region that is intrastrain conserved. Alignment gaps were seen that are consistent with illegitimate recombinant events (Jakupciak & Wells, 1999). However, we have found that interstrain variability is greatest within the segment that is conserved intrastrain, perhaps indicating that the generation of diversity in that region is not recombination-dependent.

Campylobacter is competent for transformation (Wang & Taylor, 1990) and does not appear to be clonal (Aeschbacher & Piffaretti, 1989). Therefore, it is possible that generation of variability may involve recombinant shuffling of segments of *fla* between strains. Harrington *et al.* (1997) presented convincing evidence of interstrain recombination within the *flaA* gene. There would then need to be a simultaneous or sequential insertion of the new sequence into the analogous segment of the other copy of *fla* within the strain. Intrastrain recombinant events between the *flaA* and *flaB* that cause a loss of the interstrain-conserved regions, i.e. the regions that distinguish *flaA* from *flaB* within a strain, would have to be treated as an error and lost (purged). Unequal crossing-over appears to be a prevalent mechanism of concerted evolution in eukaryotes (Li, 1997). It is unlikely that unequal crossing-over occurs with *Campylobacter flA* because the mechanism usually involves changes in the copy number. Precision splicing of regions of the genes between *flaA* and *flaB* could occur, but putative insertion sequences or *chi* sequences (West, 1992) that would facilitate such a mechanism were not conserved within the *fla* sequences examined in this study. Also, recombination between the two copies of the flagellin would have had to invoke some copy-choice mechanism that favoured the low G + C content that is characteristic of the *Campylobacter* genome. That is to say, if a mutation occurred in one copy of the flagellin that substituted a new G or C, the amelioration process of the two copies would need to favour the A or T copy. Since transitional nucleotide substitutions are biochemically more favoured than transversions (Li, 1997), failure to do so would drive towards a 50% G + C content ratio. Alternatively, there

may be non-replicative mechanisms for conversion of one *fla* sequence based on the other *fla* sequence such as a mismatch repair mechanism. If there was no preference for which version of the gene was maintained, either mechanism, recombination or mismatch repair, might increase the rate of generation of diversity of the genes within a population of the organism. Some progeny of a cell with a mutation in the intrastrain conserved segment would end up with the version that was originally *flaA* and others would end up with the *flaB* version. Gene conversion using mismatch repair of the two genes might also allow for simpler mechanisms that could favour adenosine or thymidine. The mean interstrain differences for *flaA* were slightly higher (about 162 bp difference) than the interstrain *flaB* differences (about 159 bp). If changes usually occurred first in *flaA* and are later transferred to *flaB*, a higher number of interstrain differences would be expected in *flaA*. That conclusion can not be drawn from the data because the differences are not great enough.

Examination of the amino acid sequence differences between the consensus sequences of FlaA and FlaB may explain why two copies of flagellin are maintained. In the amino acid sequence from residue 290 to residue 480, only 71 of the residues were conserved in the aligned FlaA or FlaB amino acid sequences that were analysed. This is an indication that the flagellin protein has little functional dependence on primary amino acid sequence in that region. This is the region, however, that is maintained between the *flaA* and *flaB* within an individual strain and, based on homology with the *Salmonella* flagellin, is expected to be the bearer of surface-exposed epitopes (Yamashita *et al.*, 1995). Furthermore, the high homoplasy indexes for *flaBs* imply a stronger selective pressure on *flaB* than on *flaA*. Six of the 12 amino acids that are strictly conserved in each copy of FlaA but differ between FlaA to FlaB represent differences in amino acids that can be O-linked (serine or threonine). Five of these modifiable amino acids are in the carboxy-terminal region. Doig *et al.* (1996) have demonstrated that *Campylobacter* flagellins are glycosylated. It would seem that the different copies of the flagellin gene offer the greatest opportunity for antigenic phase variation by changing the glycosylation sites.

Copy-to-copy conversion can increase the mutation rate of the gene by bringing together the mutations from two sites. This would contribute to clonal differences of the organism but at the expense of the ability to have greater phase variation. The advantage of maintaining two copies of the flagellin gene remain unclear, but it would be efficient if they contributed both to function or phase variation and to increasing the genetic pool for producing greater clone-to-clone variation. Presumably, host immune responses would exert selection for diversity that would drive the expansion of new alleles of flagellin. The two independent promoters for *flaA* and *flaB* (Guerry *et al.*, 1990; Nuijten *et al.*, 1990) would allow a mechanism for phase variation, but the host factors that might activate either promoter are not known.

The pattern of concerted evolution noted in this study is unusual for prokaryotes. Most *Salmonella* maintain two copies of flagellin genes, *fliC* and *fliB*. Okazaki *et al.* (1993) demonstrated exchange of genetic information between the phase variant flagellin genes *fliC* and *fliB* of *S. typhimurium* after selection against expression of parental *fliC* epitopes. *Neisseria gonorrhoeae* uses gene conversion to exchange sequence between the expressed pilin gene and non-expressed pseudogenes (Zhang *et al.*, 1992). We are not aware of any study to analyse the *Salmonella* flagellin or *Neisseria* pilin gene families for concerted evolution.

Concerted evolution is defined as less than expected divergence of copies of genes within an individual than the divergence of the gene from other species (Li, 1997). We have demonstrated that concerted evolution of segments of *Campylobacter fla* appears to occur at a rate that at least equals the rate of clonal divergence. There is no information currently available on the divergence rate of other genes in *Campylobacter*. Flagellin clearly has a greater diversity than expected for most of the genome. Thus it can be concluded that the intrastrain-conserved region (Block IV) in *Campylobacter fla* demonstrates concerted evolution relative to the remainder of the flagellin gene, but we do not know if this is true relative to the entire genome. The concerted evolution occurring in Block IV must happen by gene conversion events that occur almost as fast as the mutation rate since the Block IV sequences of *flaA* and *flaB* are almost identical within a strain.

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