Phenotypic characterisation of flagellin and flagella of urease-positive thermophilic campylobacters

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ABSTRACT

In this study, flagellin is purified biochemically from eight urease-positive thermophilic camplylobacters (UPTC) isolated from river water, sea water and mussels, and purified also from two isolates of *Campylobacter jejuni* and *C. coli* and fractionated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Results showed that no flagellin components were detected in the two Japanese UPTC isolates (CF89-12 and CF89-14) and the two UPTC NCTC strains (NCTC12893 and NCTC12894). Flagellin components, each consisting of a single peptide, with a heterogeneous molecular mass of approximately 52–63 kDa were demonstrated in the other four UPTC isolates (NCTC12892, NCTC12895, NCTC12896 and NI15F [from Northern Ireland]) and the two Japanese isolates of *C. jejuni* (JCM2013 and *C. coli* 27). The approximate molecular mass of flagellin from the flagellin-positive UPTC isolates was smaller than those of *C. jejuni* and *C. coli*. Flagella were not detected by electron microscopy in the four flagellin-negative UPTC isolates but they were detected in the four flagellin-positive UPTC isolates and the two isolates of *C. jejuni* and *C. coli*. Thus, significant phenotypic diversity for flagellin, which must be due to genotypic variations, was demonstrated among the UPTC isolates.

KEY WORDS: Campylobacter infections. Electrophoresis, polyacrylamide gel. Flagella. Flagellin. Microscopy, electron.

Introduction

Ten isolates of urease-positive thermophilic campylobacters (UPTC), microaerophilic and Gram-negative bacteria, were first isolated from the natural environment and identified in England.[1] Following the original description of UPTC organisms, four human isolates were reported in France.[2,3] Additional isolates of UPTC have been isolated from the natural environment, including from wild birds, in Northern Ireland,[4–7] The Netherlands[8] and Japan.[9,10] Thus, until now, almost all UPTC isolates have been found from the natural environment, including from wild birds, with the exception of the four isolation cases from humans, but none from any domestic animals.

The characterisation of UPTC as a variant of *Campylobacter lari* has been described by hybridisation dot blot assay as well as by biochemical characteristion.[2] It has been suggested that the UPTC organisms belong within *C. lari*, possibly as biovars, as a result of the numerical analysis of proteins by high-resolution polyacrylamide gel electrophoresis (PAGE).[11]

Bacterial flagella are responsible for motility and chemotaxis.[12] In addition to their motility, bacterial flagella have been shown to play important roles in adhesion, and to be one of the best-defined virulence factors.[13] They are composed of a structurally repeating protein of flagellin.[14]

In a previous study of UPTC flagellin, two *flaA*-like sequences containing two internal termination codons (TAG), the incomplete genes or pseudogenes of *flaA*, have been demonstrated in two Japanese isolates obtained from river water.[15] The shorter *flaA* genes have been demonstrated in five NCTC strains[16] and in 11 Northern Ireland isolates of UPTC isolated from the natural environment (not including wild birds) following TA cloning and nucleotide sequencing procedures (DDBJ/EMBL/GenBank accession numbers AB084911, AB084912, AB070578, AB073915–AB073918, AB080202, AB103050–AB103059).

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Here, we aim to study the characteristics of the flagellin and flagella of these isolates of UPTC in order to clarify the biological significance of their *flaA*-like genes and shorter *flaA* genes, and to phenotypically characterise flagellin and flagella of UPTC isolates from river and sea water and in mussels, using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) after biochemical purification analysis and electron microscopy (EM).

Materials and methods

Isolates of thermophilic campylobacters used in the present study are shown in Table 1. The eight UPTC isolates were obtained from river and sea water and from mussels in England, Northern Ireland and Japan. Two Japanese isolates of *C. jejuni* JCM2013 and *C. coli* 27[16] were used as non-UPTC reference organisms.

Cells were first precultured on blood agar base No. 2 (Oxoid, Hampshire, UK) containing defibrinated horse blood

Table 1. Description of isolates of thermophilic campylobacters used in the present study and the approximate molecular weight (MW) of the purified flagellin.

Isolate No.	*Campylobacter* sp.	Source	Country	MW of flagellin
NCTC12892	UPTC	River water	England	52.5 kDa
NCTC12893	UPTC	River water	England	ND
NCTC12894	UPTC	Sea water	England	ND
NCTC12895	UPTC	Mussel	England	55.1 kDa
NCTC12896	UPTC	Mussel	England	54.2 kDa
CF89-12	UPTC	River water	Japan	ND
CF89-14	UPTC	River water	Japan	ND
NI15F	UPTC	Mussel	N. Ireland	56.2 kDa
JCM2013	*C. jejuni*	Human	Japan	60.0 kDa
27	*C. coli*	Pig	Japan	63.0 kDa

"ND, flagellin not detected"

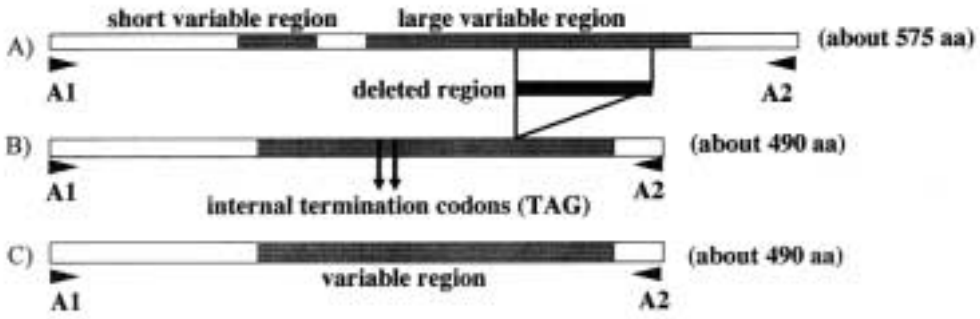

Fig. 1. Diagrams of flagellin based on the alignment information of the amino acid and nucleotide sequence of *flaA* of UPTC. A) *C. jejuni* 81116 strain (21), B) UPTC CF89-12 and CF89-14 (15) and C) UPTC NCTC12892, 12893, 12894, 12895, and 12896 (16). A1 and A2 are the primers for PCR amplification of *flaA*. Arrows are at the positions of internal termination codons.

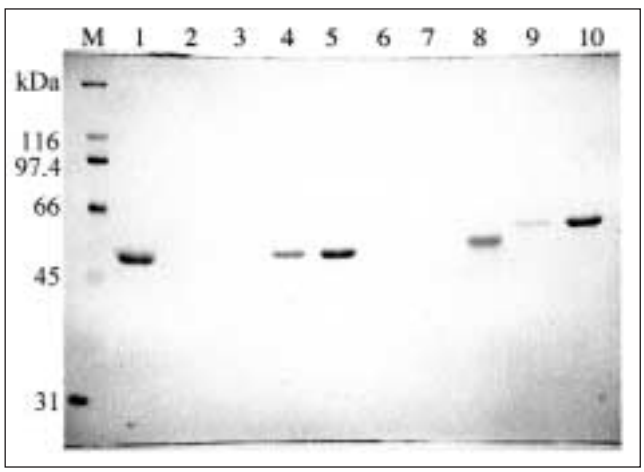

Fig. 2. Fractionation by SDS- PAGE of purified flagellin from isolates of UPTC and *C. jejuni* and *C. coli*. M: SDS-PAGE broad-range standard; lane 1: flagellin from UPTC NCTC12892; lane 2: NCTC12893; lane 3: NCTC12894; lane 4: NCTC12895; lane 5: NCTC12896; lane 6: CF89-12; lane 7: CF89-14; lane 8: NI15F; lane 9: *C. jejuni* JCM2013; lane 10: *C. coli* 27.

(7% [v/v]; Nippon Bio-Test, Tokyo, Japan) supplemented with Butzler Campylobacter-selective medium (Nissui, Tokyo, Japan) under microaerophilic conditions at 37°C for 48 h.

Flagellin from isolates of thermophilic campylobacters were purified biochemically by sequential pH dissociation, differential ultracentrifugation and neutral pH reassociation, following the procedure described by Logan and Trust[17] and Logan *et al*.[18] The purified flagellins fractionated by SDS–PAGE were stained with Coomassie brilliant blue R-250 (CBB) and their molecular weights estimated using SDS–PAGE broad-range standards (Bio-Rad, Tokyo, Japan). The protein components purified from the two strains of UPTC NCTC12895 and NCTC12896 by the biochemical procedure employed for flagellin purification were confirmed as flagellin proteins by the N-terminal amino acid sequencing analysis, as described previously.[16]

For EM observation of flagella, the fresh cultured bacterial cells were washed thoroughly with phosphate-buffered saline and incubated on the grids with collodion film for several minutes. The bacterial cells were negatively stained with a saturated solution of uranyl acetate for several seconds. Observations were made using an Hitachi H-7500 electron microscope (Hitachi, Tokyo, Japan).

Results and discussion

As previously reported, following application of a polymerase chain reaction (PCR) amplification method developed for *C. jejuni*[19,20] to amplify the *flaA* gene fragment of UPTC isolates, this group cloned and sequenced the amplicons from two Japanese UPTC isolates (CF89-12 and CF89-14) isolated from river water[15] and from five NCTC strains (NCTC12892, 12893, 12894, 12895 and 12896) isolated from river water, sea water and shellfish,[16] as well as from 11 isolates obtained from sea water and shellfish in Northern

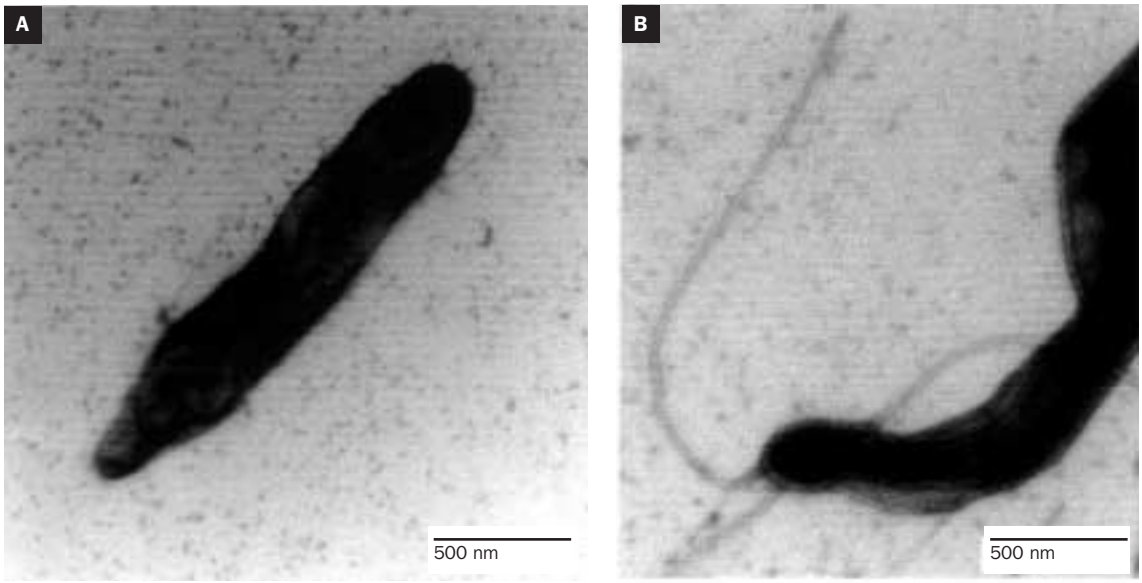

Fig. 3. Electron microscopy of examples of organisms (A) without and (B) with flagella.

Ireland. These studies showed that the two *flaA*-like sequences containing two internal termination codons at nucleotide positions from 775 to 777 and from 817 to 819 were shorter (1461 bp) than in *C. jejuni* and *C. coli*.[15] Furthermore, the shorter *flaA* genes with the possible open reading frame from 1461 to 1503 bp lacked approximately 85 amino acid residues, mainly from the approximate residue numbers 390 to 470 of the large variable region in the flaA protein of *C. jejuni* and *C. coli*, without any internal termination codons[16] (Fig. 1).

In the present study, biochemical purification of flagellin was attempted from eight isolates of UPTC and two isolates of *C. jejuni* JCM2013 and *C. coli* 27 used as reference isolates, by fractionation using SDS-PAGE. As shown in Fig. 2, no flagellin components were detected in the two Japanese UPTC isolates (CF89-12 and CF89-14) and the two NCTC strains (NCTC12893 and NCTC12894). However, flagellin components, each consisting of a single peptide band, were demonstrated in the other UPTC isolates (NCTC12892, NCTC12895, NCTC12896), a Northern Ireland isolate (NI15F) and two Japanese isolates of *C. jejuni* JCM2013 and *C. coli* 27.

The approximate molecular weight (MW) of the flagellin component is shown in Table 1 and the MWs of the NCTC12892 and NI15F isolates have been reported previously.[16] Those of the four UPTC isolates and two isolates of *C. jejuni* and *C. coli* were shown to be 52.5 kDa for NCTC12892, 55.1 kDa for NCTC12895, 54.2 kDa for NCTC12896, 56.2 kDa for NI15F, 60 kDa for a strain of *C. jejuni* JCM2013 and 63 kDa for an isolate of *C. coli* 27 (Fig. 2 and Table 1).

Thus, flagellin-negative isolates and flagellin-positive isolates represent two different groups of UPTC isolated from the natural environment (not including wild birds). The present results also demonstrated the heterogeneity found in the molecular weights of the flagellin from the six examined isolates of thermophilic campylobacters and also among four flagellin-positive isolates of UPTC.

The present results of the approximate flagellin molecular size (Table 1) also demonstrated that the flagellin in flagellin-positive isolates of UPTC was, in general, smaller than that of *C. jejuni* and *C. coli*; however, it remains unclear whether or not this applies to flagellins from all of the flagellin-positive isolates of UPTC. Although Sekizuka *et al*.[15] demonstrated flagellin biochemically in two Japanese UPTC isolates (CF89-12 and CF89-14), the present results do not support this previous work.

Consequently, EM examination of the flagella from the isolates was performed and the representative images are shown in Figures 3a and 3b. No flagella were observed in the two Japanese UPTC isolates (CF89-12 and CF89-14) and in the two NCTC strains (NCTC12893 and 12894). These results are consistent with SDS–PAGE on the biochemically purified flagellin. However, flagella were observed in the other four flagellin-positive UPTC isolates and the two isolates of *C. jejuni* and *C. coli*.

In the present study, both the phenotypic diversity of flagellin and the flagella were demonstrated among isolates of UPTC from the natural environment (not including wild birds) in England, Northern Ireland and Japan. Such significant phenotypic diversity of flagellin and flagella may be mainly due to the highly genotypic variation within the flagellin genes of UPTC, as described previously.[15,16]

Hypothetically, some UPTC biovars may carry fully-formed flagella, whereas others may carry partly formed flagella, and some may have no flagella. The switching on and off of flagellin synthesis may involve differential expression of *flaA*, the two *flaA*-like sequences and the shorter *flaA* genes. Therefore, it would be interesting to compare the differences in conventional culture techniques to determine whether or not there is a putative environmental trigger for flagella development.

Although almost all UPTC organisms have been isolated mainly from the natural environment they have rarely been isolated from warm-blooded creatures other than wild birds. Moreover, although UPTC isolates have never been associated with human gastrointestinal disease, four UPTC isolates were described in humans in France,[2,3] where they were thought to be responsible for pathology.

The characteristic isolation profiles for UPTC may be due to the phenotypic characteristics of the flagella of UPTC organisms and therefore it is highly likely that UPTC

organisms are a population of the natural mutants of bacterial flagella. If so, more extensive analysis of the flagellin and flagella of other UPTC isolates from the natural environment (including wild birds and humans) may be required in order to resolve these anomalies. □

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