The gene for *Campylobacter* trigger factor: evidence for multiple transcription start sites and protein products

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A gene encoding a protein of apparent molecular mass 56 kDa that shares 31% identity with the amino acid sequence of trigger factor from *Escherichia coli* (a protein thought to be involved in cell division), was cloned from *Campylobacter jejuni* NCTC 11168. The clone was selected from a lambda ZAP II genomic DNA library following an immuno-screen using antiserum raised against glycine-extractable proteins from *C. jejuni*. The gene has two potential initiation codons, giving rise to two possible nested protein products. Complex differential growth-phase-dependent transcripts give rise to these products.

Keywords: *Campylobacter jejuni*, trigger factor, *tig*, ClpP protease, GTP cyclohydrolase

INTRODUCTION

*Campylobacter*s are an important cause of diarrhoeal disease throughout the world (Griffiths & Park, 1990) but despite many years of research, little is known about the structure and function of the majority of membrane proteins and other important protein antigens of *Campylobacter jejuni*. Due to problems of clonal instability with *Campylobacter* DNA (Walker *et al.*, 1986) progress has not been as rapid as with other pathogenic bacteria. However, some genes, including those encoding flagellin proteins and some with housekeeping functions, have now been cloned and sequenced (reviewed by Taylor, 1992).

When examining sera obtained from human patients after a recent infection with *C. jejuni* it is not uncommon to find no discernible antibody response to *Campylobacter* antigens above control levels. For example, one study, using human volunteers (Black *et al.*, 1992), found only 8/18 (44%) of individuals seroconverted in two immunoglobulin classes by ELISA (the criterion often used to discriminate a positive seroconversion from negative). The reason for the lack of seroconversion in some individuals is not known. In those individuals who do seroconvert, the sera obtained can react with *Campylobacter* whole cells and with outer-membrane preparations in Western blotting experiments, revealing several distinct proteins to be immunogenic during infection, many of

which are common to most strains (Blaser *et al.*, 1984; Mills & Bradbury, 1984; Nachamkin & Hart, 1985). However, sera from non-infected controls may also react with *Campylobacter* proteins (Blaser *et al.*, 1984; Mills & Bradbury, 1984; Nachamkin & Hart, 1985; Panigrahi *et al.*, 1992) and therefore the reactions using convalescent sera may not always be clear-cut. The common proteins consistently recognized by infected patient sera are also to be found in acid-glycine extracts (AE) of *Campylobacter* (Blaser & Duncan, 1984) and such extracts are often used in ELISA assays as a standard antigen preparation (Svedhem *et al.*, 1983; Blaser & Duncan, 1984; Herbrink *et al.*, 1988). The major components of AE are flagellin (approximately 62 kDa), acid-extractable membrane proteins and other proteins probably derived from the periplasm (Logan & Trust, 1983). Of these components most attention has focused on flagellin and its genes, which are interesting for a number of reasons; the flagellum is thought to be important in invasion (Wassenaar *et al.*, 1991), there are two full-length flagellin genes *flaA* and *flaB*, and expression of flagella is subject to antigenic variation, as a result of rearrangement of flagellin sequence information together with rDNA sequences (Guerry *et al.*, 1990, 1992). The presence of duplicated flagellin genes is thought to confer a mechanism by which the organism can maintain motility even in the event of mutation in one copy of the gene (Alm *et al.*, 1993). In addition, the maintenance of two *fla* genes probably confers structural and regulatory advantages on the organism (Guerry *et al.*, 1991). Another component of AE that has been investigated is the 28 kDa protein PEB1 (Pei & Blaser, 1993) that was originally described as a cell

Abbreviation: AE, acid-glycine extracts.

The EMBL/GenBank/DBJ accession number for the sequence reported in this paper is X85954.

binding factor, but which has a very high degree of similarity to the transport proteins of *Escherichia coli*, *glnQ* and *bisP*.

We have initiated studies on non-flagellar genes encoding antigenic proteins in AE and their relationship to pathogenicity and protective immunity. Such antigenic proteins are interesting in their own right, but in addition they may be directly or indirectly involved in the disease process. However, it is entirely possible that the immunogenicity of such proteins is purely coincidental, occurring as a result of bacterial breakdown and antigen presentation exposing epitopes that would not normally be seen by the host immune system. It is therefore necessary to closely study this group of antigenic proteins, so as to assign possible functions and to confirm or exclude their role in disease.

METHODS

Bacterial strains and growth conditions. *Campylobacter jejuni* NCTC (National Collection of Type Cultures, Colindale, UK) strain 11168 was used to construct the lambda expression library and for the transcript mapping experiments. It was cultured on blood agar base (Oxoid, CM 271) with 5% (v/v) defibrinated horse blood (Tissue Culture Services) and incubated at 37 °C under microaerobic conditions using the partial evacuation/replacement technique (Bolton & Coates, 1993). The replacement gas mix contained 10% (v/v) H₂, 10% (v/v) CO₂ in N₂ (BOC). *Escherichia coli* strain PLKF' (obtained from Stratagene) was used as the host for the cloning and expression experiments; it was grown on Luria Bertani (LB) agar or broth (Maniatis *et al.*, 1982) and incubated at 37 °C.

Production of antiserum. Acid-glycine extraction of *C. jejuni* was carried out using the method of McCoy *et al.* (1975) and protein material inoculated into adult Dutch Half Lop rabbits using Freund's adjuvants according to standard procedures. The antiserum produced was absorbed with PLKF' whole cells prior to screening the library, to remove non-specific antibodies. Convalescent serum was obtained from six patients diagnosed as suffering from *Campylobacter* enteritis.

Isolation of DNA and construction of *C. jejuni* gene library. A lambda ZAP II (Stratagene) library was constructed using sonicated 2–10 kb DNA fragments of *Campylobacter* chromosomal DNA. Sheared ends were made flush with T4 DNA polymerase. Internal *EcoRI* sites were methylated before the fragments were ligated to phosphorylated *EcoRI* linkers and cleaved with *EcoRI* to generate 'sticky ends'. The fragments were then ligated to *EcoRI* pre-prepared lambda arms and packaged into phage heads. Recombinant phage (~2 × 10⁴) were plated out, induced with IPTG and screened with antibody using established procedures (Short *et al.*, 1988). Positive clone 10a was picked and the recombinant insert excised from lambda as a pBluescript phagemid (10a1) to enable further analysis.

DNA sequence analysis. 10a1 was sequenced as a double-stranded DNA by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the modified T7 polymerase, Sequenase (USB). Convenient restriction fragments were either subcloned into pBluescript and sequenced using universal primers or 10a1 was sequenced directly using synthetic oligonucleotide primers: F1, 5'-GGATCATACCATAGC-3'; F2, 5'-CGCTCTTACAGCGG-3'; F3, 5'-GATGAATTAAGG-C-3'; F4, 5'-CCTGGTCTAAACCCATCC-3'; AP1, 5'-GAAA-TTGTGTTAAACCAATCGTTC-3'; AP2, 5'-GCAAAAT-

TCCACGCGTTGTAGAAGTG-3'; AP3, 5'-GACATTTTG-CGTATAACCACTAG-3'; P1, 5'-CACACATATGTCTTGC-3'.

Protein techniques. Whole-cell proteins of *C. jejuni* NCTC 11168 and *E. coli* with or without phagemid 10a1 were resolved by SDS-PAGE [0.1% (w/v) SDS, 12% (w/v) polyacrylamide] and either stained with Coomassie Blue R-250 or transferred to Immobilon membranes (Millipore). Western blotting was carried out using a Bio-Rad mini-transblot system according to the manufacturer's instructions. Bound antibodies were detected using anti-rabbit and anti-human IgG (Fc fragment) conjugated to alkaline phosphatase (Life Technologies) and detected with nitro blue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) as previously described (Griffiths *et al.*, 1992).

Induction of recombinant protein. An overnight culture of *E. coli* PLKF' containing the excised phagemid was inoculated into 50 ml LB broth containing 50 µg ampicillin ml⁻¹ and incubated for 2 h with shaking, until an OD₆₀₀ of 0.5 was reached. After this time 10 mM IPTG was added and incubation continued for a further 2–3 h. The culture was then cooled on ice, centrifuged at 3000 g and the cells immediately added to SDS-PAGE loading buffer. After boiling for 3 min, samples were resolved by SDS-PAGE.

Cell fractionation studies. In order to determine the location of the recombinant protein in *E. coli*, induced cells were fractionated into outer-membrane and cytosolic fractions and examined by Western blotting. Cells were disrupted by sonication, membranes were separated by centrifugation and the supernatant fluid (cytosolic fraction) was collected. Outer membranes were prepared by the method of Carlone *et al.* (1986). Periplasmic shock proteins were prepared as described by Logan & Trust (1983).

N-terminal amino acid sequencing. Automated gas-phase N-terminal amino acid sequencing was undertaken directly on the two protein products after resolution by SDS-PAGE and transfer to Immobilon membranes (Matsudaira, 1987).

Northern blotting. This was carried out using Amersham Hybond N nylon membranes according to the protocol provided by the manufacturer. Blots were probed with ³²P-labelled 10a1 insert, prepared using the Boehringer Mannheim Random Primed DNA labelling kit according to the manufacturer's instructions.

Transcript mapping. Transcription start points were determined by primer extension. Total RNA for primer extension was extracted from cells of *C. jejuni* NCTC 11168 in the exponential, late-exponential and stationary growth phases, as determined from contemporaneous growth curves of viable counts. Briefly, cells were grown with shaking in Nutrient Broth no. 2 (Oxoid; CM67) at 37 °C in gas jars and viable counts were made at appropriate intervals. Cells from each time point were examined by light microscopy after heat fixing and staining with crystal violet as previously described (Griffiths, 1993). Approximately 100 mg cells were resuspended in 0.5 ml lysis buffer (0.6 M NaCl, 10 mM EDTA, 100 mM Tris/HCl pH 8.0), and lysed by addition of 0.25 ml 10% (w/v) SDS. An equal volume of TE (10 mM Tris/HCl pH 8.0, 1 mM EDTA) saturated phenol was then added and the tubes were shaken for 15 min at room temperature. Following centrifugation at 10000 g for 5 min the aqueous phase was transferred to a clean tube and extracted twice with diethyl ether to remove traces of phenol. An equal volume of 8 M LiCl was then added and the tubes incubated overnight at 4 °C. The tubes were vortexed briefly and centrifuged at 10000 g for 10 min. The pellet was

resuspended in a mixture of 0.3 ml H₂O, 0.03 ml 3 M sodium acetate pH 5.2 and 0.75 ml ethanol. After storage at -70 °C for 30 min the tubes were centrifuged at 10000 *g* for 10 min. The pellet was washed with 70% (v/v) ethanol then dried and redissolved in 20 µl diethyl-pyrocabonate-treated H₂O. The oligonucleotide primer F4 was radioactively end-labelled by phosphorylation with [γ -³²P]ATP (3000 Ci mmol⁻¹, 111 TBq mmol⁻¹; Amersham) using T4 polynucleotide kinase (Gibco-BRL). The radiolabelled primer was hybridized to approximately 20 µg total *Campylobacter* RNA for 2 h at 37 °C in reverse transcriptase buffer (Gibco-BRL). Following hybridization, dNTPs were added (1 mM of each) and the DNA/RNA hybrids were then incubated for 2 h at 37 °C with 100 units Superscript reverse transcriptase (Gibco-BRL). A 10 µl aliquot was added to 4 µl sequencing gel loading buffer and the samples were heated to 98 °C for 10 min before loading onto a 8% polyacrylamide/urea sequencing gel alongside a sequencing reaction of phagemid 10a1 DNA primed with the same primer. *Campylobacter* RNA samples subjected to RNase treatment did not act as a substrate for the reverse transcriptase.

Isolation of overlapping clones. In order to obtain sequence upstream from the primary clone, DNA/DNA hybridization was used to screen plaques from the lambda ZAP II library. The entire 1.6 kb fragment from phagemid 10a1 was labelled with [α -³²P]dATP using a random prime kit (Megaprime, Amersham) according to the manufacturer's instructions. DNA from 2 × 10⁴ plaques was bound to nylon membranes (Hybond-N, Amersham) according to the manufacturer's instructions. The probe DNA was hybridized to the plaque DNA at 65 °C and

washed using stringent conditions according to Amersham's protocol. Following autoradiography, positive plaques were picked and phagemids containing recombinant inserts were excised as before, in order that the DNA sequence could be determined. Southern blots of genomic DNA were also performed to demonstrate that the overlapping sequences possessed co-linear restriction sites with the cloned DNAs. These blots demonstrated that no additional unaccountable restriction fragments hybridized to the trigger factor probe. The sequences are therefore likely to be unique within the *Campylobacter* genome.

RESULTS

Immunoscreening of *Campylobacter* gene library

A recombinant library containing random chromosomal fragments of *C. jejuni* NCTC 11168 in lambda ZAP II was constructed. Approximately 20000 plaques from this expression library were screened with serum raised in rabbits to AE. The anti-AE serum detected four positive plaques from the library. *In vivo* excision of one of these positive lambda clones (10a) to give phagemid 10a1 allowed over-expression of the recombinant protein in *E. coli* in response to IPTG induction. SDS-PAGE of IPTG-induced *E. coli* demonstrated the expression of the recombinant protein, clearly showing a polypeptide of approximately 56 kDa with a second less intense band of approximately 52 kDa (Fig. 1a). Both the 56 kDa and the 52 kDa bands reacted with anti-AE serum on Western

Fig. 1. *In vivo* excision of lambda clone 10a to give a phagemid 10a1 allowed IPTG-induced expression of the recombinant protein in *E. coli* PLKF'. Lanes: C, whole cells of control strain without phagemid; I, induced whole cells of strain with phagemid 10a1. Arrowheads indicate 56 kDa and 52 kDa recombinant proteins attributable to the 10a1 coding sequences. Other additional protein bands were equally reproducible upon induction of a phagemid-containing control. (a) SDS-PAGE gel (12%) stained with Coomassie blue; (b) Western blot of whole-cell proteins incubated with rabbit anti-AE serum; (c) Western blot of whole-cell proteins incubated with human serum from a patient infected with *C. jejuni*.

Fig. 4. Morphology of *E. coli* cells expressing *Campylobacter* trigger factor. (a) *E. coli* host PLKF' stained with carbol fuchsin following induction with IPTG (0.4 mM); (b) PLKF' cells carrying the phagemid 10a1 treated as above. Bar, 10 µm. Alternative PLKF' cultures bearing the phagemid alone or bearing other recombinant *Campylobacter* phagemids did not demonstrate the elongated cell phenotype attributable to the *Campylobacter* trigger factor gene.

may be responsible for the discrepancy. The putative *Campylobacter* trigger factor was found to be associated mainly with the cytosol of *E. coli* expressing recombinant protein; it was also detected by Western blotting in outer membrane and periplasmic fractions prepared as detailed in Methods (data not shown).

Determination of the sequence upstream from *Campylobacter* *tig*

Two clones were obtained by DNA/DNA hybridization (using 10a1 insert to probe the lambda ZAP II library) that overlapped the 10a1 clone with additional sequence upstream. One of these, phagemid 7c1, contained a 1.4 kb insert which overlapped 10a1 by approximately 0.7 kb, giving 0.7 kb of new sequence. The DNA sequence of this clone was determined and analysed as before. A restriction map of 7c1 is shown in Fig. 2(a); the sequences additional to phagemid 10a1 are shown as bases -1 to -662 in Fig. 2(b). The additional DNA sequence revealed an ORF translated from the opposite direction to *tig*, encoding a protein of 178 codons. The translated sequence was found to show 53% identity to human GTP cyclohydrolase I,

which catalyses dihydroneopterin triphosphate formation from GTP (Togari *et al.*, 1992). The protein sequence also showed 48.3% and 39.4% identity, respectively, to the equivalent ORFs of *Bacillus subtilis* (*mtrA*) and *E. coli* (*folE*) (Gollnick *et al.*, 1990; Katzenmier *et al.*, 1991). The arrangement of these genes in *Campylobacter* is different from that of *E. coli*, where the gene immediately upstream, and translated in the same direction as *tig*, is *bolA*. The *bolA* gene has been shown to possess a growth-phase-dependent promoter that is activated in stationary phase (Aldea *et al.*, 1989).

Cell division defect and growth phase regulation

Trigger factor may have a role in cell division, possibly acting as a chaperone to other proteins involved in septation (Guthrie & Wickner, 1990). Incomplete septation was observed by Guthrie & Wickner (1990) in trigger-factor-depleted cells and in cells over-expressing the native *tig* gene. These effects were suppressed by co-overexpression of the crucial cell division gene *ftsZ*. *Campylobacter* have been observed to adopt filamentous forms on transition from exponential to stationary phase (Griffiths, 1993), implying that certain cell division genes are affected by growth phase. In addition, Tai *et al.* (1992) reported that levels of the protein encoded by *secI* (*tig*) increased on entry to stationary phase in *E. coli*.

We therefore examined transcription of the *Campylobacter* *tig* gene with respect to growth phase and cell division. It was also noted that overproduction of *Campylobacter* trigger factor in *E. coli* resulted in the elongation of the host cells (Fig. 4). RNAs were isolated from campylobacters harvested after culture in liquid medium during the exponential, late-exponential, stationary and death phases. As described by Griffiths (1993) the campylobacters became more filamentous in form upon entering late-exponential phase, eventually adopting coccoid morphology at death phase. We could not isolate RNA from such coccoid cultures. The total transcriptional levels detected on Northern transfers of the growth-phase-specific RNA preparations did not indicate a clear relationship between growth phase and changes in transcript levels detected. The Northern blot also failed to provide clear evidence as to the programmed use of the second AUG which gives rise to the shorter translation product observed. However, from the Northern blots the transcript size could be estimated to be 2.2 kb (Fig. 5), somewhat larger than that required for the *tig* gene alone. Significantly, the upstream ORF (GTP cyclohydrolase I) is encoded in the opposite strand, implying that two divergent transcripts arise from the intergenic region. It is therefore likely, based on the size of the *tig* transcript, that it is co-transcribed with the downstream *clpP* gene.

Transcript mapping

The *Campylobacter* *tig* gene encodes two polypeptides, of 410 and 388 amino acids, each initiated by an AUG codon (nucleotides 106 and 172) and terminated by the chain-termination codon UGA (nucleotide 1336). Putative ribosome-binding sites are indicated in Fig. 2(b) directly

Fig. 5. Northern blot of *Campylobacter* RNA probed with ^{32}P -labelled cloned DNAs. RNA was extracted and subjected to electrophoresis on a formaldehyde agarose gel as described in Methods. Molecular sizes (in kb) are indicated on the left; the positions of the *tig* transcript (T) at 2.2 kb and 235 and 16S rRNAs are indicated on the right (these were initially identified independently of the *tig* transcript and represent internal controls).

upstream from the initiation codons. As the Northern blot provided little information regarding regulation of the *Campylobacter* *tig* gene or the use of the alternative initiation codons, transcript mapping was therefore undertaken. The *tig* gene transcription start sites were determined by primer extension from RNAs isolated during the mid-exponential and early stationary phases of growth (Fig. 6). The evidence suggests there are three main transcription start sites utilized during exponential growth, the first (P1) initiating at nucleotides 29/31, the second (P2) at nucleotide 78 and the third (P3) at nucleotide 163. The P3 start site is inside the reading frame specified by the first initiation codon. This shorter transcript will therefore translate from the second in-frame AUG using the preceding putative ribosome-binding site. It is notable that this promoter continues to operate into stationary phase whereas the P1 and P2 promoters, specifying the larger translation product, begin to decline. Examination of the putative -10 and -35 promoter elements revealed no significant homology with those common for other bacterial genera. The -13 guanine-centred consensus sequence (TTGCT) attributed to σ^{54} -dependent function is present upstream of the P2 transcript. The P1 and P3 promoters also feature similar sequences further upstream, at -20 and -15, respectively. However, the consensus guanine residues at -24 and -25 for the σ^{54} promoter are either absent or shifted by 2 bp in the case of the P2 promoter. The σ^{54} -RNA polymerase holoenzyme transcribes a diverse set of genes from various bacterial species, including those whose products are involved in nitrogen assimilation, those which encode pilins necessary for human cell

Fig. 6. Mapping the transcripts of the *Campylobacter* *tig* gene at two different phases of growth. Primer extension products were generated after reverse transcription of total RNAs isolated from exponential (1) and early stationary phase (2) cells. The primer extension products were co-electrophoresed on an 8% sequencing gel against dideoxy sequencing reactions primed with the same primer (F4).

attachment in pathogenic species (Kustu *et al.*, 1989) and, perhaps significantly, the weaker of the two flagellin gene promoters (*flaB*) from campylobacters (Guerry *et al.*, 1991). The upstream sequences of *Campylobacter* *tig* and the published sequence of *flaB* are compared in Fig. 7, showing the similarity of the two sequences. The promoter structure of the *Campylobacter* *tig* gene suggests that it contains sequences that allow for closely regulated, probably growth-phase-dependent, transcription. It is notable that the amino acid homology observed with *E. coli* begins after the N-terminal extension directed from the first AUG. High-titre antiserum to purified *E. coli* trigger factor did not react with the *Campylobacter* homologue by Western blotting, despite the amino acid sequence similarity.

Fig. 7. Alignment of upstream regions of *Campylobacter tig* with the published sequence of *flaB* (Guerry *et al.*, 1991). Transcription start sites are indicated by arrows; -10 and -35 regions are boxed; putative ribosome-binding sites are underlined; also underlined are the GG pairs associated with σ^{54} function.

DISCUSSION

Trigger factor in *E. coli* was originally identified as one of a number of proteins that were important in translocation of proOmpA across the cytoplasmic membrane (Crooke & Wickner, 1987; Lill *et al.*, 1988). Later it was found that although trigger factor co-purified with proOmpA it was not in fact necessary for translocation but could maintain proOmpA in a conformation capable of membrane translocation (Crooke *et al.*, 1988a, b). When the *tig* gene was isolated, allowing construction of strains where expression of trigger factor is independently regulated, it became apparent that the main physiological effect of altering levels of trigger factor in cells was to inhibit normal cell division, leading to filamentation. Since the filamentation occurring in cells either depleted for or overproducing trigger factor can be suppressed by simultaneous over-expression of the essential cell division gene *ftsZ*, it has been suggested that trigger factor has an important role in cell division, possibly acting as a chaperone (Guthrie & Wickner, 1990). Similarly over-expression of the *Campylobacter tig* gene also leads to the filamentation of *E. coli* cells. It has also been proposed that the product of the *tig* gene (described as SecI), has a regulatory function in protein translocation across bacterial cytoplasmic membranes (Tai *et al.*, 1992). The precise physiological function of this essential gene product remains obscure.

Campylobacter trigger factor is clearly highly immunogenic in rabbits immunized with AE and reacts with human convalescent sera. The less abundant shorter version of the *Campylobacter tig* gene product was not detected on Western blots with human convalescent sera. This may reflect the relative abundance of the two proteins in the *Campylobacter* cell. However, it is notable that the 22-amino-acid sequence that distinguishes these products has little similarity with the *E. coli* protein: proper alignment of these sequences is only possible after the second *Campylobacter* initiating methionine (Fig. 3a).

As in *E. coli*, the ORF following *tig* in *Campylobacter* encodes ClpP, the proteolytic component of the ATP-dependent Clp protease which is thought to be involved in the turnover of aberrantly folded proteins (Squires & Squires, 1992). Considering the size of the *Campylobacter* mRNA (2.2 kb) and the determined transcription start sites, the *clpP* gene is likely to be co-transcribed with *tig*. Whether *clpP* is also independently transcribed from within the short non-coding region between the reading frames has not yet been determined. However, considering the relative abundance of the trigger factor

protein in the cell and its possible growth-phase-dependent regulation, the coordinate of ClpP protease is likely to have a critical physiological significance in the control and turnover of misfolded proteins under sub-optimal growth conditions.

The *Campylobacter tig* gene was expressed in *E. coli* without some of the problems encountered by others when expressing *Campylobacter* genes in foreign hosts. *C. jejuni* trigger factor has been identified as a major antigenic component in acid-glycine extract, which is often used as a standard antigenic preparation in serological studies of the species. The gene shows several interesting features, including multiple transcription start sites, co-transcription, an unusual σ^{54} -like promoter, and growth-related regulation.

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