INTRODUCTION

Campylobacters 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, 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 et al., 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
binding factor, but which has a very high degree of similarity to the transport proteins of Escherichia coli, glnQ and hisP.

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 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 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 x 10^8) 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 with crystal violet as previously described (Griaths, 1993). This was carried out using Amersham Hybond N nylon membranes according to the protocol

**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 washed with 80% ethanol and the DNA was dissolved in sterile water. The DNA was then ethanol precipitated, washed, dried, and dissolved in water to a final concentration of 50 μg ml⁻¹. DNA sequencing was performed on a 373A DNA sequencer (Applied Biosystems) using the Chain Terminator method.
Campylobacter trigger factor

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-pyrocarbonate-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). 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 lOal 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 DNA primed with the same primer. Campylobacter RNA samples subjected to RNase treatment did not act as a substrate for the reverse transcriptase.

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 (10a1) 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 PLK2. 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.](1361)
Complete nucleotide sequence of the insert of clone 10a1 (nucleotides 1 to 1583) together with the upstream region from clone 7c1 (nucleotides -1 to -662) showing that the direction of translation of the *Campylobacter folE* gene is in the opposite direction to that of the *Campylobacter tig* gene. (a) Restriction map of phagemids 10a1 and 7c1 and region of overlap showing sequencing strategy of the ORFs. (b) Nucleotide sequence and deduced amino acid...
By contrast, sera from two further convalescent patients gave no reaction with either polypeptide, which is perhaps not surprising considering the variability of the human response (see Introduction).

Characterization of cloned gene

Phagemid 10a1 contained a DNA insert of approximately 16 kb, the complete sequence of which revealed an open reading frame (ORF) of 410 codons (nucleotides 1–1583; Fig. 2a, b). The sequences were compared to existing sequence databases (PIR release 42.0 and SwissProt release 30.0) using the programs FASTA and BLASTX (Pearson & Lipman, 1988; Altschul et al., 1990). The ORF had an amino acid identity of 31% with E. coli trigger factor (Fig. 3a). Trigger factor, encoded by the tig gene, was discovered in E. coli by its ability to stabilize proOMP in a membrane assembly competent form but later was found not to play a direct role in the in vivo synthesis and export of proOMP (Guthrie & Wickner, 1990). N-terminal amino acid sequence data of the 56 kDa recombinant protein (underlined in Fig. 3a) confirmed the expression of the ORF. The N-terminal sequence was also determined for the 52 kDa protein observed by SDS-PAGE. Because this protein was found to commence with a methionine and to possess an N-terminal sequence corresponding to the initiation of E. coli trigger factor it is most likely that it arises from an internal initiation codon producing a similar protein product, less the first 22 amino acids (also underlined in Fig. 3a). However, the possibility that it is formed by proteolytic processing of the 56 kDa protein has not yet been ruled out. A smaller ORF containing a truncated sequence of 49 codons fused to the vector was also identified in the database search. The truncated reading frame had an amino acid sequence identity of 31% with ClpP of E. coli. ClpP is the proteolytic component of the ATP-dependent Clp protease of E. coli (Maurizi et al., 1990). The gene product of clpP, like that of tig, has been suggested to have a chaperone function and the two genes are similarly sequentially organized in E. coli (Squires & Squires, 1992). Their comparative conservation may be related to a need to express the chaperones coordinately in response to growth conditions. Interestingly, the pre-sequence observed in E. coli ClpP is missing from the Campylobacter N-terminal sequence.

The polypeptide product of the 410-codon ORF has a predicted molecular mass of 47,226 Da, somewhat smaller than that observed from SDS-PAGE gels. Trigger factor from E. coli also demonstrated this discrepancy, with a molecular mass of 60 kDa estimated from SDS-PAGE (Crooke & Wickner, 1990) and an ORF encoding a protein of 47,993 Da (Guthrie & Wickner, 1990), a similar size to the Campylobacter protein. The large proportion of charged residues found in the predicted protein sequences of the ORFs of the inserts in clones 10a1 and 7c1. The ORF for the folE gene homologue extends from nucleotides 70 to 596. The ORF for the tig gene homologue extends from nucleotides 106 to 1336. The ORF for the truncated clpP gene homologue starts at nucleotide 1435. Putative ribosome-binding sites are underlined. Transcription start sites determined by primer extension are marked with solid arrows and their corresponding putative promoter elements are boxed. The two potential initiation codons for the tig ORF are marked 1 and 2 in reverse field.
which catalyses dihydronopterin 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 fitZ. Campylobacters 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 coccal morphology at death phase. We could not isolate RNA from such coccal 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 cipP 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 32p-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 165 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 σ54-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 σ54 promoter are either absent or shifted by 2 bp in the case of the P2 promoter. The σ54-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 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.
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 (Crockett & 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 form capable of membrane translocation (Crockett et al., 1988a, b). When the *tig* gene was isolated, allowing construction of strains where expression 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 *fliZ*, 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 suboptimal 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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