Transposon mutagenesis in a hyper-invasive clinical isolate of *Campylobacter jejuni* reveals a number of genes with potential roles in invasion

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Transposon mutagenesis has been applied to a hyper-invasive clinical isolate of *Campylobacter jejuni*, 01/51. A random transposon mutant library was screened in an *in vitro* assay of invasion and 26 mutants with a significant reduction in invasion were identified. Given that the invasion potential of *C. jejuni* is relatively poor compared to other enteric pathogens, the use of a hyper-invasive strain was advantageous as it greatly facilitated the identification of mutants with reduced invasion. The location of the transposon insertion in 23 of these mutants has been determined; all but three of the insertions are in genes also present in the genome-sequenced strain NCTC 11168. Eight of the mutants contain transposon insertions in one region of the genome (~14 kb), which when compared with the genome of NCTC 11168 overlaps with one of the previously reported plasticity regions and is likely to be involved in genomic variation between strains. Further characterization of one of the mutants within this region has identified a gene that might be involved in adhesion to host cells.

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INTRODUCTION

Campylobacter jejuni is the main cause of human acute bacterial enteritis in the developed world. In England and Wales in 2008 there were 49 880 reported cases of *C. jejuni* gastroenteritis, according to the Health Protection Agency Centre for Infections; this is thought to be a significant underestimate (Tompkins *et al.*, 1999). The disease itself is usually self-limiting and the main symptoms in developed countries, such as the UK and USA, are abdominal pain and diarrhoea, often with mucus and blood in the stool (Ketley, 1997; Tompkins *et al.*, 1999; Wassenaar & Blaser, 1999). Nevertheless this organism is still the most common cause of food-borne intestinal infectious disease and is a significant public health burden.

C. jejuni is an invasive organism and there is much evidence, from both *in vivo* and *in vitro* studies, that invasion is a virulence mechanism used by campylobacters (De Melo *et al.*, 1989; Fauchere *et al.*, 1986; Garrity *et al.*, 2005; Klipstein *et al.*, 1985; Konkel & Joens, 1989). In the absence of a suitable animal model that mimics human disease (Newell, 2001), invasion has been studied using *in vitro* cell culture. There have been many reports of *in vitro* cell culture models of invasion for campylobacters and these have recently been reviewed (Friis *et al.*, 2005). As in

other enteropathogens, virulence varies between strains of *C. jejuni*. This has been observed for invasion and adhesion as well as toxicity (Abuoun *et al.*, 2005; Everest *et al.*, 1992; Gilbert & Slavik, 2004; Konkel & Joens, 1989; Newell *et al.*, 1985; Wassenaar, 1997). A number of hyper-invasive clinical strains of *C. jejuni* have recently been identified (Fearnley *et al.*, 2008). These isolates were found to invade the human epithelial cell lines INT-407 and Caco-2 to significantly higher levels (>25-fold) compared with a low-invasive control strain of *C. jejuni* 81116 (NCTC 11828). These strains provide a unique opportunity to investigate the molecular basis of invasion, as any reduction in invasion would be much easier to identify compared to using low-invasive strains like 81116. It should, however, be noted that these hyper-invasive strains may use different invasion strategies from those of other non-hyper-invasive strains, and so the role of any genes in invasion should be confirmed using strains with varying levels of invasion.

Molecular techniques to investigate virulence mechanisms are now available for use in *C. jejuni*. Over the last few years the development of transposon mutagenesis for *C. jejuni* has advanced significantly and several methods are now available for the random mutagenesis of this organism (Colegio *et al.*, 2001; Golden *et al.*, 2000; Golden & Acheson, 2002; Hendrixson *et al.*, 2001; Hendrixson & DiRita, 2004). One method in particular utilizes an *in vitro* method of

Abbreviation: SNP, single nucleotide polymorphism.

transposition, using a *mariner*-based transposon, followed by natural transformation to introduce the mutated genomic DNA back into the host strain (Grant *et al.*, 2005). This is a particularly useful technique, as many strains of *C. jejuni* will not take up heterologous DNA. Previously transposon mutagenesis has been limited to a handful of those strains for which genetic manipulation is relatively easy, and many of these strains have a relatively low invasion potential. However, using this *in vitro* system a transposon mutant library has been constructed in a hyper-invasive clinical isolate of *C. jejuni*, strain 01/51 (Fearnley *et al.*, 2008), providing a unique opportunity to investigate the molecular basis of invasion in a strain with a significantly high level of invasion.

The aim of this study was to investigate the molecular basis of host cell invasion in *C. jejuni* by applying transposon mutagenesis to a hyper-invasive strain. We report the screening of this mutant library in an *in vitro* assay of invasion and the identification of a number of previously uncharacterized genes that have a role in invasion. Mutants were selected for confirmatory assays and further study on the basis of their reduced level of invasion and maintenance of motility compared to the wild-type strain 01/51. We also identify a region of the *C. jejuni* genome known to be variable between strains (Pearson *et al.*, 2003) in which several genes with a role in invasion are located.

METHODS

Bacterial strains and growth media. Six clinical *Campylobacter jejuni* isolates were used in this study: 01/38, 01/41, 01/51, 01/35, 01/10 and 01/04; all of these were human faecal isolates apart from 01/10 and 01/04, which were isolated as a blood culture from patients with bacteraemia. These clinical strains were selected on the basis that they were all hyper-invasive (>25-fold more invasive than the control strain 81116) in an *in vitro* assay of invasiveness, apart from 01/38, which was highly invasive (>10-fold more invasive than the control strain 81116) (Fearnley *et al.*, 2008). Strain 01/38 was included in case it proved difficult to generate a random mutant library in one of the hyper-invasive strains. The non-motile, non-invasive mutant *C. jejuni* 81116 *flaA/flaB* (Wassenaar *et al.*, 1991) was used as a negative control in the invasion assays. NCTC 11168 and RM1221 were both used as reference strains for investigation of the TTT trinucleotide sequence (see later). All *C. jejuni* strains were routinely grown on blood agar (BA) plates (Columbia agar supplemented with 5%, v/v, sheep blood) or mCCDA agar (Oxoid) at 37 °C in a microaerobic atmosphere (10% CO₂, 5% O₂, 85% N₂, by vol.) for 24–48 h. When required, the medium was also supplemented with 10 µg chloramphenicol ml⁻¹, or 50 µg kanamycin ml⁻¹. The transposon mutants (*n*=864) were grown in each well of a 96-well plate containing 100 µl BA supplemented with 10 µg chloramphenicol ml⁻¹. *Escherichia coli* strains were grown in Luria–Bertani (LB) broth with shaking, or on LB agar plates, aerobically at 37 °C. The following antibiotics were added when required: 100 µg ampicillin ml⁻¹, 50 µg kanamycin ml⁻¹.

Generation of a random transposon mutant library. The random transposon mutant library was generated and the randomness assessed as previously described (Grant *et al.*, 2005).

In vitro adhesion and invasion assay. The semiquantitative invasion assay used was an adaptation of the gentamicin protection assay, using the human intestinal epithelial cell line INT-407

(Elsinghorst, 1994; Fearnley *et al.*, 2008). It is now generally recognized that the INT-407 cell line was contaminated with HeLa cells in the 1970s and therefore has cellular markers consistent with this contamination (Lacroix, 2008); however, it is still widely used for studying invasion and in our study it was used as a preliminary screen, with confirmation of any mutants of interest using the alternative cell line Caco-2. For preliminary screening of the transposon mutants a 96-well plate assay was adapted from that described by Golden & Acheson (2002). Briefly, the frozen transposon mutant library was thawed on ice and 10 µl of each mutant transferred to BA (100 µl) supplemented with chloramphenicol (10 µg ml⁻¹) in each well of a 96-well plate. The plates were incubated at 37 °C microaerobically for 48 h. Meanwhile 200 µl of INT-407 cells at a density of 1×10^5 cells ml⁻¹ was seeded to each well of a fresh 96-well plate and incubated for 48 h at 37 °C in 5% (v/v) CO₂ to allow the cells to grow to confluency. On the day of the assay, the cell culture medium covering the INT-407 cell monolayers was replaced with 200 µl fresh pre-warmed complete cell culture medium (CCCM). Fresh CCCM (100 µl) was also added to each well of the plate containing bacterial growth and left for 5–10 min for the colonies to soften. The bacterial cells were resuspended by pipetting and 20 µl of the suspension was added to the 200 µl of medium in each well covering the INT-407 monolayers. Given the number of mutants tested in this way it was very difficult to normalize the starting bacterial cell concentration; however, any mutants that did not grow well prior to the assay were noted and removed from further study. Infected plates were incubated for 3 h at 37 °C in 5% (v/v) CO₂. After incubation, monolayers were washed and then 200 µl CCCM supplemented with 250 µg gentamicin ml⁻¹ was added. Following 2 h of incubation, monolayers were washed and the cells lysed with 100 µl 1% (v/v) Triton X-100. The total number of bacteria per well was determined by viable count and plating onto BA plates. *C. jejuni* 01/51 and *C. jejuni* 81116 *flaA/flaB* (Wassenaar *et al.*, 1991) were also used as reference parent strain and negative control, respectively, in the assays. The preliminary invasion screening assay was repeated three times independently. Mutants that consistently showed a reduced recovery compared to the parent strain 01/51 following the assay were selected for further characterization, including motility and confirmation of the reduced invasion phenotype using a standardized assay as described below.

For a more quantitative analysis and confirmation of the invasion of selected mutants, standard gentamicin protection assays (confirmatory assay) using both INT-407 and Caco-2 cells in 24-well plates were carried out, in which each mutant was assayed in triplicate. The mutants were grown on BA plates for 48 h and were added to the monolayers at an m.o.i. of 100. To determine the number of associated bacteria, the INT-407 cells were lysed after the initial 3 h incubation period and the bacterial cells enumerated by plate count. This gives the total number of bacteria that are associated and internalized. The number of associated bacteria was then calculated by subtracting the number of internalized bacteria from the total number counted. Invasion efficiency was expressed as the percentage of the inoculum that survived the gentamicin treatment.

Motility assay. Bacterial motility was tested as described previously (Fearnley *et al.*, 2008). The parent strain 01/51 was assumed to have 100% motility (with an average diameter of the zone of growth of 5.5 cm) and only mutants with ≥75% motility as compared to the parent strain were selected for further study.

Location of transposon insertions. To determine the location of the transposon insertion point a plasmid rescue technique was carried out (Grant *et al.*, 2005). Briefly, the genomic DNA from each of the mutants was isolated and digested to completion with *Bgl*II or *Ssp*I. The genomic DNA fragments were then self-ligated and transformed by electroporation into *E. coli* S17- λ pir (Simon *et al.*, 1983). Plasmids

were prepared using the QIAprep spin miniprep kit (Qiagen) from a 10 ml overnight culture. The protocol was carried out according to the manufacturer's recommendations for large plasmids, which meant that the DNA was eluted from the spin-column using water heated to 70 °C to maximize recovery. Recovered plasmid was then concentrated by ethanol precipitation and resuspended in 5 µl distilled H₂O. The insertion site was identified by DNA sequencing using a transposon-specific primer (5'-CCCGGAATCATTTGAAG-3').

SNP detection assay. A single nucleotide polymorphism (SNP) detection assay to detect the observed polymorphisms in one of the mutated genes (Cj0490) was developed based on one previously reported (Abuoun *et al.*, 2005). Primers were designed to the region flanking the TTT trinucleotide sequence (Fig. 2a) (FWD 5'-AAAGAGCGATTGAAGC-3'; REV 5'-CATTAAGAACTTCGGTTAAGA-3') and the probe was generated from the 01/51 sequence, i.e. containing the TTT trinucleotide sequence (underlined), but on the complementary strand (5'-Cy5-GCATTTTTGCGTATTAAACTAGCT-biotin-3'). The amplification was performed as follows: an initial denaturation step for 10 min at 95 °C, followed by 41 cycles of 10 s at 94 °C, 15 s at 55 °C and 10 s at 74 °C. Melting curve analysis was performed immediately after amplification by heating the product to 94 °C (20 °C s⁻¹), cooling to 45 °C for 15 s, and then heating to 85 °C (0.1 °C s⁻¹). DNA from 01/51 and NCTC 11168, and a no-template DNA control, were included in each run as controls. NCTC 11168 was found to have a melting temperature of 61 °C, indicating the presence of the TT dinucleotide sequence, and 01/51 had a higher melting temperature of 64 °C, which indicated that the probe and sequence were identical, i.e. that 01/51 possesses the TTT trinucleotide sequence.

Mutagenesis of Cj0497. For mutagenesis of Cj0497 the gene was amplified from *C. jejuni* 01/51 and cloned into pBluescript (Stratagene) via pCR2.1TOPO (Invitrogen), a T-tailed cloning vector. The following primers were used for the amplification of Cj0497: Cj0497F (5'-TTGATTAAAGGGTTATGAAGGC-3') and Cj0497R (5'-AGCCTTAATCACATCTTTGG-3'). There was a unique *Bgl*III site in Cj0497 into which the *Campylobacter coli* kanamycin cassette, from pJMK30 (van Vliet *et al.*, 1998) (kindly provided by Professor Julian Ketley, University of Leicester, UK), was cloned. Constructs with the kanamycin cassette in the same orientation as the disrupted gene (as determined by PCR analysis), to minimize the risk of polar effects since the kanamycin cassette does not bear transcription-termination sequences, were introduced into *C. jejuni* 01/51 by electroporation. Mutants were confirmed by PCR and Southern blot analysis. The approach of inserting the kanamycin cassette, lacking transcription termination sequences, in the same orientation as the gene to be inactivated has been reported previously (Elvers *et al.*, 2004; Ge *et al.*, 2005; Linton *et al.*, 2002) and has been shown to be non-polar on downstream genes (Hickey *et al.*, 2000).

Phenotypic assays to characterize the Cj0497 mutant. In order to further characterize the mutant in Cj0497 a number of phenotypic assays were carried out, including microaerobic growth, aeration survival and autoagglutination. For all of these additional assays the bacteria were grown at 37 °C for 48 h on blood agar or mCCDA with 50 µg kanamycin ml⁻¹ added for the mutant. Bacterial growth was harvested from these plates and resuspended in 2.5 ml sterile PBS prior to use in the assays. All assays were carried out in triplicate.

For the growth curve an appropriate volume of this suspension was added to Mueller–Hinton (MH) broth in a 96-well plate to make a final volume of 100 µl in a well and an OD₅₇₀ of ~0.1. The plate was then incubated microaerobically with gentle shaking at 37 °C. Regular optical density readings were taken up to 30 h.

For the aeration stress assay 2 ml of the resuspended culture was used to inoculate 200 ml pre-warmed Mueller–Hinton broth (MHB). The culture was incubated microaerobically with gentle shaking at 37 °C for 24 h. Following overnight growth (approx. 10⁸–10⁹ c.f.u.ml⁻¹), the culture was exposed to atmospheric oxygen at 37 °C and incubation continued for a further 6 h. Samples were removed hourly and viable counts performed.

The autoagglutination assay was performed following the protocol described by Golden & Acheson (2002). The OD₆₀₀ of harvested cultures was adjusted to ~1.0 in PBS and the actual optical density was measured again. The bacterial suspension (2.0 ml) was transferred into sterile bijoux tubes and incubated undisturbed at 37 °C microaerobically for 24 h to allow the bacterial cells to autoagglutinate and settle to the bottom of the tube. One millilitre of the upper aqueous phase was then aspirated and the OD₆₀₀ was measured. The level of autoagglutination was calculated by subtracting the OD₆₀₀ of the aspirate collected after 24 h from the OD₆₀₀ measured at the start of incubation.

Statistical analysis. For this a paired Student's *t*-test was performed using MS-Excel software. A probability value *P*<0.01 indicated statistical significance.

RESULTS

Generation of a random transposon mutant library in a hyper-invasive strain

Six clinical strains (01/38, 01/41, 01/51, 01/35, 01/10 and 01/04) were tested for their ability to undergo random transposition using the *mariner*-based *in vitro* transposon system, which had been optimized for transposition of *C. jejuni* NCTC 11168, 81-176 and M1. The generation of a random transposon mutant library was only found to be successful in one of the clinical hyper-invasive strains, 01/51. This was a faecal isolate from a patient with *C. jejuni* gastroenteritis. No other information regarding the disease severity or symptoms is available. In order to hit every gene in the genome of 01/51 it would be necessary to screen around 4000–5000 mutants; however, it was decided to screen an initial batch of up to 1000 mutants in the first instance. The initial batch of mutants was picked and stored for future use.

The randomness of the transposon insertions was determined by randomly selecting ten mutants and carrying out Southern blot hybridizations, using a fragment of the transposon as a probe (data not shown), as well as sequence analysis to determine the location of the transposon insertion point. From this initial screen there did not appear to be any bias towards a particular region of the genome or particular strand of the chromosome for the insertion point (data not shown). This level of randomness was in keeping with other strains such as the genome-sequenced strain NCTC 11168, and strain M1. In addition the risk of siblings within the library was minimized by keeping the recovery time of the mutants, following transformation, to a minimum. It was anticipated, following analysis of 01/51 and the other strains to which this technique has been applied, that the maximum risk of siblings within the library is 2%.

Screening of the 01/51 transposon mutant library for defects in invasion and motility

The mutant library ($n=864$) was screened in an invasion assay using INT-407 cells, and 174 mutants that showed minimal invasion in the preliminary screening assays were selected to assess motility. The mutants showed a wide range of motility phenotypes, with nine mutants being non-motile and the remainder retaining motility ranging from 20 % to 174 % of the parent strain 01/51. As motility is important for invasion, an arbitrary cut-off for mutant selection was chosen and 40 mutants with ≥ 75 % motility as compared to the wild-type strain, 01/51, were selected. The reduced invasion capacity of these selected mutants was confirmed using INT-407 cells in a confirmatory standard invasion assay in which each mutant was tested in triplicate. A selection of mutants that showed a ≥ 60 % reduction in invasion in INT-407 cells were also checked for their ability to invade Caco-2 cells. All the tested mutants showed a similar reduction in invasion of Caco-2 cells compared to INT-407 cells (Table 1). The localization of the transposon insertions was investigated in 26 mutants.

Location of the transposon insertions

The transposon insertion point was identified in 23 of the 26 mutants by the plasmid rescue technique and sequencing using a transposon-derived primer (Table 1). The transposon insertion site could not be determined in the remaining three mutants even after several attempts with alternative restriction enzymes. Southern blot analysis was also performed on a random selection of mutants to ensure that a single transposon had inserted into each mutant. Analysis showed one band in every mutant lane, confirming that the transposon had inserted at one site in the genome of each mutant (data not shown).

All but three of the transposon insertions identified were found to be in genes which were also present in NCTC 11168 (Parkhill *et al.*, 2000) (Table 1). Of the remaining three insertions, one was in a gene, *dtpT* (di-/tripeptide transporter), which is present in other *C. jejuni* strains for which the genome sequences are now available, including *C. jejuni* strain RM1221 (CJE0757) and *C. jejuni* strain 81116 (C8J-0613). The second was annotated as a capsule polysaccharide biosynthesis protein in *C. jejuni* subsp. *doylei* strain 269.97. In the third mutant (10D12) the sequence obtained showed homology to a putative *rloE* gene (CJJ26094_0063) in *C. jejuni* strain 260.94, whose sequence was incomplete at the time of searching (July 2009). The function of this gene is unknown and in other *C. jejuni* strains, namely 84-25 and Tgh133, it is annotated as a putative hypothetical protein.

In 8 of the 23 mutants for which the transposon insertion point was determined, the transposon was located within a ~14 kb region of the genome between Cj0483 and Cj0499, according to the genome sequence of NCTC 11168 (Fig. 1). The gene order in 01/51 was found to be similar to that in

NCTC 11168 as determined by PCR analysis across this region (Fig. 1). Two of the genes located within this 14 kb region of the genome, Cj0490 and Cj0497, were identified as the site of transposon insertion in three and two separate mutants respectively. The position of the transposon in these two genes seems to have little effect on invasion, but a greater effect on motility (Table 1). For example the three mutants in Cj0490 were 16–18 % as invasive and 78–174 % as motile as the parent strain.

Genomic comparison of the 14 kb region amongst other strains of *C. jejuni* reveals some conservation in gene order

Comparison of the 14 kb region (Cj0483–Cj0499) of NCTC 11168 with strain RM1221, another available *C. jejuni* genome sequence (TIGR), indicated that this region is similar in the two strains. However, in RM1221 between Cj0493 and Cj0494 there is an insertion of a ~40 kb region of DNA (Fig. 1), which appears to have very few similarities to known sequences. This 40 kb region has a G + C content of 27.42 mol% compared with an average of 30.31 % for the RM1221 genome (Fouts *et al.*, 2005). Interestingly, on further analysis of the NCTC 11168 genome there is an additional small ORF located between Cj0494 and *fusA* (Cj0493) annotated as tRNA-Arg; however, it is not known whether this tRNA gene also exists in 01/51 at this position.

Cj0490 is annotated as two ORFs in NCTC 11168, but is a single ORF in 01/51

Cj0490 was one of the genes for which there were three individual transposon mutants. In NCTC 11168 Cj0490 encodes the aldehyde dehydrogenase C-terminus whereas Cj0489 encodes the N-terminus of this same protein. Comparison of this sequence with that in other bacteria, such as *E. coli*, *Shigella flexneri* and *Neisseria meningitidis*, indicated that it is unusual to find the two parts of this protein encoded by separate genes. The sequence of this aldehyde dehydrogenase was therefore determined in the hyper-invasive strain, 01/51, and interestingly this gene was also found to be a single ORF with both the N-terminus and C-terminus together, as in other bacteria. At the DNA level in NCTC 11168 a single T deletion appears to have resulted in the generation of a stop codon (Fig. 2a), which is followed by an intergenic region of 36 nucleotides before the start of the next ORF. At the amino acid level (Fig. 2b) the stop codon causes a frameshift and the appearance of the separate N- and C-termini.

The presence of the TTT trinucleotide sequence was investigated in seven *C. jejuni* strains by sequencing across the region between Cj0489 and Cj0490 and in a further 20 strains with known invasion potential using a SNP detection assay. There was no correlation between the presence of the TTT trinucleotide and the invasion potential; therefore further analysis of this gene was not carried out.

Table 1. Location of the transposon insertion point in 26 selected mutants with reduced invasion compared with the parent strain 01/51

Invasion potential and motility are also given. Mutants are arranged according to functional classification (<http://xbase.bham.ac.uk/campydb/>).

Mutant	INT-407 invasion*	Caco-2 invasion*	Motility†	Location	Annotation
Adaptations and atypical conditions					
10D2	40	ND	105	Cj0293	<i>surE</i> , stationary-phase survival
Central intermediary metabolism					
1C1	19	1	104	Cj0490	<i>ald</i> , aldehyde dehydrogenase C-terminus (571/1182)‡
1C5	16	ND	174	Cj0490	<i>ald</i> , aldehyde dehydrogenase C-terminus (139/1182)‡
1F4	18	ND	78	Cj0490	<i>ald</i> , aldehyde dehydrogenase C-terminus (929/1182)‡
1F3	26	ND	165	Cj0483	<i>uxaA</i> , altronate hydrolase C-terminus
Conserved hypothetical proteins					
10D10	3	2	76	Cj1555c	Hypothetical protein
Degradation – amino acids					
10H3	6	4	88	Cj1503c	Putative proline dehydrogenase
DNA replication, restriction modification, recombination and repair					
6A9	37	40	82	Cj0690c	Putative restriction/modification enzyme
Energy metabolism – electron transport					
3H7	2	<1	82	Cj1020c	Putative cytochrome <i>c</i>
Membranes, lipoproteins and porins					
1B5	14	ND	83	Cj0497	Putative lipoprotein (308/1278)‡
1D1	15	ND	100	Cj0497	putative lipoprotein (563/1278)‡
10H1	2	3	88	Cj1245c	Putative membrane protein
Signal transduction					
3E9	4	ND	100	<i>flgS</i>	Signal transduction histidine kinase
Surface polysaccharides, lipopolysaccharides and antigens					
3A10	1	2	88	Cj0685c	<i>cipA</i> , invasion protein
9D2	2	26	92	JJD26997_1801	Capsule polysaccharide biosynthesis protein of <i>C. jejuni</i> subsp. <i>doylei</i> 269.97
10E9	3	6	88	Cj1136	Putative glycosyltransferase
Transport – anions					
6A7	11	4	86	Cj1539c	Putative anion-uptake ABC- transport system permease
Transport – carbohydrates, organic acids and alcohols					
1D7	10	3	100	Cj0486	Putative sugar transporter
Transport – peptides					
1H10	0	2	92	<i>dtpT</i>	Di-tripeptide transporter protein not present in NCTC 11168
Unknown function/miscellaneous					
1H6	0	ND	75	Cj0519	Putative rhodanese-like domain protein; molybdopterin biosynthesis protein
3H8	3	3	82	Cj0499	Putative HIT family protein
10D12	5	4	76	<i>rloE</i>	Putative hypothetical protein, <i>C.</i> <i>jejuni</i> 260.94

Table 1. cont.

Mutant	INT-407 invasion*	Caco-2 invasion*	Motility†	Location	Annotation
10H12	1	2	96	Cj1305c	Hypothetical protein
Location not yet determined					
10A4	8	4	98		ND
10E11	2	3	94		ND
10H2	<1	<1	112		ND

ND, Not determined.

*Invasiveness is presented as a percentage of the invasiveness of the parent strain 01/51. The figure given is the mean of three replicates from within a single assay. Each assay was repeated at least once more.

†Motility is presented as a percentage of the motility of the wild-type. The figure given is the mean of three replicates.

‡Nucleotide position of the transposons in Cj0490 and Cj0497/the length of both genes (bp).

Fig. 1. Location of the transposon insertions in the 14 kb region between Cj0483 and Cj0499. ORFs with transposon insertions are shown as black arrows. The shaded inverted triangle represents the position in *C. jejuni* strain RM1221, where there is an insertion of ~40 kb. The direction of the arrow denotes the predicted direction of transcription. The dotted arrow at the bottom represents the region confirmed by PCR analysis in *C. jejuni* 01/51 as having the same gene order as NCTC 11168. This region, flanked by altronate hydrolase C-terminus (Cj0483) and a putative histidine triad (HIT) family protein (Cj499), contains genes encoding a putative MFS (major facilitator superfamily) transport protein (Cj0484), a putative oxidoreductase (Cj0485), a putative sugar transporter (Cj0486), a putative amidohydrolase (Cj0487), two conserved hypothetical proteins (Cj0488 and Cj0496), a putative aldehyde dehydrogenase N- and C-terminus (Cj0489 and Cj0490), 30S ribosomal proteins S12 and S7 (Cj0491 and Cj0492), *fusA* elongation factor G (Cj0493), a putative exporting protein (Cj0494), a putative methyltransferase domain protein (Cj0495), a putative lipoprotein (Cj0497) and *trpC*, an indole-3-glycerol phosphate synthase (Cj0498).

Targeted mutagenesis of Cj0497 in 01/51 reveals a potential adhesin

As the mutants with transposon insertions in Cj0497 (1B5 and 1D1) both showed reduced invasion, yet retained motility, compared to the wild-type 01/51, this gene was selected for further study. The gene was independently inactivated in *C. jejuni* strain 01/51 by insertion of a kanamycin cassette to confirm the observations with the transposon mutant. The resulting targeted mutant was tested in assays of association and invasion, and compared to the wild-type strain 01/51 it was found to be significantly reduced in overall association with INT-407 (4 % of inoculum associated for mutant vs 9.5 % for 01/51; $P < 0.01$) and Caco-2 cells (0.7 % of inoculum associated for mutant vs 3 % for 01/51, $P < 0.01$), and invasion into INT-407 (0.11 % of inoculum internalized vs 1 % for 01/51; $P < 0.01$) and Caco-2 cells (0.004 % of inoculum internalized for mutant vs 0.39 % for 01/51; $P < 0.01$); this suggests that the reduction in invasion in the Cj0497 mutant may be due to a reduction in adhesion. The mutant was also found to grow as well under microaerobic conditions as the wild-type strain and to survive as well under atmospheric oxygen conditions. In addition the targeted mutant was found to be 1.3 times more motile ($P < 0.001$) and 1.3 times better able to autoagglutinate ($P < 0.001$) than the wild-type strain.

DISCUSSION

C. jejuni is the most common cause of bacterial food-borne diarrhoea worldwide, but its pathogenic mechanisms are not clear. However, previous studies indicate that invasion and motility are important for campylobacter pathogenesis and not all strains have the same virulence potential. *C. jejuni* strain-dependent variability in invasion into eukaryotic cells has been reported by many researchers (Everest *et al.*, 1992; Konkel & Joens, 1989; Malik-Kale *et al.*, 2007; Newell *et al.*, 1985). The invasiveness of *C. jejuni* strains is generally low, making investigation of this property difficult; however, we recently reported the identification

Fig. 2. (a) DNA sequence of the Cj0489–Cj0490 region in NCTC 11168 compared with that in 01/51. The dark grey boxes highlight SNPs in the sequence between the two strains and the TTT trinucleotide present in 01/51 compared with the TT dinucleotide in NCTC 11168. The arrow highlights the deletion of a single T nucleotide in NCTC 11168 which results in a stop codon (TAA). The light grey and clear boxes highlight the primers and probe used in the melting curve assay respectively. (b) Predicted amino acid sequence of the Cj0489–Cj0490 region in NCTC 11168 compared with 01/51. The whole reading frame is open for 01/51 from the beginning to end. The underlined sequence is the NCTC 11168 sequence for Cj0489, terminating in a stop codon (.), which then brings about a frameshift before the start (arrow) of the Cj0490 sequence. The shaded box highlights an intervening sequence in NCTC 11168 which is not translated into the final protein sequence.

of hyper-invasive strains of *C. jejuni* (Fearnley *et al.*, 2008). A transposon mutant library was constructed in one of these hyper-invasive strains (01/51) in order to investigate the molecular basis of invasion, and 26 mutants with reduced invasion were selected for further study and identification of the genes inactivated.

Only one of the genes identified was found to be previously associated with invasion. Mutant 3A10 has a transposon insertion in *cipA* (Cj0685) a putative *Campylobacter* invasion protein). A previous study reported that a mutation in the *cipA* gene of *C. jejuni* TGH9011 resulted in reduced invasion of HEp-2 cells but there was no change in invasion of INT-407 and Caco-2 cells (Lynett, 1999). This gene was identified in our study as being involved in invasion of both INT-407 and Caco-2 cells. In previous annotations of the *C. jejuni* genome (Parkhill *et al.*, 2000) this gene was annotated as a sugar transferase with similarity to two genes involved in capsule biosynthesis (Cj1421c and Cj1422c). It is possible therefore that a mutation in this gene has altered some surface property of the organism resulting in reduced invasion; this gene is currently being investigated further.

The fact that 8 of the 23 mutants in this study had transposon insertions within one region of the genome is interesting. There was no apparent bias in the insertion point when an initial 10 mutants were screened to check

whether the transposition was random and none of those 10 mutants possessed a transposon insertion in this 14 kb region. It is possible that this 14 kb region has a role in the interaction of *C. jejuni* with its host; however, this region does appear to be associated with genomic variability. The sequence between Cj0483 and Cj0499 overlaps with one of the seven hypervariable plasticity regions, PR, previously described in the genome of *C. jejuni* which are likely to reflect the high level of phenotypic variation seen amongst the *C. jejuni* population and account for the ability of this organism to exist in a wide range of ecological niches (Pearson *et al.*, 2003). Moreover, further analysis of this region revealed that in *C. jejuni* RM1221 there appears to be a large DNA insertion at this point (CJIE2) which has integrated into the 3'-end of an arginyl tRNA gene (Fouts *et al.*, 2005) and may represent an intact prophage or a genomic island; this highlights the genetic diversity within this region.

Many of the genes into which the transposons have inserted are genes associated with metabolism and survival, e.g. *putA* (Cj1503c), a putative proline dehydrogenase/ Δ 1-pyrroline-5-carboxylate dehydrogenase, which catalyses the oxidation of proline into glutamate; *ald* (Cj0490), aldehyde dehydrogenase, involved in energy acquisition and amino acid transport; *uxaA* (Cj0483), altronate hydrolase, involved in carbohydrate metabolism; Cj0519, involved in

molybdopterin biosynthesis; *surE*, a putative stationary-phase survival protein; and *dtpT*, encoding a di-tripeptide transporter protein.

Interestingly, the aldehyde dehydrogenase gene (Cj0490) is polymorphic in those strains in which it is present and it is only present in five of the eight *C. jejuni* genome sequences currently available, suggesting that this gene is non-essential for metabolism and may even be a pseudogene (<http://xbase.bham.ac.uk/campydb/>).

The lack of well-defined virulence mechanisms in *C. jejuni* and the involvement of metabolism-associated genes with virulence phenotypes may reflect the possibility that *C. jejuni* is an opportunistic pathogen and does not possess specific virulence factors as in other bacteria, with disease resulting as a consequence of the need of the organism to grow and survive within the human host. The fact that many of these genes are part of different metabolic pathways highlights that invasion is a multi-faceted phenotype, involving many different pathways. In addition there was no difference in growth or survival of any of the 26 identified mutants compared to the wild-type strain in cell culture medium (data not shown), suggesting that the reduced invasion is not due to some alteration in growth pattern or survival.

The transposon in two mutants (1B5 and 1D1) was located within Cj0497, which is annotated as a putative lipoprotein. Further analysis of this gene sequence indicates that its product contains a signal peptide, suggesting that it might be located in the periplasm and is likely to be membrane-bound. It also contains a tetratricopeptide repeat (TPR) region, found in multi-protein complexes and transmembrane segments. TPR motifs are thought to mediate inter- and intramolecular protein interactions and occur widely in nature (Ohara *et al.*, 1999). In bacteria, proteins containing TPR repeats are thought to have a role in gene regulation, flagellar motor function and virulence (Newton *et al.*, 2007). Moreover in *Legionella pneumophila*, two genes encoding TPR-containing proteins, *lpnE* and *enhC*, have been shown to be associated with entry into human tissue culture cell lines (Cirillo *et al.*, 2000; Newton *et al.*, 2006).

Targeted insertional inactivation of Cj0497 resulted in a reduction in bacterial association and invasion; this suggests that this gene may have a role in host cell adhesion, which may lead to invasion. Further phenotypic studies indicated that the mutant was unaffected in its ability to grow under microaerobic conditions or to survive in air compared to the wild-type and so the reduction in invasion could not be attributed to either of these factors. The mutant was, however, more motile and better able to autoagglutinate than the wild-type, suggesting that a loss of this gene does not result in reduced motility or autoagglutination. A similar finding was reported previously, whereby a mutation in a known adhesin gene, *peb1A*, failed to reduce the level of autoagglutination compared to the wild-type strain (Misawa & Blaser, 2000). This could mean

that the mechanisms used by the bacteria to adhere to cells are different from those used to adhere to each other, and in the case of Cj0497, if it were to encode an adhesin, by removing it, autoagglutination was increased. The reason for this observation is unclear but it may indicate enhanced exposure of other surface molecules that are involved in the autoagglutination process.

A number of adhesins have been identified in *C. jejuni*, including PEB1 (Kervella *et al.*, 1993; Pei & Blaser, 1993), CadF (Konkel *et al.*, 1997) and JlpA (Jin *et al.*, 2001). The role of Cj0497 as encoding a lipoprotein and adhesin in *C. jejuni* warrants further investigation, as it does appear to be present in all *C. jejuni* strains tested to date and is not species-specific like *jlpA*: it is present in the genome sequences of *Campylobacter coli*, *Campylobacter upsaliensis* and *Campylobacter lari* as well as other members of the epsilon subdivision of the proteobacteria (<http://xbase.bham.ac.uk/campydb/>).

We have identified a number of previously uncharacterized genes with a potential role in host-cell invasion. The advantage of this study was that a hyper-invasive strain of *C. jejuni* was used for transposon mutagenesis, which facilitated the detection of mutants with reduced invasion. Many of the genes are annotated as metabolism-associated rather than 'virulence' genes per se and many appear to be conserved within the species. In addition this study has highlighted a region of the genome that may be involved in genomic variability between strains. Further investigation of some of the genes described through targeted mutation and complementation studies is currently under way, which will help to provide greater insight into the molecular basis of the observed variation in virulence in *C. jejuni*.

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