6. TetO analysis from multiple clinical isolates

6.1 Introduction

Campylobacteriosis is usually self-limiting and treated by replacing fluids and restoring electrolyte balance. Antibiotics are generally only used in severe infections in which case erythromycin is used as the drug of choice [207] but tetracycline may also be used [26]. *C. jejuni* resistance to erythromycin remains low among clinical isolates [208] while resistance to other antibiotics has increased in the last few decades [26]. Tetracycline resistance has increased dramatically over the past 20 years, with resistant isolates in Canada rising from 8% to 50%. The tetracycline resistance of clinical *C. jejuni* isolates has been recorded as 55% in North America and up to 95% in Thailand. In addition tetracycline has been used prophylactically and therapeutically as a feed additive for poultry [209;210]. In the Netherlands tetracycline resistance in *Salmonella* spp. was found to increase in poultry when tetracycline was administered, and decrease after a ban on the use of tetracycline for growth promotion in animal feed, although the link between resistant strains in poultry and clinically resistant isolates remains unproven [210]. Most tetracycline resistance is thought to be plasmid mediated although a chromosomally mediated tetracycline resistance determinant has been reported for *C. coli* [176]. This determinant could be transferred into tetracycline sensitive *C. coli* by natural transformation where it inserted into the same site, however this was deemed to be due to recombination rather than chromosomal insertion mediated for example by a transposon [58].

In strain M1 a chromosomally located *tetO* gene which encodes a tetracycline resistance determinant was located (section 5.2.6) and in order to investigate whether this represents a transposable element, it was decided to look at other strains that might also harbour *tetO* on the chromosome. A recent study showed that in tetracycline resistant

clinical isolates from Canada 67% contained plasmids. However, 32 strains contained *tetO* but plasmid DNA could not be isolated from these strains using three separate methods [207].

6.2 Methods

6.2.1 Strains and primers used in this study

Table 6.1: *C. jejuni* strains used in this study

strain	Location of isolation, Canada	Year of isolation	Blood in stool?	Contains pVir?
23-45	Spruce Grove, Alberta	2000	Yes	Yes
16-60	Fort Smith, Alberta	1999	No	No
16-48	Edmonton, Alberta	1999	No	No
24-50	Yellowknife	2001	No	No
24-34	Valemont	2001	Yes	No
16-02	St. Albert, Alberta	1999	No	No
25-69	Edmonton, Alberta	2001	No	No
25-25	Edmonton, Alberta	2001	No	No

Diane Taylor's group (University of Alberta, Canada) supplied DNA from 8 strains of *C. jejuni* for which plasmids had not been identified for this part of the project.

Table 6.2: Primer used in this study

Primer	Sequence 5`-3`	tm	notes
MP1d11L	CTAATAACATCCCTCAAATGC	54.1	*tetO*
MP1d11R	AATTATGGGAAACGATGAAC	54.1	*tetO*
5B3D12vL	ATATGCTAAAGAATCAGGAATG	53	*virB4* region
5B3D12vR	TATTGTCTATGCTCGAAACC	53	*virB4* region

6.2.2 Overview

Libraries of DNA from all the strains were constructed in pBACe3.6 with an average insert size of 10-15 Kb (section 2.3.2). These libraries were arrayed in duplicate onto nylon membranes (section 2.3.3) and hybridized (section 2.3.4) either to a probe generated from the *tetO* sequence of M1 or a probe from the contig containing a homologue of *virB4* from

the BAC clone sequence of strain 52472 which both showed high similarity to the pTet sequence. Clones which hybridized to either probe were then selected for end sequencing. The two end sequences were compared to the sequences of both strain NCTC 11168 and pTet using WUBLASTN to give an idea of the location of *tetO*. Strains that appeared to harbour a small chromosomal insertion were selected for BAC shotgun sequencing using the same approach as for sequencing the other BACs in this study (section 5.2.1).

6.3 Results

6.3.1 BAC end sequencing

In order to determine which strains harboured a small chromosomal insert containing the *tetO* gene BAC libraries were screened using radiolabelled probes generated from both *tetO* and a region containing a homologue of *virB4*. The *tetO* probe was used to locate BAC clones containing the tetracycline resistance determinant. The *virB4* probe was used to determine whether more plasmid DNA was present in these strains as the probe was designed to hybridize to a region approximately half way round the plasmid compared to *tetO*. If upon end sequencing the BAC clones only plasmid DNA was discovered there would be a high probability that these strains contained a tetracycline resistance plasmid (possibly chromosomally integrated) rather than a transposon type insert (**Table 6.3**).

Table 6.3: **End sequence data from BAC clones that hybridized to probes**. The 'well' column represents the well reference of each positive hybridization clone. The 'match to 11168' column represents the location on the NCTC 11168 chromosome in base pairs that the end sequences match to using WUBLASTN. Similarly the 'match to pTet' column represents the location of the end sequences on the pTet plasmid (45204bp). The 'notes' column shows likely locations of poor sequence quality reads on the pTet plasmid or NCTC 11168 chromosome, and also matches of novel DNA sequences that matched neither pTet nor the NCTC 11168 chromosome.

Strain	probe	well	match to 11168	match to pTet	notes
23-45	*tetO*	E21:1	-209370	-42331 (*tetO*)	
		D21:2	195474-203366		
		I1:2	187660-198835		
		J21:2	1332211-200302		
	virB4	no matches			
16-60	*tetO*	A21:3		-4389	~40000
		B11:3		38920-6960	
		G9:3		38067-4452	
		H21:3		36061-4544	
		A12:4	407763-421374		
		E2:4		-4761	~35342
	virB4	J10:3		15022-28846	
		D7:4		-29666	~19000
		M2:4		17812-30027	
16-48	*tetO*	D17:6		-4512	~40000
		M24:6		38093-6504	
	virB4	A17:5		18603-29929	
		N10:5		17085-29069	
24-50	*tetO*	H5:7		38922-5749	
		L10:7		38280-5604	
		E1:8		38799-4544	
		J5:8		38797-7541	
	virB4	H9:7		-29464	~19120
		P21:7		-26086	~18107
24-34	*tetO*	F13:1	190516-198933		
		I18:1	192590-199546		
		K19:1	192601-200225		
		L9:1	190462-		~198162
		L23:1	191476-198650		
		H20:2	195672-		~206077
		H23:2	192754-198835		
	virB4	no matches			
16-02	*tetO*	H3:4		33768-44445	
		O9:4		38922-5909	
	virB4	A24:3	-111653		
		B14:3	1307047-	-31580	
		H11:4	12644-	-28970	
25-69	*tetO*	D11:5		38295	~4700
		I21:5		38271-5801	
		N13:6		-6568	~40000
	virB4	H1:5	200752-	15022	
25-25	*tetO*	B5:7		36158-2645	
		E20:7		34415-3568	
		H18:7		35242-4387	
		I2:7		38813-5369	
		M15:7		35097-2795	
		O21:7		38156-4240	
		J10:8		35192-4453	
	virB4	C15:8		-26307	protein kinase
		I18:8		-29649	virB5 pCC31
		L12:8		-29524	~15685

Both strains 23-45 and 24-34 hybridized to the *tetO* probe but not the *virB4* probe. Sequences from the ends of BAC clones from these strains are anchored in the NCTC 11168 genome sequence suggesting that they harbour a small chromosomal insertion containing *tetO*.

Strains 16-60, 16-48, 24-50, 16-02, 25-69 and 25-25 seem to contain plasmid DNA as well as *tetO* as these strains also hybridized to the *virB4* probe. In these strains sequences from the ends of BAC clones containing DNA that hybridized to the *tetO* probe contained sequence complementary to pTet. This suggests that a small chromosomal insert is not present in strains 16-60, 16-48, 24-50, 16-02, 25-69 and 25-25. The *virB4* probe also hybridized to DNA from clones of these strains. Strain 25-25 appeared to contain sequence not contained in pTet with the BAC clone C15:8 end sequence encoding a predicted CDS with 37% aa id to a serine-threonine protein kinase from *Streptomyces avermitilis*. Further investigation would be required to explore the possibility that this strain contains novel DNA in the same region as plasmid DNA with homology to pTet. The BACs identified with the *virB4* probe from strains 16-02 and 25-69 have end sequence matches to NCTC 11168 which may indicate that plasmid DNA is inserted into the chromosome while the BAC end sequences from strains 16-48, 24-50 and 25-25 have no matches to strain NCTC 11168 chromosomal DNA.

6.3.2 BAC insert sequencing

DNA inserts from BAC clones I1:2 and I18:1 from strains 23-45 and 24-34 respectively were subcloned into pUC19 and sequenced using a shotgun strategy (section 5.2.1). These clones were selected for sequencing as the end sequence reads were complementary to regions on the strain NCTC 11168 chromosome consistent with the expected insert size of the BAC clones. In addition the end sequences from several other clones from these strains, which hybridized to the tetO probe, were complementary to the same chromosomal region.

Both strains 23-45 and 24-34 contained an approximately 5.5 kb insertion in a CDS orthologous to cj0203 from strain NCTC 11168 and showed 99% sequence similarity to each other with only 19 bp differences (**Table 6.4** and **Fig 6.1**).

Table 6.4: CDSs from strain 23-45 encoded on the *tetO* insert.

Locus_id	Length in aa	Putative function	Database match	Organism with match	SWALL	E-value	% id
2345_5	212	transposase	OrfA	*Helicobacter pylori*	Q9RMU7	4.5e-64	85.84
2345_6	430	unknown	OrfB	*Helicobacter pylori*	Q9RMU6	7e-92	62.88
2345_7c	57	unknown	hypothetical	*Enterococcus faecalis* tn916	Q56396	6e-14	66.66
2345_8c	639	Tetracycline resistance	TetO	*Campylobacter jejuni*	TETO_CAMJE	0	99.84
2345_9c	124	unknown	TnpV	*Clostridium difficile*	O05416	6.7e-15	44.91
2345_10c	53	Gene fragment	DNA relaxation protein	*Fusobacterium nucleatum*	Q9L9V7	9e-09	58.82

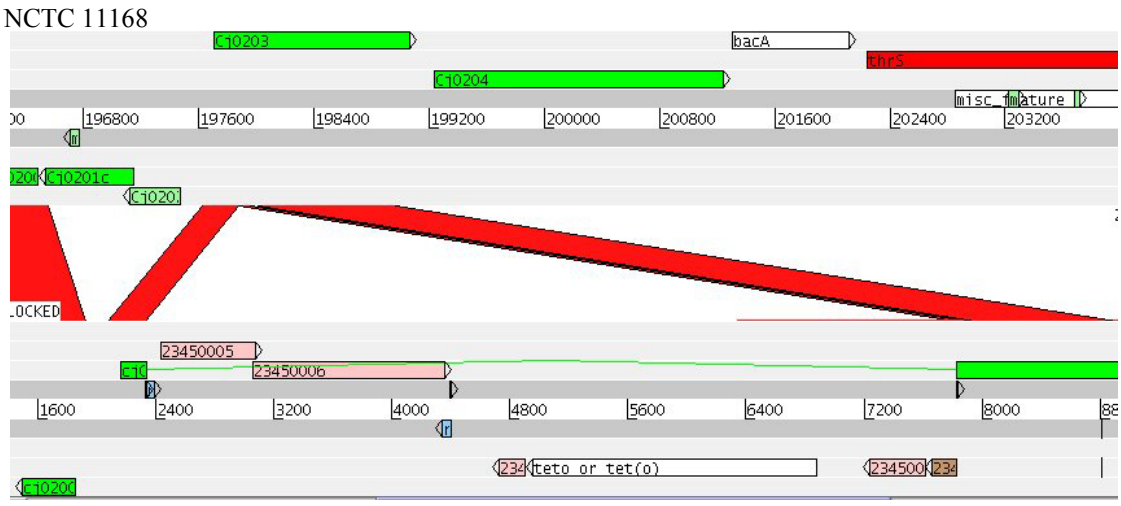

Fig 6.1: **Blastn comparison of strain NCTC 11168 compared to strain 23-45.** The comparison is viewed using ACT; blocks of red indicate sequence homology with the colour intensity proportional to the percent id of the match. Forward and reverse DNA sequences are represented by dark grey lines; the three-frame forward and reverse DNA translations are represented by light grey lines. Features are represented by open boxes: inverted repeats (blue) and target site duplications, flanking the putative transposon (2345_5 and 2345_6) and at the edges of the insert, are marked on the DNA lines; CDSs are marked on the translated frame lines. CDSs are coloured according to functional category: dark green, surface; light green, unknown; pink, bacteriophage/ IS elements; white,

pathogenicity/ adaptation/ chaperones; brown, pseudogenes and partial genes; red, information transfer/ DNA modification. In strain 23-45 cj0201c and cj0202c are missing, and 6 novel CDSs including a tetracycline resistance gene are inserted within cj0203, compared to the chromosome of strain NCTC 11168.

As the sequence from strains 23-45 and 24-34 are so similar only the sequence of strain 23-45 will be discussed further. Interestingly, within this insertion there is a putative transposon homologous to IS607 from *Helicobacter pylori* [211]. The predicted CDS 2345_5 shows 86% id to OrfA which has been shown to be necessary for transposition whilst the predicted CDS 2345_6 shows 63% id to OrfB which has no known function. These transposon associated CDSs are flanked by inverted repeats. It has also been noted that IS607 inserts either next to, or between, adjacent GG nucleotides generating a 2-bp or 0-bp target sequence duplication. This would be consistent with the insertion site in cj0203. It is possible that the transposon might have picked up the adjacent DNA from a plasmid similar to pTet containing a *tetO* homologue, although there does not appear to be an inverted repeat at the other side of the insert that could lead to extended excision.

The *tetO* gene and the CDS immediately downstream appear to be highly conserved between pTet, M1 and 23-45/24-34 while other surrounding CDSs differ. Strains 23-45 and 24-34 are both predicted to encode a CDS with homology to TnpV from *Clostridium difficile.* In strain M1 there is also a CDS with homology to TnpV although the CDS only matches to the C-terminus of TnpV suggesting that it is a fragment (**Fig 6.2**).

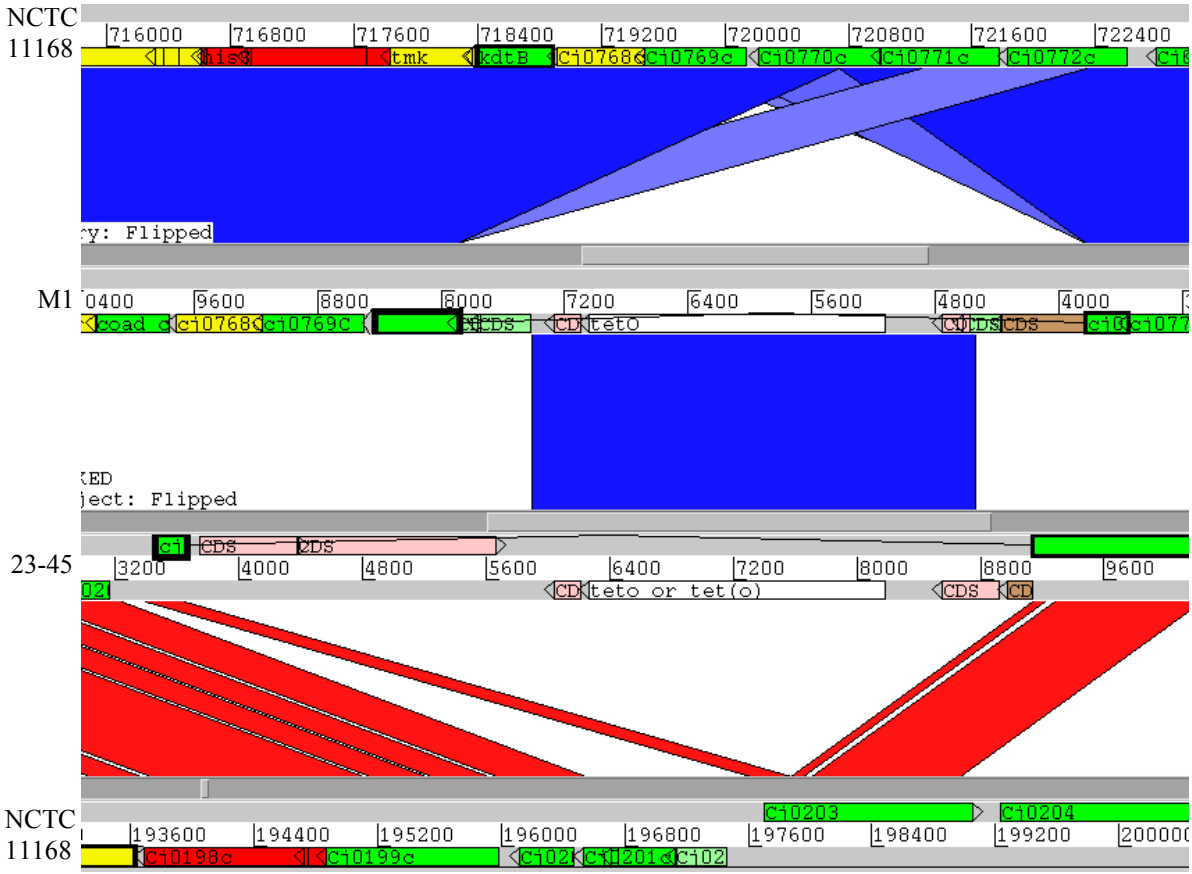

Fig 6.2: **A blastn comparison of strains NCTC 11168, M1 and 23-45**. The comparison is viewed using ACT; blocks of red or blue indicate sequence homology with the colour intensity proportional to the percent id of the match. Forward and reverse DNA sequences are represented by dark grey lines. CDSs are represented by open boxes and are coloured according to functional category: yellow, central/ intermediary/ miscellaneous metabolism; red, information transfer/ DNA modification; dark green, surface; light green, unknown; pink, bacteriophage/ IS elements; white, pathogenicity/ adaptation/ chaperones; brown, pseudogenes and partial genes. The pale blue blocks between cj0770c, cj0771c and cj0772c show that these genes have a degree of identity to each other. Both strain M1 and strain 23-45 have a chromosomal *tetO* insertion but these insertions occur at different places relative to the chromosome of strain NCTC 11168. The inserts in strain M1 and strain 23-45 share homology in the central portion with the *tetO* gene, downstream CDS and part of the upstream CDS being conserved between the two.

Unfortunately as both 23-45 and 24-34 have a very similar insert at an identical chromosomal location it is not possible to say whether the insert is mobile, whether a subsection of the insert is mobile or what would constitute a minimum mobile element. It

would be interesting to look at other strains to gather more information on insertions of this type.

6.4 Discussion

The gene *tetO* has been found in many *Campylobacter* isolates but usually associated with plasmids. Tetracycline acts by binding to the prokaryotic ribosome and inhibiting the elongation phase of protein synthesis. TetO is a ribosomal protection protein that acts by dislodging tetracycline from the ribosome and allowing aa-tRNA to bind to the A site and thus allows protein synthesis to continue [212]. Both strains 23-45 and 24-34 contain a novel region including a *tetO* gene that has been inserted on the chromosome within a homologue of CDS cj0203 which is predicted to encode a periplasmic protein. For strains 16-60, 16-48, 24-50, 16-02, 25-69 and 25-25 it appears likely that the *tetO* gene is found in conjunction with many other plasmid genes but whether this represents a cryptic plasmid or a large chromosomal insertion of plasmid related DNA remains to be established.

The novel chromosomal insertion in strains 23-45 and 24-34 appears to contain a homologue of the *Helicobacter pylori* transposable element IS607 in addition to *tetO* and other predicted CDSs but it is unclear how these two segments may interact. In addition it appears that in pTet, M1 and 23-45/24-34 the *tetO* gene is always associated with a 172 bp downstream CDS of unknown function that interestingly is present downstream of *tetM* in the transposon tn916 of *Enterococcus faecalis*. This may be required for tetracycline resistance, for transposition, or it may be physically linked to *tetO* without functional consequence. It would be interesting to explore the function of this gene further. These two CDSs are the only conserved CDSs between the insertions in strains 23-45 and 24-34 and that in M1. There are also CDSs with homology to TnpV from *Clostridium perfringens* which is present on a chloramphenicol-resistance transposon. A function for TnpV in

Clostridium perfringens was not found but it was discounted as a transposase [177]. In strain M1 there is only a partial CDS with homology to TnpV (**Fig 6.2** and section 5.2.6). It may be that a transposon is responsible for exchanging these fragments between plasmid and chromosome, picking up extra genes along the way by imperfect excision. Further study of more strains containing chromosomally located *tetO* genes would be needed to identify whether this is indeed a mobile element and if it is mobile what constitutes the minimum mobile element. It would also be interesting to explore the function of the CDS that is located downstream of *tetO* in all of the regions sequenced in this study.