7. Mutagenesis of *C. jejuni* strain 81-176 plasmid pTet

7.1 Introduction

As previously discussed in chapter 3 a predicted CDS with homology to site-specific DNA recombinase genes from other bacteria was found within the plasmid pTet from strain 81-176, flanked by 31 bp inverted repeats. Enclosed within the inverted repeats is a predicted promoter which may drive transcription of the downstream predicted type IV secretion system. It is possible that this putative DNA invertase acts on the repeats to invert the whole region, switching the type IV secretion system on and off as it switches orientation. It was therefore decided to explore the implications of this region further.

7.2 Materials and Methods

7.2.1 Reagents

Reagents for this work were purchased from Oxoid (Hampshire, U.K.), Sigma (Dorset, U.K.) or Roche (Lewes, East Sussex, U.K.) unless otherwise stated.

7.2.2 Strains and plasmids

In addition to strains 81-176 and NCTC 11168 (section 2.1.1) a strain NCTC 11168 *cj0742::cat* mutant (C. Coward and A. Grant; Department of Veterinary Medicine, Cambridge) was used for conjugation experiments (section 7.2.3.5). A kanamycin resistance cassette from plasmid pRY107 and chloramphenicol resistance cassette from plasmid pRY111 were used with permission from P. Guerry (Naval Medical Research Institute, Bethesda, USA) [213].

7.2.3 Methods

7.2.3.1 Primer sequences

Table 7.1: Primer sequences used in this part of the study. Engineered restriction sites are shown in bold.

Name	Sequence 5`-3`	Tm	notes
pTir1	ATTAAGCGTAGTGATCCAATG	54.6	Inverting region
pTir2	GTCAAAGAACTAAAGCAGGAC	53.4	Inverting region
pTir3	TTATCATTCCTGATTCTTTAGC	53.1	Inverting region
pTir3b	AACTCGTCTTTCATTTATTGG	53.7	Inverting region
pTir4	AACTCCACCAAGTGCATAC	53.3	Inverting region
pTir4b	TAAAATGATACATCCCATCG	53.5	Inverting region
Km-3.claI	CC**ATCGAT**TGCGTAAGAACATAGAAAGG	53.29	Km cassette plus ClaI site
Km-P-5.stuI	CT**AGGCCT**AAATGGCTAAAATGAGAATATCAC	55.81	Km cassette plus StuI site
Tet23-r3.claI	CT**ATCGAT**GCTATGATGGTCTATTGTGGTTTATC	59.27	Inverse PCR plus ClaI site
Tet23-r5.stuI	TC**AGGCCT**TCATCGTTCTAAATCTTTTAACTTTTG	58.56	Inverse PCR plus StuI site
Km.verif-5	AGTGGTATGACATTGCCTTC	55.06	Mutant verification
Km.verif-3	GTTCCACATCATAGGTGGTC	55.14	Mutant verification
pT23L	GCTCTAGCATTTTCTAGTCCTG	55.32	DNA invertase for Southern
pT23R	TTTGGATGAAATTTTGGAAG	55.29	DNA invertase for Southern
Ampli.Km-3	TGCGTAAGAACATAGAAAGG	53.29	Km cassette
Ampli.Km-P-5	AAATGGCTAAAATGAGAATATCAC	55.81	Km cassette
Ampli.Km-5	GCTGTTTTCTGGTATTTAAGG	53.53	Km cassette
T27L	CTTGGCAGATGGTAGAAGAC	54.94	Plasmid copy number
T27R	CAAAGGGCTAAGATCAAGAG	54.36	Plasmid copy number
1CTL	AAAGTTGGTTGAATGATTGG	55.05	Chromosomal copy number
1CTR	AGCATACCACAAGAATCCAC	55.06	Chromosomal copy number
1CKL	TTTATCACAGCAGATGCAAG	55.03	Chromosomal copy number
1CKR	GAGAGCGGAGTAACATCAAG	55.11	Chromosomal copy number

7.2.3.1 Growth of *Campylobacter jejuni*

All experiments involving growth of *C. jejuni* were carried out in the laboratory of D. Maskell (Department of Veterinary Medicine, Cambridge). *C. jejuni* was grown under microaerophilic conditions at 42°C in a microaerophilic cabinet, MAC5 VA500 microaerophilic workstation (Don Whitley Scientific, Shipley, U.K.). Microaerophilic conditions consisted of 85% Nitrogen, 5% Oxygen, 5% Hydrogen and 5% Carbon dioxide. *C. jejuni* was grown on Mueller-Hinton (MH) agar (Oxoid) containing Trimethoprim (5 μg/ml). Other supplements included defibrinated horse blood (5% v/v) (TCS Biosciences, Buckingham, U.K.), Tetracycline (20 μg/ml), Chloramphenicol (10 μg/ml) and Kanamycin (25 μg/ml) as appropriate.

7.2.3.2 Chromosomal DNA preparation

7.2.3.2.1 Phenol/Chloroform extraction

C. jejuni was grown for 24-48 hrs on MH agar plus defibrinated horse blood and trimethoprim, cells were harvested in Phosphate Buffered Saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 pH7.4) and pelleted by centrifugation for 1 min at 8000 rpm (Eppendorf 5415D) in a 1.5 ml tube (eppendorf). The supernatant was discarded and the cell pellet was resuspended in 1 ml Sucrose Tris EDTA (STE) (TE 10:1 and 25% sucrose) then spun in a centrifuge for 3 mins at 10000 rpm. This step was repeated with pelleting performed for 3 mins at 13000 rpm. The cell pellet was resuspended in 1 ml STE. The cell suspension was placed on ice and 25 μl lysozyme (40 mg/ml in 0.25 M Tris pH8) was added. The sample was incubated on ice for 5 mins then 50 μl of Proteinase K (20 mg/ml) was added and the sample was mixed by inversion. Next 15 μl of RNase (10 mg/ml) was added and the sample incubated on ice for 5mins before 200 μl EDTA (0.5 M) was added. Finally 125 μl Sarkosyl (10% w/v) was added and the sample incubated on ice for 2

hours. The sample was then incubated overnight at 50°C. The following day the sample was transferred to a 50 ml tube (Falcon) and the volume was made up to 5 ml with TE before 5 ml of phenol/chloroform (Sigma) was added. The sample was mixed by inversion then spun in a centrifuge (Eppendorf 5810R) for 40 mins at 4000 rpm and 15°C. The top layer was transferred to a new 50 ml tube (Falcon) and an equal volume of phenol/chloroform added before the sample was spun in a centrifuge for 15 mins at 4000 rpm and 15°C. The previous step was repeated with centrifugation for 10 mins at 4000 rpm and 15°C. The top layer was transferred to a new tube and an equal amount of chloroform (Sigma) was added, then the sample was spun for 1 hour at 4000 rpm. The previous step was repeated with centrifugation for 5 mins at 4000 rpm and 15°C. The top layer was transferred to a new 50 ml tube (Falcon) and 18.5 ml 100% ethanol added, and the sample was spun in a centrifuge for 3 mins at 4000 rpm 4°C. The supernatant was removed and the pellet washed with 10 ml 70% ethanol by spinning in a centrifuge for 3 mins at 4000 rpm and 4°C. The supernatant was removed and the pellet dried by incubating for 10 mins at 37 °C. The dried pellet was resuspended in an appropriate volume of buffer EB (Qiagen).

7.2.3.2.2 Qiagen column prep

Genomic DNA was purified using the Qiagen® Genomic-tip System with a 100/G genomic tip (Qiagen) according to manufacturer's specifications.

7.2.3.3 Quantitative Polymerase Chain Reaction (QPCR)

QPCR was performed using Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Reactions were thermocycled using an Mx3000P instrument (Stratagene) with an initial denaturing step of 10 mins 95°C followed by amplification steps of 92°C 30 s, 53°C 30 s and 72°C for 2 mins for 40 cycles. The data generated was analysed with the software provided.

7.2.3.4 Mutagenesis of pTet

7.2.3.4.1 Constructing a suicide vector

PCR of a promoterless kanamycin resistance gene to engineer terminal restriction enzyme sites was conducted as in (section 2.2.4) using the primers Km-3.claI and Km-P-5.stuI (section 7.2.3.1). Inverse PCR was conducted using a pUC clone, 8pT2G1, containing an insert consisting of the DNA invertase region from the pTet shotgun assembly as a template and the primers Tet23-r3.claI and Tet23-r5.stuI. DNA was amplified by thermocycling with the following conditions:-

94°C 4 mins

94°C 10 s
43-55°C 1 min
68°C 5 mins
) 10 cycles

94°C 10 s
43-55°C 1 min
68°C 5 mins
+10 s/ cycle
) 25 cycles

72°C 20 mins

10°C holding temperature

PCR products were verified and purified using agarose gel electrophoresis (section 2.2.3) followed by gel extraction (section 2.2.6.1). The purified gel fragments were digested with both ClaI and StuI (section 2.2.5), phenol extracted (section 2.2.6.2) and ethanol precipitated (section 2.2.6.3). The fragments were ligated by mixing 1.5 µl of the inverse PCR product and 1.5 µl of the kanamycin cassette with 0.4 µl of 10x ligase buffer, 0.3 µl of ligase (Roche 5 U/µl) and 0.3 µl of DDW. The ligation mixture was incubated and the ligation reaction terminated (section 2.3.1.2) then the pUC construct was transformed into an

Escherichia coli DH10B host (section 2.3.1.3). Recombinant pUC plasmid DNA was purified and knockout mutation constructs were verified by PCR (section 2.2.4), restriction enzyme digest with *Cla*I and *Stu*I or *Sac*I and *Xba*I (section 2.2.5) and sequencing (section 2.3.6).

7.2.3.4.2 Transformation

Overnight culture plates of *C. jejuni* strain 81-176 were harvested and spotted onto fresh MH agar plates containing defibrinated horse blood and trimethoprim. The plates were incubated under microaerophilic conditions for 3 hours at 42°C, then harvested and spotted onto fresh plates plus 1-10 μg of pUC construct DNA. The transformation mixtures were incubated for 4 hrs under microaerophilic conditions before serially diluting and plating onto selective MH agar plates containing defibrinated horse blood, trimethoprim, tetracycline and kanamycin.

7.2.3.4.3 Colony PCR

In order to verify the incorporation of the kanamycin resistance cassette in the correct location on pTet in *C. jejuni* strain 81-176, PCR was performed (section 2.2.4), with colonies used as templates instead of purified DNA. Colonies were suspended in water and boiled for 10 mins before being added to the reaction mix.

7.2.3.5 Southern blotting

Southern blotting was performed according to established methods: DNA was digested (section 2.2.5), fragments were separated by agarose gel electrophoresis (section 2.2.3), DNA fragments were transferred to Nytran N+ membrane (Amersham, Buckinghamshire, UK) using capillary transfer [214]. The membrane was then hybridized to a radioactively labelled oligonucleotide probe generated by PCR (section 2.3.5.2).

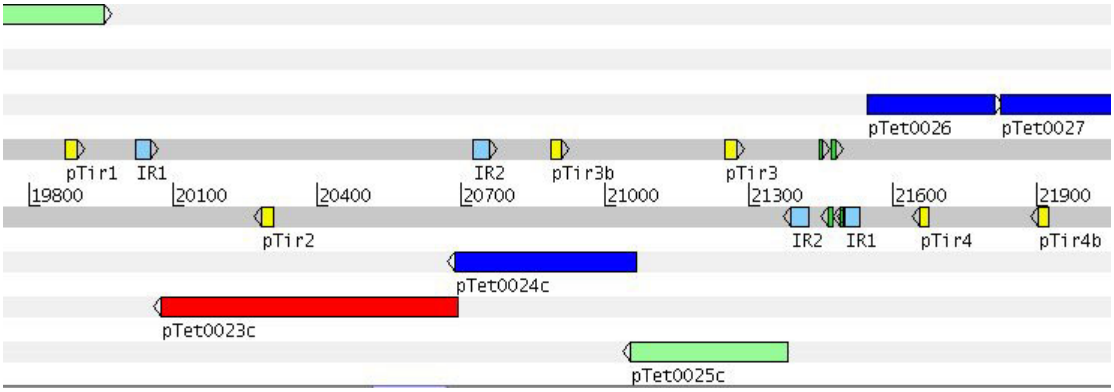
7.2.3.5 Conjugation experiments

Conjugation experiments were performed as previously described [128]. Donor strains 81-176 (pTet) or 81-176 (pTet/pTet23ΔKm), and the recipient strain NCTC 11168 *Cj0742::cat* were grown for approximately 24 hours on MH agar plus defibrinated horse blood, trimethoprim and the appropriate selective antibiotics (section 7.2.3.1). Strain 81-176 (pTet) was grown with tetracycline (20 μg/ml), strain 81-176 (pTet/pTet23ΔKm) was grown with tetracycline (20 μg/ml) and kanamycin (25 μg/ml), and strain NCTC 11168 *Cj0742::cat* was grown with chloramphenicol (10 μg/ml). Cells were harvested in MH broth at an optical density (OD) at 600 nm of 1 giving approximately 10^8 Colony Forming Units (CFU) per ml. Cells from each strain were serially diluted in MH broth and spread in triplicate on MH agar plates containing trimethoprim at this stage to determine CFU per ml. Equal quantities of donor and recipient cells (50 μl) were mixed and DNaseI (10 U/ml, Roche) was added. This conjugation mixture was then spotted onto MH agar plates containing defibrinated horse blood and trimethoprim but without selective antibiotics. Strains were also spotted individually onto MH agar plates containing defibrinated horse blood and trimethoprim as controls. The agar plates were incubated under microaerophilic conditions for 16-18 hours at 42°C. Subsequently the cells were harvested in MH broth, serially diluted and spread in triplicate onto MH agar plates containing trimethoprim with and without tetracycline and chloramphenicol and incubated under microaerophilic conditions for 2-3 days at 42°C.

7.3 Results

7.3.1 Invertible region

The putative CDS pTet23, which is predicted to encode a DNA invertase, is located on a region of the plasmid pTet that is flanked by two sets of inverted repeats (**Fig 7.1A**). In order to determine whether this region is inverting and whether inversion occurs using the outer repeats, the inner repeats, or both, a set of oligonucleotide primers were designed (**Fig 7.1**). PCR amplification using various primer combinations was conducted using prepared pTet plasmid DNA from strain 81-176 in order to identify whether inversion occurs (**Fig 7.2** and **Fig 7.3**). Fig 7.2 shows that products are obtained between primer sets pTir1-pTir2 and pTir3-pTir4 representing the invertible region in the sequenced orientation and between pTir2-pTir4 and pTir1-pTir3 representing the invertible region in the inverted orientation. The obtained product sizes are consistent with the predicted product sizes if only the outer repeats are used for inversion (**Fig 7.1**). As the product sizes for the different orientations are very similar the PCR amplifications were repeated using different primer sets to obtain different sized products to double check the inversion (**Fig 7.3**). These product sizes are again consistent with predicted product sizes if the region inverts using the outer repeats only (**Fig 7.1**).



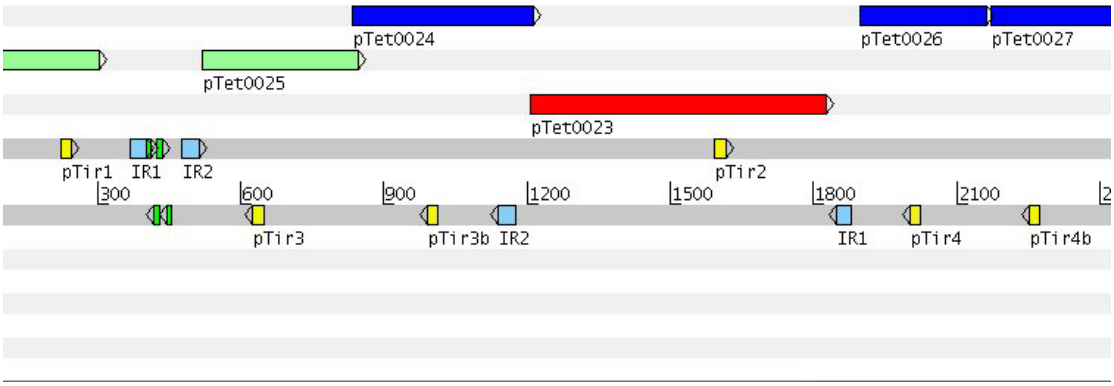
A

pTir1-pTir2 429 bp

pTir3-pTir4 422 bp

pTir3b-pTir4b 1035 bp

pTir1-pTir4 1798 bp



B

pTir1-pTir3 423 bp

pTir2-pTir4 428 bp

pTir2-pTir4b 677 bp

pTir1-pTir3b 787 bp

pTir1-pTir4b 2047 bp

Fig 7.1: *C. jejuni* strain 81-176 plasmid pTet invertible region. **A**, invertible region in the sequenced orientation; **B**, invertible region in the inverted orientation. Regions are viewed using Artemis [102]. The 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: oligonucleotide primer sequences (yellow), inverted repeats (blue) and promoters (green), are marked on the DNA lines; CDSs are marked on the translated frame lines.

CDSs are coloured according to functional category: light green, unknown; red, information transfer; blue, pathogenicity/ adaptation/ chaperones. Below the diagrams the expected sizes of PCR amplification products are indicated.

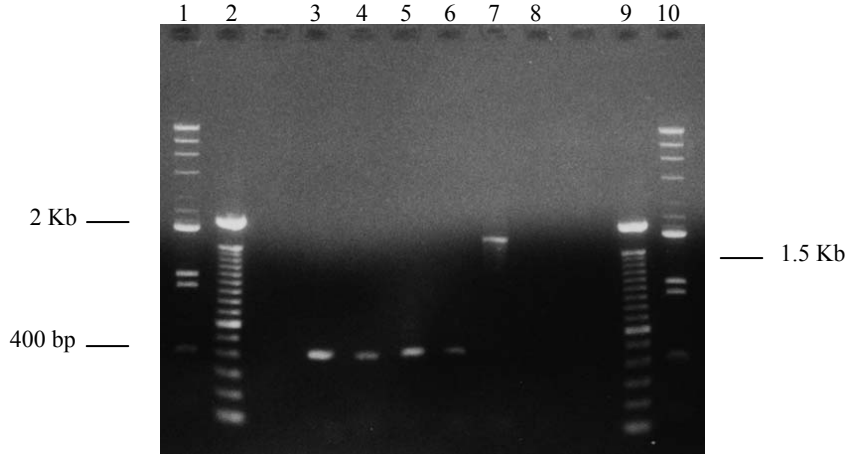


Fig 7.2: PCR of invertible region from extracted *C. jejuni* 81-176 plasmid DNA. Lanes 1 and 10 contain a mixture of λ *Hin*dIII and pBR322 *Bst*NI digested DNA, and lanes 2 and 9 contain 100 bp marker. Strain 81-176 plasmid DNA was amplified with the following primers: lane 3, pTir1-pTir2; lane 4, pTir3-pTir4; lane 5, pTir2-pTir4; lane 6, pTir1-pTir3; lane 7, pTir1-pTir4; lane 8, pTir3-pTir2. These results are consistent with the invertible region inverting between inverted repeat set 1.

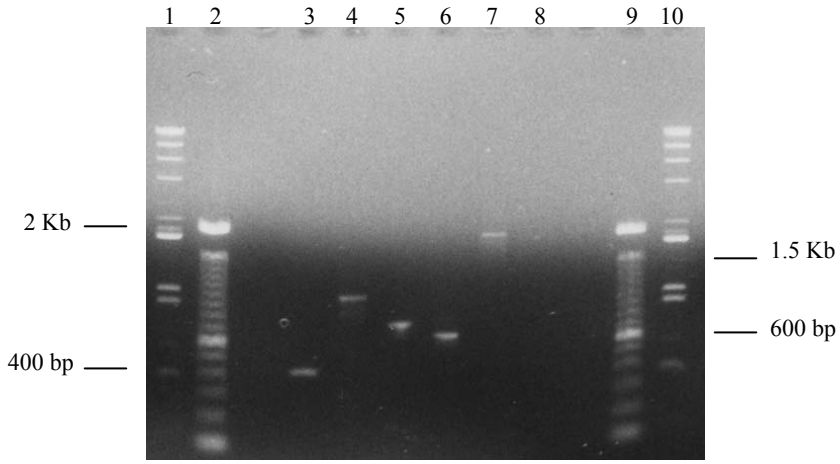


Fig 7.3: PCR of invertible region from extracted *C. jejuni* plasmid DNA. Lanes 1 and 10 contain a mixture of λ *Hin*dIII and pBR322 *Bst*NI digested DNA, and lanes 2 and 9 contain 100 bp marker. Strain 81-176 plasmid DNA was amplified with the following primers; lane 3, pTir1-pTir2; lane 4, pTir3b-pTir4b; lane 5, pTir1-pTir3b; lane 6, pTir2-pTir4b; lane 7, pTir1-pTir4b; lane 8, pTir3b-pTir2. These results suggest that the region is inverting between inverted repeat set 1.

The PCR products between primer sets pTir1-pTir2, pTir3-pTir4, pTir2-pTir4 and pTir1-pTir3 were used as sequencing templates and sequenced using each of the primers used to generate the products. These products were selected for sequencing as they were small enough to be sequenced completely from both directions and would cover the inverted repeats so the exact site of inversion could be verified. The sequences from the inverted orientation (pTir2-pTir4 and pTir1-pTir3) were then aligned using clustal X to the sequence in the 'sequenced' orientation to illustrate the inversion and to confirm that this was occurring at the inverted repeats (**Fig 7.4**). Fig 7.4 shows that the invertible region inverts within the perfect repeat area of IR1.

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primer1->primer2       AAAATCTTTATTTGATTAACATTTACAATCAAGGAGTGTTGTATGAATGAATGGGAATTA
                       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
primer1->primer3       AAAATCTTTATTTGATTAACATTTACAATCAAGGAGTGTTGTATGAATGAATGGGAATTA
                         :::         :  ::     : :::      :  :   :: :::::        :
primer4->primer3       TCAATACCGCCAGCACCAAATGCAAAAATAGGACTGATTAAAATTAATGATAATAGCCTT

                                                    IR1
                                          <----------------------------->
primer1->primer2       GAAAGACAAAGACAAATAAGATAGACAAATTGAGCGTTTTATTTTATTACAAAATTTTTT
                       ::::::::::::::::::::::::::::::::::::::::::::   :   :::::: :
primer1->primer3       GAAAGACAAAGACAAATAAGATAGACAAATTGAGCGTTTTATTTATGTCTTAAATTTGTA
                                  :    :  : ::::::::::::::::::::::::::::::::::
primer4->primer3       TTTTTCATCCATATCCTTTCGTTAAAAAATTGAGCGTTTTATTTATGTCTTAAATTTGTA


primer1->primer2       ATATTTTTCTTAAATGAAAATTTAGCTTCCTTTTAATTTTCGTTTTTTGATAAACCACAA
                         :   :   : :: : ::  :     :    :: ::::    :: ::  : :      :
primer1->primer3       TTTAATAAATAAATTAAATTTAGTTTTATAATTAAATTAATATTATTCAAAAGCTTTTAT
                       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
primer4->primer3       TTTAATAAATAAATTAAATTTAGTTTTATAATTAAATTAATATTATTCAAAAGCTTTTAT


primer1->primer2       TAGACCATCATAGCTTTTTCCATTATCTTTGTATAATAACTTCCAAATACTTTTAATAGA
                       :  :  :: :    :::::   :    :::::    :   :    ::    :  :: :
primer1->primer3       TTAAGTATTAGCTATTTTTTACTAGAATTTGTTAGGTTTTTGATGAAAGGATAAAAAAAT
                       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
primer4->primer3       TTAAGTATTAGCTATTTTTTACTAGAATTTGTTAGGTTTTTGATGAAAGGATAAAAAAAT


primer1->primer2       TAAATCTTTATCAAGCAAGGTTTGTATTTTATCTATATCCTTATCATAAA
                          ::    :             : ::  :::          :
primer1->primer3       GGCATACCCACTTTTAGGTACAAGAATAGTATTAGATGAAGAAAAGATTT
                       ::::::::::::::::::::::::::::::::::::::::::::::::::
primer4->primer3       GGCATACCCACTTTTAGGTACAAGAATAGTATTAGATGAAGAAAAGATTT
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A

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primer1->primer2       AAAATCTTTATTTGATTAACATTTACAATCAAGGAGTGTTGTATGAATGAATGGGAATTA
                         :::         :  ::     : :::      :  :   :: :::::        :
primer4->primer2       TCAATACCGCCAGCACCAAATGCAAAAATAGGACTGATTAAAATTAATGATAATAGCCTT
                       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
primer4->primer3       TCAATACCGCCAGCACCAAATGCAAAAATAGGACTGATTAAAATTAATGATAATAGCCTT

                                                    IR1
                                          <------------------------------>
primer1->primer2       GAAAGACAAAGACAAATAAGATAGACAAATTGAGCGTTTTATTTTATTACAAAATTTTTT
                                 :    :  : ::::::::::::::::::::::::::::::::
primer4->primer2       TTTTTCATCCATATCCTTTCGTTAAAAAATTGAGCGTTTTATTTTATTACAAAATTTTTT
                       ::::::::::::::::::::::::::::::::::::::::   :   :::::: :
primer4->primer3       TTTTTCATCCATATCCTTTCGTTAAAAAATTGAGCGTTTTATTTATGTCTTAAATTTGTA


primer1->primer2       ATATTTTTCTTAAATGAAAATTTAGCTTCCTTTTAATTTTCGTTTTTTGATAAACCACAA
                       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
primer4->primer2       ATATTTTTCTTAAATGAAAATTTAGCTTCCTTTTAATTTTCGTTTTTTGATAAACCACAA
                        :   :   : :: : ::  :     :    :: ::::    :: ::  : :      :
primer4->primer3       TTTAATAAATAAATTAAATTTAGTTTTATAATTAAATTAATATTATTCAAAAGCTTTTAT


primer1->primer2       TAGACCATCATAGCTTTTTCCATTATCTTTGTATAATAACTTCCAAATACTTTTAATAGA
                       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
primer4->primer2       TAGACCATCATAGCTTTTTCCATTATCTTTGTATAATAACTTCCAAATACTTTTAATAGA
                       :  :  :: :    :::::   :    :::::    :   :    ::    :  :: :
primer4->primer3       TTAAGTATTAGCTATTTTTTACTAGAATTTGTTAGGTTTTTGATGAAAGGATAAAAAAAT


primer1->primer2       TAAATCTTTATCAAGCAAGGTTTGTATTTTATCTATATCCTTATCATAAA
                       ::::::::::::::::::::::::::::::::::::::::::::::::
primer4->primer2       TAAATCTTTATCAAGCAAGGTTTGTATTTTATCTATATCCTTATCATAAA
                          ::    :             : ::  :::          :
primer4->primer3       GGCATACCCACTTTTAGGTACAAGAATAGTATTAGATGAAGAAAAGATTT
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B

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primer2->primer1       TTTATGATAAGGATATAGATAAAATACAAACCTTGCTTGATAAAGATTTATCTATTAAAA
                              :          :::  :: :             :    ::     : ::  :
primer3->primer1       AAATCTTTTCTTCATCTAATACTATTCTTGTACCTAAAAGTGGGTATGCCATTTTTTTAT
                       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
primer3->primer4       AAATCTTTTCTTCATCTAATACTATTCTTGTACCTAAAAGTGGGTATGCCATTTTTTTAT


primer2->primer1       GTATTTGGAAGTTATTATACAAAGATAATGGAAAAAGCTATGATGGTCTATTGTGGTTTA
                          ::    :   :    :::::    :   :::::    : ::  :  : :      :
primer3->primer1       CCTTTCATCAAAAACCTAACAAATTCTAGTAAAAAATAGCTAATACTTAAATAAAAGCTT
                       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
primer3->primer4       CCTTTCATCAAAAACCTAACAAATTCTAGTAAAAAATAGCTAATACTTAAATAAAAGCTT


                                                                          <------
primer2->primer1       TCAAAAAACGAAAATTAAAAGGAAGCTAAATTTTCATTTAAGAAAAATATAAAAAATTTT
                       :  :: ::    :::: ::    :     :  :: : :: :   :   :  : :::::::
primer3->primer1       TTGAATAATATTAATTTAATTATAAAACTAAATTTAATTTATTTATTAAATACAAATTTA
                       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
primer3->primer4       TTGAATAATATTAATTTAATTATAAAACTAAATTTAATTTATTTATTAAATACAAATTTA

                          IR1
                       ----------------------->
primer2->primer1       GTAATAAAATAAAACGCTCAATTTGTCTATCTTATTTGTCTTTGTCTTTCTAATTCCCAT
                         :   ::::::::::::::::::::::::::::::::::::::::::::::::::::::
primer3->primer1       AGACATAAATAAAACGCTCAATTTGTCTATCTTATTTGTCTTTGTCTTTCTAATTCCCAT
                       ::::::::::::::::::::::: :  :    :                    :
primer3->primer4       AGACATAAATAAAACGCTCAATTTTTTAACGAAAGGATATGGATGAAAAAAAGGCTATTA


primer2->primer1       TCATTCATACAACACTCCTTGATTGTAAATGTTAATCAAATAAAGATTTT
                       ::::::::::::::::::::::::::::::::::::::::::::::::::
primer3->primer1       TCATTCATACAACACTCCTTGATTGTAAATGTTAATCAAATAAAGATTTT
                       ::::: ::   :  :      ::: :     ::  :         :::
primer3->primer4       TCATTAATTTTAATCAGTCCTATTTTTGCATTTGGTGCTGGCGGTATTGA
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C

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primer2->primer1       TTTATGATAAGGATATAGATAAAATACAAACCTTGCTTGATAAAGATTTATCTATTAAAA
                       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
primer2->primer4       TTTATGATAAGGATATAGATAAAATACAAACCTTGCTTGATAAAGATTTATCTATTAAAA
                              :          :::  :: :             :    ::     : ::  :
primer3->primer4       AAATCTTTTCTTCATCTAATACTATTCTTGTACCTAAAAGTGGGTATGCCATTTTTTTAT


primer2->primer1       GTATTTGGAAGTTATTATACAAAGATAATGGAAAAAGCTATGATGGTCTATTGTGGTTTA
                       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
primer2->primer4       GTATTTGGAAGTTATTATACAAAGATAATGGAAAAAGCTATGATGGTCTATTGTGGTTTA
                          ::    :   :    :::::    :   :::::    : ::  :  : :      :
primer3->primer4       CCTTTCATCAAAAACCTAACAAATTCTAGTAAAAAATAGCTAATACTTAAATAAAAGCTT


                                                                            <------
primer2->primer1       TCAAAAAACGAAAATTAAAAGGAAGCTAAATTTTCATTTAAGAAAAATATAAAAAATTTT
                       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
primer2->primer4       TCAAAAAACGAAAATTAAAAGGAAGCTAAATTTTCATTTAAGAAAAATATAAAAAATTTT
                       :  :: ::    :::: ::    :     :  :: : :: :   :   :  : ::::::
primer3->primer4       TTGAATAATATTAATTTAATTATAAAACTAAATTTAATTTATTTATTAAATACAAATTTA

                          IR1
                       ----------------------->
primer2->primer1       GTAATAAAATAAAACGCTCAATTTGTCTATCTTATTTGTCTTTGTCTTTCTAATTCCCAT
                       ::::::::::::::::::::::: :  :    :                    :
primer2->primer4       GTAATAAAATAAAACGCTCAATTTTTTAACGAAAGGATATGGATGAAAAAAAGGCTATTA
                         :   ::::::::::::::::::::::::::::::::::::::::::::::::::::
primer3->primer4       AGACATAAATAAAACGCTCAATTTTTTAACGAAAGGATATGGATGAAAAAAAGGCTATTA


primer2->primer1       TCATTCATACAACACTCCTTGATTGTAAATGTTAATCAAATAAAGATTTT
                       ::::: ::   :  :      ::: :     ::  :         :::
primer2->primer4       TCATTAATTTTAATCAGTCCTATTTTTGCATTTGGTGCTGGCGGTATTGA
                       ::::::::::::::::::::::::::::::::::::::::::::::::::
primer3->primer4       TCATTAATTTTAATCAGTCCTATTTTTGCATTTGGTGCTGGCGGTATTGA
```

D

Fig 7.4: clustal alignments of DNA sequence from inverted orientation PCR products. The middle sequence represents the invertible region sequenced in the inverted orientation between the primers specified to the left of the sequence. The outer sequences represent the invertible region in the sequenced orientation between the primers specified to the left of the sequence. The inverted repeat, IR1 is marked above the alignments; **A** and **B** show the left hand repeat, and **C** and **D** show the right hand repeat. The alignments show that the region is inverting within the perfect repeat area of IR1.

The sequenced orientation of this invertible region appears to be predominant within the purified DNA sample used for these experiments as during the shotgun assembly this region was not identified in the inverted orientation and all attempts to sequence products obtained with primers pTir1 and pTir4 showed the region in the sequenced orientation. However a BAC clone was obtained from 81-176 DNA that contained the region in the inverted orientation. If the plasmid is high copy number then the putative variable promoter may have no observable effect at the bacterial cell level, also if the plasmid is multi-copy then any mutant construct may be compromised by homologous recombination with a wild type plasmid if the strains are *recA* positive. For this reason it was decided to experimentally determine the plasmid copy number.

7.3.2 Determination of plasmid copy number

Usually low copy number plasmids have partition genes which allow the plasmid to be incorporated into daughter cells whereas high copy number plasmids can rely upon random distribution between daughter cells [114]. As no homologues of partition genes were discovered in pTet it was decided to determine the relative plasmid copy number using QPCR to amplify regions from the chromosome and from the plasmid.

The fluorescence of SYBR Green dye increases when bound to double stranded DNA allowing the accumulation of PCR product to be measured. To determine the relative copy number the threshold cycle (Ct) is used which is the lowest cycle of amplification at which the fluorescence is detectable above the background level and therefore product is starting to be formed. The more initial template DNA there is the fewer cycles are necessary to produce fluorescence that is detectable above the background level. The value Ct has been shown to be inversely proportional to the log of the initial copy number of the starting material [215]. Comparing measurements from the initial amplification stage is more

accurate than from subsequent cycles when the availability of reaction components can be a limiting factor in product amplification.

QPCR (section 7.2.3.3) was performed using total DNA extracted by a phenol/chloroform method (section 7.2.3.2.1) as a template. Primer sets 1CTL/R (designed from *pheT* of NCTC 11168) and 1CKL/R (designed from *dnaK* of NCTC 11168) were used to amplify regions of the chromosome and primer set T27L/R (designed from pTet27) was used to amplify a region from pTet. Primers were designed from the chromosome in genes not likely to be variable between strains 81-176 and NCTC 11168 as they perform housekeeping functions, at different positions around the chromosome. Primers were designed to give products of approximately equal sizes: 1CTL/R 340 bp; 1CKL/R 341 bp; T27L/R 367 bp. The PCRs were performed in triplicate using 1/10, 1/20 and 1/50 dilutions of the 81-176 total DNA as a template. Amplification plots from the QPCR reaction (**Fig 7.5**) indicate that for each of the dilutions the fluorescence produced using each of the primer pairs begins to exceed the background level at approximately the same amplification cycle and that there is a difference between each of the dilutions. The relative quantities were determined using the amplification product from the primer set 1CKL/R with a 1/50 dilution of the template DNA as a reference to which all other values were compared (**Fig 7.6**). The relative quantities for each primer set show that a 1/20 dilution of template is approximately 2.5x greater than 1/50 dilution of template, and 1/10 dilution of template is approximately 5x greater than 1/50 dilution of template. The relative quantities between primer sets for each dilution are approximately equal which shows that pTet is a low copy number plasmid.

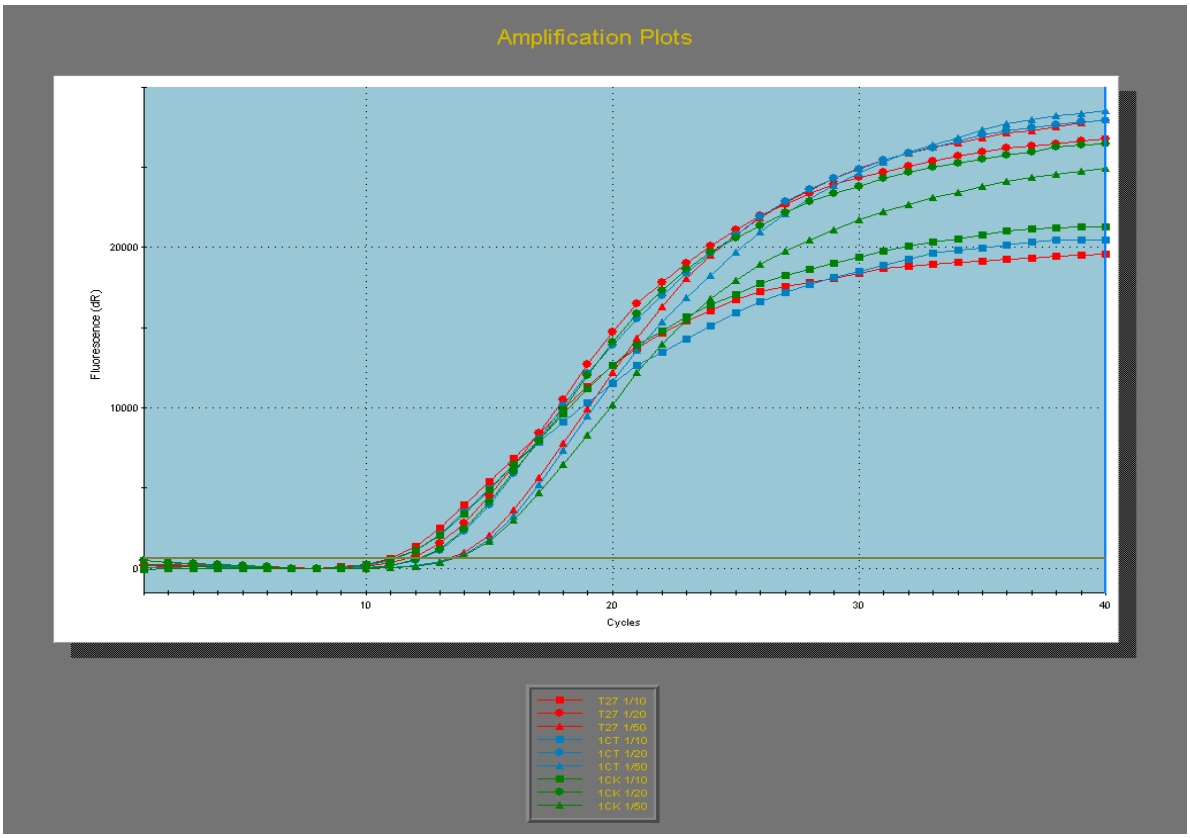

Fig 7.5: QPCR amplification plots for the determination of plasmid copy number. Primer sets (pTet, T27; chromosomal, 1CT and 1CK) and dilutions of template DNA are indicated in the key. The horizontal gold line indicates the threshold fluorescence value. The threshold cycle (Ct), where the fluorescence from an amplification reaction is just detectable above the threshold value, has been shown to be inversely proportional to the log of the initial copy number of the starting material [215]. From this graph it can be seen that fluorescence for each of the primer pairs, at the same dilution of template DNA, begins to exceed the threshold level at approximately the same amplification cycle. There is a visible difference in Ct for each of the template dilutions, with the lower dilutions producing detectable fluorescence after fewer cycles, as expected.

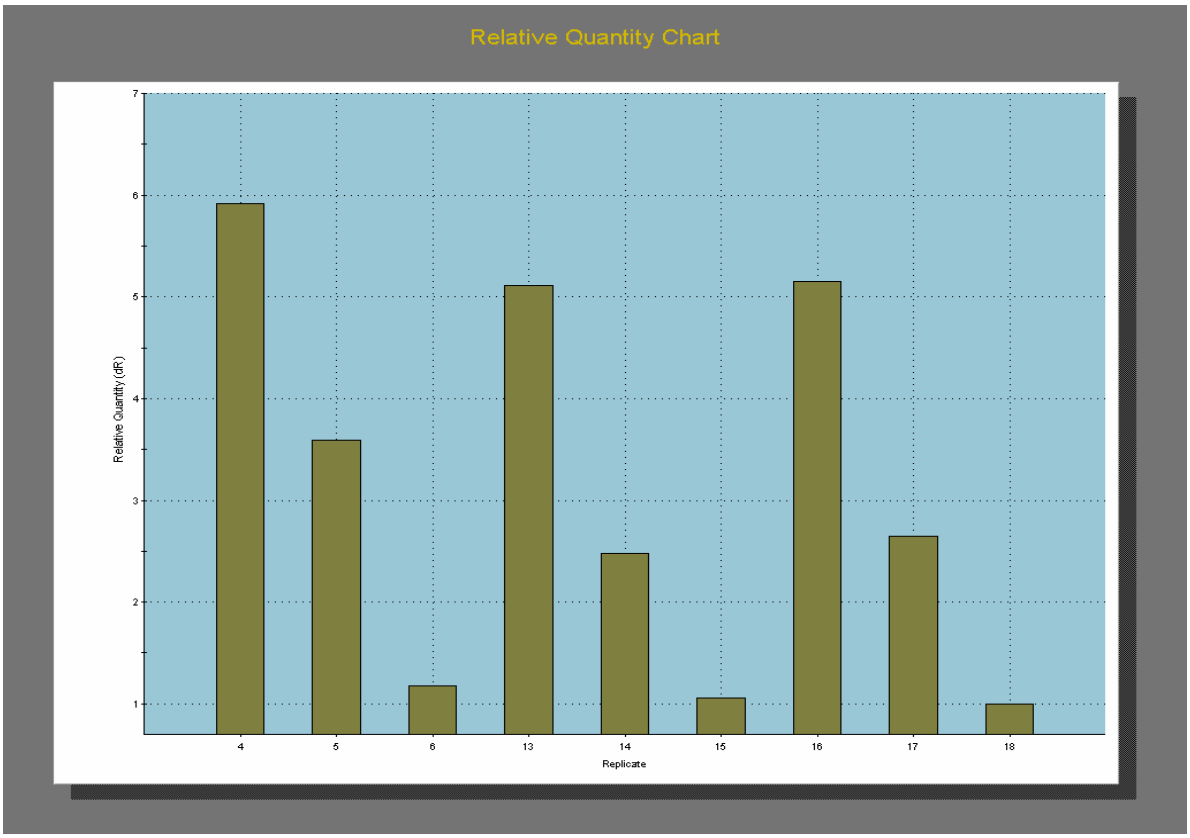

Fig 7.6: QPCR Relative Quantity Chart. Relative quantities are determined from the Ct values of each amplification compared to the Ct value of replicate 18 which is given a value of 1. Primer sets to amplify a region from pTet, Tet27, and regions from the chromosome, 1CT and 1CK, were used at various concentrations of extracted total DNA from strain 81-176. Each of the replicates is based on an average of the results for each primer set at each dilution of template DNA. Relative quantities of pTet DNA, determined from primer set Tet27, are shown in replicates 4, 5 and 6, at a 1/10, 1/20 and 1/50 dilution of template DNA respectively. Relative quantities of chromosomal DNA, determined from primer set 1CT, are shown in replicates 13, 14 and 15, at a 1/10, 1/20 and 1/50 dilution of template DNA respectively. Relative quantities of chromosomal DNA, determined from primer set 1CK, are shown in replicated 16, 17 and 18, at a 1/10, 1/20 and 1/50 dilution of template DNA respectively. The 1/20 dilutions are roughly 2.5 x the 1/50 dilutions, and 1/10 dilutions are roughly 5 x the 1/50 dilutions, within one primer set. The comparison of quantities between primer sets indicates that pTet is a low copy number plasmid.

7.3.3 Knockout mutagenesis of the site specific recombinase pTet0023

A pUC plasmid, 8pT2G1, from the library used for shotgun sequencing (chapter 3) containing the invertible region was used to create an antibiotic cassette knockout of the invertase in order to identify whether this gene is responsible for the inversion seen in the plasmid. The plasmid 8pT2G1 contains an insert of 2615 bp from base 19297-21911 on the pTet plasmid (**Fig 7.7**). Inverse PCR with the primers Tet23-r3.claI and Tet23-r5.stuI was used to amplify 8pT2G1 minus the invertase and to engineer terminal *Cla*I and *Stu*I restriction enzyme sites respectively. A kanamycin resistance gene (*apha-3)* from plasmid pRY107 was amplified by PCR without its promoter using the primers Km-P-5.stuI and Km-3.claI to engineer terminal restriction enzyme sites that would be complementary to the amplified plasmid 8pT2G1. Both of these products were digested with *Stu*I and *Cla*I then ligated together resulting in a pUC plasmid, 8pT2G1ΔKm, with the putative DNA invertase, pTet0023, replaced with a promoterless kanamycin resistance gene (section 7.2.3.4.1) (**Fig 7.8**). A promoterless kanamycin resistance gene was used so as not to affect transcription of downstream genes by introducing a strong promoter that could cause read through transcription. Knockout constructs were verified by PCR, restriction enzyme digests and sequencing. The invertible region was shown by PCR to invert in the plasmid 8pT2G1 within the *Escherichia coli* host (**Fig 7.9**). Once the putative DNA invertase, pTet23c, had been replaced with a kanamycin resistance gene the region no longer inverted within the *Escherichia coli* host suggesting that this region was now fixed in one orientation (**Fig 7.10**). As the wild-type region inverts in *Escherichia coli* some of the 8pT2G1 template used for the inverse PCR would have had the invertible region in the inverted orientation. Attempts were made to isolate an 8pT2G1ΔKm clone containing an insert with the invertible region fixed in the alternative direction. DNA from pools of colonies was analysed by restriction enzyme digests, PCR and sequencing but a mutant plasmid with the invertible region fixed in

the inverted orientation could not be purified from the background of clones with the invertible region in the sequenced orientation.

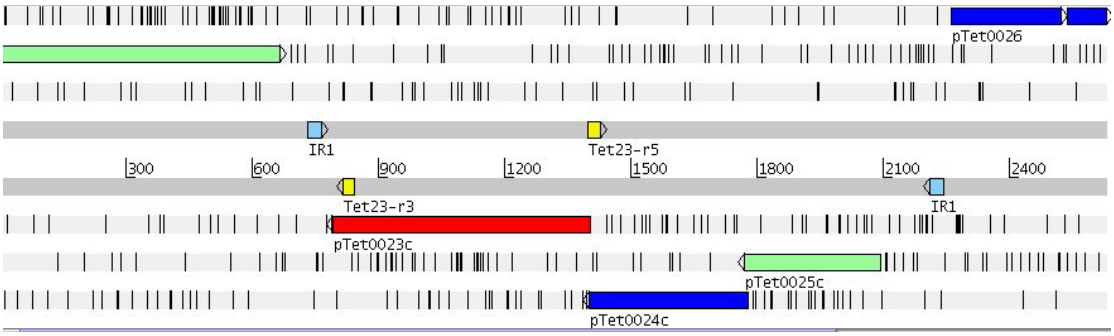

Fig 7.7: Insert sequence of the pUC clone 8pT2G1. The region is viewed using Artemis [102]. The forward and reverse DNA sequences are represented by dark grey lines. The three-frame forward and reverse DNA translations are represented by light grey lines with stop codons indicated by vertical black lines. Features are represented by open boxes: oligonucleotide primer sequences (yellow) and inverted repeats (blue) are marked on the DNA lines; CDSs are marked on the translated frame lines. CDSs are coloured according to functional category: light green, unknown; red, information transfer; blue, pathogenicity/ adaptation/ chaperones. A mutant was constructed using inverse PCR (from the sites indicated) to amplify this region without the invertase, pTet23, and to engineer restriction enzyme sites in order to attach a kanamycin resistance cassette in its place.

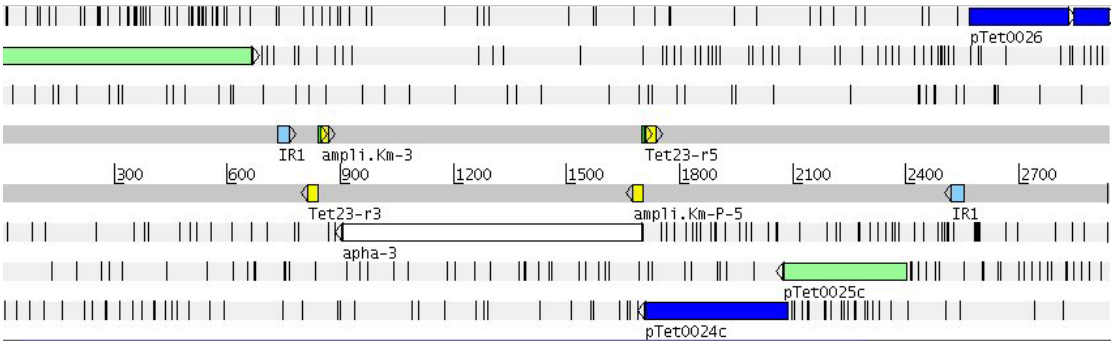

Fig 7.8: Insert sequence of the pUC clone 8pT2G1ΔKm. This region is viewed using Artemis [102]. The forward and reverse DNA sequences are represented by dark grey lines. The three-frame forward and reverse DNA translations are represented by light grey lines and stop codons are marked by vertical black lines. Features are represented by open boxes: oligonucleotide primer sequences (yellow), inverted repeats (blue) and promoters (green), are marked on the DNA lines; CDSs are marked on the translated frame lines. CDSs are coloured according to functional category: light green, unknown; red, information transfer; blue, pathogenicity/ adaptation/ chaperones. The kanamycin resistance cassette is coloured white and replaces the invertase in this mutant construct.

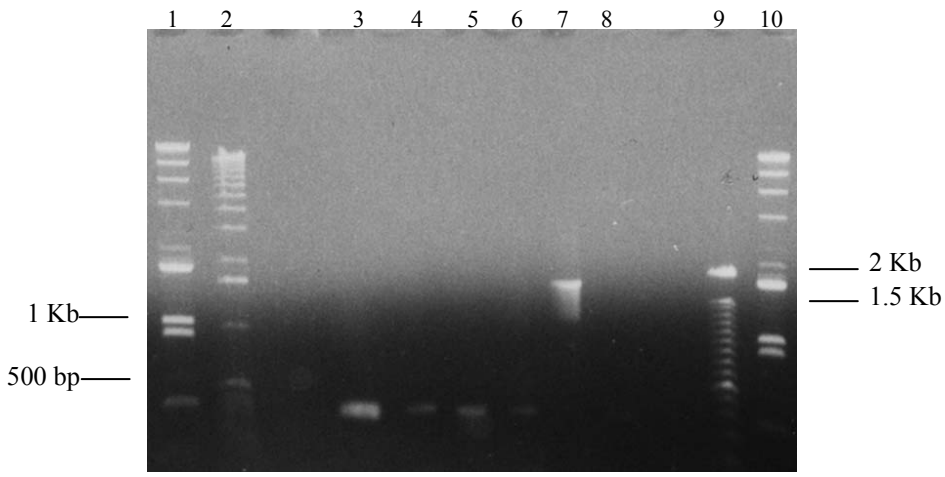

Fig 7.9: PCR of invertible region from extracted 8pT2G1 DNA. Lanes 1 and 10 contain a mixture of λ *Hin*dIII and pBR322 *Bst*NI digested DNA, lane 2 contains 1 Kb marker DNA, and lane 9 contains 100 bp marker DNA. Sample lanes contain 8pT2G1 plasmid DNA amplified with the following primers; lane 3, pTir1-pTir2; lane 4, pTir1-pTir3; lane 5, pTir3-pTir4; lane 6, pTir2-pTir4; lane 7, pTir1-pTir4; lane 8, pTir3-pTir2. The fact that bands are produced from combinations of primers spanning the inverted repeats shows that the region is able to invert within an *Escherichia coli* host.

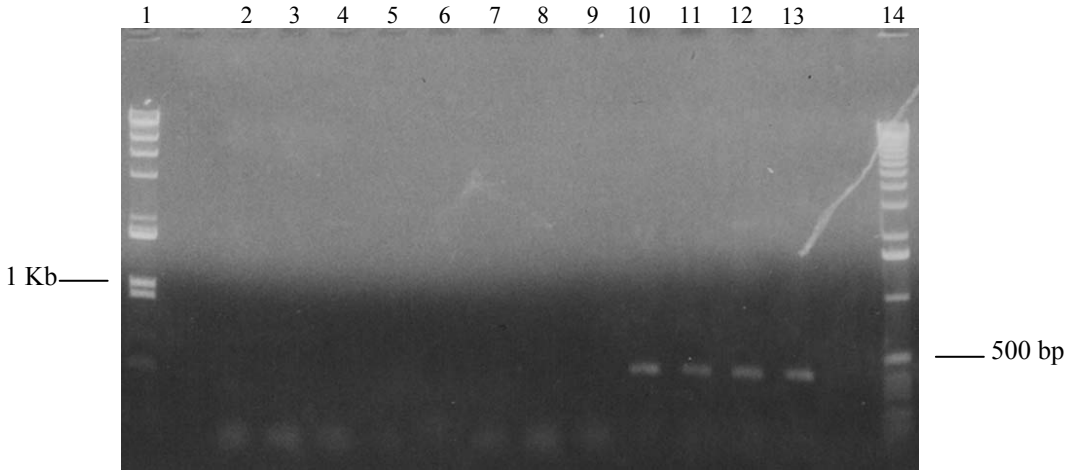

Fig 7.10: PCR amplification between various primers in 8pT2G1ΔKm colonies. Lane 1 contains a mixture of λ *Hin*dIII and pBR322 *Bst*NI digested DNA and lane 14 contains 1 Kb marker DNA. Lanes 2, 3, 4 and 5 contain amplifications products produced using primers pTir1-pTir2 in 8pT2G1ΔKm colonies A11, E2, D3 and G6 respectively; lanes 6, 7, 8 and 9 contain amplification products produced using primers pTir1-pTir3 in 8pT2G1ΔKm colonies A11, E2, D3 and G6 respectively; lanes 10, 11, 12 and 13 contain amplification products produced using primers pTir3-pTir4 in 8pT2G1ΔKm colonies A11, E2, D3 and G6 respectively. Bands are only produced with the primers pTir3-pTir4 showing that the invertase has been deleted and the invertible region has been fixed in the sequenced orientation.

The purified pUC plasmid, 8pT2G1ΔKm, was used as a suicide vector to transform *C. jejuni* strain 81-176 (section 7.2.3.4.2) with the intention that the knockout mutation would be transferred to the indigenous pTet plasmid. Cells were then plated onto MH agar plates containing defibrinated horse blood, trimethoprim, kanamycin and tetracycline to select for recombinant knockout mutants. *C. jejuni* colonies that grew on these selective plates were checked by colony PCR to assess whether the putative DNA invertase gene on the mutagenized pTet had been replaced with the kanamycin resistance gene, by homologous recombination with the suicide vector. Primers flanking the invertible region (pTir1-pTir4) were used to amplify DNA from 12 colonies (**Fig 7.11**).

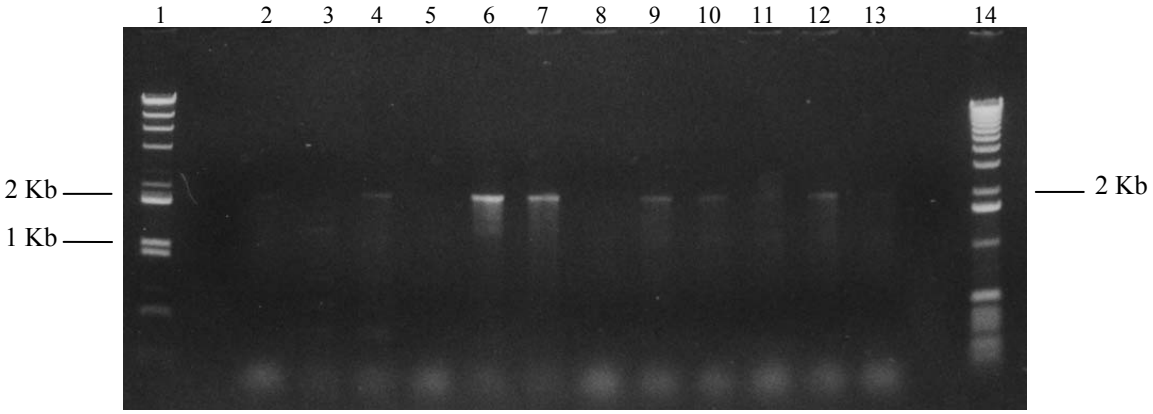

Fig 7.11: Colony PCR from 12 colonies that grew on selective media after transformation with mutant construct using primers pTir1-pTir4. Lane 1 contains a mixture of λ *Hin*dIII and pBR322 *Bst*NI digested DNA and lane 14 contains 1 Kb marker DNA. Lanes 2-13 contain amplification products, from 12 colonies that grew on selective media, produced using primers pTir1-pTir4. Only colonies 5 and 6 (products in lanes 6 and 7) were selected for further analysis as the bands were of the right size (approximately 2 Kb) and lanes did not appear to contain spurious products.

The colonies with amplification products in lanes 6 and 7 (**Fig 7.11**) were selected for further experiments as the amplification products were of the expected size, 2108 bp, compared to wild type, 1799 bp. DNA was extracted using a genomic DNA kit (7.2.3.2.2) and PCR was used to verify the position of the kanamycin resistance gene insert. **Fig 7.12** shows 81-176 (pTet/pTet23 Km) mutant 5 compared to 81-176 (pTet) using primers pTir1-pTir4. There is a larger band for the mutant as the kanamycin resistance gene is larger than the DNA invertase gene it replaces (2108 bp compared to 1799 bp). **Fig 7.13** shows that the kanamycin gene has inserted in the expected orientation and that the kanamycin gene is present without promoter, the band size of 855 bp is consistent with the kanamycin gene having no promoter (**Fig 7.14**). **Fig 7.15** shows that the region no longer inverts within strain 81-176 indicating that the DNA invertase gene is indeed responsible for the inversion in the *C. jejuni* background.

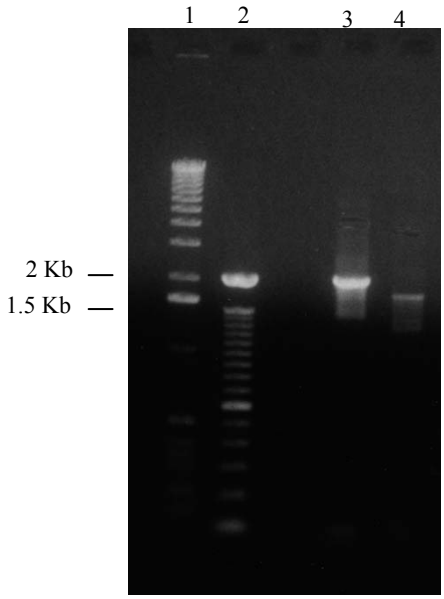

Fig 7.12: PCR amplification of the predicted invertible region from pTet. Lane 1 contains 1 Kb DNA marker and lane 2 contains 100 bp DNA marker. Lanes 3 and 4 contain PCR amplification products produced using primers pTir1-pTir4 from template DNA: lane 3, 81-176 (pTet/pTet23ΔKm) mutant 5; lane 4, wild type 81-176 (pTet). The PCR product from the mutant is larger (approx. 2 Kb) than the wild type (approx 1.8 Kb) which is expected if the kanamycin resistance cassette has replaced the invertase.

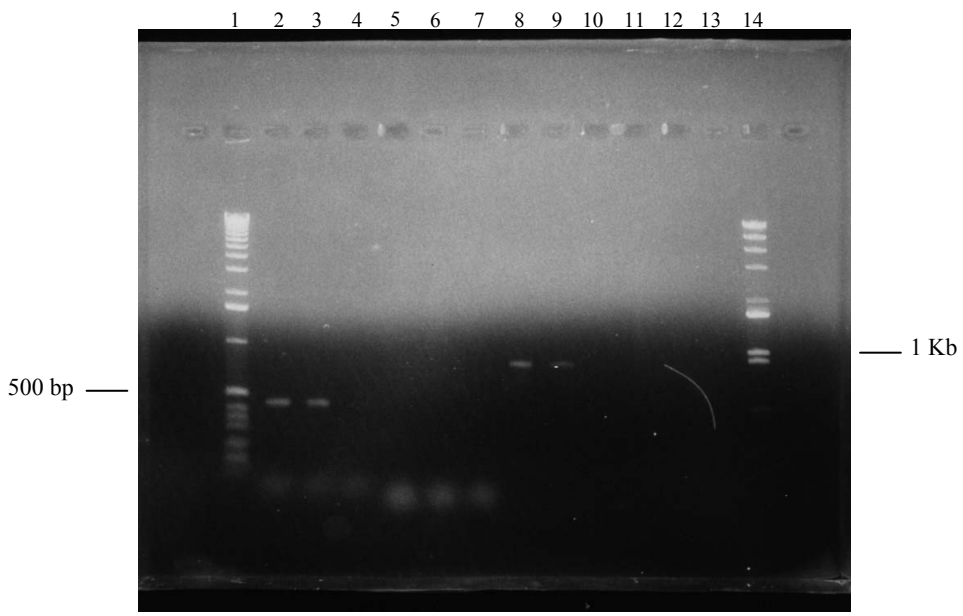

Fig 7.13: PCR amplification of pTet mutants and WT. Lane 1 contains 1 Kb DNA marker and lane 14 contains a mixture of λ *Hin*dIII and pBR322 *Bst*NI digested DNA. Lanes 2, 3 and 4 contain amplifications using primers Km.verif-5-pTir1 in strain 81-176 (pTet/pTet23ΔKm) mutant 5 and 6, and wild type 81-176 (pTet) respectively. There is no band produced with wild type template DNA as there is no kanamycin resistance cassette. Lanes 5, 6 and 7 contain amplifications using primers Km.verif-5-pTir4 in strain 81-176 (pTet/pTet23ΔKm) mutant 5 and 6, and wild type 81-176 (pTet) respectively. There are no bands for wild type or mutant as in the wild type there is no kanamycin resistance cassette and in the mutants the invertible region is fixed in one orientation. Lanes 8, 9 and 10 contain amplifications using primers ampli.Km-3-ampli.Km-P-5 in strain 81-176 (pTet/pTet23ΔKm) and wild type 81-176 (pTet) respectively. Bands are only produced for the mutants as the wild type has no kanamycin resistance cassette. Lanes 11, 12 and 13 contain amplifications using primers ampli.Km-3-ampli.Km-5 in strain 81-176 (pTet/pTet23ΔKm) and wild type 81-176 (pTet) respectively. No bands are produced for the mutants as the inserted kanamycin resistance cassette has no promoter.

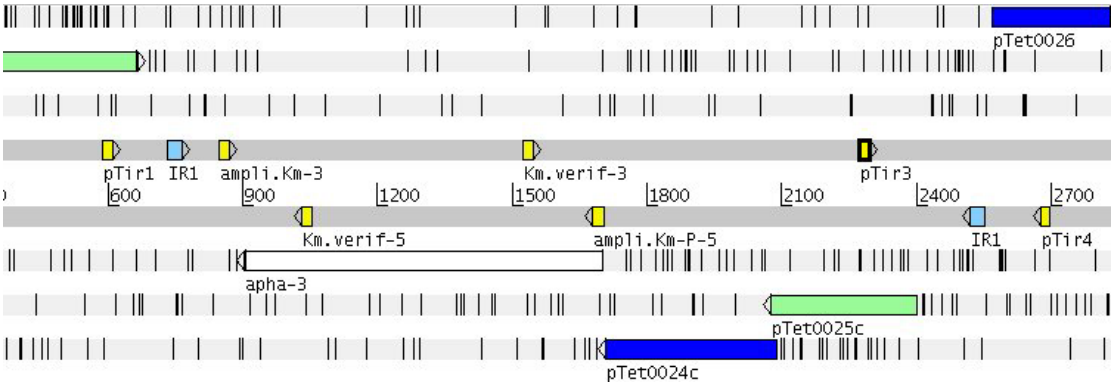

Fig 7.14: Representation of the invertible region of *C. jejuni* strain 81-176 (pTet/pTet23ΔKm). The region is viewed using Artemis [102]. The forward and reverse DNA sequences are represented by dark grey lines. The three-frame forward and reverse DNA translations are represented by light grey lines. Features are represented by open boxes: oligonucleotide primer sequences (yellow) and inverted repeats (blue) are marked on the DNA lines; CDSs are marked on the translated frame lines. CDSs are coloured according to functional category: light green, unknown; blue, pathogenicity/ adaptation/ chaperones. The kanamycin resistance gene *apha-3* is coloured white.

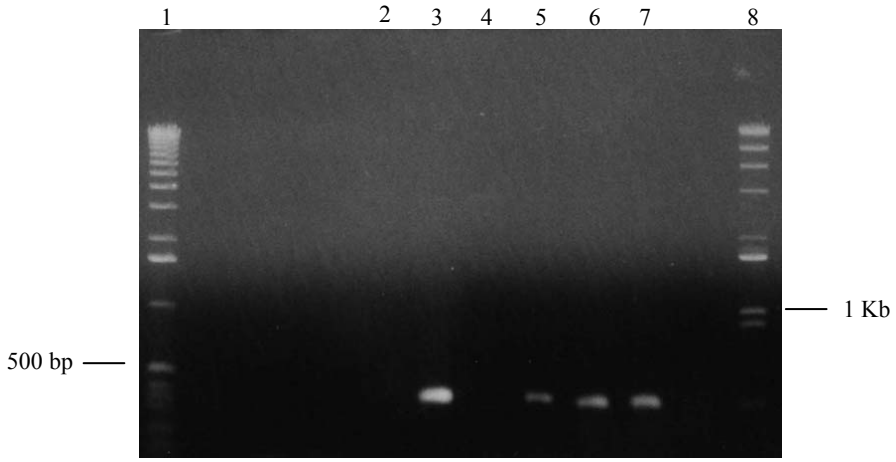

Fig 7.15: PCR amplification between various primers in mutant and wild type 81-176 strains. Lane 1 contains 1 Kb DNA marker and lane 8 contains a mixture of λ *Hin*dIII and pBR322 *Bst*NI digested DNA. Lanes 2 and 3 contain amplifications using primers pTir1-pTir2 in strain 81-176 (pTet/pTet23ΔKm) and wild type 81-176 (pTet) respectively. Lanes 4 and 5 contain amplifications using primers pTir1-pTir3 in strain 81-176 (pTet/pTet23ΔKm) and wild type 81-176 (pTet) respectively. Lanes 6 and 7 contain amplifications using primers pTir3-pTir4 in strain 81-176 (pTet/pTet23ΔKm) and wild type 81-176 (pTet) respectively. This shows that the invertible region no longer inverts in the mutant.

In order to show that the kanamycin resistance gene had inserted in the right location in pTet and that there was only a single copy, purified total DNA from wild type 81-176 (pTet) and 81-176 (pTet/pTet23ΔKm) mutants 5 and 6 were analysed by Southern blotting. DNA was digested with enzymes *Bgl*II and *Hin*dIII. *Bgl*II has been used previously for analysis of pTet [55] and the restriction sites are located outside the invertible region (**Fig 7.16 A**). *Hin*dIII sites are present both within and outside the invertible region resulting in the DNA invertase being located on different size fragments in the wild type pTet depending on which orientation the invertible region is in (**Fig 7.16 A** and **B**). For the pTet/pTet23ΔKm mutant an extra *Hin*dIII site had been introduced downstream of the kanamycin resistance gene (*apha-3*) which means there are two *Hin*dIII sites within the invertible region in the mutant (**Fig 7.16 C**). The undigested and digested DNA was separated by agarose gel electrophoresis in duplicate (**Fig 7.17**). The DNA was then transferred to a Nytran + membrane by capillary transfer (section 7.2.3.5). The membrane was cut in two and each section was hybridized in two parallel reactions to radiolabelled probes generated by PCR amplification from the kanamycin resistance gene, using primers ampli.Km-P-5 and ampli.Km-3, and the putative DNA invertase gene, using primers pT23L and pT23R (**Fig 7.14** and **Fig 7.18**).

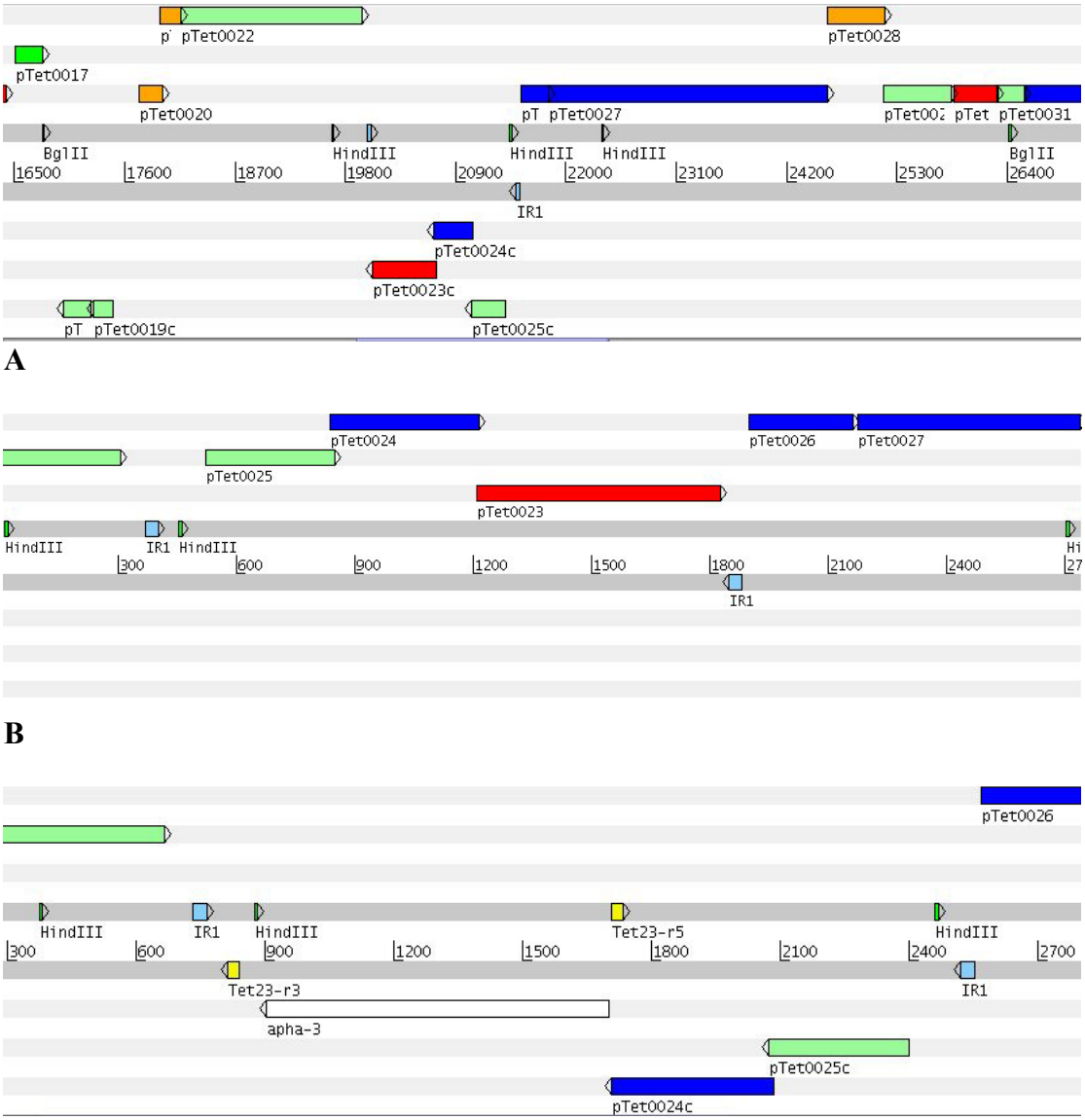

Fig 7.16: Representation of restriction sites within pTet. **A** is 81-176 (pTet) in the 'sequenced' orientation, **B** is 81-176 (pTet) in the 'inverted' orientation, **C** is 81-176 (pTet/pTet23ΔKm). The regions are viewed using Artemis [102]. The forward and reverse DNA sequences are represented by dark grey lines. The three-frame forward and reverse DNA translations are represented by light grey lines. Features are represented by open boxes: oligonucleotide primer sequences (yellow), inverted repeats (blue) and restriction enzyme sites (green), are marked on the DNA lines; CDSs are marked on the translated frame lines. CDSs are coloured according to functional category: light green, unknown; red, information transfer; blue, pathogenicity/ adaptation/ chaperones; orange, conserved hypothetical. The kanamycin resistance gene, *apha-3*, is coloured white. The *Bgl*II restriction sites are located outside the region of inversion, **A**. The *Hin*dIII restriction sites are located such that different size fragments will be produced depending on the orientation of the invertible region, **B**. For the mutant an extra *Hin*dIII restriction site has been introduced downstream of the *apha-3* gene, **C**.

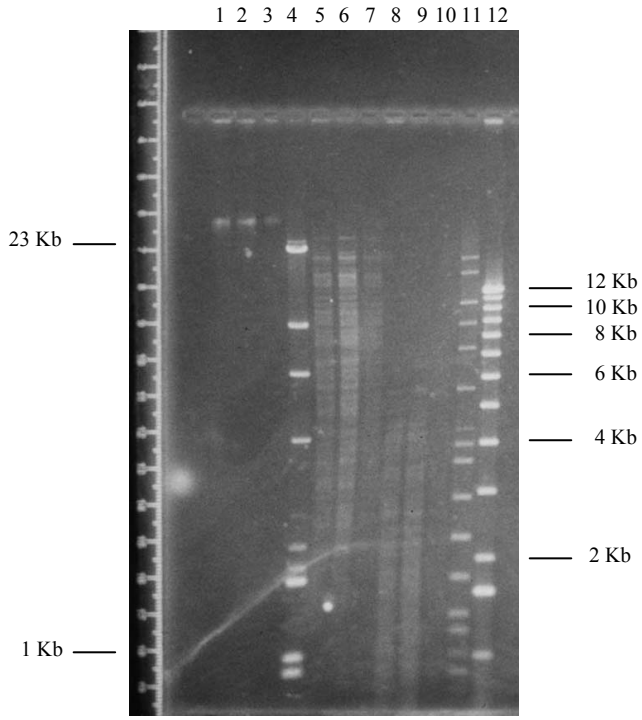

Fig 7.17: Mutant and wild type restriction enzyme digests used for southern transfer. Lanes 1, 2 and 3 contain undigested DNA from 81-176 (pTet/pTet23ΔKm) mutants 5 and 6, and wild type 81-176 (pTet); lane 4 contains marker λ *Hin*dIII/pBR322 *Bst*NI; lanes 5, 6 and 7 contain *Bgl*II digested DNA from 81-176 (pTet/pTet23ΔKm) mutants 5 and 6, and wild type 81-176 (pTet); lanes 8, 9 and 10 contain *Hin*dIII digested DNA from 81-176 (pTet/pTet23ΔKm) mutants 5 and 6, and wild type 81-176 (pTet); lane 11 contains Raoul marker and lane 12 contains 1 Kb marker.

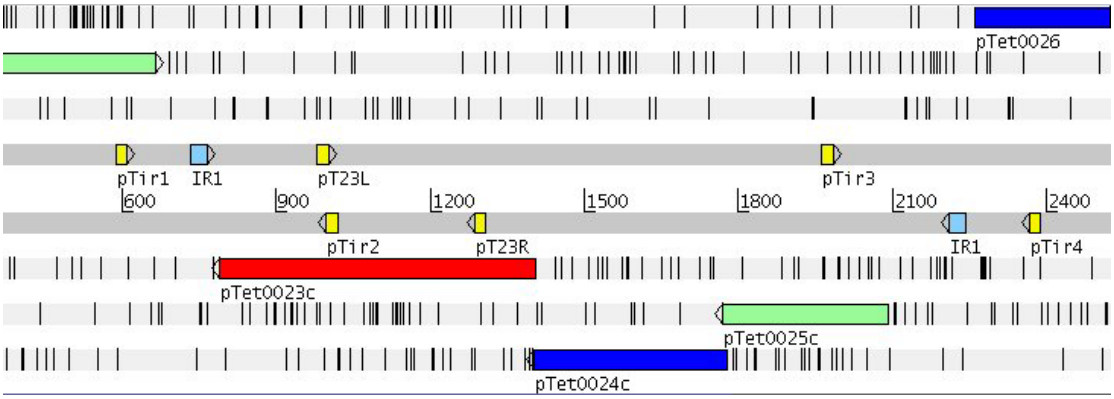

Fig 7.18: Sequence of the wild type *C. jejuni* strain 81-176 pTet with the invertible region in the sequenced orientation viewed using Artemis. The forward and reverse DNA sequences are represented by dark grey lines. The three-frame forward and reverse DNA translations are represented by light grey lines with stop codons indicated by vertical black lines. Features are represented by open boxes: oligonucleotide primer sequences (yellow) and inverted repeats (blue) are marked on the DNA lines; CDSs are marked on the translated frame lines. CDSs are coloured according to functional category: light green, unknown; red, information transfer; blue, pathogenicity/ adaptation/ chaperones. The primers pT23L and pT23R were used to amplify a region from the invertase gene for use as a template to construct a radiolabelled probe for the Southern blot.

Only DNA from 81-176 (pTet/pTet23ΔKm) mutants 5 and 6 hybridized to the kanamycin probe and only the wild type 81-176 (pTet) DNA hybridized to the DNA invertase probe (**Fig 7.19**). The *Bgl*II digested DNA from the wild type has a band size of 9644 bp while the *Bgl*II digested mutant DNA has a band size of 9953 bp. The *Hin*dIII digested wild type DNA has band sizes of 2255 bp for the invertible region in the inverted orientation and 1780 bp for the invertible region in the sequenced orientation. It is apparent that the hybridization signal from the band in the sequenced orientation is much stronger than that for the inverted orientation. The *Hin*dIII digested mutant DNA has a band size of 1588 bp. The observed and expected band sizes correlate.

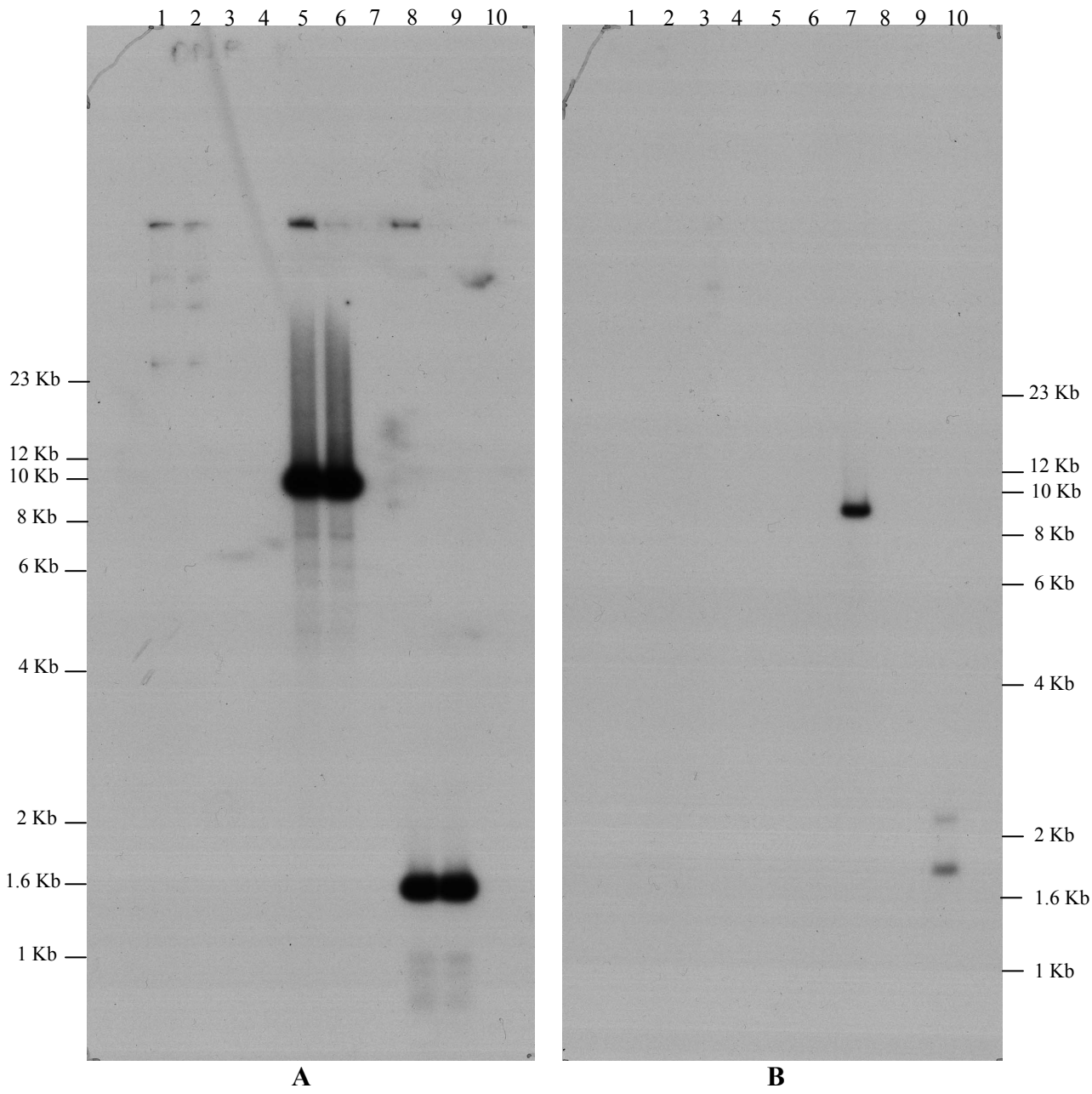

Fig 7.19: Southern blot showing pTet23 knockout mutant. Probing was performed in duplicate. **A** is using kanamycin gene probe. **B** is using invertase gene probe. Lanes 1, 2 and 3 contain undigested DNA from 81-176 (pTet/pTet23ΔKm) mutants 5 and 6, and wild type 81-176 (pTet); lane 4 contains marker λ *Hin*dIII/pBR322 *Bst*NI; lanes 5, 6 and 7 contain *Bgl*II digested DNA from 81-176 (pTet/pTet23ΔKm) mutants 5 and 6, and wild type 81-176 (pTet); lanes 8, 9 and 10 contain *Hin*dIII digested DNA from 81-176 (pTet/pTet23ΔKm) mutants 5 and 6, and wild type 81-176 (pTet). From these blots it can be seen that in the mutant the invertase has been replaced with a kanamycin resistance cassette. Also from the two bands seen in lane 10, where the wild type DNA was digested with *Hin*dIII and hybridized with the invertase gene probe, it appears that the inverted orientation is less common (weaker, upper band).

7.3.4 Conjugation experiments

Strain NCTC 11168 *cj0742::cat* was used as the recipient strain for conjugation experiments, as a strain with an alternative chromosomal antibiotic resistance marker to tetracycline and kanamycin was needed so that conjugation events could be measured by selecting for recipient cells that had received pTet from donor cells (section 7.2.3.5). Both 81-176 (pTet) and strain (pTet/pTet23ΔKm) were used as donor strains. Conjugation reactions were performed in the presence of DNase I to prevent uptake of the plasmid by natural transformation as opposed to transfer by conjugation. Transconjugants were grown on plates containing tetracycline and chloramphenicol to select for NCTC 11168 cells that had received pTet or pTet23ΔKm. In addition single strains were plated on double selective plates (tetracycline and chloramphenicol) to check for spontaneous resistance mutants. The frequency of transconjugation was expressed as the number of NCTC 11168 cells that had received pTet or pTet23ΔKm per donor cell in the initial conjugation mix. All serial dilutions were plated in triplicate and conjugations between donor and recipient were performed in duplicate. The results from conjugation experiments show that there is no significant difference between conjugation frequencies of wild type 81-176 (pTet) and knockout mutant 81-176 (pTet/pTet23ΔKm) mutants 5 and 6 (**Fig 7.20**). This indicates that the putative promoter may be in the "on" orientation and therefore drive transcription of the down stream type IV secretion system homologues when the invertible region is in the 'sequenced' orientation.

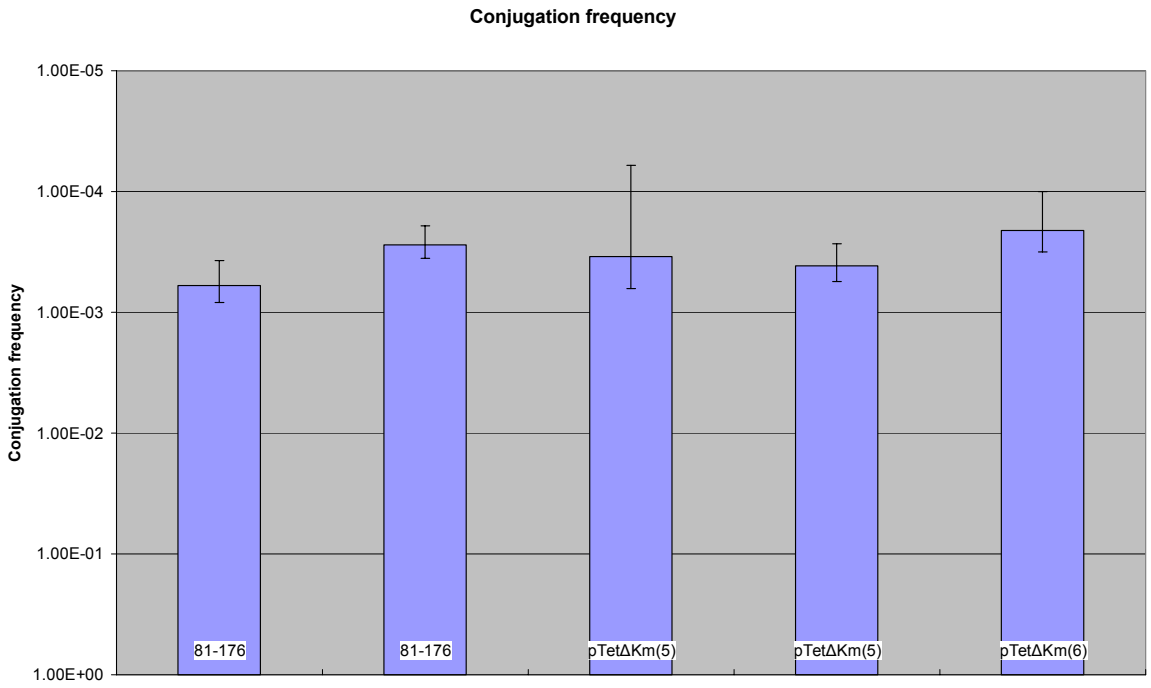

Fig 7.20: Transfer of pTet and pTet23ΔKm to strain NCTC 11168 (*cj0742::cat*). Conjugation frequencies are expressed as the number of transconjugants obtained per donor cell in the initial conjugation culture. Error bars indicate one standard error either side of the mean value. Two biological replicates are shown for wild type and pTet23Km mutant 5, only one for pTet23Km mutant 6. This indicates that there is no significant difference between wild type and mutant conjugation frequencies.

7.4 Discussion

The aims of this section of the project were to assess whether the region in pTet containing a putative DNA invertase, flanked by 31 bp inverted repeats, does invert in the plasmid pTet, whether the DNA invertase is responsible for the inversion and what the implications of this are for the plasmid and host.

Site-specific DNA recombinases can be involved in many biological functions; the family includes DNA invertases, resolvases and integrases. In a multicopy plasmid of *Clostridium perfringens* a resolvase gene has been proposed to act in resolving plasmid multimers into monomers. This is thought to stabilize the plasmid and allow more efficient partitioning [216]. The resolvase of the transposon γδ resolves cointegrate intermediates formed during intermolecular transposition of the parent transposons [217]. Inversion of DNA segments has also been linked to alternation of expression between sets of genes when promoters are located on the ends of invertible regions. In *Salmonella enterica* Typhimurium and other closely related Salmonella spp. a recombinase, Hin, is responsible for inverting a region of DNA that places a promoter upstream of one of two copies of a flagellar gene [218]. This inversion requires a host protein, factor II, and a histone-like protein, and the rate of inversion is increased by an additional enhancer element [219]. This enhancer is located within the N-terminal coding region of the recombinase and has been shown to be cis-acting. The cis-acting sequence is also present in Gin, a recombinase from bacteriophage Mu [219] which controls host range alternation [217]. In bacteriophage Mu two sets of proteins involved in tail fibre production are alternately expressed, thereby altering host range. The rate of inversion is low, presumably to keep one phenotype through one infectious cycle [220]. Expression linked to the G(+) orientation of the invertible region is required for infection of *Escherichia coli* K12 whereas expression linked to the G(-) orientation is required for infection of *Shigella sonnei* and *Escherichia coli* C [221].

Inversion of a region within *Campylobacter fetus* has been proposed to vary surface layer proteins. The *sapA* promoter is located on an inverting stretch of DNA which upon inversion is positioned upstream of two different oppositely orientated gene cassettes [123]. Some systems are more complex with different gene cassettes being inverted upstream of static promoters, such as the Min-like system in R64-related Inc plasmids [123]. The IncIα plasmid R64 contains seven 19bp repeats which upon inversion orientate different C-terminal regions in frame downstream of a fixed N-terminal region to create seven different genes [222].

Site specific recombinases have conserved regions (**Fig 7.21**); the carboxy terminal region is involved in DNA recognition whereas the amino terminal region is responsible for mediating inversion [217]. The amino terminus is highly conserved but the carboxy terminus is more divergent as might be expected with the different target specificities displayed by the recombinases [216]. The mechanism of action of serine recombinases is not clearly understood [223]. The recombinases interact with short DNA repeats, bringing them together, and then all four strands are cleaved and re-ligated. In serine recombinases a staggered break is generated at a 2 bp sequence and transient linkages are formed between the phosphate groups at the recessed 5`-ends of the newly broken DNA and serine residues within the catalytic domain of the recombinase. The strands are exchanged and then re-ligated *via* an unknown mechanism [223].

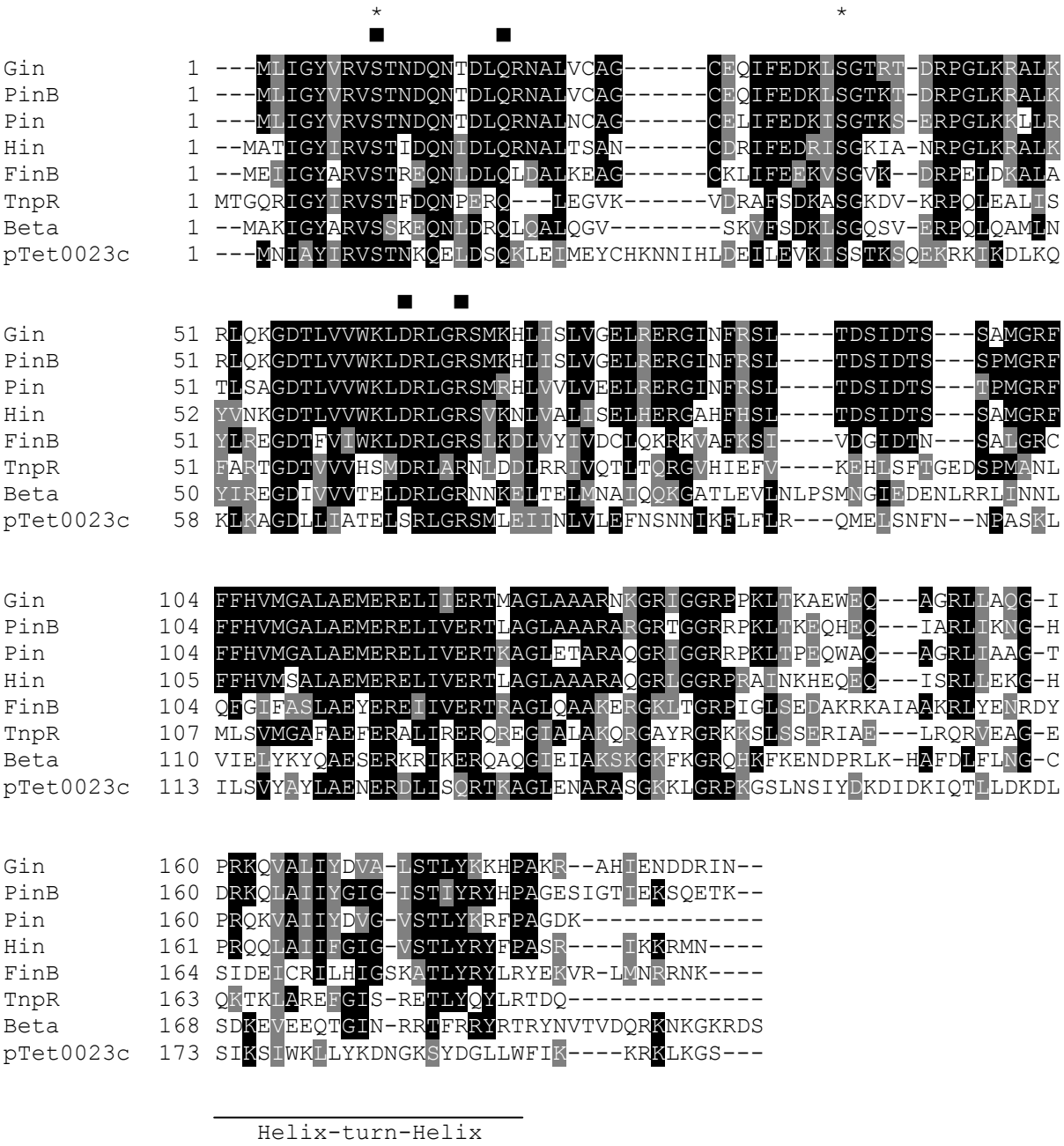

Fig 7.21: Site specific DNA recombinase alignments. The protein sequences were aligned using clustal X. * = serine residues likely to be covalently linked to DNA during recombination. ■ = functionally important as identified by missense mutation analysis of γδ resolvase as identified by Newman and Grindley, 1984 [217]. The amino acid sequence of pTet0023c is compared with the sequences of site-specific recombinases from: Bacteriophage Mu, Gin (accession JWBPU); *Shigella sonnei*, PinB (accession BAA00556); *Escherichia coli*, Pin (accession AAA24391); *Salmonella enterica* Typhimurium LT2, Hin (accession NP_461699); *Bacteroides fragilis*, FinB (accession YP_209695); *Escherichia coli* Tn21, TnpR (accession RPEC21); *Streptococcus pyogenes*, Beta (accession AAR27194).

In this study PCR and sequencing was used to confirm the fact that the pTet0023-pTet0025 region flanked by inverted repeats does invert in *C. jejuni* and in *Escherichia coli*. In addition it was proved that the DNA invertase gene is responsible for this inversion. When the DNA invertase was knocked out inversion could not be demonstrated. The frequency of DNA inversion in the *C. fetus* study was found to be 1.3-5.7 $x10^{-4}$ per generation [123]. The Southern blot showed that there was a much weaker signal from the band corresponding to the invertible region in the inverted orientation compared to in the sequenced orientation which suggests that inversion of this region does not occur very often. This is backed up by the observation that the opposite orientation was not seen in the shotgun sequence assembly of pTet (chapter 3). It may be that when the invertible region is in the inverted orientation that the inverted repeats represent a better substrate for the DNA invertase than the inverted repeats when the invertible region is in the sequenced orientation. As the inverted repeats are imperfect it is possible that the sequence surrounding the repeats in the inverted orientation alters the secondary structure of the DNA making the repeat more accessible to the invertase than when the invertible region is in the sequenced orientation. If this is the case then the forward and reverse inversion reactions may proceed at different rates which would explain why the invertible region has been predominantly found in the sequenced orientation in this study.

There is a predicted promoter located between pTet0025 and pTet0026 which would be upstream of the type IV secretion gene homologues when the invertible region is in the sequenced orientation. It would appear that, as there was no difference in conjugation frequencies when the inverted region was fixed in this orientation, this represents the promoter "on" position and that in the inverted orientation the promoter could be in an "off" position. If the conjugation genes are under the control of a variable promoter then it may be beneficial to the bacterium, and therefore the plasmid, under some circumstances to not have

a type IV pilus. This could be because the pilus may present a target for the immune system during infection of a host. A type IV pilus is needed for conjugation and transfer of the plasmid so it is beneficial for plasmid propagation. By controlling its conjugation system with a phase variable promoter, the plasmid could maximise its opportunity for transfer, whilst minimising its risk to the bacterial host. To prove that this is a phase-variable promoter, the conjugation experiments would need to be performed with the invertible region fixed in the opposite orientation in order to see if this has the predicted effect on conjugation frequencies. Unfortunately attempts to isolate a clone fixed in the inverted orientation were unsuccessful; this may be due to the unequal inversion frequencies discussed above.

In addition experiments could be done to determine the exact location of a promoter in the region upstream of the type IV secretion system gene homologues using RNase partial digestion and S1 nuclease digestion assays [224;225]. The promoter could also be used to drive expression of a promoterless reporter gene to prove it is functional and to determine the rate of inversion of the DNA segment [126]. Another possibility would be to extract RNA from wild type 81-176 and mutants with the invertible region fixed in both orientations and use reverse transcriptase PCR (RT-PCR) to see whether the mutant in the opposite orientation has decreased transcription of conjugation genes and to see how far into the type IV secretion system this effect extends.