Beta-lactam resistance in *Campylobacter*

By

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A thesis submitted to the University of Birmingham for the degree of DOCTOR OF PHILOSOPHY

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October 2010

Abstract

Resistance to β-lactam antibiotics in *Campylobacter* is often associated with the production of a β-lactamase; to date the genomically encoded bla_{OXA-61} and the closely related *cj0299* are the only described β-lactamase genes of *Campylobacter*.

Cj0299 of *C. jejuni* NCTC11168 was assigned a novel oxacillinase number OXA-193.

Previously, a novel β-lactamase (CjBla2) was identified in two $bla_{OXA-193}$ negative isolates, P843 and P854. Southern blotting with a probe for $bla_{OXA-193}$ confirmed that Bla2 is not the product of a mutated $bla_{OXA-193}$ gene. A further thirteen veterinary isolates of *Campylobacter* have been identified that have the same phenotype as P843 and P854.

Whole genome sequencing of P854 revealed four putative beta-lactamase genes, one of which, *P854_1490*, encodes a completely novel oxacillinase (OXA-184). PCR screening and sequencing of the other putative CjBla2 producers revealed six to contain the novel oxacillinase. A further five encode a variant of this novel OXA in which a point mutation has led to an amino acid coding change from leucine to isoleucine (OXA-185). These isolates represent a selection of *flaA* types and were isolated from two separate locations at different times, therefore it is unlikely that they are a clonal population.

Two conjugative plasmids, each approximately 45Kb in size, have been identified from two veterinary isolates of *Campylobacter.* These plasmids have been shown to horizontally transfer resistance to tetracycline and to the β-lactams penicillin, ampicillin and oxacillin, between *Campylobacter*, a process never previously described. The two β-lactam resistance encoding plasmids thought to contain a β-lactamase gene(s) have been named pBla1 and pBla2.

The role of efflux in beta-lactam resistance has also been investigated; inactivation of the efflux pump gene *cmeB* in the reference strain NCTC11168 resulted in increased susceptibility to a range of beta-lactams including the cephalosporins which *Campylobacter* are reported to be innately resistant to and have been included in some *Campylobacter* selective media.

The work completed as part of this PhD program has furthered the understanding of beta-lactam resistance in *Campylobacter* and has demonstrated that it is clearly a multi-faceted mechanism incorporating various chromosomally encoded beta-lactamases, putative transferable beta-lactamases and efflux.

For Daniel and Ella

Acknowledgements

I am extremely grateful to my supervisor Laura Piddock for giving me this opportunity and for being behind me every step of the way. I would like to thank all the past and present members of ARG for their help and support during the last three years, and for making me laugh every day. In particular I would like to thank the past and present postdocs Mark Garvey, Jon Caddick, Mark Webber, Vito Ricci and Andrew Bailey for answering what must have seemed like a never ending list of questions. Thank you to Jess for her understanding and support which helped me to keep going and to Jen for her excellent proof reading skills and eagerness to 'talk science'.

I would also like to thank Chris Thomas from Biosciences for his time in teaching me how to extract difficult plasmids and also Lisa Williams at Bristol for helping me set up my growth kinetics.

Finally, I would like to thank my parents and family for their continued support during my long university days and for always believing in me, in particular my Grandma Marlene for checking up on me at least once a week. Thank you to all my friends for helping me through and supplying wine where necessary and an especially big thank you to Bec for dealing with more than her fair share. Most of all I would like to thank my husband, for everything.

Clarification of Contribution to Collaborative work

The following two figures were the result of collaborations. Details of the collaboration and the contribution by both parties are clarified below.

Figure 5.7 Growth kinetics of pBla1 donor, recipient and transconjugant (p 158)

Figure 5.8 Growth kinetics of pBla2 donor, recipient and transconjugant (p 160)

Leanne Stones was responsible for culture of the organisms and setting up microtitre trays under the supervision of Lisa Williams (Bristol) and for analysing growth kinetics data. Lisa was responsible for loading the tray onto the BioScan and for retrieving data.

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List of Abbreviations

Abbreviation	Definition
µg	Microgram
µl	Microlitre
µM	Micromole
aph	Aminoglycoside phosphotransferase
bp	Base pair
BSAC	British Society for Antimicrobial Chemotherapy
cDNA	Complementary DNA
Cfu	Colony forming units
CO_2	Carbon dioxide
CsCl	Caesium chloride
CTX-M	Cefotaximase
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EtBr	Ethidium bromide
ESBL	Extended spectrum beta-lactamase
g	Gram
g	Gravity
HCl	Hydrochloric acid
Kb	Kilobase
kDa	Kilodalton
L	Litre
LB	Luria Bertani
M	Molar
MIC	Minimum Inhibitory Concentration
min	Minute
mg	Milligram
MH	Mueller Hinton
ml	Millilitre
mM	Millimolar
Mr	Molecular mass
NCTC	National collection of typed cultures
ng	Nanogram
nm	Nanometre
OD	Optical density
OXA	Oxacillinase
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pI	Isoelectric point

RNA	Ribonucleic acid
RND	Resistance nodulation division
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase PCR
SD	Standard deviation
SDW	Sterile distilled water
sec	Second
TBE	Tris-Borate EDTA buffer
TE	Tris EDTA buffer
UV	Ultraviolet
VBNC	Viable but non culturable

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Chapter 1 – Introduction

1.1 *Campylobacter jejuni*

Campylobacter jejuni sub species *jejuni* (*C. jejuni*) is the most commonly isolated species of the *Campylobacter* genera (HPA 2006; HPA 2007; ECDC 2009). Other clinically important species include *Campylobacter coli* and *Campylobacter foetus*. Two other closely related genera, *Helicobacter* and *Wolinella* along with the *Campylobacter* genera make up the order of the *Campylobacterales* which belong to the epsilon class of proteobacteria.

Although *Campylobacter* have only been recognised as human pathogens for the last 40 years, their presence may have been noted in clinical samples as early as 1886 when Theodore Escherich described un-culturable *Campylobacter*-like organisms in the stools of infants who had died of 'cholera infantum' (Kist 1985). From this time until the 1960s, all the information about *Campylobacter* was gained through the field of veterinary medicine.

A five year survey into the causes of abortion in sheep was set up in 1906 by the English board of agriculture and fisheries and was led by John McFadyean and Stewart Stockholm; they were the first to define "vibrionic abortion" after isolating a "vibrio" from the uterine mucus of a calf and the stomach contents of her foetus (McFadyean and Stockholm 1913). One hundred years later, it is clear that what they were actually describing is *Campylobacter foetus*.

In 1931, Jones *et al.,* described an organism isolated from the jejunal mucosa of calves and cattle with an enteric fever or "winter dysentery" similar to "*Vibrio foetus"* but distinct antigenically, which they termed "*Vibrio jejuni*"(Jones, Orcutt et al. 1931). This name persists in the form of *Campylobacter jejuni* although none of the original isolates do; some

speculate this is because no-one was willing to continue with time consuming work of isolating these organisms without the advantage of selective media (Skirrow and Butzler 2000).

A major contribution to the history of *Campylobacter* was made by Elizabeth King who studied isolates labelled as *"Vibrio foetus"* and made the distinction between the usual isolates e.g. *Vibrio cholerae* and those with a higher optimal growth temperature, *Campylobacter jejuni and coli*, which she gave the temporary name of "related *Vibrios"* (King 1957; King 1962). In 1963, the term *Campylobacter* (Greek for 'curved rod') was suggested to re-classify those "*vibrios*" that were both serologically and biochemically different from the classical *Vibrios* (e.g. *Vibrio cholerae*) (Sebald and Veron 1963).

The isolation of *Campylobacter* from the faeces of patients with enteritis was first achieved by Dekeyser and Butzler in 1972 (Dekeyser, Gossuin-Detrain et al. 1972; Butzler, Dekeyser et al. 1973) by using the filtration method that Dekeyser, as a veterinary microbiologist, was familiar with, as it was used routinely to isolate "*Vibrio foetus*" from cattle suffering septic abortion (Dekeyser, Gossuin-Detrain et al. 1972). These experiments were repeated by Skirrow in 1977 and *Campylobacter* was shown to be the most common bacterial pathogen isolated from the stool samples of patients with diarrhoea (Skirrow 1977). Since then the understanding of *Campylobacter* has benefited dramatically from the sequencing of an exemplar genome and other genetic elements (Parkhill, Wren et al. 2000; Bacon, Alm et al. 2002).

1.1.1 Microbiology

C. jejuni is a small (1.5-6.0µm in length and 0.2-0.5µm in width), Gram negative, spiral bacterium with a characteristic corkscrew motility that can distinguish it from other bacteria

when viewed under a phase contrast microscope. Single, un-sheathed polar flagella are usually present at one or both ends of the cell (Figure 1.1) (Snelling, Matsuda et al. 2005; LaGier, Pratt et al. 2009). *C. jejuni* is microaerophilic and optimal growth of the organism is achieved in an atmosphere of 3-15% O_2 and 3-5% CO_2; the 42°C optimal growth temperature of this thermophile is thought to be a consequence of the natural habitat of *C. jejuni* being within the gastrointestinal tract of birds (and other animals) whose body temperature is higher than that of humans (Ketley 1997; Snelling, Matsuda et al. 2005).

The process of obtaining pure cultures of *Campylobacter* is simplified by the use of compounds in medium that inhibit the growth of other bacterial species and so promotes the growth of *Campylobacter.* Common *Campylobacter* selective media include modified CCDA, a blood free agar which contains instead charcoal, ferrous sulphate and sodium pyruvate; this medium with the selective supplements amphotericin and cefoperazone is recommended for the isolation of *Campylobacter* from clinical specimens plus food and water specimens (HPA 2004).

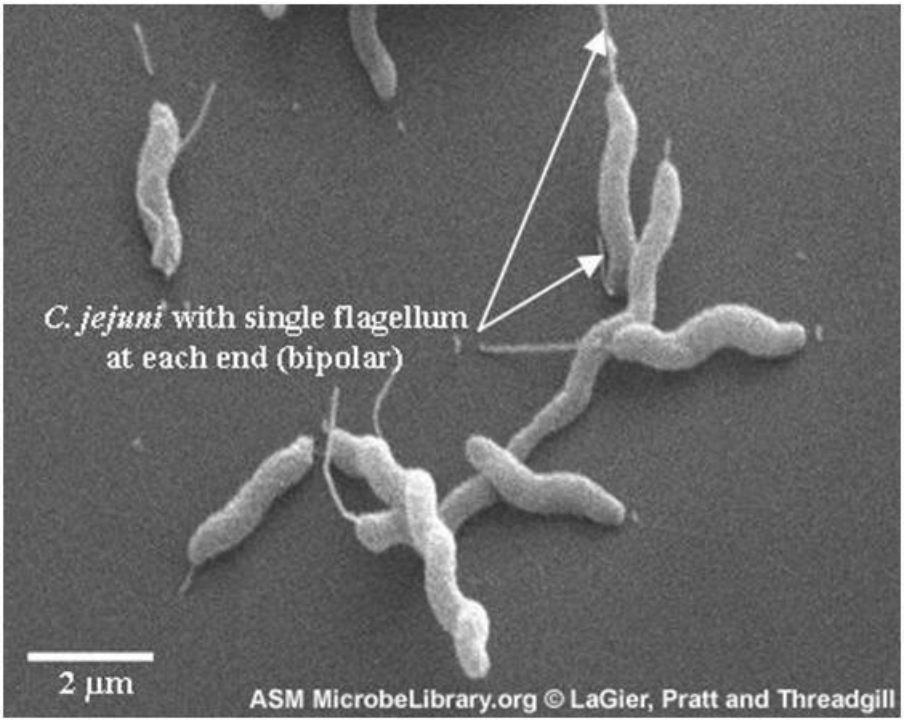
Skirrow's selective supplement contains vancomycin, polymyxin and trimethoprim and is added to a blood agar or Columbia agar base to make Skirrow's medium which is also recommended for the isolation of *Campylobacter* from clinical specimens (HPA 2004).

1.1.2 Campylobacter genetics

1.1.2.1 Sequenced genomes

The genome of *C. jejuni* NCTC 11168 was sequenced in 2000 (Parkhill, Wren et al. 2000) and re-annotated in 2007 (Gundogdu, Bentley et al. 2007), as a result considerable amount data is available about the genome of this pathogen. This exemplar genome of *C. jejuni* is 1.6Mb

Figure 1.1 Scanning electron micrograph of *Campylobacter jejuni*



Scanning electron micrograph of *Campylobacter jejuni* with labelled bipolar terminal flagella (LaGier, Pratt et al. 2009).

in size and is now thought to encode 1643 proteins (initially thought to be 1654). Few insertion sequences or phage-associated sequences have been found and it has been noted that the genome has a lower than average GC content (30.6% G+C) (Parkhill, Wren et al. 2000; Gundogdu, Bentley et al. 2007). Hyper variability was found in several genes relating to surface proteins, Parkhill *et al.,* postulated that this was likely to be a survival mechanism of *C. jejuni* (Parkhill, Wren et al. 2000).

The NCBI Entrez Genome Project now lists twenty *C. jejuni* sequencing projects (Table 1.1) and five *Campylobacter* plasmid sequencing projects (NCBI 2010).

1.1.2.2 Mechanisms of horizontal gene transfer in bacteria

The ability of bacteria to acquire genes from within and between bacterial species has enabled them to adapt to and exploit a multitude of ecological niches. The proportion of horizontally acquired genes varies greatly between species, some organisms with smaller genomes e.g. *Mycoplasma genitalium*, contain virtually no acquired genes whereas in others non-native DNA can contribute to nearly 17% of the organism's genome e.g. *Escherichia coli* K12 and *Synechocystis* (Figure 1.2) (Ochman, Lawrence et al. 2000; Pallen and Wren 2007). There are three main ways in which bacteria can acquire 'foreign' DNA by horizontal transfer; transformation, transduction and conjugation.

Transformation

Transformation does not require the donor and recipients to be in physical contact with each other as the process involves the recipient cell taking up 'naked DNA' from the environment. This process has the potential to allow un-related species to exchange DNA. It has been demonstrated that during natural transformation *Campylobacter* show a preference for the

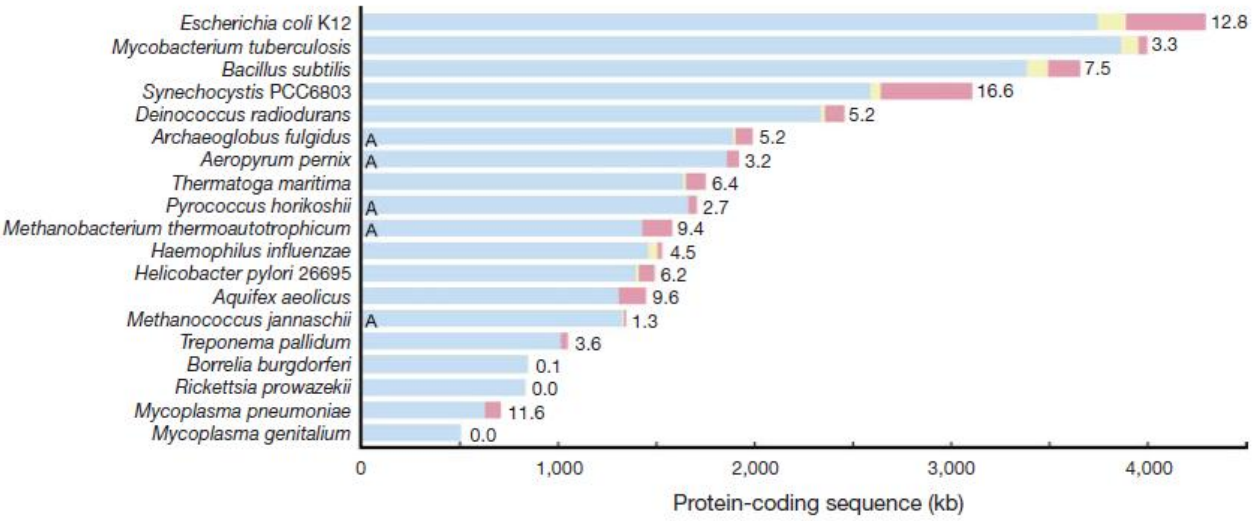
Table 1.1 *Campylobacter jejuni* genome sequencing projects as of September 2010

Isolate	Sequencing Body	Status	NCBI Project ID:
Campylobacter jejuni subsp. jejuni NCTC 11168	Sanger Institute	Complete	8
Campylobacter jejuni subsp. jejuni 81116	BBSRC Institute of Food Research	Complete	17953
Campylobacter jejuni subsp. jejuni 81-176	TIGR	Complete	16135
Campylobacter jejuni subsp. doylei 269.97	TIGR	Complete	17163
Campylobacter jejuni RM1221	TIGR	Complete	303
Campylobacter jejuni subsp. jejuni HB93-13	TIGR	Draft Assembly	16267
Campylobacter jejuni subsp. jejuni CG8486	NMRC	Draft Assembly	17055
Campylobacter jejuni subsp. jejuni CG8421	NMRC	Draft Assembly	21037
Campylobacter jejuni subsp. jejuni CF93-6	TIGR	Draft Assembly	16265
Campylobacter jejuni subsp. jejuni BH-01-0142	NMRC	Draft Assembly	27797
Campylobacter jejuni subsp. jejuni 84-25	TIGR	Draft Assembly	16367
Campylobacter jejuni subsp. jejuni 81-176	Yale University	Draft Assembly	17341
Campylobacter jejuni subsp. jejuni 260.94	TIGR	Draft Assembly	16229
Campylobacter jejuni subsp. jejuni M1	Danish Technical University	In Progress	38041
Campylobacter jejuni subsp. jejuni IA3902	Iowa State University	In Progress	28907
Campylobacter jejuni subsp. jejuni DFVF1099	Copenhagen University	In Progress	41639
Campylobacter jejuni subsp. jejuni 327	Copenhagen University	In Progress	41643
Campylobacter jejuni subsp. jejuni 305	Copenhagen University	In Progress	41641
Campylobacter jejuni 414	University of Liverpool	In Progress	33821
Campylobacter jejuni 1336	University of Liverpool	In Progress	33777

Information from the NCBI as of 20/09/2010 (NCBI 2010).

BBSRC; Biotechnology and Biological Sciences Research Council, TIGR; The institute for genomic research, NMRC; US Naval Medical Research Centre.

Figure 1.2 Distribution of horizontally acquired (foreign) DNA in sequenced bacterial genomes



Lengths of bars denote the amount of protein-coding DNA. For each bar, the native DNA is blue; foreign DNA identifiable as mobile elements, including transposons and bacteriophages, is yellow and other foreign DNA is red. The percentage of foreign DNA is noted to the right of each bar. `A' denotes an Archaeal genome (Ochman, Lawrence et al. 2000).

uptake of its own DNA (Wang and Taylor 1990; Wilson, Bell et al. 2003). Surface polysaccharides have recently been shown to limit natural transformation in *C. jejuni*; mutations in both lipooligosaccharide and capsular polysaccharide genes in *C. jejuni* results in a 97 fold increase in transformation frequency (Jeon, Muraoka et al. 2009).

Campylobacter is one of over 40 species of bacteria that are naturally competent. Others include *Neisseria gonorrhoeae, Haemophilus influenzae* and *Streptococcus pneumoniae* (Dubnau 1999). However, it has become common laboratory practice to make bacteria that are not naturally competent, such as *Escherichia coli*, competent by the process of heat shocking or electroporation or can be made chemically competent so that they can take up not only linear fragments of DNA but also circular DNA i.e. plasmids.

Transduction

The introduction of DNA into a bacterium by a bacteriophage is termed transduction. When replicating within the 'donor' bacterium the phage can package either random pieces of its host DNA (generalised transduction) or host DNA adjacent to the phage attachment site (specialized transduction) into its capsid and can then transfer this DNA into the next bacterium it infects. This process is limited by (i) the amount of DNA that can fit within the phage capsid (although this may be greater than 100 kilobases) and (ii) by the need for the 'recipient' to express the receptors the phage requires for attachment (Ochman, Lawrence et al. 2000). When the *Campylobacter jejuni* NCTC11168 genome was sequenced virtually no phage associated sequences were found (Parkhill, Wren et al. 2000). Sequencing of further *Campylobacter* strains revealed the presence of a small number of phage associated regions; *C. jejuni* RM1221 was found to contain four phage associated regions, *C. lari* RM2100 and *C.*

upsaliensis RM3195 each contain one phage associated region whereas *C. coli* RM2228 contains no phage associated regions at all (Fouts, Mongodin et al. 2005).

Conjugation

Conjugation or bacterial 'mating' allows the transfer of plasmids, autonomous, self replicating pieces of DNA from a donor to a recipient cell. Plasmids are usually closed, circular double stranded DNA molecules, although linear double stranded DNA molecules have also been described, for example in *Borrelia burgdorferi* (Hinnebusch and Tilly 1993; Stewart, Byram et al. 2005).

The size range of *Campylobacter* plasmids has been shown to be 3kb to 178kb (Ansary and Radu 1992; Aquino, Filgueiras et al. 2002; Fouts, Mongodin et al. 2005) and these are thought to constitute up to nine incompatibility groups (plasmids from the same incompatibility group have too similar mechanisms of replication to allow them to co-exist in the same bacterial cell) (Miller, Heath et al. 2007). Plasmids can be either self transmissible or mobilisable; the former are usually larger and have a low copy number, the latter usually small and of a high copy number. Hfr (high frequency of recombination) plasmids that integrate into the host chromosome can also be responsible for the transfer of bacterial genomic DNA from one bacterium to another when they are excised imprecisely (Ramirez-Arcos, Fernandez-Herrero et al. 1998; Papke, Koenig et al. 2004).

Besides plasmids other conjugative elements exist including conjugative transposons which encode proteins required for their own excision from the donor bacterium's chromosome, formation of a 'conjugative bridge' and transfer to the recipient bacterium (Bellanger, Roberts et al. 2009).

1.1.3 Typing

Due to the high numbers of human cases of *Campylobacter* infection and the many possible sources of those infections, it is vital to establish typing methods capable of determining the sources of outbreaks of *Campylobacter* illness. Traditional phenotypic methods such as phage typing and Penner serotyping (which is based on heat-stable antigens) are becoming less popular as laboratories switch to molecular typing methods (On, McCarthy et al. 2008). Two such commonly used molecular typing methods are described below.

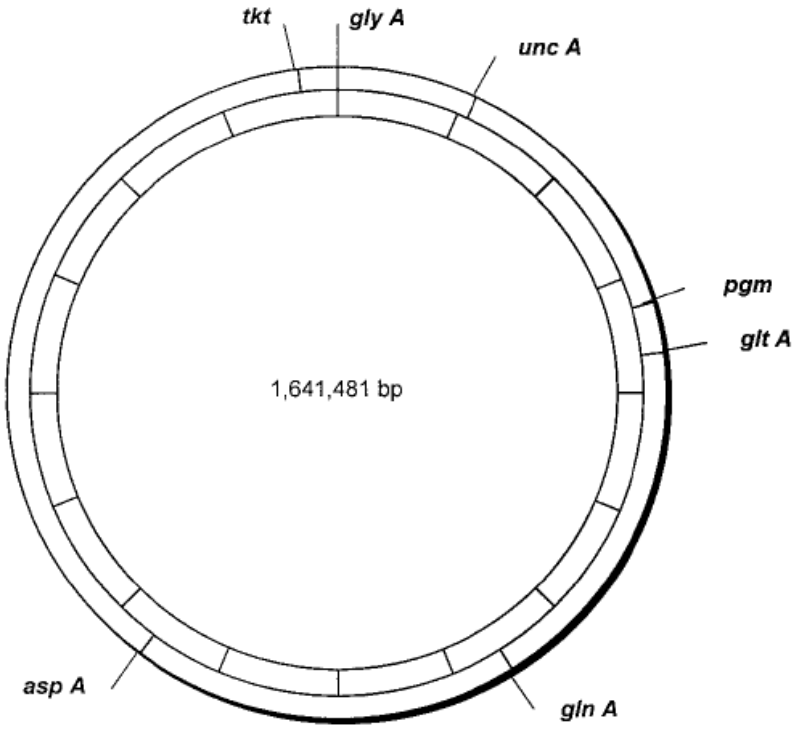
1.1.3.1 *flaA*

Campylobacter possess two flagellin genes, *flaA* and *flaB* which can be used to type isolates. Originally *flaA* RFLP (restriction fragment length polymorphism) was the only available method of *flaA* typing; the *flaA* gene and its flanking region was amplified by PCR, digested with a single or combination of restriction enzymes and then electrophoresed (Nachamkin, Ung et al. 1996). A molecular method of *flaA* analysis known as *flaA* SVR (short variable region) sequencing is now favoured over RFLP, as this method relies on sequencing of the SVR region of *flaA* it is much more reproducible (Meinersmann, Helsel et al. 1997; Ragimbeau, Schneider et al. 2008). The *Campylobacter flaA* database (http://pubmlst.org/campylobacter/) currently lists 1429 *flaA* alleles coding for 332 peptides (last accessed 29/10/2010).

1.1.3.2 Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) exploits the natural sequence variation of housekeeping genes to establish relationships between isolates. An MLST system has been established in *Campylobacter* based on seven 'housekeeping' genes (Figure 1.3)

Figure 1.3 Locations of MLST loci on the chromosome of *Campylobacter jejuni* NCTC 11168



(Dingle, Colles et al. 2001)

Housekeeping Gene	Gene product
aspA	aspartase A
glnA	glutamine synthetase
gltA	citrate synthase
glyA	serine hydroxymethyltransferase
pgm	phosphoglucomutase
tkt	transketolase
uncA	ATP synthase α subunit

The inner circle shows the 1,641,481-bp genome divided into sixteen segments with each segment representing 164,148-bp. The loci of the seven genes targeted for MLST are indicated on the diagram and the products of these genes are listed in the table.

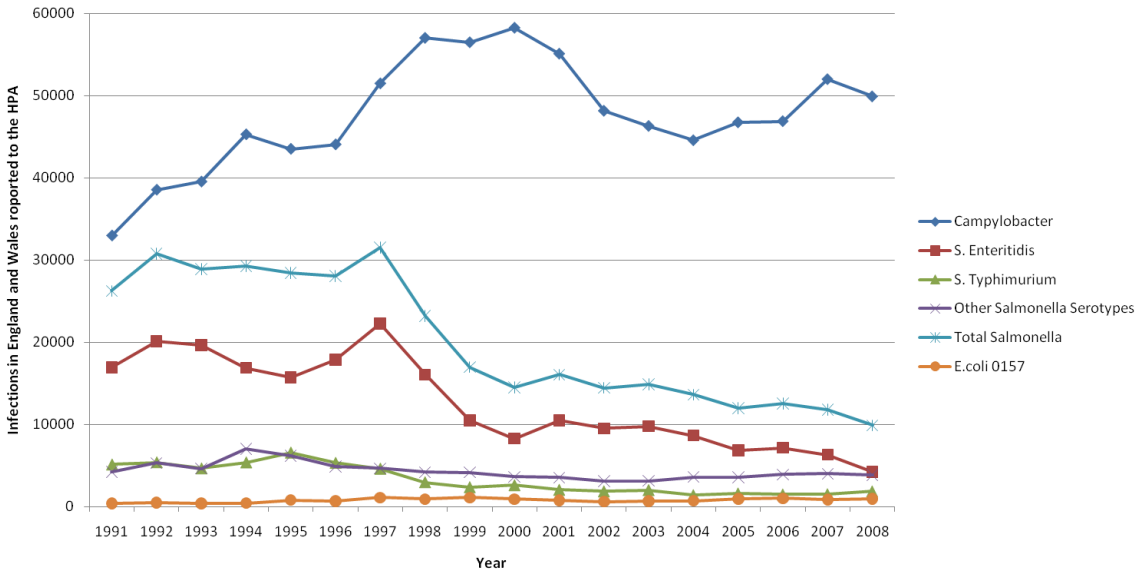
(Maiden, Bygraves et al. 1998; Dingle, Colles et al. 2001; Maiden 2006). Each allelic variant of a gene is assigned a different integer; the seven integers representing each gene are then combined to define the sequence type of a particular isolate. As the results rely on DNA sequence data they are unambiguous and are easily reproducible by different laboratories. Advantages of this technique include excellent reproducibility and the ever decreasing cost of DNA sequencing. The *Campylobacter jejuni* and *Campylobacter coli* MLST isolate database (http://pubmlst.org/campylobacter/) developed by Keith Jolley and Man-Suen Chan (Jolley, Chan et al. 2004) currently contains information on 10580 isolates of *Campylobacter jejuni* and *Campylobacter coli* (last accessed 29/09/2010).

1.1.4 Epidemiology and transmission of *Campylobacter*

For 2008, the HPA reported that there were 49,880 laboratory identified cases of *Campylobacter* infection in the England and Wales; and provisional data published by the HPA suggest that the infection rate rose 19% in 2009 (HPA 2009; HPA 2009). Although large, this number is thought to be a gross under estimation of actual cases as the vast majority require no medical treatment and so go un-reported (HPA 2007). Despite suspected under-reporting the number of cases of bacterial gastroenteritis caused by *Campylobacter* vastly outnumbers those caused by *Salmonella enterica* (serovar Enteritidis plus serovar Typhimurium) and *Escherichia coli* (Figure 1.4).

In the developed world most *Campylobacter* infections are sporadic and peak during the summer and early autumn although some outbreaks do occur (Blaser and Reller 1981; Finch and Riley 1984; Skirrow 1987; Humphrey, O'Brien et al. 2007). In developing countries there has been no proven link to seasonality (Biswas, Lyon et al. 1996).

Figure 1.4 Number of gastrointestinal infections in England and Wales between 1991 and 2008 published by the HPA



Based on data from HPA (HPA 2009).

Campylobacteriosis is a zoonotic disease. The most common route of human infection in developed countries is through contact with contaminated meat, especially under cooked poultry (Sahin, Morishita et al. 2002; Humphrey, O'Brien et al. 2007). Fifty seven and a half percent of chicken at retail was positive for *Campylobacter* (Humphrey, O'Brien et al. 2007) but waterborne transmission is also a problem in both developed and developing nations (Blaser, Taylor et al. 1983; Schonberg-Norio, Takkinen et al. 2004; Carrique-Mas, Andersson et al. 2005). *C. jejuni* is a commensal and can colonise the gastrointestinal tracts of food animals (including cattle, sheep and poultry) and also domestic animals (including cats and dogs), making transmission from animal-human either directly or in-directly (i.e. water contaminated with animal faeces) a common occurrence (Blaser, Taylor et al. 1983; Humphrey, O'Brien et al. 2007).

Other foods that have been implicated in sporadic outbreaks include bird-pecked milk (Neal and Slack 1997), bottled mineral water (Evans, Ribeiro et al. 2003), salad vegetables (Evans, Ribeiro et al. 2003), un-pasteurised milk (Neimann, Engberg et al. 2003) and grapes (Neimann, Engberg et al. 2003).

As chickens clearly play an important role in *Campylobacter* infection in humans, limiting colonisation of poultry is seen as one possible way of controlling *Campylobacter* infection in humans. It has been demonstrated there are various routes by which broilers can become colonised by *Campylobacter,* these include horizontal transmission, via vectors including flies (Hald, Skovgard et al. 2004) and from protozoa found in the birds drinking water which harbour internalised *Campylobacter* (Axelsson-Olsson, Waldenstrom et al. 2005; Snelling, McKenna et al. 2005; Snelling, McKenna et al. 2006; Snelling, Stern et al. 2008).

Although *C. jejuni* has fastidious growth requirements in the laboratory it has been recovered from various harsh environmental sources including river water, sea water, sand and sewage treatment plants (Rollins and Colwell 1986; Bolton, Coates et al. 1987; Jacob, Bindemann et al. 1991; Frias-Lopez, Zerkle et al. 2002). There is some dispute as to whether stressful environments (low temperature and low nutrient availability, high oxygen concentrations) cause *Campylobacter* to enter a 'viable but non-culturable' (VBNC) state, which can be seen microscopically as a change from their usual spiral, motile form to a coccoid from which, it is postulated, they revert back to the infective, spiral form following passage in animals (Rollins and Colwell 1986; Murphy, Carroll et al. 2006). It has been reported that VBNC forms microscopically detected in water could colonise chickens; other groups reported similar findings within laboratory animals (Saha, Saha et al. 1991; Pearson, Greenwood et al. 1993; Stern, Jones et al. 1994).

Contradictory reports show that VBNC cannot initiate colonisation or disease (Beumer, de Vries et al. 1992; Medema, Schets et al. 1992; Hazeleger, Janse et al. 1995) and are in fact a degenerative rather than dormant stage of *C. jejuni's* life cycle (Vandamme 2000).

1.1.5 Disease and Pathogenesis

1.1.5.1 Pathogenesis

Immune Avoidance

Campylobacter has several highly variable regions in its genome due to the presence of homopolymeric tracts. These are runs of approximately eight to thirteen base pairs of a single nucleotide, commonly guanine/cytosine often in a coding region and with guanine on the coding strand; the number of residues in the tract varies at a high frequency typically 10^{-}

^{3} per generation, due to strand or polymerase slippage during transcription or replication (Parkhill, Wren et al. 2000). These regions contain the genes encoding proteins that are responsible for the production/modification of the bacteria's surface carbohydrates including lipooligosaccharide (LOS), capsular polysaccharide and flagella; phase switching by this mechanism is often involved in glycan synthesis or modification (e.g. glycosyl transferases) thereby allowing the organism to vary its immune exposed surface antigens (Parkhill, Wren et al. 2000).

Some strains of *Campylobacter* also contain unusual capsular polysaccharides containing sugars such as 6‑deoxy‑d-manno-heptose and d‑xylose which have been shown to be present in the capsule of strain RM1221 (Gilbert, Mandrell et al. 2007).

Adhesion and Invasion

Campylobacter are orally acquired pathogens so before they are able to access their usual site of infection (the intestine) they first have to survive the acidity of the stomach and the highly alkaline secretions of the bile duct in the small intestine.

Colonisation of the gastrointestinal tract by adhesion to intestinal epithelial cells and components of the extracellular matrix has long been considered essential for disease production (Fauchere, Rosenau et al. 1986; Konkel, Garvis et al. 1997). The bacterial polysaccharide capsule is essential for adhesion (Bacon, Szymanski et al. 2001; Watson and GalÃ¡n 2008) but several specific adhesins have also been identified; these include PEB-1, CadF and JlpA (Kervella, Pages et al. 1993; Pei and Blaser 1993; Konkel, Garvis et al. 1997; Jin, Joe et al. 2001).

Motility has been demonstrated to be essential for invasion of epithelial cells in many studies (Wassenaar, Bleumink-Pluym et al. 1991; Grant, Konkel et al. 1993; Carrillo, Taboada

et al. 2004), although one theory suggested that the flagellum itself is required for invasion; a study in which non-motile mutant with an intact flagellum was created demonstrated that such mutants are not able to invade epithelial cells (Yao, Burr et al. 1994).

Cytolethal distending toxin

Cytolethal distending toxin (CDT) is produced by most *Campylobacter* strains and causes cell cycle arrest at the G2/M phase which leads to progressive cellular distension and apoptotic cell death (Lara-Tejero and Galan 2000; Lara-Tejero 2001; Frisan, Cortes-Bratti et al. 2002). CDT has been proven to be immunogenic in human infection as pooled human sera from patients previously infected with *Campylobacter* has been shown to neutralise CDT (Abuoun, Manning et al. 2005). CDT does not appear to be necessary for colonisation of chickens (Biswas, Fernando et al. 2006).

1.1.5.2 Gastrointestinal Disease

C. jejuni illness usually presents as an acute gastroenteritis. Incubation times have been estimated to be between 2-11 days, with an estimated average of 3 days (Skirrow and Blaser 2000); a twenty four hour period of generalised fever and malaise often precedes the onset of diarrhoea which is typically bile stained and can become bloody, leukocytes are often present upon microscopic examination. Diarrhoea usually lasts from 2-5 days (but can become chronic and last for 7 days or more) and the patient can continue to suffer from abdominal pain for a further week. Relapses often occur (25% of cases) and so the average time away from work is between 10-14 days (Skirrow 1977; Blaser, Taylor et al. 1983; Snelling, Matsuda et al. 2005). Infective doses have been shown to be as low as 500-800 organisms (Robinson 1981; Black, Levine et al. 1988).

The sometimes extreme abdominal pain caused by *Campylobacter* infection can be misdiagnosed as appendicitis, termed 'pseudo-appendicitis' as the appendix is usually not involved, although there have been some cases of *Campylobacter* enteritis linked appendicitis (Blaser, Berkowitz et al. 1979; Skirrow and Blaser 2000).

Socio-economic status has been found to affect the presentation of *Campylobacter* enteritis; in less developed countries infection is much more common in children and diarrhoea is typically watery (rather than bile stained and bloody). It is thought that this type of childhood infection may help to immunise against adult disease (Blaser, Glass et al. 1980; Blaser, Taylor et al. 1983; Young, Davis et al. 2007).

1.1.5.3 Non-gastrointestinal *Campylobacter* disease and sequelae

Infection with *C. jejuni* outside of the gastrointestinal tract including; bacteraemia, hepatitis, peritonitis and myocarditis, have all been previously reported (McKendrick, Geddes et al. 1982; Florkowski, Ikram et al. 1984; McNeil, Buttoo et al. 1984; Skirrow, Jones et al. 1993).

A major complication post-infection with *C. jejuni* is Guillain-Barré syndrome (Rhodes and Tattersfield 1982), an auto-immune disease of the peripheral nervous system that results in a flaccid paralysis similar to polio (Nachamkin, Allos et al. 2000). Although this is a self limiting disease, recovery may take months and during this time the patient may need to be ventilated (which carries risks of its own) and between 15-20% may suffer with neurological deficits (Briscoe, Mcenamia et al. 1987; Rantala, Uhari et al. 1991; Adams and Victor 1993). Guillain-Barré syndrome is thought to arise following 1 in every 1000 *Campylobacter* infections and arises because *Campylobacter* LOS (lipooligosaccharide) can display molecular mimicry to human neuronal gangliosides (Yu, Usuki et al. 2006) .

Reactive arthritis (previously known as Reiter syndrome) is also thought to be a post-infection complication associated with *C. jejuni.* Reactive arthritis is an auto-immune disease that results from infection and usually has three symptoms; inflammation of the eye, urethritis and inflammation of the joints (Snelling, Matsuda et al. 2005). It is still somewhat unclear how *Campylobacter* infection triggers this disease (Hill Gaston and Lillicrap 2003).

1.1.6 Treatment

Campylobacter infections in healthy individuals are usually self-limiting, meaning that the disease is resolved without the need for antibiotic treatment; although electrolyte replacement and re-hydration therapy may be required (Peterson 1994; Koenraad, Rombouts et al. 1997).

Hospitalisation for *Campylobacter* infection is uncommon, except in patients with persistent or unusually severe disease or patients such as HIV/AIDs patients in whom *Campylobacter* infection can prove fatal (Manfredi, Nanetti et al. 1999).

The British National Formulary (BNF) recommends the use of ciprofloxacin (a fluoroquinolone) or erythromycin (a macrolide) for the treatment of 'severe' *Campylobacter* enteritis. However most treatment for diarrhoea is empirical (i.e. before any diagnosis has been made) and so ciprofloxacin is usually favoured as it has activity against all the major bacterial causes of diarrhoea, including shigellosis, and salmonellosis (BNF 2009). Treatment can differ in pregnant women as the BNF recommendations regarding antibiotics and pregnancy discourage the use of macrolides (BNF 2009).

Beta-lactams may be prescribed when treatment with the preferred agents, ciprofloxacin and erythromycin, is not possible due to allergy or when underlying illness makes treatment with a particular class of antibiotic unwise. For instance, patients with renal failure in whom

the use of aminoglycosides could be problematic may be prescribed the beta-lactam imipenem as an alternative (Nachamkin, Engberg et al. 2000). Although the prescribing of beta-lactams is not currently routine practice, the increasing amounts of fluoroquinolone resistance may make exploration of other previously un-favoured classes such as the beta-lactams, a viable and even necessary step.

1.2 Antibiotic resistance

1.2.1 Determining and defining resistance

Susceptibilities to antibiotics in *Campylobacter* are usually determined by the agar dilution method (McDermott, Bodeis et al. 2004) because the disc diffusion method can be unreliable due to the un-even growth of *Campylobacter*. Mueller Hinton agar plates that contain a doubling dilution series of an antibiotic are inoculated with bacterial test strains. Control plates that do not contain any antibiotics are also inoculated. All plates are incubated for 24-48 hours then the test plates are compared to the control. The minimum inhibitory concentration (MIC) of an antibiotic for a bacterial strain is usually defined as the concentration at which growth is inhibited by 90% compared to the antibiotic free control plate.

Bacteria are defined as 'suceptible' or 'resistant' to an antibiotic based on the value of their MIC and whether it falls above or below a pre-determined value or 'breakpoint' concentration; these differ between species and agent. Breakpoint concentrations are assigned by institutions such as the British Society for Antimicrobial Chemotherapy (BSAC), the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute in the US (CLSI, previously known as NCCLS) to

standardise the reporting of strains as ‘resistant’ or ‘sensitive’. However, breakpoints issued by the different institutions vary and so this must be taken into account when comparing the studies that use different guidelines.

There are very few breakpoint concentration guidelines for *Campylobacter*. The only breakpoint concentrations given by BSAC for *Campylobacter* are for erythromycin (resistant: MIC>0.5mg/L) and ciprofloxacin (resistant: MIC>1mg/L)(BSAC 2008). EUCAST also give breakpoint concentrations for erythromycin (resistant: MIC>4mg/L) and ciprofloxacin (resistant: MIC>1), plus levofloxacin (resistant: MIC>2mg/L) and ofloxacin (resistant: MIC>1mg/L) (EUCAST 2008). Tentative *Campylobacter* breakpoint MICs were given for erythromycin, ciprofloxacin, tetracycline, and doxycycline in a 2007 CLSI document (M45-A), but the breakpoint concentration values they give for *Enterobacteriaceae* are sometimes applied for example, ciprofloxacin (resistant: MIC≥4). It is worthy of note that beta-lactam breakpoint concentrations for *Campylobacter* are not given by BSAC, EUCAST or CLSI, so if resistance in *Campylobacter* to beta-lactams is to be reported, it must first be defined.

1.2.2 Mechanisms of antimicrobial resistance

There are several mechanisms by which bacteria can become resistant to an antibiotic; destruction or inactivation of the antibiotic, alteration or protection of the target site of the antibiotic, reduction of intracellular concentration either by reduced entry into, or enhanced efflux from the cell, and metabolic bypass (so the enzyme the drug targets is no longer necessary). *C. jejuni* utilizes a combination of these mechanisms for different agents (Table 1.2).

Table 1.2 Mechanisms of antibiotic resistance in *C. jejuni*

Type of Resistance	Antibiotic Class	Mechanism	Resistance genes/mutations	Reference
Destruction or inactivation of antibiotic	β-lactams	Inactivation by β-lactamase	bla_{OXA-61}	(Taylor, Gradis et al. 1981; Alfredson 2004; Alfredson and Korolik 2005)
	Aminoglycosides	Inactivation by aminoglycoside phosphotransferase	*aph*	(Tenover, Gilbert et al. 1989)
	Chloramphenicol	Inactivation by chloramphenicol acetyl transferase	*cat*	(Wang and Taylor 1990)
Alteration or protection of target site	Macrolides	Mutation in target	A to G at position 2230 in the 23S rDNA gene	(Engberg, Aarestrup et al. 2001; Jensen and Aarestrup 2001)
	Sulphonamides	Mutation in target	Mutation in *folP*	(Gibreel and Skold 1999)
	Tetracyclines	Protection of target	*tet(O)*	(Manavathu, Hiratsuka et al. 1988)
Prevention of accumulation	Multiple classes	Multidrug efflux	*cmeABC, cmeDEF*	(Pumbwe and Piddock 2002; Pumbwe, Randall et al. 2004)
Metabolic bypass	Trimethoprim	Replacement of sensitive enzyme	*dfr*	(Gibreel and Skold 1998; Gibreel and Skold 2000)

Adapted and updated from (Aarestrup and Engberg 2001).

1.2.3 Beta-lactam antibiotics

Beta-lactams are a group of antibiotics (Table 1.3) that share a common structural feature, a highly reactive four-member, or beta-lactam, ring (Donowitz and Mandell 1988). Their antibacterial effect is achieved by interfering with the final stage of bacterial cell wall synthesis. Peptidoglycan is the main component of bacterial cell walls and is made up of alternating units of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG); adjacent strands are cross-linked to give the peptidoglycan a flexible, 'net-like' structure. The final cross-linking step is catalysed by transpeptidases (also known as penicillin-binding proteins or PBPs). Beta-lactam antibiotics are competitive inhibitors of the bacterial transpeptidase due to their structural similarity to the terminal D-Ala D-Ala motif of NAM. When a transpeptidase uses a beta-lactam as a substrate it becomes acylated and so is unavailable to perform its usual biological function and cell wall synthesis is hindered. Consequently the cell wall becomes compromised allowing an influx of water into the cell which eventually leads to cell-lysis and cell death (Ghuysen, Charlier et al. 1996; Goffin and Ghuysen 1998).

1.2.4 Beta-lactamases

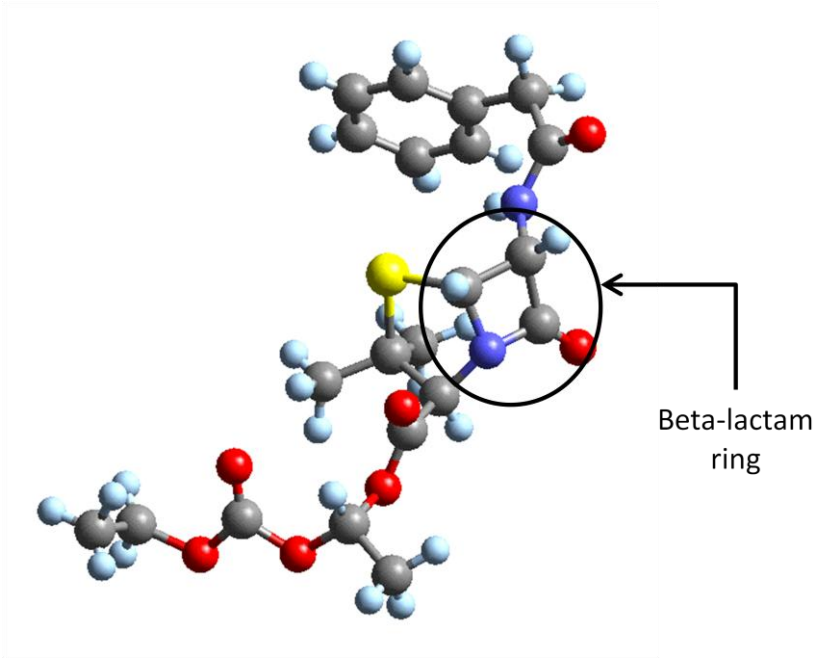
The most important mechanism of beta-lactam resistance in Gram negative bacteria is the production of beta-lactamases. Beta-lactamases are enzymes with structural similarity to PBPs but with a higher affinity for beta-lactams, allowing them to act as antagonists. Beta-lactamases hydrolyse the beta-lactam ring (Figure 1.5) of the antibiotic, as this site is crucial in modifying the PBP, the functionality of the drug is lost (Medeiros 1997; Bush and Mobashery 1998; Massova and Mobashery 1998; Bush 2001). The first beta-lactamase was described in an article in 1940, before the first beta-lactam, penicillin, was readily available (Abraham and Chain 1940).

Table 1.3 Beta-lactam antibiotics

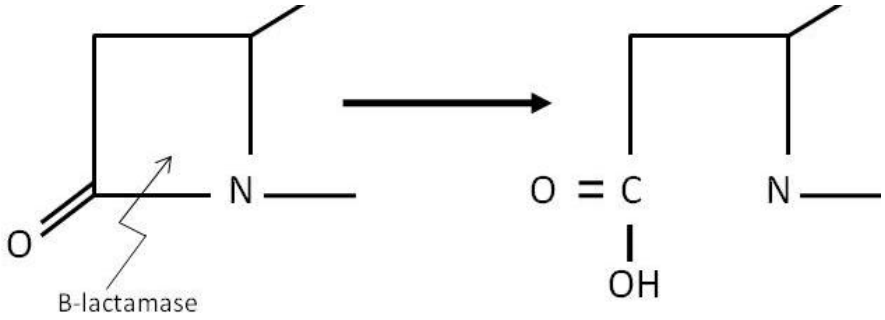
Penicillins	Cephalosporins	Carbapenems	Monobactams	Cephamycins
Natural penicillins Benzylpenicillin (penicillin G) Phenoxymethylpenicillin (penicillin V)	**1st generation (narrow spectrum)** Cephalothin Cephalexin	Meropenem Ertopenem Imipenem	Aztreonam	Cefmetazole Cefoxitin
Extended spectrum penicillins Aminopenicillins (Ampicillin, Amoxicillin) Carboxypenicillins (Carbenicillin, Ticarcillin)	**2nd generation (extended spectrum)** Cefuroxime Cefamandole Cefaclor			
Anti-staphylococcal (to target staphylococcal penicillinase producers) Cloxacillin Meticillin Oxacillin	**3rd generation (broad spectrum)** Cefotaxime Ceftriaxone			
	4th generation (broad spectrum) Cefepime Cefpirome			
	5th generation (anti-MRSA) Ceftobiprole			

Figure 1.5 Beta-lactams and their hydrolysis by beta-lactamases

a)



b)



a) Adapted 3D-structure of Benzylpenicillin showing the beta-lactam ring (Csoregh and Palm 1977).

b) Mode of action of a β-lactamase on a β-lactam ring, the core molecule of all β-lactam antibiotics. Hydrolysis of the β-lactam (e.g. penicillin) results in the production of the corresponding acid (e.g. penicilloic acid). The same is true for all classes of β-lactams including the cephalosporins, although they may produce an unstable acid that is then broken down into smaller fragments (Greenwood, Finch et al. 2007).

It is thought that beta-lactamases are an evolutionary response first elicited when some microorganisms gained the ability to produce beta-lactams (which would give them an advantage in their environmental niche) (Medeiros 1997). Structurally all beta-lactamases are very similar, typically containing both alpha-helices and beta-pleated sheets. The enzymes vary in their substrate specificities, response to inhibitors and even in the nature of their active site which is either serine or metallo (requiring two zinc ions for activation).

Over 700 distinct beta-lactamases have been described (Perez, Endimiani et al. 2007) and with them several classification systems. Both the more traditional Ambler class system and the Bush-Medeiros-Jacoby functional classification system are commonly used in the literature (Ambler 1980; Ambler, Coulson et al. 1991; Bush 1995) (Table 1.4).

1.2.4.1 Beta-lactamase inhibitors

Beta-lactamase inhibitors structurally resemble penicillin but on their own have little discernable antimicrobial activity; they are often referred to as 'suicide inhibitors' as they have a much higher affinity for beta-lactamases, occupy the active site of the enzyme for significantly longer and are essentially 'trapped' by the enzymes allowing an antibiotic delivered alongside the inhibitor to interact with the penicillin binding proteins (Helfand, Totir et al. 2003; Pagan-Rodriguez, Zhou et al. 2004; Babic, Hujer et al. 2006).

Three such inhibitors are currently available clinically; clavulanic acid, sulbactam and tazobactam. These inactivate serine type beta-lactamases (such as the TEM series enzymes found in Gram negative bacteria) but have no activity against metallo-beta-lactamases. Metal chelators such as EDTA and dipicolinic acid inhibit metallo-beta-lactamases but are not relevant clinically as they cannot be used medically for treatment (Bebrone 2007).

Table 1.4 Classification of beta-lactamases

Ambler Class	Structural Class	Preferred substrates	Type of β-lactamase	Inhibited by: Clavulanic acid	Inhibited by: EDTA	Representative enzymes
A	2a	Penicillins	Serine	✓	×	Penicillinases from Gram positive bacteria
	2b	Penicillins, cephalosporins	Serine	✓	×	TEM-1 series and SHV series
	2c	Penicillins, carbenicillin	Serine	✓	×	PSE series
	2e	Cephalosporins	Serine	✓	×	Cephalosporinases from *Proteus vulgaris*
	2f	Carbapenems	Serine	✓	✓	Sme-1 from *Serratia marcescens*
B	3a, 3b, 3c	All classes except monobactams	Metallo	×	✓	L1 from *Xanthormas maltophilia*, CcrA from *Bacteroides fragilis*
C	1	Cephalosporins	Serine	×	×	AmpC enzymes from Gram negative bacteria, MIR 1
D	2d	Penicillins, cloxacillin	Serine	✓ (weak)	×	OXA series
Not included	4	Penicillins	Serine	×	×	Penicillinase from *Pseudomonas cepacia*

Adapted from (Bush 1995; Bush 2001; Greenwood, Finch et al. 2007)

Common beta-lactam plus inhibitor combinations (that can be used against beta-lactamases such as TEM and SHV groups) include augmentin/co-amoxyclav (ampicillin + clavulanic acid), Tazocin™ (piperacillin + tazobactam) and Unasyn™ (ampicillin + sulbactam).

1.2.4.2 OXA beta-lactamases

Oxacillinase (OXA) type beta-lactamases belong to Ambler class D and are named for their ability to hydrolyse oxacillin (and cloxacillin) at a rate that is 50% higher than the rate at which they hydrolyse penicillin G (Naas and Nordmann 1999). Historically, they are narrow spectrum beta-lactamases, giving resistance to penicillin, ampicillin, amoxicillin, cloxacillin and oxacillin; they may show limited ability to hydrolyse first generation cephalosporins (such as cephalothin) but are unable to hydrolyse extended spectrum cephalosporins and they are poorly inhibited by clavulanic acid (Naas, Poirel et al. 2008). OXA type beta-lactamases have been found in many Gram negative organisms including the *Enterobacteriaceae* (usually associated with a plasmid) but are most commonly found in *Pseudomonas aeruginosa* (Naas and Nordmann 1999). The Lahey clinic currently list 193 distinct (by at least one amino acid) OXA beta-lactamases as of 01/10/2010 (Jacoby and Bush 2010). OXA enzymes with extended substrate profiles (often these are derivatives of OXA-10) that encompass the extended spectrum cephalosporins are becoming more common (Jacoby and Bush 2010).

The original definition of an extended spectrum beta-lactamase (ESBL) cannot be applied to OXA type enzymes with extended spectra, as the original definition stated that ESBLs were susceptible to inhibitors (such as clavulanic acid), to which OXA enzymes show only weak susceptibility. Nonetheless; OXA type enzymes with activity against second and third generation cephalosporins are often referred to as ESBLs (Naas, Poirel et al. 2008).

1.2.4.3 Plasmids containing genes encoding beta-lactamases

Plasmid mediated beta-lactam resistance began to arise in the 1960s following the introduction of ampicillin. By 1968, plasmid mediated resistance to ampicillin was being reported in 17% of surgical patients in a London hospital (found to be carrying ampicillin resistant *Enterococci* in their faeces) (Datta 1969). Conjugation experiments using *E. coli* K12 revealed that the majority of isolates could transfer resistance to other strains (Datta 1969).

The release of third generation (extended spectrum) cephalosporins in the 1980s was soon followed by the selection of variants of plasmids encoding TEM and SHV enzymes able to hydrolyse an 'extended spectrum' of beta-lactam substrates including monobactams and the new third generation cephalosporins such as cefotaxime and ceftazidime. These 'extended spectrum' beta-lactamases (ESBLs) were spread throughout the world, particularly by *Klebsiella spp.* and *E. coli* (Jacoby and Medeiros 1991). Another class of ESBL is currently causing a significant clinical problem worldwide, the CTX-M enzymes in particular CTX-M-14 and CTX-M-15 (Hawkey 2008).

As yet there have been no instances where plasmid encoded beta-lactam resistance has been described in *Campylobacter*.

1.3 Antibiotic resistance in *Campylobacter jejuni*

1.3.1 *Campylobacter* plasmids giving antimicrobial resistance

Transferrable antimicrobial resistance in *Campylobacter* was first described by Taylor in 1980 for tetracycline resistance (Taylor, DeGrandis et al. 1980). In 1981, they further showed that plasmid associated tetracycline resistance could be transferred from *C. jejuni* to both *C. jejuni* and *C. coli* but not to *E. coli* strains K-12 and J53-1 (Taylor, Gradis et al. 1981). The gene

responsible for tetracycline resistance, *tet(O)*, was identified from a conjugative plasmid conferring tetracycline resistance in 1988 (Manavathu, Hiratsuka et al. 1988).

The *Campylobacter* tetracycline resistance gene, *tet(O),* can be located either chromosomally or on a plasmid. One report showed that 54% of clinical isolates of *C. jejuni* that carried the *tet(O)* gene carried it on a plasmid whereas all of the *C. coli* isolates carried *tet(O)* on their chromosome (Dasti, Grob et al. 2007).

Tetracycline resistance is *Campylobacter* is usually, but not exclusively, associated with *tet(O)*. For instance, Randall et al., demonstrated that *tet(O)* could not be amplified from 24% (31/128) of tetracycline resistant isolates in their study (Randall, Ridley et al. 2003). Similarly Piddock *et al.,* found eight isolates of tetracycline resistant *Campylobacter* from which *tet(O)* could not be amplified.

Although tetracycline resistance is the most well characterised plasmid encoded antibiotic resistance in *Campylobacter,* transferrable resistance to several other classes of antibiotics has also been described.

In 1990, Wang and Taylor described the cloning of a chloramphenicol acetyl transferase (*cat*) gene from *Campylobacter coli* plasmid pNR9589 originally identified in an isolate from Japan (Wang and Taylor 1990). A kanamycin resistance determinant 3'-aminoglycoside phosphotransferase (*aphA-3*) was also shown to be located on this plasmid downstream of the *cat* gene (Wang and Taylor 1990). Previously, Tenover and Elvrum had identified two distinct plasmid encoded kanamycin resistance genes (*aphA-3* and *aphA*-7) in *Campylobacter* (Tenover and Elvrum 1988). The streptomycin resistance gene 6'-adenyl transferase (*aadE)* has also been described as part of a gene cluster alongside *aphA-3* and streptothricin acetyl transferase (*sat*) on a *Campylobacter* resistance plasmid (Gibreel, Skold et al. 2004).

It has been suggested that the resistance genes on some *Campylobacter* plasmids may have originated in *Enterococcus* spp.; for example the resistance gene cluster of *Campylobacter* plasmid pCJE8480 is similar to that of an *Enterococcus faecalis* (Taylor, Garner et al. 1983; Taylor and Courvalin 1988; Gibreel, Skold et al. 2004).

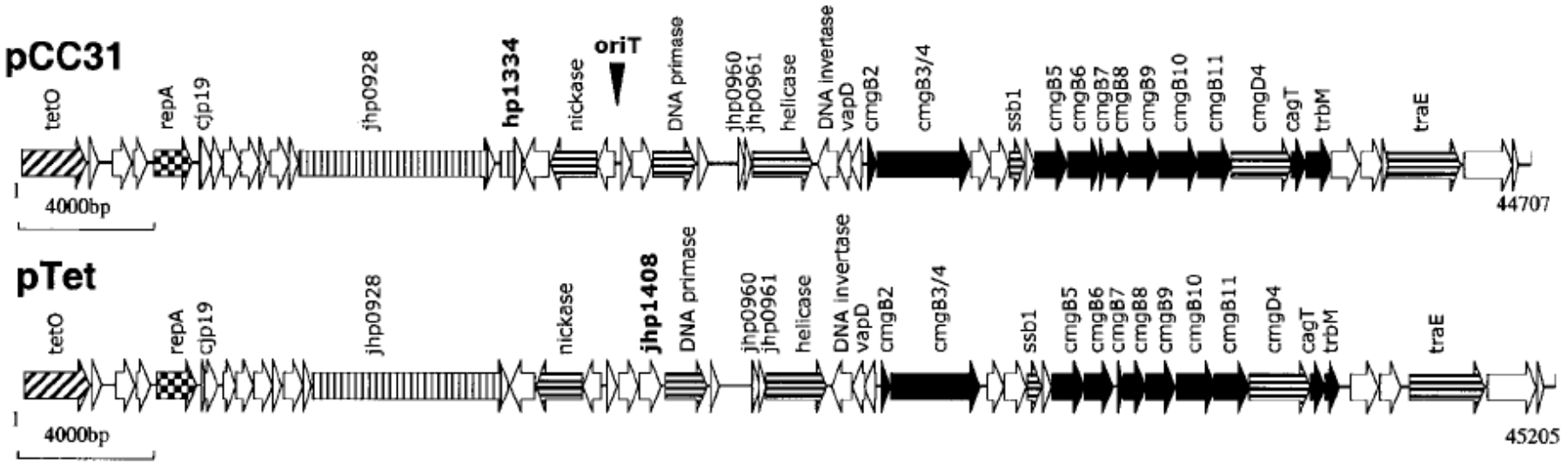
Aminoglycoside–inactivating enzymes (such as *aph2* and *aadE*) encoded on *Campylobacter* plasmids with origins from both Gram positive and Gram negative sources have been described elsewhere (Nirdnoy, Mason et al. 2005).

Although there are numerous *Campylobacter* plasmids that contain *tet(O)*, it is thought that the plasmids share some homology to one another. A well described *C. jejuni* strain 81-176 contains two plasmids, pVir a 37kb non-conjugative plasmid encoding a type IV secretion system that may be associated with virulence (Bacon, Alm et al. 2000; Bacon, Alm et al. 2002) and pTet a 45kb, *tet(O)* containing conjugative plasmid (Bacon, Alm et al. 2000). Sequence analysis of pTet and another smaller plasmid pCC31 also containing *tet(O)* revealed that despite both being mosaic in structure (having homologues of genes found in different commensal and pathogenic bacteria that inhabit the oral and intestinal tract of animals) and being isolated nearly 20 years apart they share a high identity (94.3%) with each other and also show similar gene organisation (Figure 1.6) (Batchelor, Pearson et al. 2004).

1.3.2 Why study beta-lactam resistance in *C. jejuni*?

Erythromycin (a macrolide) and the fluoroquinolones (in particular ciprofloxacin) are the drugs of choice for treating campylobacteriosis, not beta-lactams (Aarestrup and Engberg 2001; Allos 2001; BNF 2009). Nonetheless *Campylobacter* surveillance schemes have shown that ciprofloxacin resistant *Campylobacter* are widespread. For instance, 45% of isolates

Figure 1.6 Genetic map of plasmids pTet and pCC31 showing differences in gene organization



Genes are identified by their predicted function as follows: DNA transfer functions, horizontal stripes; *Campylobacter* mating gene (*cmg*) homologues of T4SS, filled; *repA*, chequered; restriction modification, vertical stripes; tet(O), diagonal stripes; genes with unknown role, open. Genes that are only found in one of the two plasmids are labelled in bold (Batchelor, Pearson et al. 2004).

were reported as resistant from EnterNet participating laboratories in Europe between 2005-2006 to ciprofloxacin (HPA 2006; HPA 2007). The percentage of isolates resistant to beta-lactams is also high. Enter-net reported that in 2007 34% of *C. jejuni* isolated from its participating European laboratories, were resistant to ampicillin (HPA 2007). The number of ampicillin resistant isolates of *C. jejuni* isolated from poultry and clinical isolates has been shown to be increasing (Corcoran, Quinn et al. 2006; McGill, Cowley et al. 2006).

Nonetheless beta-lactams could be used in the treatment of infections with *Campylobacter*. Augmentin, a combination of ampicillin and the beta-lactamase inhibitor clavulanic acid (also called co-amoxyclav), could be used as resistance was detected in only 0.4% of *C. jejuni* screened by Enter-Net in 2007 (HPA 2007) and may become a viable alternative to erythromycin and ciprofloxacin.

1.3.3 Beta-lactamases of *C. jejuni*

Beta-lactamases are thought to be the main mechanism of beta-lactam resistance in *C. jejuni* and their production of these enzymes has been noted for over 20 years (Fliegelman, Petrak et al. 1985). In 1985 Lucain *et al.,* reported in a conference abstract the presence of four antigenically distinct beta-lactamases that had differing pI values, molecular weights and activity profiles (Lucain, Goosses et al. 1985). Ninety six percent (22/23) of the isolates Lucain studied produced a beta-lactamase; the most prevalent was termed type A and was the product of over 78% (18/23) of the beta-lactamase producing isolates. Type A had a pI of 8.3 and a distinct narrow-spectrum penicillinase profile i.e. only active against penicillin, ampicillin, oxacillin and carbenicillin. The type A beta-lactamase had no activity for the cephalosporins (cephaloridine, cefuroxime and cefotaxime), but weak activity for cephalothin.

The presence of beta-lactamases in a high proportion of *C. jejuni* isolates has been documented on several further occasions. In 1985, Fliegelman *et al.* detected the production of a beta-lactamase in 93% (25/27) of the isolates studied (Fliegelman, Petrak et al. 1985), Lariviere *et al.* reported that 89.3% (142/159) of the strains they tested were beta-lactamase producers (Lariviere, Gaudreau et al. 1986) and 88% (88/100) of isolates produced a beta-lactamase in the study described by Tajada *et al.* in 1996 (Tajada, Gomez-Garces et al. 1996). Production of a beta-lactamase was also linked to amoxicillin and ampicillin resistance (Fleming, D'Amico et al. 1882; Maia, Rubin et al. 1983).

In 1991, Lachance *et al.* partially characterised a beta-lactamase isolated from clinical isolates of *C. jejuni* from the Montreal region of Canada. Again a distinct pattern of activity was noted with the penicillins; ampicillin, amoxicillin, penicillin G and cloxacillin being the preferred substrate. As with Lucain's type A beta-lactamase, cephalothin was weakly hydrolysed whilst activity against other cephalosporins and imipenem was lacking (Lachance, Gaudreau et al. 1991). Clavulanic acid and tazobactam were found to significantly inhibit the enzyme ($P<0.05$ Mann-Whitney U test) and one microgram per millilitre of clavulanic acid was shown to cause all beta-lactamase positive strains to become susceptible to both amoxicillin and ampicillin (susceptible when MIC$\leq$8µg/ml).

Conflicting reports about the effectiveness of inhibitors on beta-lactamases from *C. jejuni* have been published. Some are in agreement with Lachance *et al.,* (Fleming, D'Amico et al. 1882; Maia, Rubin et al. 1983) whilst others are in disagreement having found no significant effect of inhibitors (Van der Auwera and Scorneaux 1985; Alfredson and Korolik 2005).

1.3.3.1 The chromosomally encoded *Campylobacter* oxacillinases

A 257 amino acid Ambler class D beta-lactamase was isolated from a human clinical isolate of *C. jejuni* and was assigned the number OXA-61 by the Lahey clinic (Alfredson and Korolik 2005; Jacoby and Bush 2010). When a non-beta-lactamase producing isolate of *C. jejuni* was transformed with the bla_{OXA-61} gene (and its conserved 122bp promoter region), a ≥32 fold increase in the MIC of ampicillin, piperacillin and carbenicillin was conferred. When *E. coli* HB101 was transformed with bla_{OXA-61} , there was a less prominent increase in MIC. Alfredson and Korolik hypothesised that this may be due to *E. coli* only weakly recognising the bla_{OXA-61} promoter (Alfredson and Korolik 2005). OXA-61 was found not to be significantly affected by clavulanic acid at a concentration of 2µg/ml.

The product of the Cj0299 gene of the sequenced *Campylobacter jejuni subspecies jejuni* NCTC11168 was shown to vary from OXA-61 by only one amino acid (Griggs, Peake et al. 2009). The *cj0299* gene was found to be present in 91% of ampicillin resistant (MIC≥16µg/ml) poultry isolates (Griggs, Peake et al. 2009). However, unlike Alfredson and Korolik, Griggs *et. al.,* found that the beta-lactamase inhibitors clavulanic acid and tazobactam had a significant effect on Cj0299, reducing the MIC of amoxicillin by 4-64 and 16-32 fold respectively.

1.3.4 A novel beta-lactamase in *Campylobacter*

Following the Defra funded studies OZO501 and V02200 between 2000 and 2006 (Humphrey, Jorgensen et al. 2005; Piddock, Griggs et al. 2008), 1288 veterinary isolates of *Campylobacter* were isolated from chicken flocks in the UK, fifty two percent of which were found to be resistant to ampicillin (Griggs, Peake et al. 2009).

Twenty six of the isolates that were ampicillin resistant (MIC≥16μg/ml), tested positive (by nitrocefin testing) for the presence of a beta-lactamase and did not produce an amplimer following PCR designed to amplify the *cj0299* gene. From two of these isolates, P843 and P854, a novel protein with beta-lactamase activity was isolated (Griggs, Peake et al. 2009); this was the first report of more than one beta-lactamase being isolated from *C. jejuni* since the conference abstract of Lucain (Lucain, Goosses et al. 1985). This second beta-lactamase (CjBla2) was distinct from OXA-61 and Cj0299 as it had a pI of 9.21 and a molecular mass of 32.4kDa. Analysis by QTOF-MS and FTICR-MS yielded no homology to known β-lactamases of *Campylobacter, Helicobacter or Pseudomonas*. BLAST searching revealed the closest hit in the *Campylobacter* protein database was with formyltetrahydrofolate deformylase (accession number CAB73055), thought to be the product of the *purU* gene (Cj0790). This protein has no predicted β-lactamase activity.

1.3.5 Non-beta-lactamase mediated beta-lactam resistance in *Campylobacter*

Resistance to ampicillin and other β-lactams had also been found in isolates that do not produce a β-lactamase suggesting that other mechanisms of beta-lactam resistance are employed by *Campylobacter* (Griggs, Peake et al. 2009).

Intrinsic resistance to some β-lactams may in part be due to the size and charge restrictions that outer membrane porins impose on molecules trying to enter the cell, it had been suggested that this may be the case with resistance to the cephalosporins (Page, Huyer et al. 1989).

Alternatively beta-lactams could be substrates of efflux pumps. Two RND type multi-drug efflux pumps, CmeB (encoded by the *cmeABC* operon) and CmeF (encoded by the *cmeDEF*

operon) have been described in *C. jejuni* (Lin, Michel et al. 2002; Pumbwe and Piddock 2002; Pumbwe, Randall et al. 2004; Pumbwe, Randall et al. 2005). Inactivation of *cmeB* was shown to give a 2-fold increase in susceptibility of NCTC11168 to ampicillin (MIC reduced from 4μg/ml to 1μg/ml), whilst over expression decreased susceptibility three fold (MIC increased from 4μg/ml to 32μg/ml (Pumbwe, Randall et al. 2004). It has also been shown that inactivation of *cmeF* also increases susceptibility to ampicillin, but this time a threefold increase in susceptibility was found (MIC reduced from 4μg/ml to 0.5μg/ml) (Pumbwe, Randall et al. 2005).

1.4 Aim

The aim of this study is to better understand the various mechanisms contributing to beta-lactam resistance in *Campylobacter* with particular focus on the role of efflux and both genomically and plasmid encoded beta-lactamases.

1.5 Hypotheses to be investigated during this thesis

- A second beta-lactamase (Bla2) can confer resistance to beta-lactams in *Campylobacter.*
- The *purU* gene codes for the novel beta-lactamase, Bla2.
- The c*j0299* gene can be present on the genome but not expressed.
- Beta-lactam resistance can be transmissible between strains of *Campylobacter.*
- Beta-lactam resistance can be conferred by the CmeABC and/or the CmeDEF efflux pump(s).
- Presence of the CmeABC and/or the CmeDEF efflux pump(s) is required for Cj0299 mediated resistance to be conferred.
- Over-expression of *cj0299* can confer resistance to all beta-lactams except carbapenems.
- Cj0299 confers resistance due to altered kinetic properties and not over-production of enzyme.

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Chapter 2 – Materials and Methods

2.1 Bacterial strains

Veterinary *Campylobacter* isolates from Defra funded studies OZO501 (Griggs, Johnson et al. 2005; Humphrey, Jorgensen et al. 2005) and VM2200 (Piddock, Griggs et al. 2008) were selected for study if they fell in to one of two categories:

i. No amplimer was produced following PCR for the $bla_{OXA-193}$ gene (*C. jejuni* NCTC11168 c*j0299*) and beta-lactamase activity was detectable (Table 2.1)

ii. An amplimer was produced following PCR for the $bla_{OXA-193}$ gene and no beta-lactamase activity was detectable (Table 2.2).

Details of $bla_{OXA-193}$ PCR are given in Section 2.4.5. Other bacterial isolates/strains used in this study include those required for investigations into transferrable beta-lactam resistance, the role of efflux in beta-lactam resistance and the identification of the novel beta-lactamase, CjBla2 (Table 2.3).

2.2 Growth, storage and identification of bacterial strains

All *Campylobacter* strains were grown routinely on Mueller-Hinton (MH) agar (Oxoid Ltd., Basingstoke, UK, Catalogue No: CM0337B) supplemented with 5% defibrinated horse blood (TCS Bioscience Ltd., Buckingham, UK, Cat. No: HB034) with antibiotics where necessary. On occasion *Campylobacter* agar base (Oxoid Ltd., Basingstoke, UK, Catalogue No: CM0689) with 5% horse blood (lysed by addition to hot agar) and Preston supplement (which contains the antibiotics rifampicin, trimethoprim and cycloheximide to select for the growth of *Campylobacter*) (Oxoid Ltd., Basingstoke, UK, Catalogue No: SR0117) was used in place of MH agar to reduce the risk of contamination. Cultures were grown for 48 hours (unless

Table 2.1 Isolates selected for study as they do not produce an amplimer following PCR of *bla*$_{OXA-193}$ (*C. jejuni* NCTC11168 *cj0299)* but did produce a beta-lactamase phenotype

ARG	Species	Defra Study	Farm	Rx Phase	Serotype	Phage type	*bla*$_{OXA-193}$	Nitrocefin	Ampicillin MIC (µg/ml)
P339	*C. jejuni*	OZO501	Farm 1	1 week post	HS8	1	-	+	128
P843	*C. jejuni*	OZO501	Farm 4	During	HS37	1	-	+	32
P852	*C. jejuni*	OZO501	Farm 4	1 week post	UT	1	-	+	128
P853	*C. jejuni*	OZO501	Farm 4	1 week post	HS2	UT	-	+	128
P854	*C. jejuni*	OZO501	Farm 4	1 week post	UT	UT	-	+	128
P858	*C. jejuni*	OZO501	Farm 4	1 week post	HS50	1	-	+	128
P859	*C. jejuni*	OZO501	Farm 4	1 week post	UT	UT	-	+	128
P862	*C. jejuni*	OZO501	Farm 4	1 week post	UT	UT	-	+	256
P888	*C. jejuni*	OZO501	Farm 4	2 week post	HS50	1	-	+	128
P891	*C. jejuni*	OZO501	Farm 4	2 week post	UT	1	-	+	128
P1007	*C. jejuni*	OZO501	Farm 5	Pre	HS3	UT	-	+	128
P1052	*C. jejuni*	OZO501	Farm 5	During	UT	UT	-	+	128
P1058	*C. jejuni*	OZO501	Farm 5	During	UT	UT	-	+	128
P1170	*C. jejuni*	OZO501	Farm 4	2 week post	HS50	UT	-	+	64
P1177	*C. jejuni*	OZO501	Farm 4	2 week post	HS50	UT	-	+	128

(Griggs, Johnson et al. 2005; Humphrey, Jorgensen et al. 2005)

UT = un-typable

Table 2.2 Isolates selected for study as they do produce an amplimer by PCR of *bla*$_{OXA-193}$ but do not produce a beta-lactamase phenotype

ARG Code	Species	Defra Study	Farm	Treatment Phase	Serotype	Phage type	*bla*$_{OXA-193}$	Nitrocefin	Ampicillin MIC (µg/ml)
P323	*C. coli*	OZO501	Farm 1	1 week post	HS56	2	+	-	8
P324	*C. coli*	OZO501	Farm 1	1 week post	HS48	44	+	-	8
P851	*C. jejuni*	OZO501	Farm 4	1 week post	UT	UT	+	-	8
P1102	*C. jejuni*	OZO501	Farm 6	During	HS4	19	+	-	8
P1154	*C. jejuni*	OZO501	Farm 6	4 week post	HS42	44	+	-	8
P1197	*C. jejuni*	OZO501	Farm 5	Pre	UT	31	+	-	8
P1199	*C. jejuni*	OZO501	Farm 5	2 week post	UT	33	+	-	8

(Griggs, Johnson et al. 2005; Humphrey, Jorgensen et al. 2005)

UT = un-typable

Table 2.3 All other isolates used in this study

ARG Code	Species	Section of work	Description	Reference
P270	*C. jejuni*	Throughout study	NCTC11168	National Collection of Type Cultures
P2252	*C. jejuni*	Efflux (Chapter 3)	P270*cmeB::Magellan3(aph)*	This study
P2253	*C. jejuni*	Efflux (Chapter 3)	P270*cmeF::Magellan3(cat)*	This study
P2224	*C. jejuni*	CjBla2 (Chapter 4)	P270*purU::Magellan3(cat)*	This study
P2247	*C. jejuni*	CjBla2 (Chapter 4)	P854*purU::Magellan3(cat)*	This study
I819	*E. coli*	CjBla2 (Chapter 4)	DH5α + pHSG398(P854_1490)	This study
L823	*S. enterica* serovar Typhimurium	CjBla2 (Chapter 4)	*S. enterica* serovar Typhimurium SL1344 *acrA::aph*	(Blair, La Ragione et al. 2009)
P1131	*C. coli*	Transferrable beta-lactam resistance (Chapter 5)	Poultry isolate	(Griggs, Johnson et al. 2005; Humphrey, Jorgensen et al. 2005)
P1931	*C. coli*	Transferrable beta-lactam resistance (Chapter 5)	Poultry isolate	(Piddock, Griggs et al. 2008)
P1983	*C. jejuni*	Transferrable beta-lactam resistance (Chapter 5)	Kanamycin resistant *fla*B mutant of NCTC81116	Donated by A.Ridley
P1984	*C. jejuni*	Transferrable beta-lactam resistance (Chapter 5)	NCTC81-176	National Collection of Type Cultures
P2238	*C. jejuni*	Transferrable beta-lactam resistance (Chapter 5)	Transconjugant (P1984 donor, P1983 recipient)	This study
P2241	*C. jejuni*	Transferrable beta-lactam resistance (Chapter 5)	Transconjugant (P1131 donor, P1983 recipient)	This study
P2244	*C. jejuni*	Transferrable beta-lactam resistance (Chapter 5)	Transconjugant (P1931 donor, P1983 recipient)	This study

method required otherwise) in a CO_2 incubator (7.5% CO_2) at a temperature of 37°C. For some experiments strains were cultured in a microaerophilic atmosphere created using a gas generating system (BD Bioscience, Oxford, UK, Catalogue No: 260628) and microaerophilic sachets (BD Bioscience, Oxford, UK, Catalogue No: 260680) incubated for 48 hours at 37°C. Liquid cultures were prepared in 5 ml of MH broth (Oxoid Ltd., Basingstoke, UK, Catalogue No: CM0405) in T25 Falcon™ tissue culture flasks (BD Bioscience, Oxford, UK, Catalogue No: 353108) and were cultured overnight in 7.5% CO_2 at 37°C with shaking (80 rpm).

For long term storage strains were put onto Protect ™ beads (Technical Service Consultants Ltd., Heywood, UK, Catalogue No: TS70) and kept at -80°C.

Microscopic examination (at X100 magnification with an oil immersion lens) was performed routinely for identification. Colonies were Gram stained (see below) and observed to confirm that the curved-spiral Gram negative rods, characteristic of *Campylobacter jejuni*, were present.

When Gram staining, approximately five large colonies were removed from the surface of an agar plate with a sterile loop and emulsified onto a microscope slide with 1 ml of sterile distilled water and left to air dry. Once dry, the slide was passed through the flame of a Bunsen burner two-to-three times to fix the slide. The slide was flooded; 1. with crystal violet for 30 seconds to stain, 2. with Grams iodine for 30 seconds to fix, 3. with alcohol decolouriser (methanol and acetone mix) for ten seconds then finally, 4. counter stained with safranin for 30 seconds, the slide was rinsed with water between each stage. Reagents were purchased together in a Gram staining kit (Fisher Scientific, Loughborough, Catalogue No: P/L505/15).

2.3 Investigating the role efflux plays in beta-lactam resistance in *Campylobacter*

2.3.1 PCR amplification of *cmeB* and *cmeF*

PCR primers were designed to amplify the entire *cmeB/F* gene plus extensive flanking sequence, including large parts of upstream and downstream genes using the PRIMER v2.00 program (Scientific and Educational Software, Cary, USA) (Table 2.4). Strains to be tested were grown for 48 hours on MH agar + 5% horse blood, cells were then harvested and used to form a turbid suspension in 200 µl of sterile distilled water. The suspension was heated to 99°C for five minutes in a heated block to form a crude boiled lysate, and then pulsed in a micro-centrifuge to pellet cell debris. The supernatant was used as template DNA.

PCR reactions contained 45 µl of PCR ReddyMix (containing a *Taq* polymerase) (Thermo Scientific, Waltham, USA, Catalogue No: AB0794), 1 µl of forward primer, 1 µl of reverse primer (all primers used in this study were at a working concentration of 25 µM) and 1 µl of template DNA. A contamination control that contained primers, master mix and water (in place of template DNA) was included in all PCRs undertaken in this study as was a positive control strain (in this instance NCTC11168) and negative control strain where applicable. The PCR parameters varied depending upon which primers were used (Table 2.4.).

2.3.1.1 Agarose gel electrophoresis

PCR amplimers were viewed by agarose gel electrophoresis to check for the expected amplimer. Gel electrophoresis was performed using 1% (w/v) agarose gels made by combining electrophoresis grade agarose (Invitrogen Ltd., Paisley, UK, Catalogue No: 10975-

Table 2.4 *cmeB* and *cmeF* primers and PCR parameters for *cmeB* and *cmeF PCR*

Primer	Target gene	5'→3' Sequence	Product Size	Initial denaturation	PCR Parameters X 30 cycles			Final extension
					Denaturation	Annealing	Extension	
297	*cmeB*	AGCTGGAGCTATAGGTCT	2921 bp	95°C (5 min)	95°C (1 min)	47°C (1 min)	72°C (4 min)	72°C (10 min)
298	*cmeB*	GTCTTGAAGCACTTCCTG						
291	*cmeF*	AACCGACCTATTACCGT	2932 bp	95°C (5 min)	95°C (1 min)	47°C (1 min)	72°C (5 min)	72°C (10 min)
633	*cmeF*	GTGCAGGTACTACGAGTAAG						

035) with Tris-Boric acid-EDTA (TBE) (Invitrogen Ltd., Paisley, UK, Catalogue No: 15581-028). Ten times TBE stock was diluted one in ten to give a working concentration of 0.1 M Tris, 0.09 M Boric Acid, and 0.001 M EDTA. The agarose was dissolved by heating for 90 seconds in 650 W microwave and allowed to cool before ethidium bromide was added to a final concentration of 0.1 µg/ml. Molten agarose was poured into a gel tray (to which combs were added to create wells) and allowed to set before being immersed in working concentration TBE in a gel tank. Five microlitres of each PCR product were loaded into a well in the gel, 5 µl of HyperLadder 1 (BioLine, London, UK, Catalogue No: BIO-33025), (separation range of 200-10,000bp), was also loaded into a well so the PCR product sizes could be determined. The gel was electrophoresed at 120 V until the dye front was close to the bottom of the gel at which time the gel was removed from the gel tray and placed on the viewing platform of a Gene genius image analyser (Syngene, Cambridge, UK) which was used to visualise the gel.

2.3.2 DNA sequencing of *cmeB* and *cmeF* PCR amplimers

PCR amplimers were column purified using Qiagen's QIAquick PCR Purification Kit (Qiagen, Crawley, UK, Catalogue No: 28104) and eluted in 50 µl of the supplied elution buffer. Five microlitres was electrophoresed on a 1% (w/v) agarose gel alongside HyperLadder I, and GeneTools software (Syngene, Cambridge, UK) was used to quantify the DNA. An image of the DNA was captured and the software was used to compare the sample DNA to a known standard (the HyperLadder) in order to determine the concentration of DNA in the samples. Sequencing was performed at the Functional Genomics department at Birmingham University and sequencing reactions were set up according to their protocol (Appendix A). The BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems Ltd., Foster City, USA) was used to perform the PCR sequencing reaction. Sequence data were compared to

published genome sequences using MEGA 3.1 software (Center for Evolutionary Functional Genomics, Tempe, USA).

2.3.3 Natural transformation

The method employed was adapted from Wang and Taylor (Wang and Taylor 1990). The insertionally inactivated *cmeB* or *cmeF* gene was amplified from P2252 or P2253 respectively, purified using a QIAquick kit by Qiagen, and then kept at -20°C until needed. The strain to be transformed was cultured on four MH agar plates for 16 hours at 37°C in an atmosphere of 7.5% CO_2. All cells were then harvested and re-suspended in MH broth to an OD_{600} of 0.5 (≈$3x10^9$ cells/ml); 0.5 ml of the cell suspension was added to a T25 Falcon™ tissue culture flask, already containing 1 ml of MH broth. The transformation mixture was cultured at 37°C in 7.5% CO_2 with shaking (80 rpm). After three hours incubation 3 µg of purified PCR amplimer (*cmeB::aph* or *cmeF::cat*) was added to the mixture which was then left to incubate for a further five hours. A control flask was set up to which water was added in place of DNA. Following incubation, 100 µl of each transformation mixture was plated out onto MH + 5% horse blood plates containing 50 µg/ml of kanamycin or 20 µg/ml of chloramphenicol (Table 2.5); the plates were incubated at 37°C in 7.5% CO_2 and were examined for colonies for up to 5 days.

2.3.4 Determination of the minimum inhibitory concentrations (MICs) of antibiotics

The agar doubling-dilution method recommended by the NCCLS Campylobacter Working Group (McDermott, Bodeis et al. 2004) was followed throughout this study to determine the minimum inhibitory concentrations (MICs) of a range of antibiotics (Table 2.5). Strains to be

Table 2.5 Antibiotics used in this study

Antibiotic	Antibiotic Class	Supplier	Product Number	Solubilised in
Ampicillin	β-lactam, penicillin	Sigma Aldrich, UK	A9393	Sodium bicarbonate
Cefotaxime	β-lactam, 3rd generation cephalosporin	Sigma Aldrich, UK	C7912	Distilled water
Cefoxitin	β-lactam, 2nd generation cephalosporin	Sigma Aldrich, UK	C4786	Distilled water
Cephalothin	β-lactam, 1st generation cephalosporin	Sigma Aldrich, UK	C4520	Distilled water
Chloramphenicol	-	Sigma Aldrich, UK	C-0378	70% Methanol
Kanamycin	Aminoglycoside	Sigma Aldrich, UK	K1876	Distilled water
Meropenem	β-lactam, carbapenem	Purchased from Heartlands Hospital pharmacy (distributed by AstraZeneca)		Distilled water
Oxacillin	β-lactam, penicillin	Sigma Aldrich, UK	28221	Distilled water
Penicillin	β-lactam, penicillin	Sigma Aldrich, UK	P7794	Distilled water
Tetracycline	Tetracycline	Sigma Aldrich, UK	T8032	Distilled water

tested were grown on MH agar + 5% horse blood plates for 48 hours in a microaerophilic atmosphere at 37°C. Colonies were harvested and emulsified in 5 ml of MH broth to give turbidity equal to a 0.5 McFarland standard (measured spectrophotometrically as an absorbance of 0.13 at OD_{600}).

Antibiotic stocks (10,000 µg/ml, 1,000 µg/ml and 100 µg/ml) were made up on the day of the experiment. Appropriate volumes of antibiotic stock solutions to create a doubling dilution series (Appendix B) were aseptically pipetted into sterile universal tubes to which 20 ml of molten MH agar (containing 5% horse blood) was dispensed using a peristaltic pump dispenser (Jencons Scientific Ltd., UK). The antibiotic and agar were mixed and poured into sterile tri-vented Petri dishes and allowed to set. Plates were dried in a 50°C oven for 15 minutes before being inoculated. Two plates that contained 20 ml of MH agar + 5% horse blood but did not contain any antibiotics were created and inoculated at the start of replica plating to create a 'start plate' and at the finish of replicator plating to create a 'finish plate'. Two hundred microlitres of each bacterial suspension was added to the wells of a multi-point inoculator template and then replica plated with a multi-point inoculator (that delivered approximately 1 µl of culture) onto the test and control plates. NCTC11168 whose antimicrobial resistance profile is well documented was included as a control. The test plates and the start and finish plates were incubated for 48 hours at 37°C in an atmosphere of 7.5% CO_2. After 48 hours test plates were compared to the start and finish plates, the MIC of an agent was defined as the concentration at which an approximately 90% reduction in growth of the organisms could be seen when compared to the start/finish plate.

2.4 Identification of novel *Campylobacter* beta-lactamases

2.4.1 PCR amplification of *purU* (*C. jejuni* NCTC11168 *cj0790*)

A *C. jejuni* mutant in which the *purU* gene had been disrupted by a *Magellan3 -Himar* based transposon containing a chloramphenicol acetyl transferase (*cat*) gene was obtained from Dr A Grant (Table 2.3) and given the laboratory code P2224.

PCR reactions were performed using 12.5 µl of Extensor PCR master mix (containing ThermoPrime® *Taq* and a proof reading enzyme) (Thermo Scientific, Waltham, USA, Catalogue No: AB-0794), 2 µl of the supernatant of boiled cell lysate, 5 µl of UltraPure™ DEPC-Treated water (Invitrogen Ltd., Paisley, UK, Catalogue No: 750023) and 1 µl each of the forward and reverse primers. Primers were designed to amplify the entire *cmeB/F* gene plus extensive flanking sequence, including large parts of upstream and downstream genes. Cycles were modified for the use of Extensor PCR master mix (Table 2.6).

2.4.2 DNA sequencing of the *cj0790* PCR amplimer

Performed as described previously (Section 2.3.2) but with *purU* primers (Table 2.6).

2.4.3 Transformation of P854 with *purU::cat*

Method performed as described previously (Section 2.3.3) using the insertionally inactivated *purU::cat* (which was amplified from P2224) (Section 2.4.1) to transform P854. Chloramphenicol was used to select transformants.

Table 2.6 *purU* primers and PCR parameters for *purU* PCR

Primer	Target gene	5'→3' Sequence	Product Size	Initial denaturation	PCR Parameters X 30 cycles			Final extension
					Denaturation	Annealing	Extension	
665	*purU*	GAAGGAATGGCTTGGACT	1192 bp	95°C (5 min)	95°C (1 min)	47°C (1 min)	72°C (4 min)	72°C (10 min)
666	*purU*	CTGGATGTGCTTGTGCTG						

2.4.4 Detection of beta-lactamase activity

2.4.4.1 Nitrocefin spot test

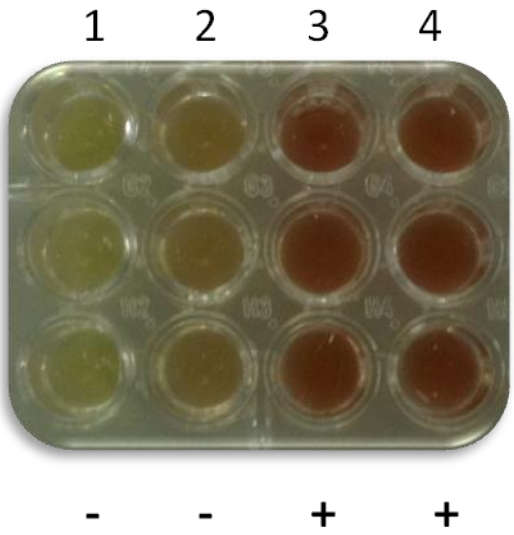
Strains to be tested were grown on MH agar then harvested after 48 hours and added to 1 ml of sterile water to form a turbid suspension. An MSE Soniprep 150 microprobe (Sanyo Biomedical, Loughborough, UK) was used to lyse the suspensions by sonicating (on ice) for a total of 2 minutes (4 X 30 second pulses with 3 X 30 second pauses). When large numbers of samples needed to be processed simultaneously a sonicating waterbath was used in place of a sonicating probe to allow batch processing. In order to ensure that these different methods of sonicating did not affect the outcome of the assay, positive (P854) and negative (NCTC1168) control strains were processed using each method to ensure the outcomes were consistent between the two methods of sonicating (Figure 2.1).

Fifty microlitres of sonicate was added to a well in a 96 well microtitre tray. Ten microlitres of freshly made nitrocefin (500 µg/ml) (Fisher Scientific, Loughborough, UK, Catalogue No: SR112C) was added to the sonicate (to a final concentration of 83 µg/ml) then observed. A colour change from yellow to red within 5 minutes denoted a beta-lactamase positive strain (Figure 2.1). Isolates that had previously had their beta-lactamase profiles characterised in this laboratory by nitrocefin testing were used as positive (P843, P854) and negative (NCTC11168) controls (Griggs, Peake et al. 2009).

2.4.4.2 Development of a quantitative nitrocefin assay

A method was developed using a spectrophotometer in order to quantify beta-lactamase activity with nitrocefin. Test isolates were grown for 48 hours on MH agar as described previously (Section 2.2). Cells were harvested and emulsified in 1 ml distilled water to an

Figure 2.1Detecting beta-lactamase production by nitrocefin spot test



1. *C. jejuni* NCTC11168 (sonication achieved using sonicating waterbath) + nitrocefin
2. *C. jejuni* NCTC11168 (sonication achieved using microprobe) + nitrocefin
3. *C. jejuni* P854 (sonication achieved using sonicating waterbath) + nitrocefin
4. *C. jejuni* P854 (sonication achieved using microprobe) + nitrocefin

Following the addition of 10 µl of nitrocefin (500 µg/ml) to 50 µl of sonicated bacterial cells (sonication achieved using either a microprobe or sonicating waterbath) a colour change from yellow to red can be observed if the bacteria are beta-lactamase positive.

Shown above is the colour change seen after five minutes for *C. jejuni* NCTC11168, a strain that does not produce a beta-lactamase (nitrocefin negative) and *C. jejuni* P854, a strain that does produce a beta-lactamase (nitrocefin positive).

OD_{600} of 0.6. Cells were then sonicated as described in section 2.3.3. Two hundred and fifty microlitres of sonicate was added to a cuvette (Fisher Scientific, Loughborough, UK, Catalogue No: 14-955-125). Fifty microlitres of freshly made nitrocefin (500 µg/ml) (Fisher Scientific, Loughborough, UK, Catalogue No: SR112C) was added to the sonicate (to a final concentration of 83 µg/ml). The change in optical density at OD_{486} for each isolate (compared to a blank containing water in place of sonicate) was recorded every five minutes for half an hour. A positive control (P843 or P854) and negative control (NCTC11168) was always included and the test performed in duplicate or triplicate.

Automation of this nitrocefin assay using a FLUOstar Optima (BMG Labtech, Aylesbury, UK) with a 485 nm filter was also investigated. Fifty microlitres of sonicate was added in triplicate to the wells in a 96 well plate (Sterilin, Aberdeen, UK, Catalogue No: 611U96) and then loaded in to a FLUOstar Optima which was programmed to record the absorbance at OD_{485} every five minutes for one hour. The FLUOstar was programmed to inject ten microlitres of working concentration nitrocefin into each well at T = 10 minutes.

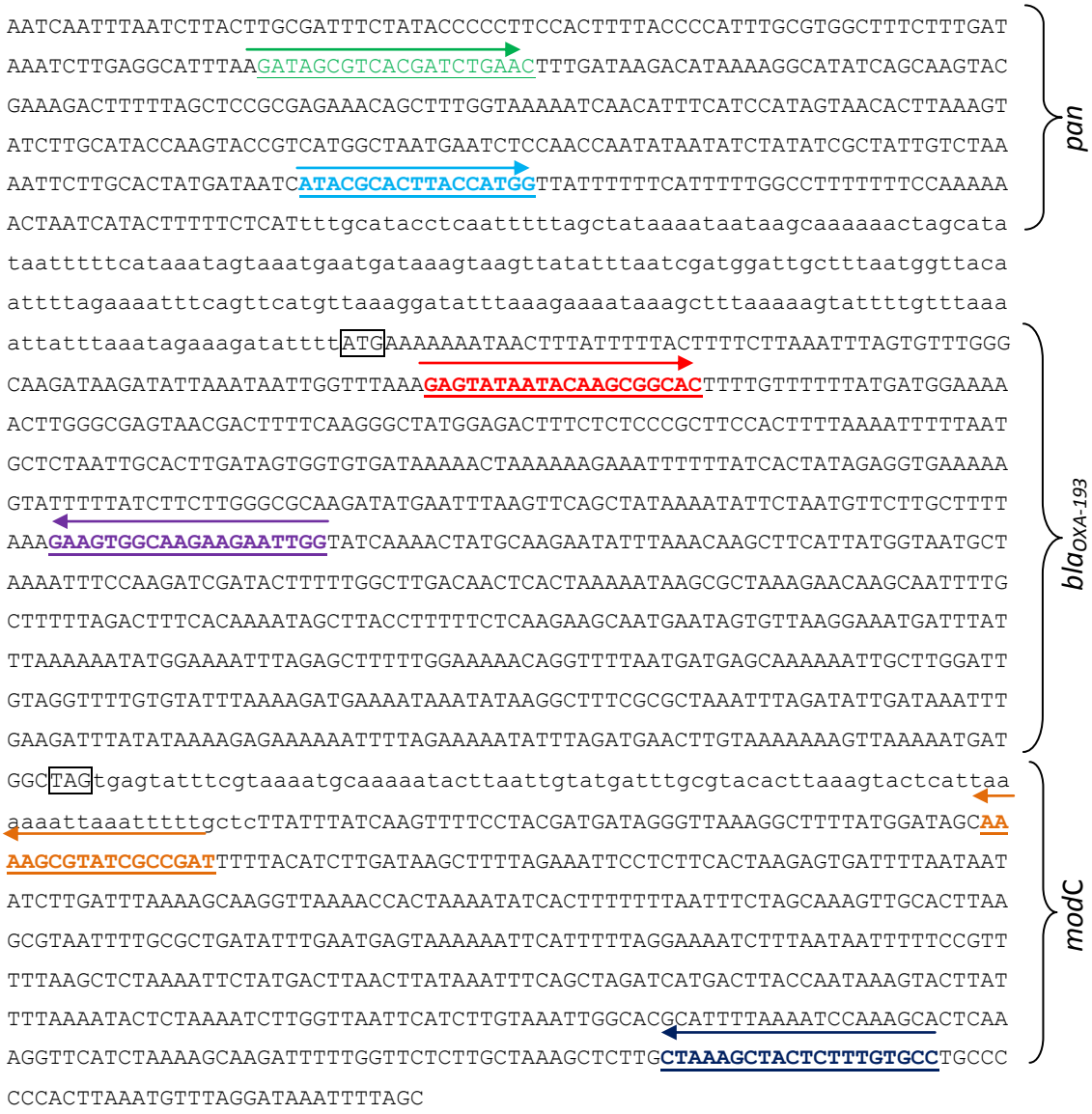
2.4.5 PCR amplification of *bla*$_{OXA\text{-}193}$ and flanking sequences

PCR reactions were set up as described previously (section 2.3.1) but primers and cycle conditions were varied (Figure 2.2 and Table 2.7). PCR primers were designed that amplified the entire gene plus extensive flanking sequence, including large parts of upstream and downstream genes; primers that bound internally were also designed.

2.4.6 Sequencing of *bla*$_{OXA\text{-}193}$ PCR amplimers

Sequencing reactions were performed as described previously (Section 2.3.2) but with *bla*$_{OXA\text{-}193}$ primers (Figure 2.2 and Table 2.7).

Figure 2.2 *bla*$_{OXA-193}$ primers



Primer Number	5'→3' Sequence	Co-ordinates on NCTC11168 genome
821	GATAGCGTCACGATCTGAAC	272811.272830
467	ATACGCACTTACCATGG	273029.273679
469	GAGTATAATACAAGCGGCAC	273399.273418
470	CCAATTCTTCTTGCCACTTC	273660.273679
468	ATCGGCGATACGCTTTT	274231. 274247
822	GGCACAAAGAGTAGCTTTAG	274640.274659

The sequence of *bla*$_{OXA-193}$ (*cj0299*) *Campylobacter jejuni subsp. jejuni* NCTC 11168 (position 273321..274094) and 600 bp of sequence both upstream and downstream is shown above. Coding sequence is shown in uppercase text whereas non-coding intergenic regions are shown in lowercase text, genes are labeled on the right of the diagram.
Primers used in this study are highlighted in various colours and detailed in the above table. Primer direction is indicated by the arrows above the primer sequence in the main diagram. *bla*$_{OXA-193}$ start and stop codons are boxed.

Table 2.7 *bla*$_{OXA-193}$ primers and PCR parameters for *bla*$_{OXA-193}$ PCR

Primer 1	Primer 2	Expected product size	Initial denaturation	PCR parameters X 30 cycles			Final extension
				Denaturation	Annealing	Extension	
821	822	1849bp	95°C (5 min)	95°C (30 sec)	45°C (45 sec)	72°C (2 min)	72°C (10 min)
467	468	1218bp	95°C (5 min)	95°C (30 sec)	45°C (45 sec)	72°C (90 sec)	72°C (10 min)
469	470	281bp	95°C (5 min)	95°C (30 sec)	45°C (45 sec)	72°C (30 sec)	72°C (10 min)
467	470	650bp	95°C (5 min)	95°C (30 sec)	45°C (45 sec)	72°C (45 sec)	72°C (10 min)
469	468	849bp	95°C (5 min)	95°C (30 sec)	45°C (45 sec)	72°C (1 min)	72°C (10 min)

2.4.7 Southern blotting for *bla*$_{OXA-193}$

There was no information in the literature regarding Southern blotting for *bla*$_{OXA-193}$ therefore this protocol was developed. Many of the conditions were varied during optimisation and are detailed in this section.

2.4.7.1 Genomic DNA extraction and quantification

Test strains were cultured on MH agar for 48 hours. All the colonies on the plate were harvested and used to make a suspension in 1 ml of sterile distilled water. A Wizard® Genomic DNA Purification Kit (Promega, Madison, USA, Catalogue No: A1120) was used and the manufacturer's instructions for extracting genomic DNA from Gram negative bacteria were followed. Re-hydration of DNA was at 65°C for 1 hour. Five microlitres of sample was run on a 1% (w/v) gel alongside HyperLadder I (BioLine, London, UK, Catalogue No: BIO-33025) and the gel was visualised using a Gene genius image analyser (Syngene, Cambridge, UK). DNA concentrations were determined by pipetting 1 µl of sample onto a Nano Drop™ spectrophotometer (Thermo Scientific, Waltham, USA).

2.4.7.2 Restriction enzyme digestion of genomic DNA

During method development the restriction endonucleases *Cla*I (Promega, Madison, USA, Catalogue No: R6551) and *Hind*III (Promega, Madison, USA, Catalogue No: R6045) were tested at concentrations of 5 U, 10 U and 30 U/µg of DNA with digestion times of 1, 2, 3 and 4 hours.

Following these experiments the restriction endonuclease *Hind*III was used to digest genomic DNA. Reactions contained 2-3 µg of genomic DNA, 30 U of enzyme, bovine serum albumin (BSA) to a final concentration of 0.1 µg/ml and 10 x Buffer E (Promega, Madison,

USA, Catalogue No: R6045); sterile distilled water was used to create a final reaction volume of 50 µl. Reactions were incubated at 37°C for 4 hours. The enzyme was inactivated by heating to 65°C for 20 minutes. Negative (contamination) controls containing no restriction endonuclease (difference in volume made up with sterile distilled water) were included in each experiment. Controls and digests were loaded onto a 0.8% (w/v) agarose gel and electrophoresed at 60 V for 4 hours and viewed with a Gene genius image analyser (Syngene, Cambridge, UK) for visual confirmation of digestion.

2.4.7.3 Transfer of DNA to blotting membrane

DNA was transferred from the agarose gel to a Hybond^{TM} H^{+} membrane (GE Healthcare, Little Chalfont, UK, Catalogue No: RPN303B) under vacuum at 80 mbar for 2 hours using a VacuGene vacuum blotting system (GE Healthcare, Little Chalfont, UK, Catalogue No: 80-1266-24). The unit was assembled by placing the gel supporting screen onto the base unit with the shiny side facing upwards, the support was wetted with distilled water and a piece of Hybond H+ membrane (2 cm larger than the gel in length and width) was placed onto the support screen. A mask was placed over the support screen and membrane ensuring that the cut-out in the mask was over the membrane. The lid of the apparatus was added and clasped down and the apparatus was connected to a vacuum tap with silicone tubing.

After the agarose gel was visualised to confirm digestion, it was placed onto the VacuGene Unit and was first flooded with depurination solution (0.125 M HCl) for 10-20 minutes, then washed with sterile distilled water. Two further 10-20 minute washes followed, one with a denaturing solution (0.5M NaOH 1.5M NaCl) and then a neutralising solution (1.5M NaCl, 0.5M Tris, 1mM EDTA, pH7.2) with washes of distilled water in between. Finally, the blot

was flooded with 20 x SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0) for 1-1.5 hours. Recipes for all solutions are listed in Appendix C.

Following transfer the gel was visualised using the Gene Genius Image Analyser to ensure all DNA has been transferred. The apparatus was dismantled and the location of the gel lanes was marked onto the membrane with a blunt pencil so that the top side of the membrane could be identified. The membrane was removed with either blunt ended forceps or gloved hands. DNA was then fixed to the membrane by placing the membrane in a UV cross linker (Stratagene, California, USA) and irradiating with 12000 Joules of UV light for 45 seconds.

2.4.7.4 Hybridisation buffer

The agarose gel was measured to determine its area and 0.25ml of hybridisation buffer (containing 4%w/v blocking reagent and NaCl to a final concentration of 0.5M) was used per cm^2 of blot.

2.4.7.5 Generating a labelled probe for *bla*$_{OXA-193}$

A 281 bp fragment of *bla*$_{OXA-193}$ was amplified by PCR from NCTC11168 (primers 467 and 468, Table 2.7) and labelled to use as a Southern blotting probe. The 'Gene Images AlkPhos Direct Labelling and Detection System' (GE Healthcare, Little Chalfont, UK, Catalogue No: RPN3690) was used to create an alkaline phosphatase labelled probe. For every millilitre of hybridisation buffer 5-10 ng of DNA was used to create labelled probe. Twenty microlitres of cross-linker solution (supplied with kit) was diluted with 80 µl of sterile water to give the working concentration. Probe DNA was diluted with sterile water to a concentration of 5-10 ng/ml. Ten microlitres of diluted DNA was denatured by heating for 10 minutes in a boiling water bath following which the DNA was immediately placed on ice for five minutes. Ten

microlitres of reaction buffer (supplied with kit) was added to the cooled DNA and mixed by gentle pipetting followed by 2 µl of labelling reagent (supplied with kit) which was mixed by gentle pipetting. Finally, 10µl of cross linker working solution was added to the DNA, mixed thoroughly by pipetting then the reaction was heated to 37°C for 30 minutes. Probe DNA was kept on ice and always used within 2 hours.

2.4.7.6 Hybridisation

The blot was pre-hybridised with the hybridisation buffer in a hybridisation oven at 50-65°C before probe labelled with alkaline phosphatase was added to a concentration of 5-10 ng/µl of hybridisation buffer. The probe was left to hybridise to the blot for 28-36 hours at 50-60°C in a rotisserie hybridisation oven.

Following hybridisation the blot was washed with both primary and secondary wash buffers. The first wash was for 10 minutes with 5ml/cm^2 of primary wash buffer (pre-heated to 55°C) with gentle agitation, this was repeated with fresh primary wash buffer for a further 10 minutes. The next wash was with sufficient secondary wash buffer (Appendix C) to cover the blot, for 5 minutes, at room temperature, again with gentle agitation. This wash was also repeated with fresh secondary wash buffer under the same conditions.

2.4.7.7 Signal detection

After being placed on Saran Wrap^{TM} (SC Johnson, Racine, USA) 40 µl/cm^2 of detection reagent (GE Healthcare, Little Chalfont, UK) was pipetted onto the blot and left for five minutes. The blot was then covered in a clean piece of saran wrap and placed DNA side up in a light proof film cassette. A sheet of Hyperfilm^{TM} (GE Healthcare, Little Chalfont, UK, Catalogue No: 28-9068-37) was placed over the blot in a dark room and the film was locked

into the cassette and left to develop for 45 minutes initially and repeated with a longer time if necessary. Film was developed in a darkroom with Kodak GBX Developer and Fixer (Sigma, Gillingham, UK, Catalogue No: P7402-1GA and P7167-1GA). The film was removed from the cassette in the darkroom and placed in a tray of photographic developer; the tray was agitated for 90 seconds then the film was removed and rinsed with water. The film was then placed in a tray of photographic fixer and agitated for 30 seconds then removed and rinsed. The film was then viewed by eye and photographed.

2.4.8 *flaA* SVR (short variable region) typing

2.4.8.1 PCR amplification of *flaA*

PCR reactions were set up as described previously (section 2.3.1) but primers and cycle conditions were varied (Table 2.8).

2.4.9 Sequencing of *flaA*

Sequencing reactions were performed as described previously (Section 2.3.2) but with *flaA* primers (Table 2.8).

2.4.9.1 Determination of *flaA* type

Sequencing data was aligned to representative *flaA* SVR types downloaded from the *Campylobacter* online *flaA* database (http://pubmlst.org/campylobacter/) using MEGA 3.1 software (Center for Evolutionary Functional Genomics, Tempe, USA). Once identified the *flaA* region of each test isolate was queried against the *Campylobacter* online *flaA* database to determine its *flaA* SVR sequence type.

Table 2.8 *flaA* primers and PCR parameters for *flaA* PCR

Primer	Target gene	5'→3' Sequence	Product Size	Initial denaturation	PCR Parameters X 30 cycles			Final extension
					Denaturation	Annealing	Extension	
985	*flaA*	GGATTTCGTATTAACACAAATGGTGC	1713 bp	95°C (5 min)	95°C (30 sec)	45°C (45 sec)	72°C (1 min 45 sec)	72°C (5 min)
986	*flaA*	CTGTAGTAATCTTAAAACATTTTG						

2.4.10 Whole genome DNA sequencing and analysis of *C. jejuni* P854

2.4.10.1 Isolation of genomic DNA

P854 genomic DNA was isolated and quantified as described previously (Section 2.4.7.1).

2.4.10.2 SOLEXA sequencing of P854 genomic DNA

Genomic DNA was sent for Illumina - SOLEXA Sequencing at the School of Biological Sciences Sequencing Service, University of Edinburgh.

2.4.10.3 Assembly and annotation of SOLEXA sequencing reads

Sequencing data was uploaded to '*my xBASE*' (http://ng.xbase.ac.uk/my/) and was assembled into 621 contigs comprising of 83 scaffolds using the SOAP de novo program (http://soap.genomics.org.cn/soapdenovo.html) with assistance from Dr Nick Loman.

Several randomly selected segments of P854 nucleotide sequence (selected by Nick Loman) were used to interrogate sequenced *Campylobacter* genomes using the Basic Local Alignment Search Tool, BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and these searches revealed *Campylobacter jejuni* RM1221 to be the most similar to P854. The P854 assembled contigs were then run through the *xBASE* annotation pipeline using *C. jejuni* RM1221 as a reference genome.

2.4.10.4 Identification of putative beta-lactamase genes

The Artemis Comparison Tool, ACT, (http://www.sanger.ac.uk/resources/software/artemis/) was used to view the annotated P854 genome and compare it to the *C. jejuni* RM1221 genome. The P854 genome was then queried for genes that had been annotated as putative beta-lactamase genes. The DNA and amino acid sequences of any putative beta-lactamase

genes identified were queried against known beta-lactamase genes/proteins in GenBank using BLAST. The product of the *P854_1490* gene was identified as a putative novel oxacillinase, the sequence of this protein was submitted to the Lahey clinic (http://www.lahey.org/studies/) who confirmed it to be a novel and assigned it the unique oxacillinase number, OXA-184. From here on the gene *P854_1490* is described as $bla_{OXA\text{-}184}$ and the product of this gene is described as OXA-184.

2.4.11 Screening isolates for the presence of $bla_{OXA\text{-}184}$

2.4.11.1 PCR amplification of $bla_{OXA\text{-}184}$

PCR reactions were set up as described previously (Section 2.3.1) but primers and cycle conditions were varied (Figure 2.3 and Table 2.9). Primers that bound within the genes up and downstream of the target gene (to amplify the whole $bla_{OXA\text{-}184}$) and that bound within the target gene itself were designed.

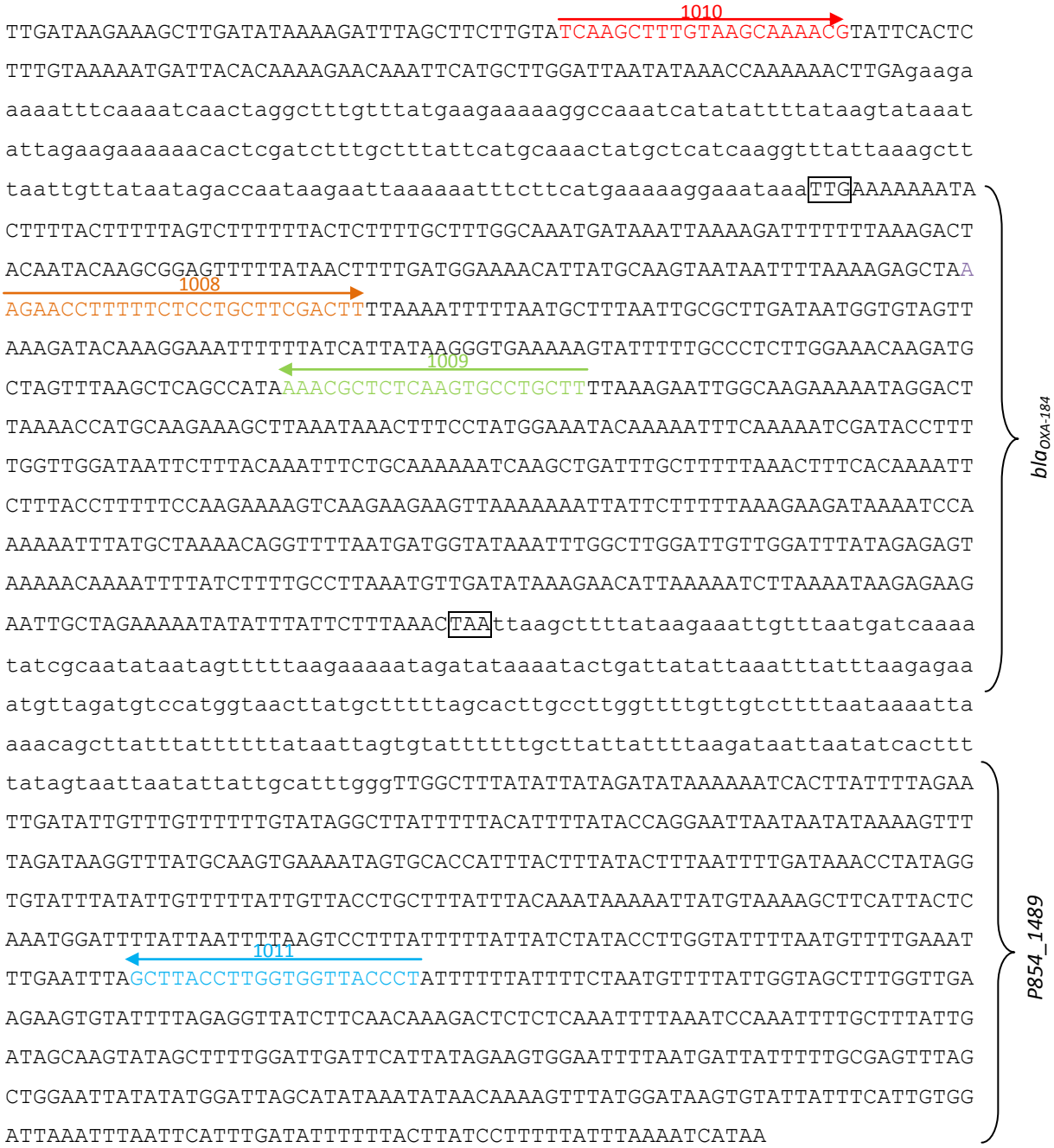
2.4.11.2 Sequencing of $bla_{OXA\text{-}184}$ PCR amplimers

Sequencing reactions were performed as described previously (Section 2.3.2) but with $bla_{OXA\text{-}184}$ primers (Table 2.9).

2.4.11.3 Analysis of $bla_{OXA\text{-}184}$ screening results

Sequence data were compared to $bla_{OXA\text{-}184}$ using MEGA 3.1 software (Center for Evolutionary Functional Genomics, Tempe, USA). Six isolates contained an oxacillinase gene which produced a protein with one amino acid difference to OXA-184. The sequence of this protein was submitted to the Lahey clinic (http://www.lahey.org/studies/) who confirmed it to be a novel and assigned it the unique oxacillinase number, OXA-185. From here on any genes encoding the OXA-185 protein are described as $bla_{OXA\text{-}185}$.

Figure 2.3 *bla*$_{OXA-184}$ primers



TTGATAAGAAAGCTTGATATAAAAGATTTAGCTTCTTGTATCAAGCTTTGTAAGCAAAACGTATTCACTC
TTTGTAAAAATGATTACACAAAAGAACAAATTCATGCTTGGATTAATATAAACCAAAAAACTTGAgaaga
aaaatttcaaaatcaactaggctttgtttatgaagaaaaaggccaaatcatatattttataagtataaat
attagaagaaaaaacactcgatctttgctttattcatgcaaactatgctcatcaaggtttattaaagctt
taattgttataatagaccaataagaattaaaaaatttcttcatgaaaaaggaaataaaTTGAAAAAAATA
CTTTTACTTTTTAGTCTTTTTTACTCTTTTGCTTTGGCAAATGATAAATTAAAAGATTTTTTTAAAGACT
ACAATACAAGCGGAGTTTTTATAACTTTTGATGGAAAACATTATGCAAGTAATAATTTTAAAAGAGCTAA
AGAACCTTTTTTCTCCTGCTTCGACTTTTAAAATTTTTAATGCTTTAATTGCGCTTGATAATGGTGTAGTT
AAAGATACAAAGGAAATTTTTTATCATTATAAGGGTGAAAAAGTATTTTTGCCCTCTTGGAAACAAGATG
CTAGTTTAAGCTCAGCCATAAAACGCTCTCAAGTGCCTGCTTTTAAAGAATTGGCAAGAAAAATAGGACT
TAAAACCATGCAAGAAAGCTTAAATAAACTTTCCTATGGAAATACAAAAATTTCAAAAATCGATACCTTT
TGGTTGGATAATTCTTTACAAATTTCTGCAAAAAATCAAGCTGATTTGCTTTTTAAACTTTCACAAAATT
CTTTACCTTTTTTCCAAGAAAAGTCAAGAAGAAGTTAAAAAAATTATTCTTTTTAAAGAAGATAAAATCCA
AAAAATTTATGCTAAAACAGGTTTTAATGATGGTATAAATTTGGCTTGGATTGTTGGATTTATAGAGAGT
AAAAACAAAATTTTATCTTTTGCCTTAAATGTTGATATAAAGAACATTAAAAATCTTAAAATAAGAGAAG
AATTGCTAGAAAAATATATTTATTCTTTAAACTAAttaagctttataagaaattgtttaatgatcaaaa
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atgttagatgtccatggtaacttatgctttttagcacttgccttggttttgttgtcttttaataaaatta
aaacagcttatttatttttttataattagtgtatttttttgcttattattttaagataattaatatcacttt
tatagtaattaatattattgcatttgggTTGGCTTTATATTATAGATATAAAAAATCACTTATTTTAGAA
TTGATATTGTTTGTTTTTTTGTATAGGCTTATTTTTACATTTTATACCAGGAATTAATAATATAAAAGTTT
TAGATAAGGTTTATGCAAGTGAAAATAGTGCACCATTTACTTTATACTTTAATTTTGATAAACCTATAGG
TGTATTTATATTGTTTTTATTGTTACCTGCTTTATTTACAAATAAAAATTATGTAAAAGCTTCATTACTC
AAATGGATTTTATTAATTTTAAGTCCTTTATTTTTATTATCTATACCTTGGTATTTTAATGTTTTGAAAT
TTGAATTTAGCTTACCTTGGTGGTTACCCTATTTTTTATTTTCTAATGTTTTATTGGTAGCTTTGGTTGA
AGAAGTGTATTTTAGAGGTTATCTTCAACAAAGACTCTCTCAAATTTTAAATCCAAATTTTGCTTTATTG
ATAGCAAGTATAGCTTTTGGATTGATTCATTATAGAAGTGGAATTTTAATGATTATTTTTGCGAGTTTAG
CTGGAATTATATATGGATTAGCATATAAATATAACAAAAGTTTATGGATAAGTGTATTATTTCATTGTGG
ATTAAATTTAATTCATTTGATATTTTTTACTTATCCTTTTTATTTAAAATCATAA

Primer Number	5'→3' Sequence	Product
1008	AAGAACCTTTTTCTCCTGCTTCGACTT	183bp
1009	AAGCAGGCACTTGAGAGCGTTT	
1010	TCAAGCTTTGTAAGCAAAACG	1670bp
1010	AGGGTAACCACCAAGGTAAGC	

The sequence of *bla*$_{OXA-184}$ *Campylobacter jejuni* P854 sequence both upstream and downstream is shown above. Coding sequence is shown in uppercase text where as non-coding intergenic regions are shown in lowercase text, genes are labeled on the right of the diagram. Primers used in this study are highlighted in various colours and detailed in the above table. Primer direction is indicated by the arrows above the primer sequence in the main diagram. *bla*$_{OXA-184}$ start and stop codons are boxed.

Table 2.9 *bla*$_{OXA-184}$ primers and PCR parameters for *bla*$_{OXA-184}$ PCR

Primer 1	Primer 2	Expected product size	Initial denaturation	PCR parameters X 30 cycles			Final extension
				Denaturation	Annealing	Extension	
1008	1009	183 bp	95°C (5 min)	95°C (30 sec)	50°C (30 sec)	72°C (20 sec)	72°C (10 min)
1010	1011	1670 bp	94°C (2 min)	94°C (10 sec)	42°C (30 sec)	68°C (1 min)	68°C (7 min)

2.4.12 Cloning *bla*$_{OXA-184}$ into *E. coli* (α-Select)

2.4.12.1 Cloning strategy (including selection of a suitable cloning vector)

A vector required for cloning that was suitable for expression in *E. coli*, had a multiple cloning site and a selective marker other than ampicillin resistance. The pHSG398 plasmid (Takara Biosciences, Product number 3398), which is a pUC18 based vector with a chloramphenicol resistance marker was selected for use as it met all requirements (Figure 2.4). The intention was to clone the coding sequence of *bla*$_{OXA-184}$ only (i.e. no *Campylobacter* expression signals) into the multiple cloning site of the plasmid, allowing the gene to be controlled by the *lacZ* promoter and ribosome binding site.

2.4.12.2 Creation of a *bla*$_{OXA-184}$ PCR amplimer suitable for cloning

PCR primers were designed to amplify the *bla*$_{OXA-184}$ gene (excluding expression signals) and to introduce an *EcoR*I restriction site at the start of the amplimer and an *Xba*I restriction site to the end of the amplimer (Table 2.10). The PCR reactions were set up as described previously (Section 2.3.1) but primers and cycle conditions were varied (Table 2.10).

2.4.12.3 Cloning into vector pHSG398

The *bla*$_{OXA-184}$ PCR amplimer with *EcoR*I/*XbaI* ends (Table 2.10) and pHSG398 DNA were both digested with EcoRI high fidelity restriction enzyme (New England Biolabs, Product number R3101S) and XbaI restriction enzyme (New England Biolabs, Product number R0145S) (Table 2.11). Digests and controls (lacking any restriction enzymes) were incubated at 37°C for 1 hour then heat inactivated at 65°C for 20 minutes.

2.4.12.4 Transformation of *E. coli* with pHSG398 (*bla*$_{OXA-184}$)

One 200 µl aliquot of BioLine bronze efficiency α-Select competent cells (Product number BIO-85025) was thawed on ice then split into four 50 µl aliquots in pre-chilled eppendorfs. Two microlitres of pHSG398(*bla*$_{OXA-184}$) was added to the first two eppendorfs containing α-

Figure 2.4 The pHSG398 cloning vector

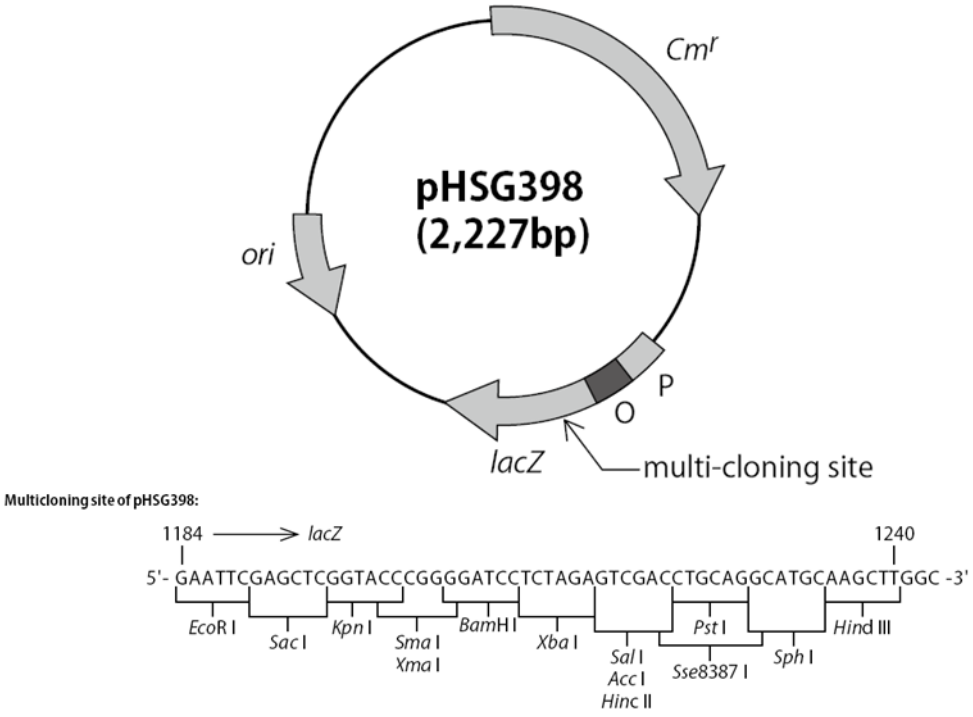


Image modified from product information sheet (Takara Biosciences).

Table 2.10 *bla*$_{OXA-184}$ cloning primers and PCR parameters for *bla*$_{OXA-184}$ cloning PCR

Primer	Target gene	5'→3' Sequence	Product Size	Initial denaturation	PCR Parameters X 20 cycles			Final extension
					Denaturation	Annealing	Extension	
1012	*bla*$_{OXA-184}$	GCGGAATTCTTGAAAAAAATACTTTTACT	879 bp	94°C (2 min)	94°C (10 sec)	45°C (30 sec)	68°C (1 min)	68°C (7 min)
1014	bla$_{OXA-184}$	GCGTCTAGAATCTAACATTTCTCTTAAAT						

TTG = Start codon

GAATTC = EcoRI restriction site

TCTAGA = XbaI restriction site

Table 2.11 Restriction enzyme digestion of *bla*$_{OXA-184}$ cloning PCR amplimer and pHSG398

Digestion of the *bla*$_{OXA-184}$ (EcoRI/XbaI) PCR amplimer		
	Digestion	**Control reaction**
DNA	30 µl	15 µl
NEBuffer 4*	5 µl	2.5 µl
EcoRI-HF restriction enzyme**	5 µl	0 µl
XbaI restriction enzyme***	5 µl	0 µl
Bovine serum albumin**	1 µl	0.7 µl
Water	4 µl	7 µl
TOTAL	50 µl	25 µl
Digestion of pHSG398 DNA		
	Digestion	**Control reaction**
DNA	2 µl	1 µl
NEBuffer 4*	2 µl	1 µl
EcoRI-HF restriction enzyme**	5 µl	0 µl
XbaI restriction enzyme***	5 µl	0 µl
Bovine serum albumin****	1 µl	0.5 µl
Water	5 µl	7.5 µl
TOTAL	20 µl	10 µl

* NEBuffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM Magnesium acetate, 1 mM DTT, pH 7.9 at 25°C)

**20,000 U/ml

***20,000 U/ml

****BSA (200 µg/ml)

Select cells, 2µl of pHSG398 was added to the third tube and 2µl of water to the fourth and final tube. Reactions were incubated on ice for 30 minutes. Cells were then heat-shocked by heating the eppendorfs to 42°C for 30 seconds then returning to incubation on ice for a further two minutes. Reactions were diluted with 950µl of SOC (Appendix D), transferred to a Universal tube and incubated at 37°C with shaking for one hour. Seventy five microlitres of each of the reactions was transferred to the surface of eight LB (Luria-Bertani) (Oxoid, UK, Product No: CM1021) agar plates containing 0.5 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside), 80 µg/ml X-gal (5-bromo-4- chloro-3-indolyl- beta-D-galactopyranoside) and 35 µg/ml chloramphenicol. Plates were incubated overnight at 37°C then examined for the presence of transformants (white colonies). Candidate colonies were streaked onto fresh LB plates (containing the same concentrations of IPTG, X-gal and chloramphenicol). Putative mutants were stored at -80°C on Protect™ beads (Service Consultants Ltd., Heywood, UK, Catalogue No: TS70).

2.4.13 Characterisation of α-Select pHSG298 (*bla*$_{OXA-184}$) mutants

2.4.13.1 PCR amplification of *bla*$_{OXA-184}$

All putative α-Select pHSG298 (*bla*$_{OXA-184}$) mutants were screened for the presence of *bla*$_{OXA-184}$ using the PCR described previously (Section 2.4.12.2).

2.4.13.2 Sequencing of *bla*$_{OXA-184}$ PCR amplimers

Sequencing reactions were performed as described previously (Section 2.3.2) but with *bla*$_{OXA-184}$ primers (Table 2.9).

2.4.13.3 Detection of beta-lactamase activity

Beta-lactamase production was determined as described previously (Section 2.4.4).

2.4.13.4 Determination of the MICs of antibiotics

MICs of antibiotics were determined as previously described (Section 2.3.4) with the addition of 0.5 mM IPTG to each agar plate.

2.4.13.5 RNA isolation

RNA was isolated from three technical replicates of each isolate, on three separate occasions. Single colonies of isolates to be tested were inoculated into 10 ml LB broths which incubated at 37°C, overnight with shaking. One millilitre of each overnight culture was inoculated into 24 ml of fresh LB broth and incubated at 37°C with shaking until mid-logarithmic phase was achieved (OD_{600} of 0.6). Five millilitres of each culture was added to 1 ml of a 95% ethanol, 5% phenol mixture and incubated on ice for 30 minutes. Samples were centrifuged at 2250 x *g* at 4°C for 10 minutes and the supernatants were discarded. Pellets were re-suspended in 100 µl of TE buffer containing 50 µg/ml lysozyme and incubated for 5 minutes at room temperature. The Promega SV Total RNA purification kit (Promega, UK, Catalogue No: Z3100) was then used, according to manufacturer's instructions. Isolated RNA was viewed by gel electrophoresis as described previously (Section 2.3.1.1). RNA concentrations were determined by pipetting 1 µl of sample onto a Nano Drop™ spectrophotometer (Thermo Scientific, Waltham, USA).

2.4.13.6 cDNA synthesis

Superscript III Reverse transcriptase (Invitrogen, UK) was used to synthesise cDNA. First strand DNA synthesis was achieved as follows; 1µg of RNA was combined with 1µl of 50 ng/µl random length primers, 1 µl dNTP mix and was made up to 10 µl with RNase free sterile water in microcentrifuge tubes. The reaction was incubated at 65°C for five minutes

followed by one minute of incubation on ice. Ten microlitres of the first strand synthesis mix was combined with 2 µl of first strand buffer, 4 µl of 25mM $MgCl_2$, 2 µl 0.1M STT, 1 µl 40 U/µl RNase Out and 1 µl of 100 U/µl Superscript III Reverse Transcriptase. The reaction was incubated at 25°C for 10 minutes then at 50°C for 50 minutes. The reaction was terminated by an inactivation step of heating to 85°C for 5 minutes.

2.4.13.7 RT-PCR

PCRs for *bla*$_{OXA-184}$ and 16S were performed using cDNA as a template. PCR amplification of *bla*$_{OXA-184}$ has been described previously (Section 2.4.11.1). PCR amplification of 16S was performed as described previously (Section 2.3.1) but primers and cycle conditions were varied (Table 2.12).

2.4.14 Inactivation of *bla*$_{OXA-184}$ in *C. jejuni* P854

2.4.14.1 Using PCR to create *bla*$_{OXA-184}$::*aph*

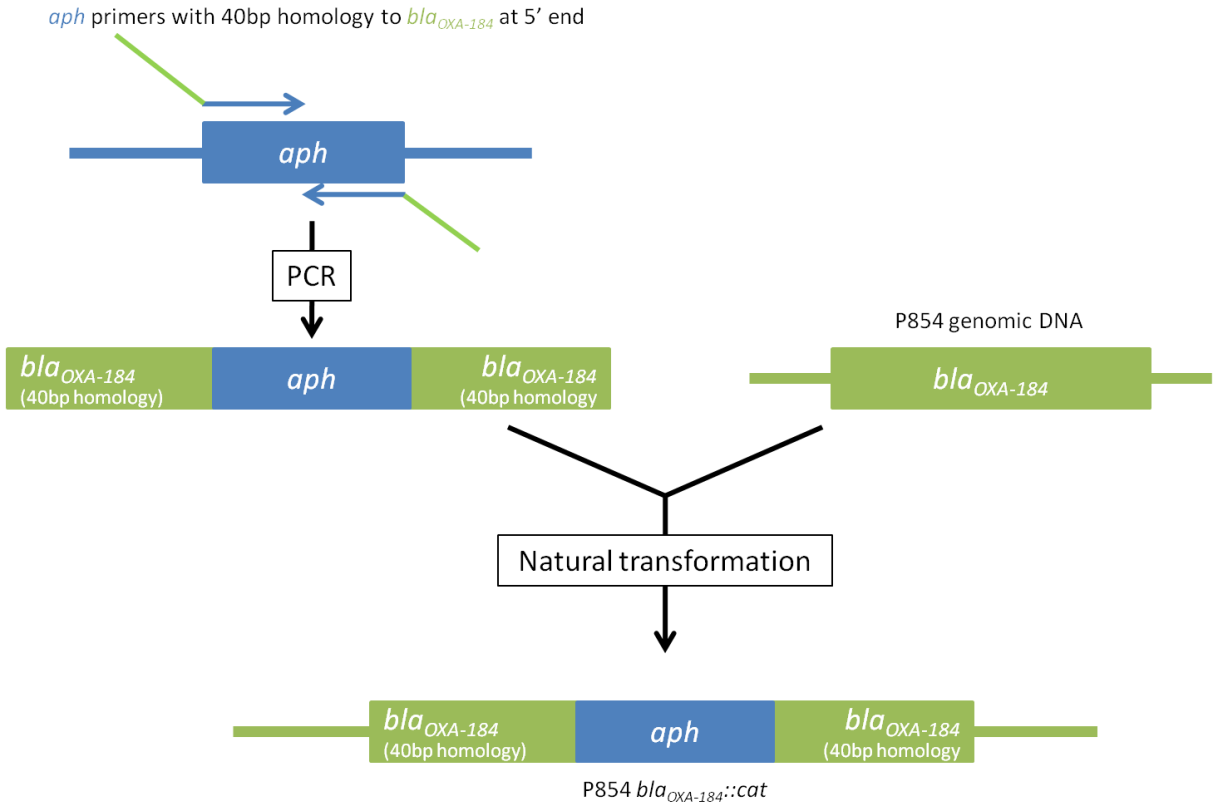
An inactivation strategy was devised based on part of the Datsenko and Wanner method of gene inactivation (Datsenko and Wanner 2000) but employing the natural competency of *Campylobacter* (Figure 2.5). In the Dasenko and Wanner method an antibiotic resistance genes flanked by an FRT (FLP recognition target) is amplified with primers that have 5' homology (30-50 nucleotides) to genes adjacent to the target; this PCR amplimer is used in transformation with a λ Red recombinase to inactivate the target gene. The resistance cassette can then be removed using a FLP expression plasmid (Datsenko and Wanner 2000).

PCR amplification of *aph* was performed as described previously (Section 2.3.1) but primers and cycle conditions were varied (Table 2.13). The *Salmonella* strain L823 (*acrA::aph*) (Table 2.3) was used as a source of template DNA.

Table 2.12 *16S* primers and PCR parameters for *16S* PCR

Primer	Target gene	5'→3' Sequence	Product Size	Initial denaturation	PCR Parameters X 30 cycles			Final extension
					Denaturation	Annealing	Extension	
147	*16s*	GGTGCAAGCGTTAATCGGAA	541 bp	95°C (5 min)	95°C (30 sec)	50°C (30 sec)	72°C (20 sec)	72°C (10 min)
148	*16s*	TCGTCAGCTCGTGTTGTGAA						

Figure 2.5 Strategy for inactivation of *bla*$_{OXA-184}$



A modified Datsenko and Wanner method employing natural transformation (Datsenko and Wanner 2000).

Table 2.13 *aph+ bla*$_{OXA-184}$ cloning primers and PCR parameters for *aph+ bla*$_{OXA-184}$ cloning PCR

Primer	Target gene	5'→3' Sequence	Product Size	Initial denaturation	PCR Parameters X 30 cycles			Final extension
					Denaturation	Annealing	Extension	
1051	*aph* + 40bp of *bla*$_{OXA-184}$	ATACTTTTACTTTTTAGTCTTT TTTACTCTTTTGCTTTGGGTGT AGGCTGGAGCTGCTTC	1574 bp	95°C (5 min)	95°C (30 sec)	45°C (30 sec)	72°C (1 min 45 sec)	72°C (10 min)
1052	*aph* + 40bp of *bla*$_{OXA-184}$	ATGGACCATGGCTAATTCCCG CTTGGATTGTTGGATTTATAG AGAGTAAAAACAAAATTT						

GAATTC = homology to *bla*$_{OXA-184}$

TCTAGA = homology to *aph*

2.4.14.2 Natural transformation of P854 with *bla$_{OXA-184}$*::*aph*

Natural transformation of P854 was performed as described previously (Section 2.3.3) using the *bla$_{OXA-184}$*::*aph* amplimer produced above to inactivate the *bla$_{OXA-184}$* gene. Transformants were selected on MH agar plates containing 50 µg/ml of kanamycin.

2.5 Conjugal transfer of beta-lactam resistance in *Campylobacter*

2.5.1 Conjugation

Conjugation reactions were set up between previously characterised tetracycline resistant, kanamycin sensitive donor strains P1131, P1931, and P1984 (conjugation positive control) and a tetracycline sensitive, kanamycin resistant recipient strain P1983 (Table2.3) to determine whether beta-lactam resistance could be transferred conjugatively alongside tetracycline resistance. The method that was followed is based on a published method (Taylor, Gradis et al. 1981).

Donor and recipient strains were cultured as described previously on MH agar for 48 hours. Two methods of conjugation were followed, one in liquid and the other on agar.

2.5.1.1 Liquid conjugation

Bacterial cells were harvested and a suspension was made in MH broth to an OD_{600} of 0.1. Five hundred microlitres of the donor solution was mixed with 1 ml of the recipient solution and added to a T25 Falcon^{TM} tissue culture flask already containing 1 ml of fresh MH broth. After overnight incubation at 37°C in 7.5% CO_2 cultures were serially diluted and 100 µl of the un-diluted, 10^{-1}, 10^{-2} and 10^{-3} dilutions were plated out onto MH agar containing 10 µg/ml of tetracycline and 50 µg/ml of kanamycin, and incubated for up to 5 days at 37°C in 7.5% CO_2.

2.5.1.2 Agar conjugation

Bacterial cells were harvested and a suspension was made in phosphate buffer (Sigma, Gillingham, UK, Catalogue No: D8537) to an OD_{600} of 0.1. One hundred and fifty microlitres of the donor solution was mixed with 150 µl of the recipient solution. The mating mixture was spread onto MH agar and cultured overnight at 37°C in CO_2. After overnight incubation the bacterial cells were harvested from the surface of the agar plate by flooding with 2 ml of phosphate buffer. The suspension was serially diluted and 100 µl each of the un-diluted, 10^{-1}, 10^{-2} and 10^{-3} dilutions were plated out onto MH agar containing 10 µg/ml of tetracycline and 50 µg/ml of kanamycin, and incubated for up to 5 days at 37°C in CO_2.

2.5.2 Plasmid Isolation

Various protocols were used so as to isolate a good quantity of high quality plasmids.

2.5.2.1 Mini-preparations

Qiagen's QIAprep Miniprep kit (Qiagen, Crawley, UK, Product No: 27104) and Promega's PureYield™ Plasmid Miniprep System (Promega, Southampton, UK, Product Number: A1221) were tested, in both instances the manufacturer's instructions were followed including modifications for large, low copy number plasmids.

2.5.2.2 Other (non-kit based) plasmid preparations

A modified Kado and Liu alkaline lysis (Kado and Liu 1981) method was carried out. Bacterial cultures were grown on MH agar for 48 hours in a CO_2 incubator (7.5% CO_2) at a temperature of 42°C. Cultures were Gram stained for purity before a loop-full of cells was used to inoculate 10 ml of MH broth. Cultures of P2241 and P2244 also contained 40 µg/ml of ampicillin. Broth cultures were incubated overnight at 37°C in 7.5% CO_2 with shaking. The

following morning 1.5 ml aliquots were centrifuged at 12,000 x *g* (in a Mistral centrifuge) for three minutes and the pellet re-suspended in 150 µl of suspension buffer (40 mM Tris, 2 mM Sodium EDTA pH7.9) followed by 300 µl of lysis buffer (100 mM SDS, 50 mM Tris pH 12.6). Tubes were mixed by gentle inversion until the suspension became clear. Following incubation at 60°C in a water bath for one hour 600 µl of phenol chloroform (Sigma, Gillingham, UK, Product No: P3803) was added; the tubes were then gently inverted and then kept on ice for 20 minutes. The upper phase from each tube were removed gently with a pipette (being careful not to disturb the interface) and 600 µl aliquots were transferred to clean microcentrifuge tubes. Twenty microlitres of 3 M potassium acetate was added to each 600 µl aliquot followed by 450 µl of 100% ethanol, the mixture was then left at -20°C overnight. Following overnight incubation tubes were centrifuged at 12,100 x *g* in a microcentrifuge for five minutes. The supernatant was discarded and the precipitate washed three times in 70% ethanol in water. The pellet was left to air dry for two hours then was re-suspended in 50 µl of water.

Another protocol, the Birnboim and Doly maxi-preparation method with modifications by Smith and Thomas (Birnboim and Doly 1979; Smith and Thomas 1983), was also followed. Bacterial cultures were grown on MH agar for 48 hours in a CO_2 incubator (7.5% CO_2) at a temperature of 37°C. Cultures were Gram stained for purity before a loop-full of cells was used to inoculate 400 ml of MH broth. Cultures of P2241 and P2244 also contained 40 µg/ml of ampicillin.

Cultures were incubated overnight at 37°C in 7.5% CO_2 with shaking (80 rpm). Bacterial cells were pelleted by centrifugation at 2,250 x *g* for 15 minutes (in a Mistral centrifuge), pellets were then re-suspended in 25 ml of ice cold Lysis Solution 1 (25 mM Tris-pH 8.5, 10 mM

EDTA-pH 8, 50 mM glucose). Fifty microlitres of Lysis Solution 2 (0.2 M NaOH, 1% SDS) was then added and the tube inverted several times to ensure complete lysis. Following incubation on ice for five minutes 37.5 ml of neutralising solution 3 (3 M Sodium acetate-pH 5) was added and the contents mixed by carefully inverting. Preparations were centrifuged for 15 minutes at 2,250x*g* (in a Mistral centrifuge) at 4°C to pellet cell debris. The supernatant was filtered using filter paper and a funnel to remove any remaining debris. Plasmid DNA was precipitated by adding 100 ml of isopropanol and pelleted by centrifugation at 2,250 x *g* for minutes at room temperature. The supernatant was discarded and the pellet re-suspended in 3 ml of TNE buffer (100 mM Tris-pH 8, 50 mM NaCl, 5 mM EDTA-pH 8).

2.5.2.3 Caesium chloride density gradient centrifugation

The 3 ml plasmid preparation from the modified Birnboim and Doly maxi-preparation was purified by density gradient centrifugation. First, 4.72 g of caesium chloride (CsCl) was accurately weighed out into a glass tube and the 3 ml of plasmid preparation was added. The volume was made up to 4.5 ml with TNE buffer (100 mM Tris-pH 8, 50 mM NaCl, 5 mM EDTA) and the solution was gently inverted until all the CsCl dissolved. Five hundred microlitres of 10 mg/ml ethidium bromide (EtBr) was then added. The sample was split equally between two Beckman 3.5 ml centrifugation tubes (Beckman Coulter, High Wycombe, Product No. 349621) using a Pasteur pipette, the tubes were weighed to ensure they were within 0.1 g of each other then the tubes were topped-up to the bottom of the neck of the tube with TNE. Tubes were then heat sealed with a QuickSeal® tube topper (Beckman Coulter, High Wycombe, UK, Product No: 360976**)** and centrifuged overnight at 801,920 x *g* in an ultra centrifuge (Beckman Coulter, Hugh Wycombe, Product No. TL-100) at

20°C using a Type 100 Ti rotor (Beckman Coulter, High Wycombe, Product No: 363013) with spacer (Beckman Coulter, High Wycombe, UK, Product No: 360270). Following centrifugation sealed caps were removed with scissors to prevent tubes collapsing when liquid was removed with a needle. Tubes were then visualised under UV light and fluorescent DNA bands were extracted and transferred to a clean tube with a needle and syringe.

Isopropanol saturated with $CsCl/H_20$ was added to the extracted plasmid with a Pasteur pipette and was gently agitated until the bottom layer became clear and the top layer pink-red. The isopropanol and EtBr (top layer) was removed with a pipette and discarded. This process was repeated three times to ensure all EtBr was removed from the plasmid DNA. Four hundred microlitre aliquots were transferred to microcentrifuge tubes; 500 µl of nuclease free water and 100 µl of 3M sodium acetate were added to each tube, followed by 530 µl of isopropanol. Tubes were centrifuged in a microcentrifuge for 45 minutes at 12,100 x *g*. The supernatant was gently removed and the pellet re-suspended in 200 µl of TNE. Twenty five microlitres of 3 M sodium acetate and 500 µl of 70% ethanol were added before centrifuging for 10 minutes in a microcentrifuge at 12,100 x *g* to pellet DNA. Supernatant was gently removed and the pellet washed with 70% ethanol. The pellet was air-dried for one hour then re-hydrated in 50 µl of TNE. Five microlitres of each plasmid preparation and 5 µl of HyperLadder 1 were electrophoresed on a 0.8% (w/v) agarose gel at 70 V for four hours.

2.5.2.4 Maxi Preparations

The QIAGEN Large Construct Kit (Qiagen, Crawley, UK, Product No:12462) was tested and the manufacturer's instructions were followed. A Beckman JA25.50 rotor (Beckman Coulter, London, UK, Catalogue No: 363058) was used for centrifugation of volumes of 50-250 ml and

a JLA16.250 rotor (Beckman Coulter, London, UK, Catalogue No: 363930) was used for centrifugation of volumes up to 50 ml.

2.5.3 Restriction enzyme digestion of plasmid DNA

The restriction endonuclease *Hind*III (Promega, Madison, USA, Catalogue No: R6045) was selected to digest plasmid DNA as a literature search revealed this enzyme had been used previously with *Campylobacter* plasmids (Jesse, Pittenger-Alley et al. 2006). Reactions contained approximately 0.5 µg of plasmid DNA, 40 U of enzyme, BSA to a final concentration of 0.1 µg/ml and 10 x Buffer E (Promega, Madison, USA, Catalogue No: R6045), sterile distilled water was used to create a final reaction volume of 40 µl. Reactions were incubated at 37°C for one and a half hours then the enzyme was inactivated by heating to 65°C for 20 minutes. Negative controls containing no restriction endonuclease (difference in volume made up with sterile distilled water) were included in each experiment. Controls and digests were loaded onto a 0.8% (w/v) agarose gel at 60 \V for 4 hours and viewed with a Gene genius image analyser (Syngene, Cambridge, UK) for visual confirmation of digestion.

2.5.4 Growth kinetics

Campylobacter isolates were grown at the University of Bristol on MH agar for 48 hours at 37°C in a microaerobic atmosphere (5% O_2, 10% CO_2 2% H_2 in a balance of N_2). Several colonies were used to inoculate 10 ml of MH broth and incubated overnight at 37°C in a microaerobic atmosphere. One millilitre of overnight culture was diluted to an OD_{600} of 0.05. Twenty microlitres of this diluted culture was added to a 100-well honeycomb plate (Thermo labsystems, UK) and diluted with a further 200 µl of MH broth. The plates were sealed inside a variable atmosphere cabinet (Don Whitley Scientific Ltd, UK, Product: MAC 10000) after

being incubated there for one hour in a microaerobic atmosphere. Plates were incubated at 37°C using a Bioscreen C plate reader (Thermo scientific, UK) and the absorbance (at OD_{600}) was measured every hour after shaking for 48 hours.

2.6 The oxacillinases of *Campylobacter*

2.6.1 Comparison of oxacillinases

The amino acid sequences of oxacillinases proteins were aligned and dendograms were produced based on the Neighbour-Joining method using MEGA 3.1 software (Center for Evolutionary Functional Genomics, Tempe, USA). The genomic context of the genes encoding these oxacillinases was compared using xBASE (http://www.xbase.ac.uk/).

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Chapter 3 – Results and Discussion

What contribution does efflux make towards beta-lactam resistance in *Campylobacter*?

3.1 Background

Efflux has previously been shown to play a role in innate antibiotic resistance in *Campylobacter jejuni*, including resistance to the beta-lactam ampicillin (Lin, Michel et al. 2002; Pumbwe and Piddock 2002; Pumbwe, Randall et al. 2005). *Campylobacter* are also innately resistant to cephalosporin type beta-lactams and previous studies have suggested that low permeability of the *Campylobacter* cell wall and/or poor binding affinity of the antibiotics to the bacterial penicillin binding proteins may be the cause (Lachance, Gaudreau et al. 1991).

3.2 Aims and Hypothesis

The hypothesis to be investigated was that: "the efflux pumps CmeB and/or CmeF are responsible for the innate resistant to cephalosporins in *Campylobacter jejuni*".

The aims of the experiments in this section were to:

- Confirm the insertional inactivation of *cmeB* and *cmeF* in *C. jejuni* NCTC11168 mutants
- Determine the MICs of a range of beta-lactam antibiotics for the mutants in which *cmeB* and *cmeF* were inactivated.

3.3 Confirmation of inactivation of *cmeB* and *cmeF*

Mutants previously created in *C. jejuni* NCTC11168 by Vito Ricci (unpublished data) were given the laboratory codes P2252 (*cmeB::aph*) and P2253 (*cmeF*::*cat*) respectively. The *cmeB*

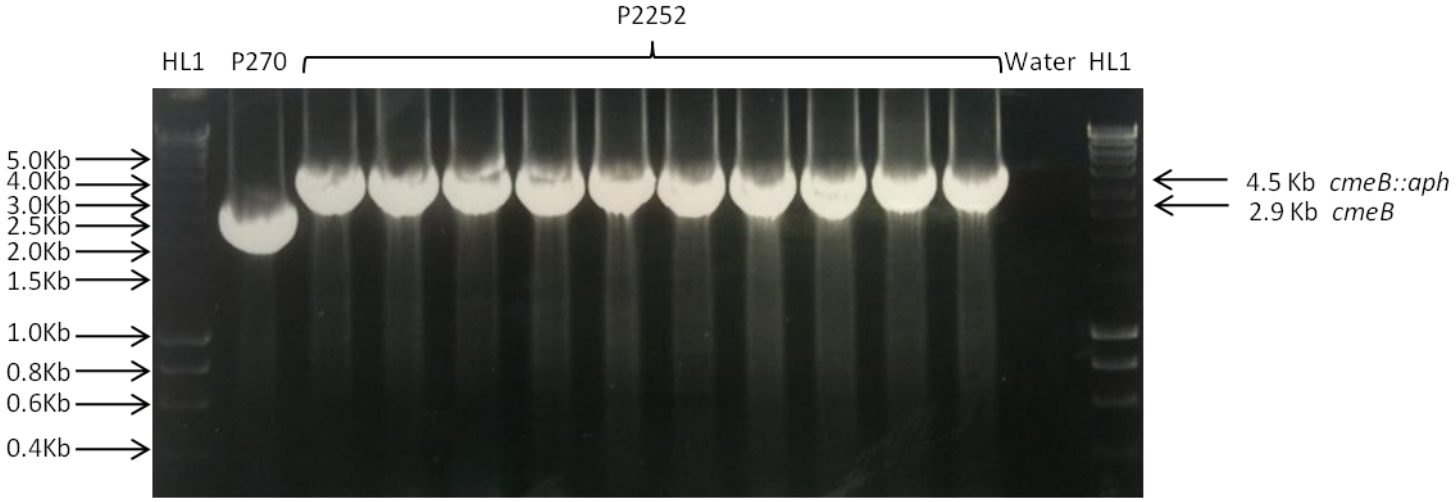
mutant (NCTC11168 *cmeB::aph*) was created by natural transformation using *cmeB::aph* DNA supplied by Professor X. Zhang (Lin, Michel et al. 2002) and the *cmeF* mutant (NCTC11168 *cmeF::cat*) was created in the same way using *cmeF::cat* DNA supplied by Dr A. Grant (Grant, Coward et al. 2005).

PCR was used to confirm the *cmeB/F* gene(s) had been disrupted. The *cmeB* gene was amplified using primers 297 and 298 (Materials and Methods, Table 2.4) and produced a 2.9Kb amplimer when the parental strain (NCTC 11168) was used as the DNA template. An amplimer of approximately 4.5Kb was predicted when *cmeB* was inactivated by mutational insertion of the *aph* gene (which encodes aminoglycoside phosphotransferase conferring kanamycin resistance). P2252 was shown to have an insertionally inactivated *cmeB* gene as it produced an amplimer of approximately 4.5Kb during PCR (Figure 3.1).

The primers 291 and 633 (Materials and Methods, Table 2.4) were used to amplify *cmeF*; this primer pair produces a 2.3Kb amplimer when the parental strain (NCTC11168) was used as the DNA template. An amplimer of approximately 4.5Kb was predicted when *cmeF* had been successfully inactivated by mutational insertion of the *cat* gene (encoding chloramphenicol acetyl transferase which confers chloramphenicol resistance). P2253 was shown to have an insertionally inactivated *cmeF* gene as PCR with the primers 291 and 633 produced an amplimer of approximately 4.5Kb (Figure 3.2).

Both the P2252 *cmeB* amplimer and the P2253 *cmeF* amplimer were sequenced using their respective primers. Sequencing confirmed that the *cmeB* gene of P2252 contained the *aph* gene and that the *cmeF* gene of P2253 contained the *cat* gene.

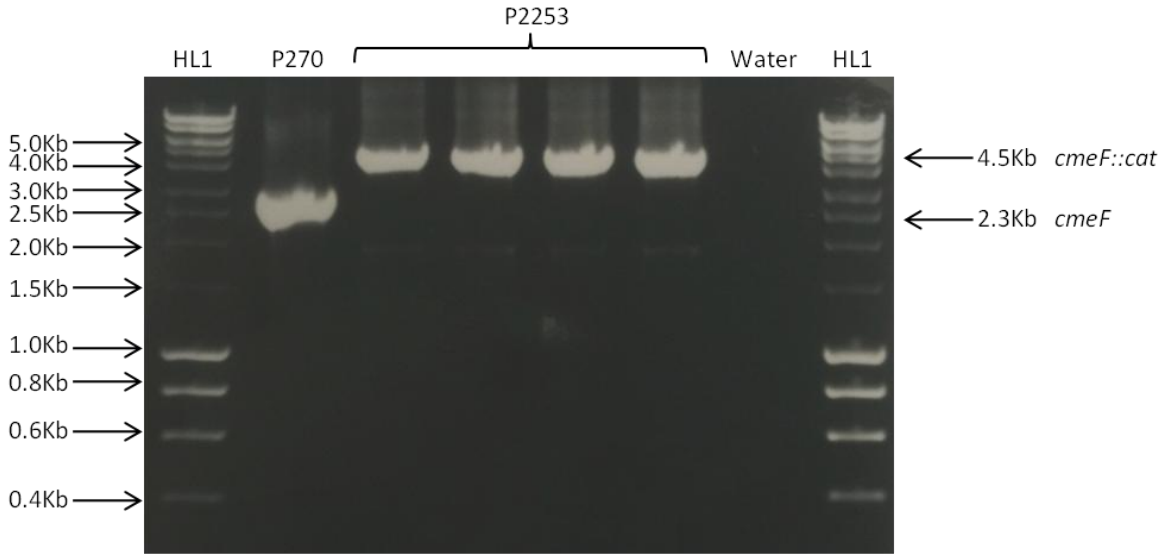
Figure 3.1 PCR amplification of *cmeB*



HL1 = HyperLadder 1

Following PCR amplification with primers 297 and 298 (Materials and Methods, Table 2.4) 5µl of each PCR reaction was loaded on to a 1% (w/v) agarose gel and electrophoresed at 100 V for approximately 45 minutes. The amplimer obtained for P270 (NCTC11168) is approximately 2.9 Kb in size, amplimers from P2252 are approximately 4.5 Kb. The ten P2252 PCR replicates were combined and used as a source of DNA to inactivate *cmeB* (by natural transformation) in other isolates.

Figure 3.2 PCR amplification of *cmeF*



HL1 = HyperLadder 1

Following PCR amplification with primers 291 and 633 (Materials and Methods, Table 2.4) 5 µl of each PCR reaction was loaded on to a 1% (w/v) agarose gel and electrophoresed at 100 V for approximately 45 minutes. The amplimer obtained for P270 (NCTC11168) is approximately 2.3 Kb in size, amplimers from P2253 are approximately 4.5 Kb. The four P2253 PCR replicates were combined and used as a source of DNA to inactivate *cmeF* (by natural transformation) in other isolates.

3.4 The effect of efflux pump inactivation on resistance to beta-lactams

The agar doubling-dilution method recommended by the CLSI *Campylobacter* Working Group (McDermott, Bodeis et al. 2004) was used to determine the minimum inhibitory concentration (MIC) of a range of beta-lactam antibiotics for the *cmeB* and *cmeF* insertionally inactivated mutants (Table 3.1).

Inactivation of *cmeB* led to a significant decrease (≥ two doubling dilutions) in the MIC of oxacillin, cephalothin, cefuroxime, cefotaxime, cefoxitin and Aztreonam. The MIC values decreased between five and nine doubling dilutions. The MIC of penicillin and ampicillin also decreased upon inactivation of *cmeB* but only by one doubling dilution. Inactivation of *cmeF* caused no significant change in the MIC of any of the beta-lactam antibiotics tested.

3.5 Inactivation of *cmeB* in ampicillin resistant *C. jejuni* isolates

On six occasions attempts were made to inactivate *cmeB* in two ampicillin resistant, beta-lactamase producing isolates (P854 and P305) by natural transformation with a *cmeB::aph* PCR amplimer created using primers 297 and 298, and using kanamycin to select for insertionally inactivated mutants (Materials and Methods, Table 2.1 and Table 2.4). However, despite repeating this experiment numerous times and varying conditions (such as the quantity of DNA used in the transformation and recovery time), no transformants could be obtained.

3.6 Discussion

The efflux mutants P2252 and P2253 were shown by both PCR and sequencing as having *cmeB* and *cmeF,* respectively, inactivated.

Table 3.1 Minimum inhibitory concentrations (MICs) of a panel of beta-lactam antibiotics for *C. jejuni* NCTC 11168 in which *cmeB* or *cmeF* had been inactivated

	Minimum Inhibitory Concentration (µg/ml)							
	Penicillin	**Ampicillin**	**Oxacillin**	**Cephalothin**	**Cefuroxime**	**Cefotaxime**	**Cefoxitin**	**Aztreonam**
P270 (NCTC11168)	8	4	64	128	64	2	64	64
P2252 (NCTC11168 *cmeB::aph*)	**4 (1)**	**2 (1)**	**2 (6)**	**4 (5)**	**0.12 (9)**	**0.03 (6)**	**0.5 (7)**	**0.5 (7)**
P2253 (NCTC11168 *cmeF::cat*)	8	4	64	256	64	2	64	64

MICs are the mode value of at least three biological repeats using the agar doubling-dilution method recommended by the CLSI Campylobacter Working Group (McDermott, Bodeis et al. 2004). Values in bold text indicate a decrease in MIC when compared to that for the parental strain, bold red text indicates a significant decrease in MIC when compared to the parental strain (≥ two doubling dilutions). Numbers in parentheses represent change (in doubling dilutions) in MIC compared with that for the parental strain.

Inactivation of *cmeB* resulted in a small, but reproducible, reduction in the MIC of penicillin and ampicillin. The relatively low MIC of penicillin and ampicillin in the parental strain could be responsible for this effect; a small decrease in MIC may always be produced but the error of the method used does not allow this decrease to be deemed significant. Others have shown inactivation of *cmeB* in *C. jejuni* NCTC11168 to cause a similar decrease in the MIC of ampicillin, a two-fold doubling dilution reduction compared to the one doubling dilution reduction found in this study (Pumbwe and Piddock 2002; Guo, Lin et al. 2010). Inactivation of *cmeB* appears to confer a host-dependent effect in *C. jejuni* for instance; inactivation of *cmeB* in *C. jejuni* 81-176 has been shown to cause a reduction of five doubling dilutions in the MIC of ampicillin, whereas inactivation of the same gene in *C. jejuni* 211190 caused no change in the MIC of ampicillin (Lin, Michel et al. 2002).

Inactivation of *cmeB* resulted in a decrease of five to nine doubling dilutions in MIC of a range of cephalosporins plus aztreonam. The innate resistance of *Campylobacter* to cephalosporins has previously been thought to be associated with low uptake into the *Campylobacter* cell and/or poor binding affinity of the antibiotics to the bacterial penicillin binding proteins (Lachance, Gaudreau et al. 1991). However, data from this study suggests that the efflux pump CmeABC confers the innate resistance of *Campylobacter* to this class of drugs.

Interestingly, it was also noted throughout this study that the *cmeB* mutant, P2252, was unable to grow on agar containing Preston supplement (Oxoid Ltd., Basingstoke, UK, Catalogue No: SR0117) which is used for the selective culture of *Campylobacter* in clinical microbiology laboratories. Preston supplement contains polymyxin B, rifampicin, trimethoprim, and cycloheximide; all agents to which *Campylobacter* are usually innately

resistant. This study suggests that the innate resistance of *Campylobacter* to one or more of these compounds is due to efflux by the CmeABC efflux pump.

Following the completion of these experiments in parallel another group published similar findings (Guo, Lin et al. 2010), suggesting that CmeABC is involved in the efflux of cephalosporins in several *Campylobacter* species. They also note that the MIC of rifampicin (one of the components of Preston supplement) decreased when the CmeABC efflux pump was inactivated (Guo, Lin et al. 2010).

Data presented herein suggested that the efflux pump CmeDEF does not play a role in resistance to beta-lactam antibiotics in *C. jejuni* NCTC 11168 as the MIC of all beta-lactams tested were unchanged in the *cmeF* mutant when compared to the parental strain.

In *Salmonella* it has been shown that an intact RND MDR efflux pump (homologous to *cmeABC*) is required for resistant bacteria to be selected (Ricci, Tzakas et al. 2006; Ricci and Piddock 2009). In addition, in several bacterial species, an intact RND MDR efflux pump is required for "full" resistance to be observed (Li and Nikaido 2009). To determine whether the same is true for beta-lactam resistance in *Campylobacter* an attempt was made to inactivate the *cmeB* gene in multiple ampicillin resistant, beta-lactamase producing isolates (it had previously been shown for one beta-lactamase producing *Campylobacter* isolate that inactivation of *cmeB* did not cause a reduction in the MIC of ampicillin) (Lin, Michel et al. 2002). However, no *cmeB* mutants could be selected from beta-lactamase producing strains. Currently it cannot be determined whether mutants could not be selected due to (i) the isolates used not being naturally competent or having low-levels of competency or (ii) because inactivating *cmeB* in these isolates was a lethal event. Further experiments to determine whether other genes in these bacteria can be inactivated by natural

transformation may determine the cause of the inability to produce *cmeB* mutants. It cannot be determined whether the efflux pump CmeABC is required for ampicillin resistance despite the presence of a beta-lactamase.

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Chapter 4 – Results and Discussion

Do some *Campylobacter* isolates encode a novel beta-lactamase?

4.1 Background

Campylobacter jejuni is known to encode a Class D beta-lactamase OXA-61 and *C. jejuni* NCTC11168 has been shown to encode a beta-lactamase (the product of the *Cj0299* gene) with just one amino acid difference to OXA-61 (Alfredson and Korolik 2005; Griggs, Peake et al. 2009). Although the product of the *cj0299* gene had been referred to as OXA-61 in the past (Griggs, Peake et al. 2009), due to its similarity to the oxacillinase, it is not OXA-61 and so for clarity another OXA number was requested from the Lahey clinic (http://www.lahey.org/studies/) during the course of this study. The Lahey clinic assigned the number OXA-193 to the product of *cj0299* and so from here on the oxacillinase gene of *C. jejuni* NCTC11168 (previously known as *cj0299*) will be referred to as $bla_{OXA\text{-}193}$.

In the prior work pertaining to this thesis it was observed that two ampicillin resistant veterinary isolates of *Campylobacter*, P843 and P854 (Materials and methods, Table 2.1), produced no amplimer from a PCR for $bla_{OXA\text{-}193}$ but produced a beta-lactamase as determined by the nitrocefin test (Griggs, Peake et al. 2009). The beta-lactamases from P843 and P854 had an isoelectric point of 9.21 and a molecular mass of 32.4kDa. The mass and isoelectric point of this protein were different to that of OXA-61 and OXA-193 so the novel beta-lactamase was termed CjBla2. Analysis by QTOF-MS and FTICR-MS yielded no homology to known β-lactamases of *Campylobacter, Helicobacter or Pseudomonas*. BLAST searching revealed no homology to any known beta-lactamases and the most protein with the highest identity in the *Campylobacter* protein database was with formyltetrahydrofolate

deformylase (accession number CAB73055), thought to be the product of the *purU* gene (*cj0790*) (Griggs, Peake et al. 2009). This protein has no predicted β-lactamase activity.

4.2 Aims and Hypotheses

There were three hypotheses:

1. The PurU protein (Cj0790) is CjBla2.
2. CjBla2 is the product of a mutated $bla_{OXA-193}$ gene.
3. CjBla2 is a novel beta-lactamase encoded by a novel gene.

The aims of the experiments in this section were to:

1. Determine whether *cj0790* encodes CjBla2.
2. Determine whether CjBla2 is the product of a mutated $bla_{OXA-193}$ gene.
3. Ascertain how many veterinary *Campylobacter* isolates have the CjBla2 phenotype ($bla_{OXA-193}$ negative, beta-lactamase producing and ampicillin resistant).
4. If CjBla2 is not the product of *cj0790* or a mutated $bla_{OXA-193}$ gene, to identify the gene responsible.

4.3 Does purU (*cj0790*) encode the novel beta-lactamase CjBla2?

4.3.1 Creation of an insertionally inactivated purU mutant

A *purU* mutant, NCTC11168 *purU::*Magellan3(*cat*), was donated by Dr A. Grant (Grant, Coward et al. 2005) and given the laboratory code of P2224. The presence of the *cat* (chloramphenicol acetyl transferase) gene within P2224 allowed the strain to be selectively cultured on MH agar plus five percent horse blood plates containing 15 µg/ml of chloramphenicol.

PCR using primers 665 and 666 that bind upstream and downstream of the *purU* gene (Materials and Methods, Table 2.6) was used to confirm that the gene was disrupted. When the *purU* gene was amplified from NCTC 11168 an 1192bp amplimer was detected whereas when the same region is amplified from P2224 the amplimer was approximately 3 Kb in size (Figure 4.1) confirming that the region has been disrupted.

Having confirmed that P2224 contains *purU::Magellan3*(*cat*) the amplimer from the *purU* PCR of P2224 was used to naturally transform P854 in order to inactivate *purU.*

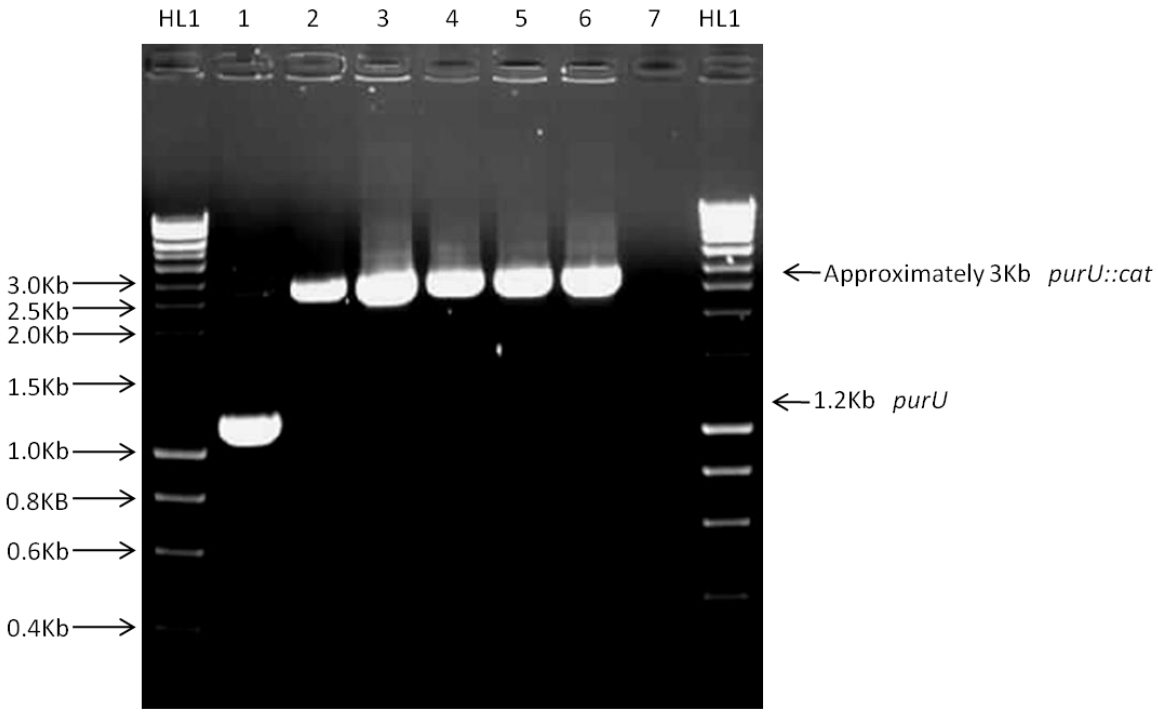
Transformants were selected on MH agar plus 5% horse blood plates containing 15 µg/ml of chloramphenicol.

4.3.2 Characterisation of P854 *purU::*Magellan3(*cat*)

PCR with external gene primers 665 and 666 was used to confirm that four representative P854 transformants (assigned laboratory codes: P2247, P2248, P2249, P2250) contained disrupted *purU* genes (Figure 4.1). The PCR amplimer of one transformant, P2250, was sequenced using primers 665 and 666. The disruption of *purU* in P2250 was confirmed as the mariner inverted repeat sequence of the transposon was identified in the sequencing read (Figure 4.2).

Nitrocefin, a chromogenic cephalosporin, was used to test for beta-lactamase activity in the P854 *purU::cat* (P2250 and the other three) insertionally inactivated candidates. All mutants retained their beta-lactamase activity (Figure 4.3).

Figure 4.1 PCR amplification of *purU* using external gene primers

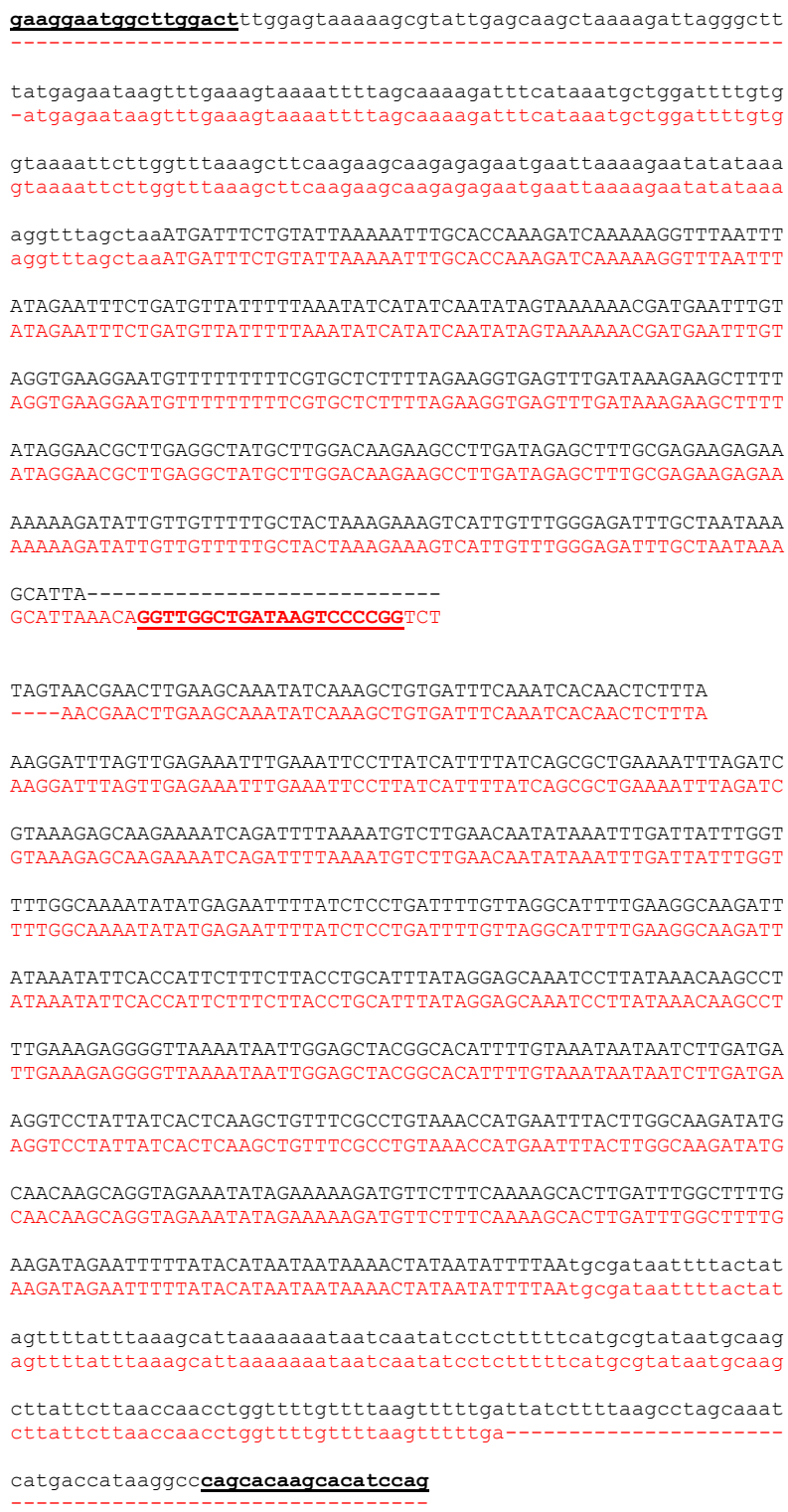


HL1 = HyperLadder1

1.	NCTC11168		
2.	P2224	NCTC11168 *purU::*Magellan3(*cat*)	
3.	P2247	P854 *purU::*Magellan3(*cat*)	candidate 1
4.	P2248	P854 *purU::*Magellan3(*cat*)	candidate 2
5.	P2249	P854 *purU::*Magellan3(*cat*)	candidate 3
6.	P2250	P854 *purU::*Magellan3(*cat*)	candidate 4
7.	Negative control		

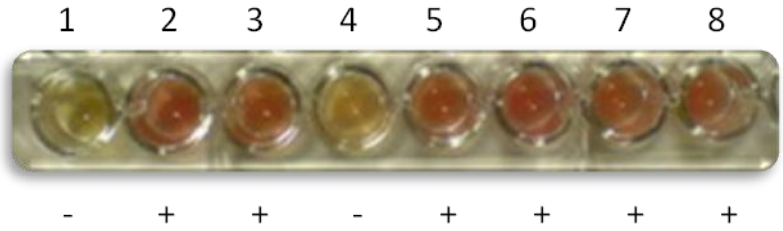
Following PCR amplification of *purU* using the external gene primers 665 and 666, 5 µl of each PCR reaction was loaded on to a 1% (w/v) agarose gel and electrophoresed at 100 V for approximately 45 minutes. The amplimer obtained for NCTC11168 is approximately 1.2 Kb in size, amplimers from all other reactions are approximately 3 Kb.

Figure 4.2 DNA sequencing of the *purU* region of P2250



Black text, published *purU* gene (Parkhill, Wren et al. 2000); black bold underlined text, *purU* external gene primers; red text, P2250 sequence read; red, bold, underlined text, Mariner inverted repeat .

Figure 4.3 Determining whether insertionally inactivated *purU* mutants have beta-lactamase activity (detectable by nitrocefin)



Yellow = Nitrocefin negative, no beta-lactamase activity detectable

Red = Nitrocefin positive, detectable beta-lactamase activity

Number	Strain		Beta-lactamase activity
1	NCTC11168	Reference strain	Not detectable
2	P843	CjBla2 producer	Detectable
3	P854	CjBla2 producer	Detectable
4	P2224	NCTC11168*purU::Magellan3(cat)*	Not detectable
5	P2247	P854 *purU::Magellan3(cat)*	Detectable
6	P2248	P854 *purU::*Magellan3(*cat*)	Detectable
7	P2249	P854 *purU::*Magellan3(*cat*)	Detectable
8	P2250	P854 *purU::*Magellan3(*cat*)	Detectable

Bacterial cultures were sonicated for two minutes then centrifuged to pellet cell debris, 50 µl of the supernatant was transferred to a well in a 96 well microtitre tray and 10 µl of nitrocefin was added to each sample. A colour change from yellow to red is a positive nitrocefin reaction indicating the presence of beta-lactamase activity.

4.4 Is CjBla2 the product of a mutated *bla$_{OXA-193}$* gene?

4.4.1 PCR of *bla$_{OXA-193}$*

Previously both P843 and P854 had been identified as giving no amplimer during PCR of the *bla$_{OXA-193}$* gene (Table 2.7). In order to confirm these findings PCRs were repeated using primers both internal and external to the gene in case mutations in one or more of the primer binding sites had led to the previous lack of amplimers. None of the primer combinations yielded amplimers following PCR of *bla$_{OXA-193}$* in either isolate (Figure 4.4).

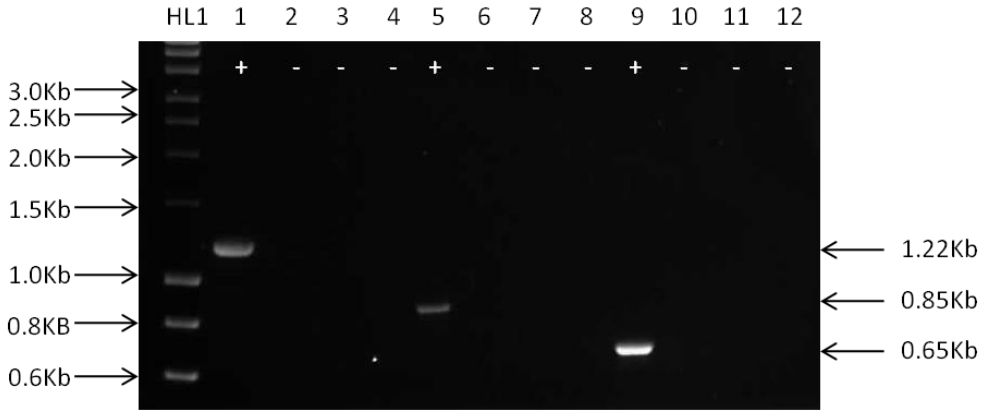
4.4.2 Southern blotting of *bla$_{OXA-193}$*

Southern blotting was carried out to determine whether P843 and P854 contained a mutated beta-lactamase gene that was sufficiently different from *cj0299* to prevent primer binding and subsequent PCR amplification but still shared enough homology to *cj0299* to be considered a variant form.

A search of the literature revealed that *Hind*III and *Cla*I are commonly used to digest *Campylobacter* genomic DNA (Korolik, Moorthy et al. 1995; Ahmed, Manning et al. 2002). A 281 bp probe was designed that spanned the active site of the OXA-193 enzyme (Figure 4.5). Both enzymes were tested at a range of concentrations and incubation times (Figure 4.6) to determine the optimal digest conditions. A four hour incubation with *Hind*III at a concentration 30 U/µg gave the clearest digest pattern so was used for all blots.

Blotting was initially performed following the manufacturers protocol, however when following this method the resulting blots were unreadable as they contained too much background signal (Figure 4.7a). Various changes were made to the protocol in order to decrease background signal and increase signal from the probe (Table 4.1). These changes

Figure 4.4 PCR amplification of *bla*$_{OXA-193}$ from NCTC11168, P843 and P854



PCR of *bla*$_{OXA-193}$ with primers 467 and 468	PCR of *bla*$_{OXA-193}$ with primers 469 and 468	PCR of*bla*$_{OXA-193}$ with primers 467 and 470
1. NCTC 11168	5. NCTC 11168	9. NCTC 11168
2. P843	6. P843	10. P843
3. P854	7. P854	11. P854
4. Negative control (water)	8. Negative control (water)	12. Negative control (water)

White + or -, presence or absence of amplimer. HL1, HyperLadder1.

Following PCR amplification of *bla*$_{OXA-193}$ with three combinations of *bla*$_{OXA-193}$ primers (Materials and Methods, Table 2.7), 5 µl of each PCR reaction was loaded on to a 1% (w/v) agarose gel and electrophoresed at 100V for approximately 45 minutes. Amplimers were obtained for NCTC11168 from PCR with all three primer combinations. P843 and P854 did not produce amplimers for any of the PCRs.

Figure 4.5 Southern blotting probe

gagtataatacaagcggcacttttgttttttatgatggaaaaacttgggcgagtaacgac

E Y N T S G T F V F Y D G K T W A S N D

Ttttcaagggctatggagactttctctcccgct**tccacttttaaaa**ttttaatgctcta

F S R A M E T F S P A **S T F K** I F N A L

Attgcacttgatagtggtgtgataaaaactaaaaaagaaatttttatcactatagaggt

I A L D S G V I K T K K E I F Y H Y R G

Gaaaaagtatttttatcttcttgggcgcaagatatgaatttaagttcagctataaaatat

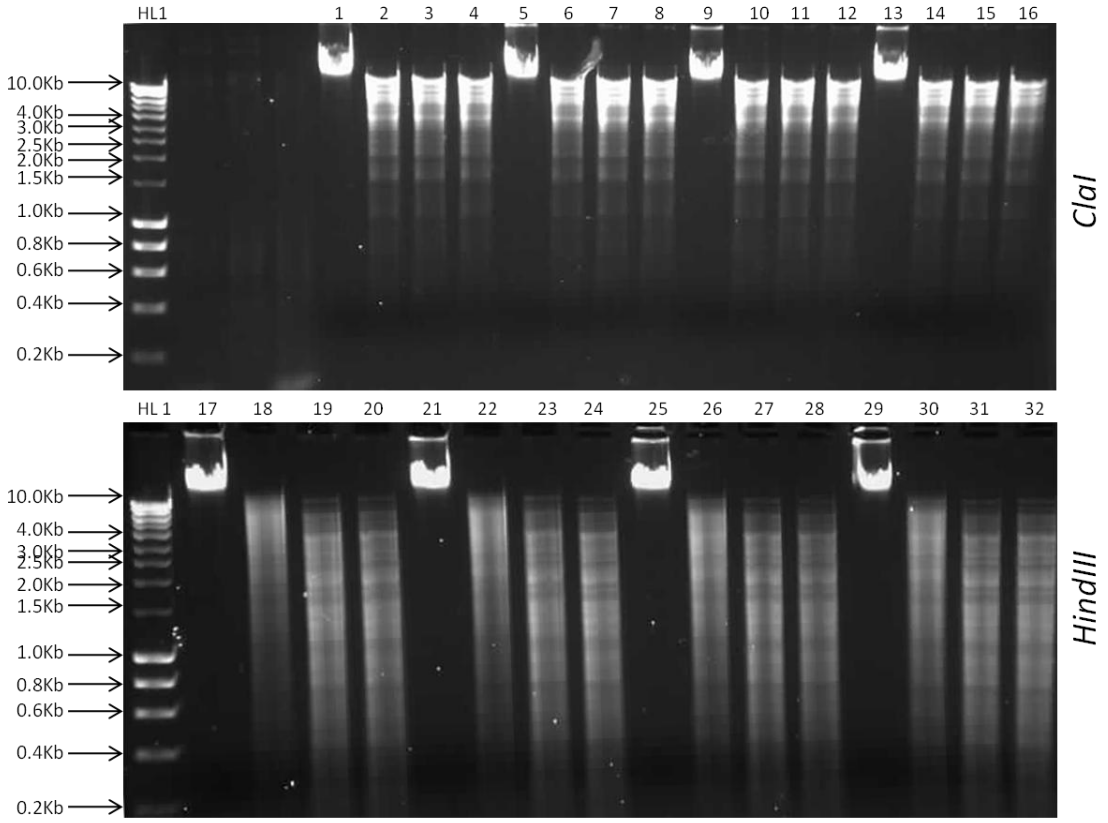
E K V F L S S W A Q D M N L S S A I K Y

tctaatgttcttgcttttaaagaagtggcaagaagaattgg

S N V L A F K E V A R R I

The DNA and amino acid sequence of the 281bp Southern blotting probe, boxed text indicates the active site tetrad STFK and the Amino Acid sequence coding for it.

Figure 4.6 Optimisation of restriction enzyme digestion of NCTC11168 genomic DNA

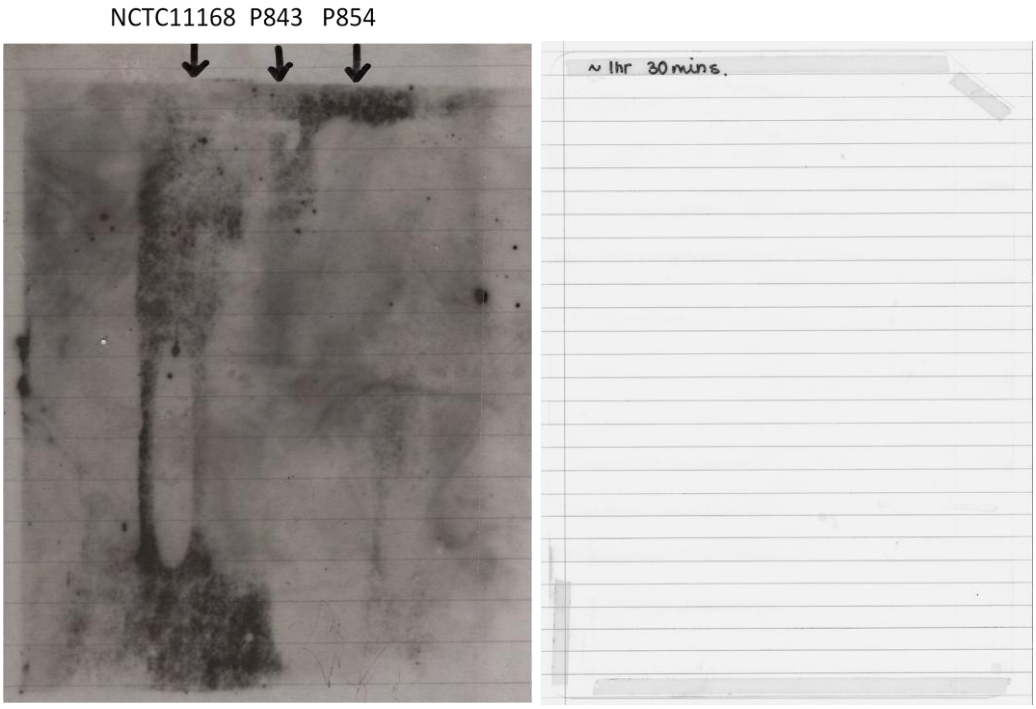


Lane	Restriction Enzyme	Enzyme Concentration	Digestion Time	Lane	Restriction Enzyme	Enzyme Concentration	Digestion Time
1	None*	-	-	17	None*	-	-
2	*Cla*I	5 U/µg DNA	1 hour	18	*Hind*III	5 U/µg DNA	1 hour
3	*Cla*I	10 U/µg DNA	1 hour	19	*Hind*III	10 U/µg DNA	1 hour
4	*Cla*I	30 U/µg DNA	1 hour	20	*Hind*III	30 U/µg DNA	1 hour
5	None*	-	-	21	None*	-	-
6	*Cla*I	5 U/µg DNA	2 hours	22	*Hind*III	5 U/µg DNA	2 hours
7	*Cla*I	10 U/µg DNA	2 hours	23	*Hind*III	10 U/µg DNA	2 hours
8	*Cla*I	30 U/µg DNA	2 hours	24	*Hind*III	30 U/µg DNA	2 hours
9	None*	-	-	25	None*	-	-
10	*Cla*I	5 U/µg DNA	3 hours	26	*Hind*III	5 U/µg DNA	3 hours
11	*Cla*I	10 U/µg DNA	3 hours	27	*Hind*III	10 U/µg DNA	3 hours
12	*Cla*I	30 U/µg DNA	3 hours	28	*Hind*III	30 U/µg DNA	3 hours
13	None*	-	-	29	None*	-	-
14	*Cla*I	5 U/µg DNA	4 hours	30	*Hind*III	5 U/µg DNA	4 hours
15	*Cla*I	10 U/µg DNA	4 hours	31	*Hind*III	10 U/µg DNA	4 hours
16	*Cla*I	30 U/µg DNA	4 hours	32	*Hind*III	30 U/µg DNA	4 hours

*Un-digested DNA control

Lane 32 is the clearest digest.

Figure 4.7 Examples of Southern blots during development



a **b**

Southern-blot hybridisation of *Hind*III digested genomic DNA from *C. jejuni* NCTC11168, P843 and P854 probed with bla_{OXA-61} gene.

a. Too much background to determine whether or not there are any signals.

b. Blot is completely blank; no signal has been produced even for the bla_{OXA-61} positive control (NCTC 11168 digested genomic DNA). Blot was left for 1 hour 30 minutes before the film was developed.

Table 4.1 Steps taken to optimise Southern blot

Problem	Adjustment	Reason
Too much background	Use new photographic developer/fixer	In case the developer/fixer were contaminated.
	Increase hybridisation temperature	At a higher temperature the stringency is increased, this should help to eliminate none-specific binding of the probe.
	Decrease development time	Leaving the film in photographic developer for too long increases the amount of background.
	Filter the detection reagent	Filtering detection reagent through a 0.2 micron filter removes any contamination (step recommended by manufacturer).
	Increase concentration of probe DNA	Concentration of DNA used to make probe was increased from 10ng/microlitre to 40ng/microlitre.
No signal	Lower hybridisation temperature	At a lower temperature the stringency is decreased, this should increase binding of the probe.
	Increase ratio of probe:hybridisation buffer	To increase the signal generated by the probe.
	Increase the amount of target DNA loaded onto gel.	The amount of genomic DNA that was digested and loaded onto gel was increased from 2µg to 3µg in order to increase the amount of target DNA available for the probe to bind to.
	Increase the length of each transfer stage.	The time taken to perform each wash during transfer of DNA from gel to membrane was increased to allow maximum transfer.

were implemented incrementally and were successful in removing non-specific signal (Figure 4.7b). Once background signal had been eradicated it became clear that the positive control (NCTC11168) did not generate a signal (Figure 4.7b). Further modifications were made to the protocol (Table 4.1) to enhance the probe signal.

Once the Southern blot for *bla*$_{OXA-193}$ was sufficiently optimised it was used to test for the presence of *bla*$_{OXA-193}$ in P843 and P854. Genomic DNA from NCTC11168 (positive control), P843 and P854 was digested with *Hind*III and electrophoresed through a 0.8% agarose gel (Figure 4.8a).

Southern blotting was performed using the modified protocol. The 281bp *bla*$_{OXA-193}$ probe hybridised to NCTC11168 DNA (positive control) but did not hybridise to the DNA of either P843 or P854 (Figure 4.8b).

4.5 Do any other veterinary isolates of *Campylobacter* posses the 'CjBla2 phenotype'?

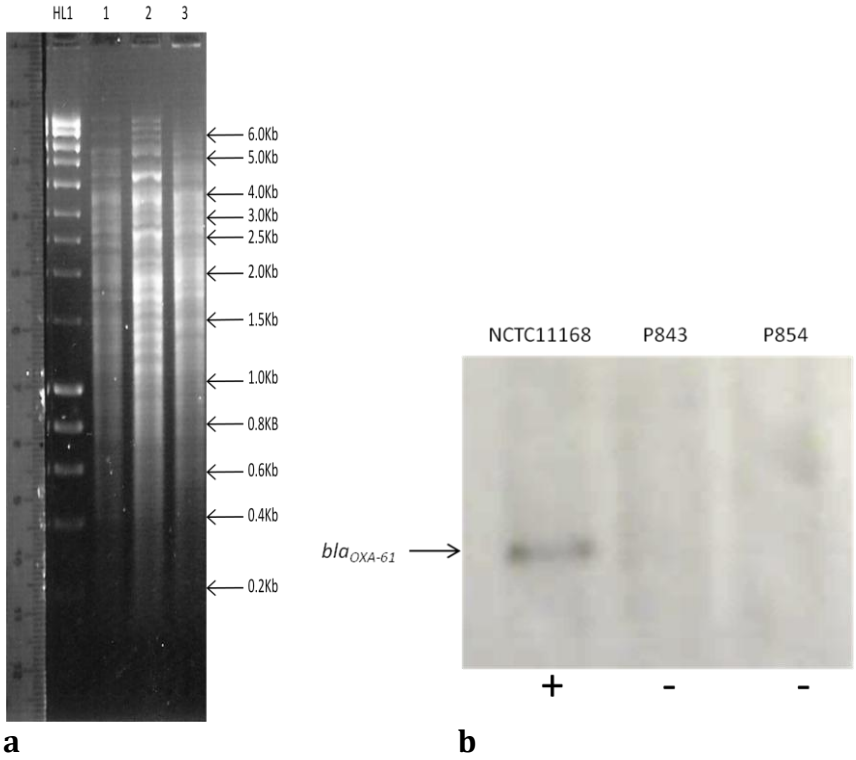
Prior to this project only two veterinary isolates had been identified that were ampicillin resistant, had beta-lactamase activity detectable by nitrocefin and did not produce amplimers during *bla*$_{OXA-193}$ PCR.

Initial screening data relating to veterinary isolates of *Campylobacter* collected during two Defra funded studies VM0220 and OZ0501 (Griggs, Johnson et al. 2005; Humphrey, Jorgensen et al. 2005; Piddock, Griggs et al. 2008) was queried to produce a list of potential isolates with the 'CjBla2 phenotype'.

These isolates were then checked to determine whether they

1. produced amplimers during *bla*$_{OXA-193}$ PCR

Figure 4.8 Southern blot hybridization for *bla*$_{OXA-193}$



Southern-blot hybridisation of *Hind*III digested genomic DNA from *C. jejuni* NCTC11168, P843 and P854 probed with *bla*$_{OXA-61}$ gene.

a. Digested genomic DNA of 1. NCTC11168, 2. P843 and 3. P854.

b. Probe was able to bind to NCTC11168 genomic DNA (arrow on diagram), the *bla*$_{OXA-193}$ probe was unable to bind to either P843 or P854 genomic DNA.

2. hydrolysed nitrocefin
3. were resistant to ampicillin (MIC≥ 16µg/ml)

Thirteen isolates were identified in addition to the initial two, P843 and P854, from the collection as non-replicate isolates (Materials and Methods, Table 2.1). The *flaA* type of each isolate was determined (Table 4.2). All isolates were shown to not produce amplimers during $bla_{OXA-193}$ PCR (Figure 4.9), were able to hydrolyse nitrocefin (Figure 4.10) and were resistant to ampicillin (MIC of ampicillin ranged from 128-256 µg/ml) (Table 4.3).

Four isolates from the CjBla2 panel were subjected to a Southern hybridisation with a 281bp probe for $bla_{OXA-193}$. The probe was unable to bind to the DNA of any of these isolates (Figure 4.11).

4.6 Sequence analysis of the P854 genome

DNA was isolated from a culture of P854 that had its $bla_{OXA-193}$ negative status checked by PCR and had also been shown to have beta-lactamase activity. DNA was then sent to The Gene Pool (University of Edinburgh) to be sequenced using an Illumina GA II (SOLEXA) platform.

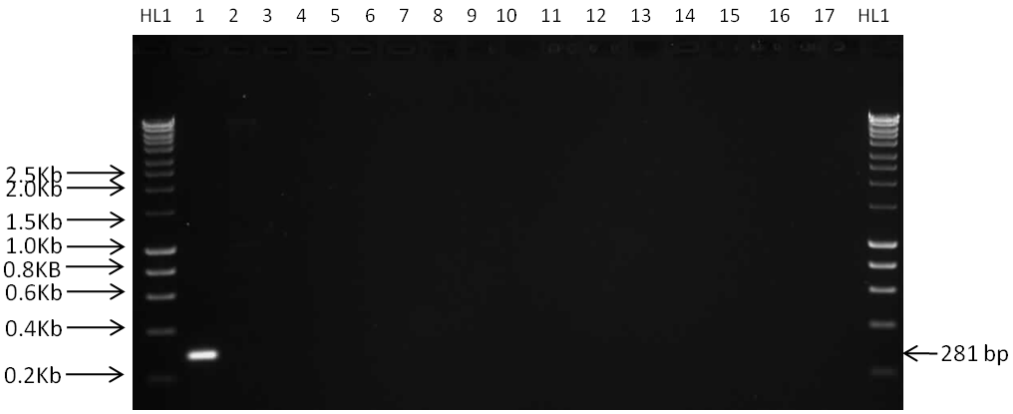
With the help of Dr Nick Loman of the University of Birmingham, the sequencing data received was assembled into 621 contigs which made up 83 scaffolds. The P854 genome was run through an annotation pipeline using *Campylobacter jejuni* RM1221 as the reference strain as preliminary BLAST searching had revealed that this was the most closely related fully annotated *Campylobacter* genome. It is important to note that although *C. jejuni* RM1221 is not identical to NCTC11168 (the reference strain used in other aspects of this work), RM1221 and NCTC11168 have similar genomic organisation in the

Table 4.2 *flaA* types of putative CjBla2 producers

Laboratory code	*flaA* type
P339	*flaA*100
P843	***flaA*21**
P852	*flaA*617
P853	*flaA*18
P854	*flaA*18
P858	*flaA*616
P859	*flaA*18
P862	*flaA*18
P888	*flaA*18
P891	*flaA*617
P1007	*flaA*34
P1052	*flaA*34
P1058	*flaA*616
P1170	*flaA*34
P1177	*flaA*350

Previously characterised isolates (P843 and P854) are illustrated in bold text.

Figure 4.9 PCR amplification of *bla*$_{OXA-193}$ from potential CjBla2 producers

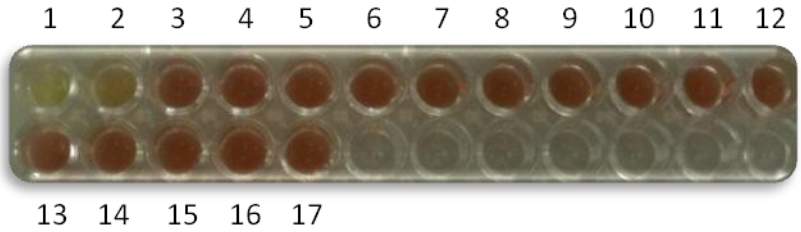


HL1 = HyperLadder1

1. NCTC11168
2. P339
3. P843
4. P852
5. P853
6. P854
7. P858
8. P859
9. P862
10. P888
11. P891
12. P1007
13. P1052
14. P1058
15. P1170
16. P1177
17. Water (negative)

Following PCR amplification of *bla*$_{OXA-193}$ using the internal gene primers 467 and 468 (Materials and Methods, Table 2.7) 5 µl of each PCR reaction was loaded on to a 1% (w/v) agarose gel and electrophoresed at 100 V for approximately 45 minutes. The amplimer obtained for NCTC11168 is the correct size (281bp), no amplimers were obtained for any of the CjBla2 panel.

Figure 4.10 Detection of beta-lactamase activity in CjBla2 producers



Number	Isolate	Beta-lactamase activity	Number	Isolate	Beta-lactamase activity
1	Water	Not detectable	10	P862	Detectable
2	NCTC11168	Not detectable	11	P888	Detectable
3	P339	Detectable	12	P891	Detectable
4	P843	Detectable	13	P1007	Detectable
5	P852	Detectable	14	P1052	Detectable
6	P853	Detectable	15	P1058	Detectable
7	P854	Detectable	16	P1170	Detectable
8	P858	Detectable	17	P1177	Detectable
9	P859	Detectable			

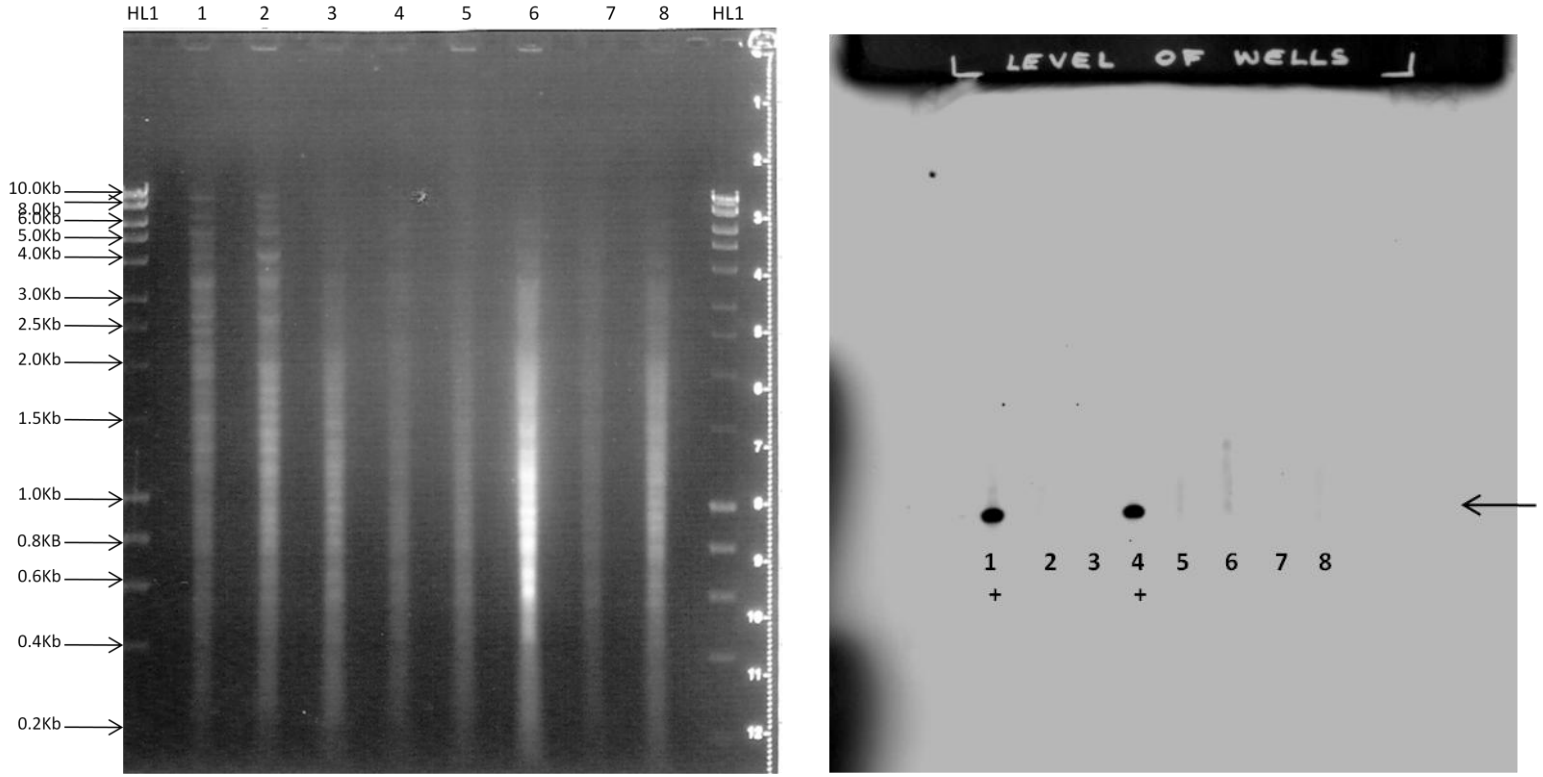
Bacterial cultures were sonicated for two minutes then centrifuged to pellet cell debris, 50 µl of the supernatant was transferred to a well in a 96 well micro titre tray and 10 µl of nitrocefin was added to each sample. A colour change from yellow to red is a positive nitrocefin reaction indicating the presence of beta-lactamase activity.

Table 4.3 MIC of a range of beta-lactams for putative CjBla2 producers

Isolate	Minimum Inhibitory Concentration (MIC) (µg/ml)						
	PEN	AMP	OXA	CEF	CFX	CTX	FOX
P339	128	256	256	512	128	4	256
P843	64	64	512	1024	256	16	256
P852	256	256	256	512	128	16	256
P853	256	128	512	1024	128	16	256
P854	>256	>256	512	1024	256	16	256
P858	>256	256	512	1024	128	16	256
P859	128	128	256	256	128	4	128
P862	256	256	256	512	128	4	128
P888	256	128	128	256	128	4	128
P891	256	256	256	512	128	16	256
P1007	>256	256	512	512	128	8	256
P1052	128	128	256	256	64	8	128
P1058	128	128	128	256	64	4	128
P1170	256	128	128	256	64	4	128
P1177	>256	256	512	256	128	16	256

MICs are the modal value of at least three biological repeats.
PEN, penicillin; AMP, ampicillin; OXA, oxacillin; CEF, cephalothin; CFX, cefuroxime; CTX, cefotaxime; FOX, cefoxitin.

Figure 4.11 Southern blot hybridization for *bla*$_{OXA-193}$



a **b**

+ Positive control (known to contain *bla*$_{OXA-193}$).

a. Digested genomic DNA. **b.** *bla*$_{OXA-193}$ probe was only able to bind to the DNA of the two positive controls, arrow on diagram.

1. NCTC11168	*bla*$_{OXA-193}$ positive	5. P339	*bla*$_{OXA-193}$ negative
2. P843	*bla*$_{OXA-193}$ negative	6. P859	*bla*$_{OXA-193}$ negative
3.P854	*bla*$_{OXA-193}$ negative	7. P1007	*bla*$_{OXA-193}$ negative
4. P305	*bla*$_{OXA-193}$ positive	8. P1052	*bla*$_{OXA-193}$ negative

region surrounding the beta-lactamase gene *bla*$_{OXA-193}$ of NCTC11168 and *cje0344* of RM1221 (Figure 4.12).

An alignment of P854 with RM1221 was created using the Artemis Comparison Tool (Figure 4.13) allowing the P854 genome to be interrogated.

4.7 Identification of putative beta-lactamases

Four P854 genes were annotated as being putative beta-lactamase genes (Table 4.4). Three of these (*P854_0720, P854_0903* and *P854_1029*) were annotated as metallo beta-lactamases and had homologues within RM1221. The remaining gene, *P854_1490*, was simply annotated as a 'beta-lactamase' and there were no significantly homologous genes in RM1221 (Table 4.4).

4.8 *In silico* analysis of P854_1490

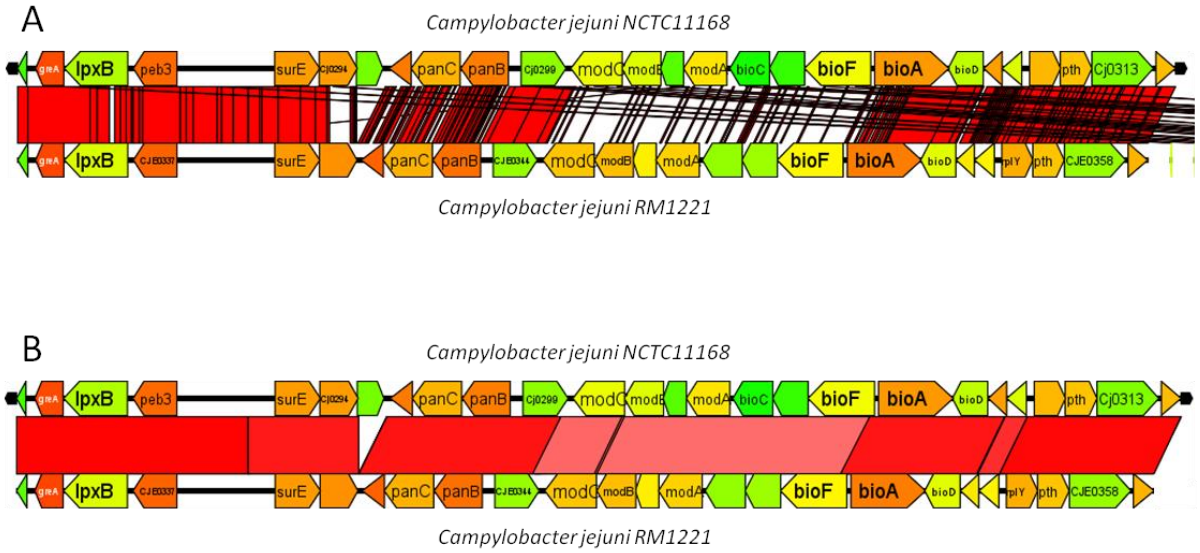
4.8.1 The genomic context of *P854_1490*

When the area containing *P854_1490* was aligned with the corresponding region of RM1221 despite some similarities there are also some clear differences (Figure 4.14).

When considering *P854_1490,* the most noteworthy point is that it is in a similar position to the *cje0344* gene of RM1221. However, protein-protein BLAST searching revealed that the P854_1490 protein shares only 64% identity with Cje0344 and 65% identity with OXA-193.

Downstream of *P854_1490* the genomic arrangement is typical of *Campylobacter jejuni* as the *mod* operon is present. The *mod* genes encode an ABC transport system that transports molybdenum and to a lesser extent, tungsten; both of these metals co-factors for enzymes involved in the *Campylobacter* respiratory chain (Taveirne, Sikes et al. 2009). The Artemis Comparison Tool did not identify the *mod* operon as being homologous to the same

Figure 4.12 Area surrounding *bla*$_{OXA-193}$ in NTC11168 compared to that same region in *Campylobacter jejuni* RM1221

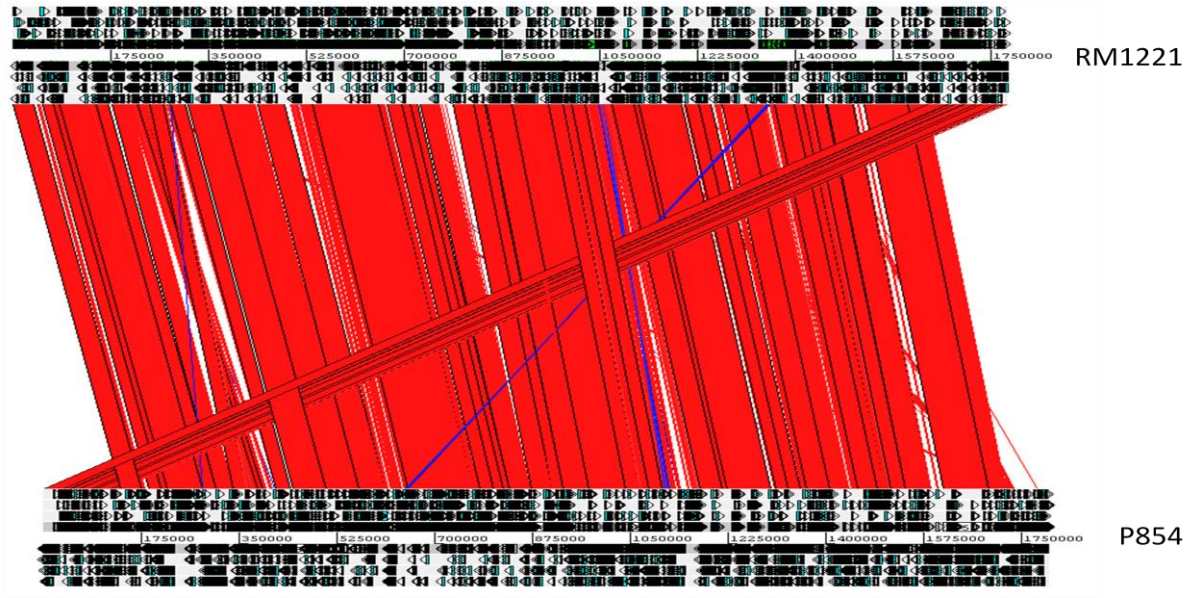


Campylobacter jejuni RM1221 contains a *bla*$_{OXA-193}$ homologue *cje0344*, RM1221 also contains the *pan* operon upstream and the *mod* operon downstream of the beat-lactamase as does NCTC11168.

Genomes are aligned based on their nucleotide (A) or amino acid (B) sequence. Homologous regions of nucleotide (A) and amino acid (B) sequence are connected with red lines.

Figure was produced using the nucmer and promer tools on Xbasse (http://xbase.bham.ac.uk/campydb/).

Figure 4.13 Alignment of P854 with *Campylobacter jejuni* RM1221produced using the Artemis Comparison Tool

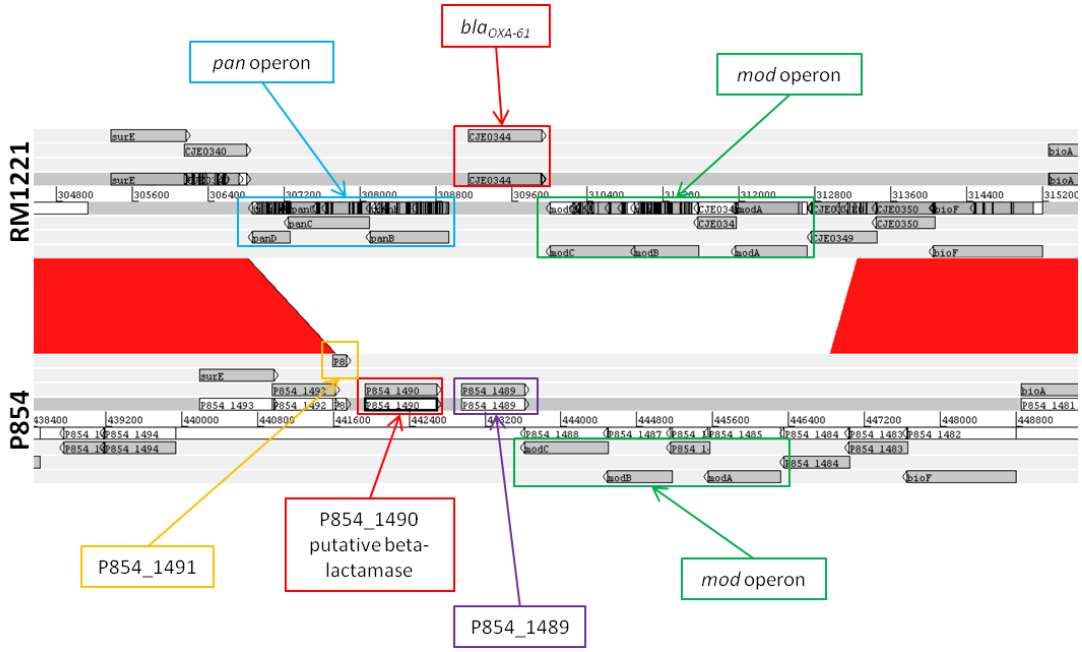


Homologous genes are connected by red lines; blue lines represent genes that are inverted in P854 when compared to the reference strain RM1221. Areas that are not connected represent genes that are unique to the strain.

Table 4.4 Putative beta-lactamase genes in the P854 genome

Gene	Annotation	Protein sequence	Homologue in RM1221?	Protein sequence of RM1221 homologue
P854_0720	Metallo-beta-lactamase family	MQIIKQACGAYETNCYILFSEHGEIIIDPGFDALNFIKKHVKNPLAILNTH GHYDHVWDNEKVKQAYQIPIYIHKNDAFMLEDPFNQGFMPSKADYLI DDENIISIGGLDFKFHFLPGHTPGCTMIEIVGKNIMFSGDFLFYRSIGRW DFPYSDANLMKQSLEKVMTYKEDFKLLPGHGQETTLKEEQVHLPSWLR YF	Yes, homology to *cje0900*	MQIIKQACGAYETNCYILFGEHGEIIIDPGFDALNFIKKHVKNPLAILNTH GHYDHVWDNEKVKQAYQIPIYIHKNDTFMLEDPFNQGFMPSKANYLI DDENIISIGGLDFKFHFLPGHTPGCTMIEIVGKNIMFSGDFLFYRSIGRW DFPYSDANLMKQSLEKVMTYKEDFKLLPGHGQETTLKEEQVHLPSWLR YF
P854_0903	Metallo-beta-lactamase domain-containing protein	MFNFITTLLKTNLYFLEEKTTSKNSNQTHIISLKQSDDEFLNLLFSFLVPQS DEDKNFIGKTRTKIKRNEHNIALIKNPNFNALIDTGFLDTIDTLKEKLHIHK TDFKDITHIILTHAHPDHIGALMSEENLFPKAQILIDKKEYDFWIKSDRQEI KNTLLKLKNIEFIDHSKDLIFQNSGIKAIPAYGHTPGQNAIIIDDKIVFWGD LLHLYDIQIPKPKIAIKFDIDQNEAIQTREKLLKEFKERKLKVIGTHVPFIKPK FLD	Yes, homology to *cje1761*	MSEENLFPKAQILIDKKEYDFWIKSDRQEIKNTLLKLKNIEFINHSKDLIFQ NSGIKAIPAYGHTPGQNAIIIDDKIVFWGDLLHLYDIQIPKPKIAIKFDIDQ NEAIQTREKLLKEFKERKLKVIGTHVPFIKPKFLD
P854_1029	Metallo-beta-lactamase family	MDENKEINKNEQNPNSNLKNNKRYKYKNRRKKLADSLQNENDTPKID QNSNKEISENSENKTEKKKKKNRNLPSKLTGNEDQIALAECIEANRVSHE NRLHPLKYNNSSEHKIRITPLGGLGEIGGNISVFETNKDAIIVDIGMSFPD GTMHGVDIIIPDFDYVRKIKDKIRGIVITHAHEDHIGAVPYFFKEFQFPIYA TPLALGMISNKFEEHGLKAERKWFRPVEKRRVYEIGEFDIEWIHITHSIID ASALAIKTKAGTIIHTGDFKIDQTPIDGYPTDLGRLAHYGEEGVLCLLSDS TNSYKEGYTKSESSVGPTFDQIFSRTKGRVIMSTFSSNIHRVYQAITYGLK YGRKVCVIGRSMERNLYTTMELGYIKLDRKIFIDADEVSKYKDNEVLIVTT GSQGETMSALYRMATDEHKFIKIKPTDQVIISAKAIPGNEASVSAVLDYL LKAGAKVAYQEFSEIHVSGHASIEEQKLMLTLTKPKFFLPVHGEYNHITK HKETAMKCGIPERNIYLMSDGDQVELCQKYVKRIKTVKTGKVFVDNQI NKQIADDVVIDRQKLADSGIVVIIAQIDKTTKTLINKPRVFSYGLVADKHD HAFSKDMAEVLGQFFINVKDEVLNDPRFLENQIRQVLRKHIFRKIKKYPT IVPTIFVM	Yes, homology to *cje1879*	MDENKEINKNEQNPNSNSKNNKRYKYKNRRKKLADSLQNENDTPKID QNSNKENSENSENKTEKKKKKNRNLPSKLTGNEDWQIALAECIEANRIS HENRLHPLKYNNSSEHKIRITPLGGLGEIGGNISVFETNKDAIIVDIGMSF PDGTMHGVDIIIPDFDYVRKIKDKIRGIVITHAHEDHIGAVPYFFKEFQFP IYATPLALGMISNKFEEHGLKAERKWFRPVEKRRVYEIGEFDIEWIHITHS IIDASALAIKTKAGTIIHTGDFKIDQTPIDGYPTDLGRLAYYGEEGVLCLLS DSTNSYKEGYTKSESSVGLTFDQIFARTKGRVIMSTFSSNIHRVYQAITYG LKYGRKVCVIGRSMERNLYTTMELGYIKLDRKIFIDADEVSKYKDNEVLIV TTGSQGETMSALYRMATDEHKFIKIKPTDQVIISAKAIPGNEASVSAVLD YLLKAGAKVAYQEFSEIHVSGHASIEEQKLMLTLTKPKFFLPVHGEYNHIT KHKETAMKCGIPERNIYLMSDGDQVELCQKYVKRIKTVKTGKVFVDNQI NKQIADDVVIDRQKLADSGIVVIIAQIDKATKTLINKPRVFSYGLVADKH DHAFSKDMAEVLGQFFINVKDEVLNDPRFLENQIRQVLRKHIFRKIKKYP TIVPTIFVM
P854_1490	Beta-lactamase	LKKILLLFSLFYSFALANDKLKDFFKDYNTSGVFITFDGKHYASNNFKRAK EPFSPASTFKIFNALIALDNGVVKDTKEIFYHYKGEKVFLPSWKQDASLSS AIKRSQVPAFKELARKIGLKTMQESLNKLSYGNTKISKIDTFWLDNSLQIS AKNQADLLFKLSQNSLPFSKKSQEEVKKIILFKEDKIQKIYAKTGFNDGINL AWIVGFIESKNKILSFALNVDIKNIKNLKIREELLEKYIYSLN	No	

Figure 4.14 Alignment of the region surrounding *P854_1490* with the corresponding region in *Campylobacter jejuni* RM1221 (produced using the Artemis Comparison Tool)



Homologous genes are connected by red lines. Areas that are not connected represent genes that are unique to the strain. The genes in the P854 and RM1221 *mod* operons share only 82% identity (at the amino acid level) with one another.

operon in RM1221. This is not surprising as the sequence of the *mod* genes vary between *Campylobacter* species.

Upstream of *P854_1490* there were clear differences between P854 and RM1221. Upstream of the oxacillinase gene of RM1221 is the *pan* operon which contains the pantothenate biosynthesis genes *panD, panC* and *panB*. In higher organisms pantothenic acid is a precursor of coenzyme A that is required for fatty acid biosynthesis although its role in bacteria is less well defined (Pearson, Pin et al. 2003). The PanD protein has also been shown to be involved in amino acid catabolism (production of alanine from aspartate) (Fouts, Mongodin et al. 2005). The *pan* operon is completely lacking in P854. The putative beta-lactamase gene *P854_1490* is flanked by two genes that are not found in this position in any other sequenced *Campylobacter jejuni* isolates. The gene upstream, *P854_1491*, may be a pseudogene, or a gene encoding a truncated form of the ThiF (thiamine biosynthesis) protein encoded by the gene that is usually between the *pan* operon and the *surE* gene upstream. The protein product of the *P854_1491* gene is 44 amino acids in length and shares 55% identity with ThiF (Cj0295) protein of *C. jejuni* NCTC11168.

The protein product of P854_1489 shows little similarity to any *Campylobacter* protein, protein BLAST revealed a maximum of 27% identity with *Campylobacter* proteins. Using a blastn search one gene was similar, the *cla0303* gene (a metal dependent membrane permease) of *Campylobacter lari* RM2100. When this gene is viewed in context in *C. lari* RM2100, it can be seen downstream of a putative beta-lactamase gene, *cla0304* (Figure 4.15).

4.8.2 The OXA-184 (P854_1490) protein

The *P854_1490* gene has a GC content of 25.7% and encodes a protein of 248aa in length.

Figure 4.15 The region of *Campylobacter jejuni* RM1221 corresponding to the P854_1490 containing region of P854 (produced using the Artemis Comparison Tool)

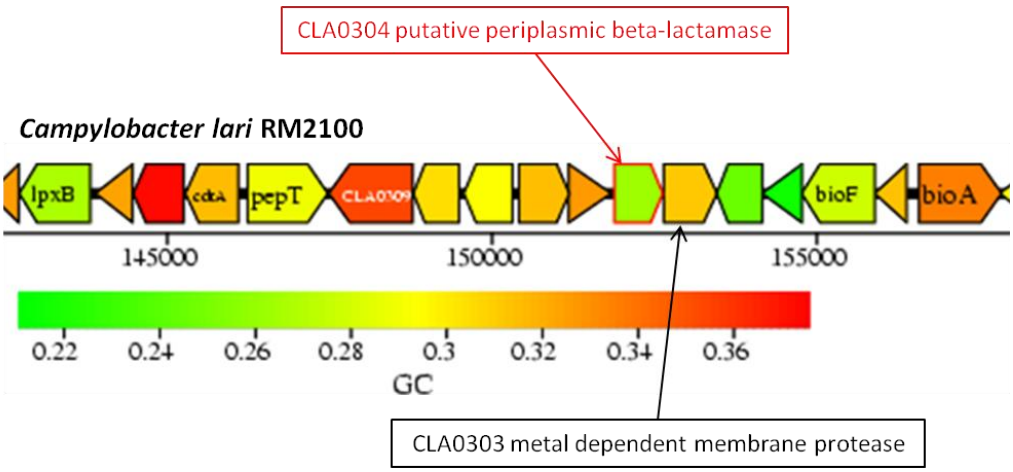


Diagram produced using CampyDB (http://xbase.bham.ac.uk/campydb/).

Protein BLAST searches reveal that the P854_1490 protein is most similar to class D beta-lactamases, also known as Oxacillinases (OXAs). The amino acid sequence of P854_1490 was submitted to the Lahey clinic who confirmed it to be a novel oxacillinase sequence and assigned it the number OXA-184. From here on the gene encoding OXA-184 (previously referred to as *P854-1490*) will be referred to as $bla_{OXA-184}$.

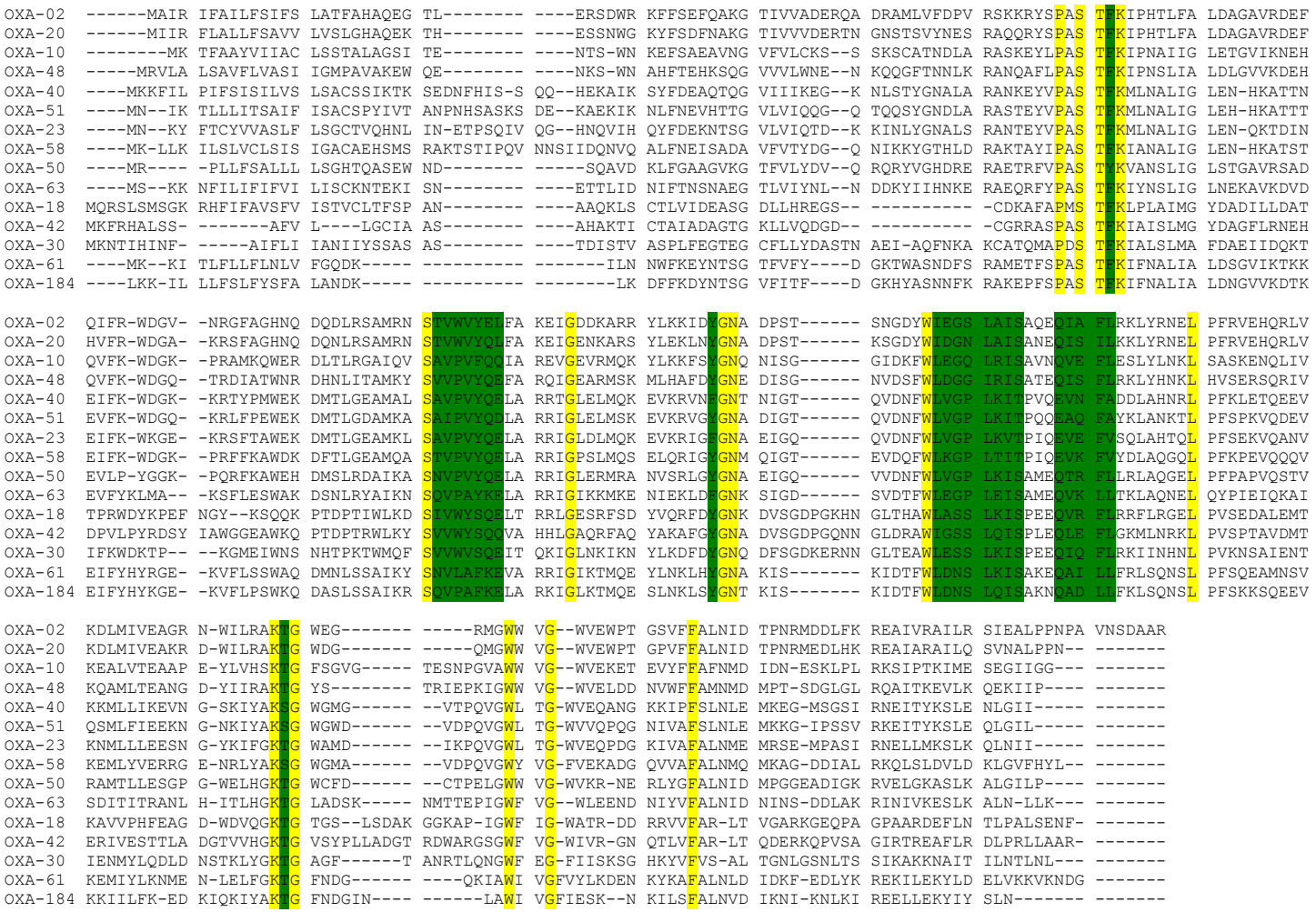
When OXA-184 is aligned with other class D beta-lactamases including representatives of the OXA sub groups and the *Campylobacter jejuni* beta-lactamase OXA-61, it can be seen to fit within the expected patterns and contains all the conserved regions of an OXA including the active site tetrad STFK (Figure 4.16).

Analysis in the form of a neighbour-joining tree (Figure 4.17) illustrates that the closest relative of OXA-184 is OXA-61 and that the beta-lactamase of *C. lari* RM2100 also clusters with them to form a group of *Campylobacter* beta-lactamases.

4.9 Screening the CjBla2 panel for $bla_{OXA-184}$

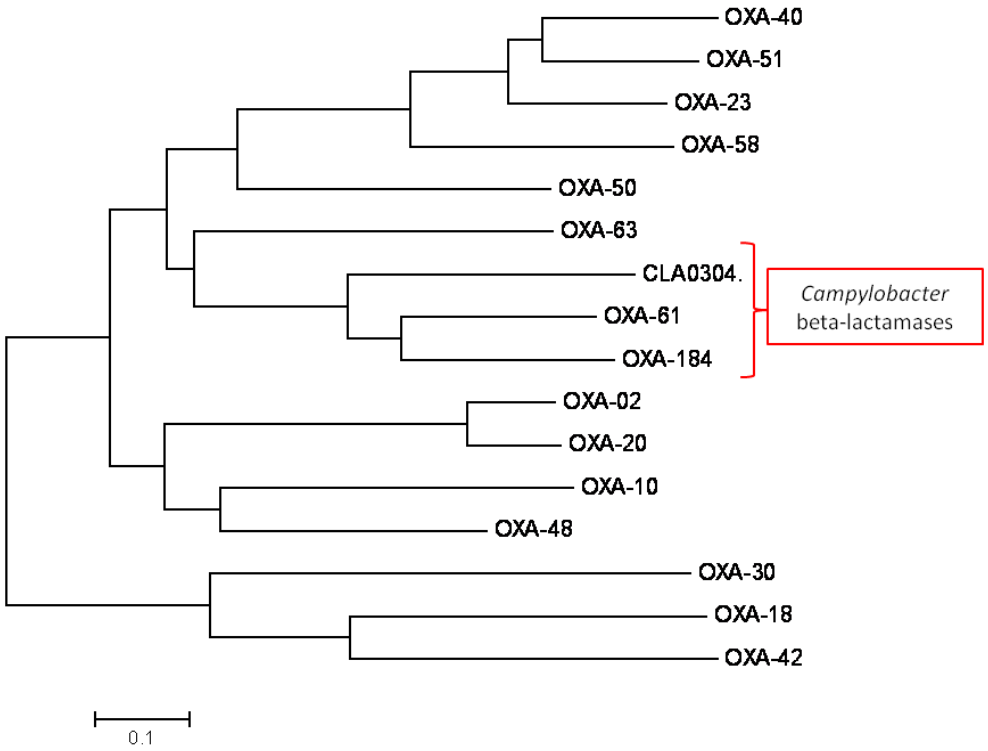
One pair of primers was designed to amplify an 183bp fragment of the $bla_{OXA-184}$ gene, another pair was designed to amplify a 1670bp product that included the whole $bla_{OXA-193}$ gene. The forward primer was within the gene upstream (*P854_1491*) and the reverse primer was within the gene downstream (*P854_1489*) (Materials and Methods, Figure 2.3). The internal primer pair may not be specific to OXA-184 and may amplify other Class D beta-lactamase genes due to sequence similarity. The external primer pair which bind in the genes up and downstream will be able to identify isolates which have this unusual arrangement of genes in the OXA region. During PCR using the internal primer pair, all thirteen isolates in the 'CjBla2 panel' produced amplimers of the expected size (Figure 4.18).

Figure 4.16 Amino acid alignments of thirteen representative class D β-lactamases from the different subgroups with OXA-61 and the novel β-lactamase from P854, OXA-184



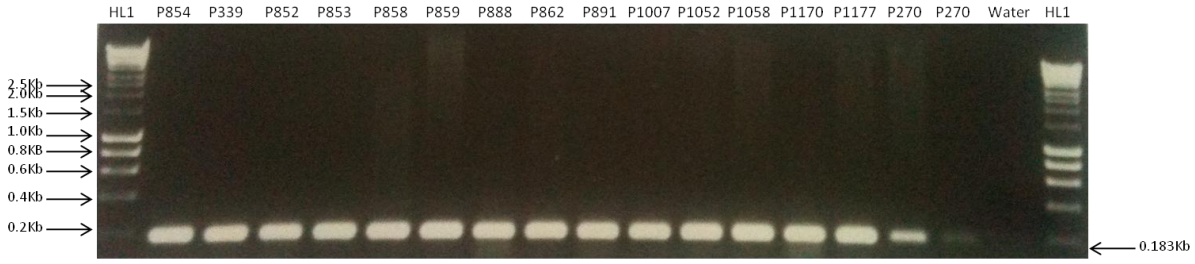
Yellow highlighting -identical amongst all the amino acid sequences; green highlighting - conserved (even if variable) among all class D β-lactamases.

Figure 4.17 Neighbour joining tree of thirteen representative class D β-lactamases from all the different subgroups with OXA-61, the beta-lactamase of *C. lari* RM2100 (CLA0304) and the novel β-lactamase from P854 (OXA-184)



The putative beta-lactamase of P854 is outlined in red (OXA-184). The *Campylobacter* beta-lactamase group is also illustrated. Diagram produced using MEGA 4.1.

Figure 4.18 PCR amplification of a 183bp fragment of *bla*$_{OXA-184}$ from potential CjBla2 producers using internal primers 1008 and 1009



HL1, HyperLadder 1.

Following PCR amplification of *bla*$_{OXA-184}$ using the internal gene primers 1008 and 1009 (Materials and Methods, Table 2.9) 5 µl of each PCR reaction was loaded on to a 1% (w/v) agarose gel and electrophoresed at 100 V for approximately 45 minutes. The amplimer obtained for P854 and all thirteen members of the CjBla2 panel is 183bp in size; the amplimers from P270 (NCTC11168) were the same size but did not amplify as well.

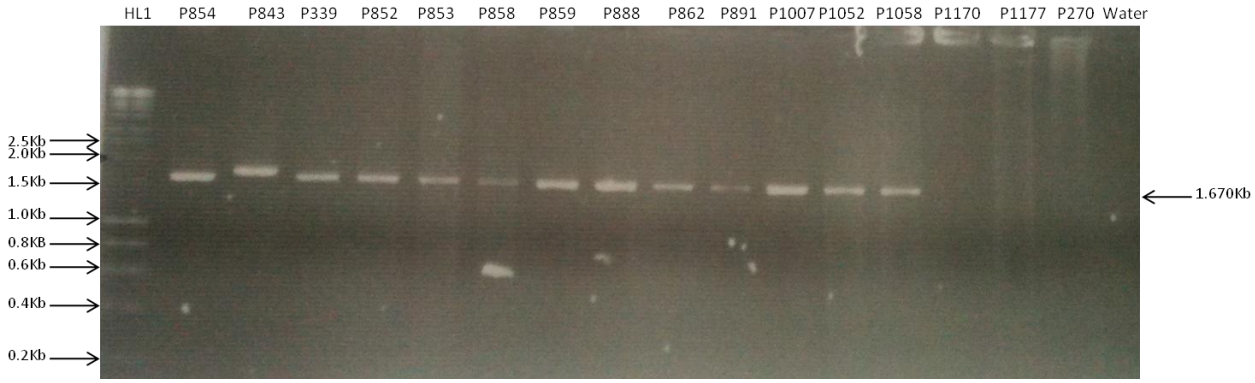
As expected the primer pair was also able to amplify the *bla*$_{OXA-193}$ gene from NCTC11168, although the amplification was less efficient due to mismatch primer binding.

Two of the isolates, P1170 and P1177 did not produce an amplimer during PCR with the external primer pair (Figure 4.19). The remaining eleven isolates produced amplimers of the expected size. P843 a previously characterised 'CjBla2 producer' produced a slightly larger amplimer than expected.

The amplimers produced (by the twelve strains) during PCR amplification with external primers were sent for DNA sequencing. These data revealed that the isolates can be divided into three distinct groups (Figure 4.20). 'Group 1' consisted of five isolates (P339, P853, P859, P862 and P1007) which contained *bla*$_{OXA-184}$ in the same genomic context as P854. 'Group 2' contained only P843, this isolate had a 108bp insertion in the non-coding region upstream of *bla*$_{OXA-184}$ and a C → G point mutation in the non-coding region downstream of *bla*$_{OXA-184}$. Six isolates (P852, P858, P888, P891, P1052 and P1058) made up 'Group 3'. These isolates contained a C→ A point mutation in *bla*$_{OXA-184}$ which resulted in an amino acid change from leucine to isoleucine at amino acid position 233/248. Isolates in this group also contained a point mutation in the non-coding region downstream of *bla*$_{OXA-184}$ and seven point mutations in *P854_1489*. As this oxacillinase was distinct from OXA-184, its amino acid sequence was also submitted to the Lahey clinic and was assigned the number OXA-185.

As the amplimer for isolates P1170 and P1177 could not be classified into any of these groups they could perhaps be considered to be a fourth group. DNA sequencing of the 183bp internal PCR amplimer showed the sequence to be identical to *bla*$_{OXA-184}$ but as this amplimer does not cover the region containing the C→A (Leu→Ile) point mutation present

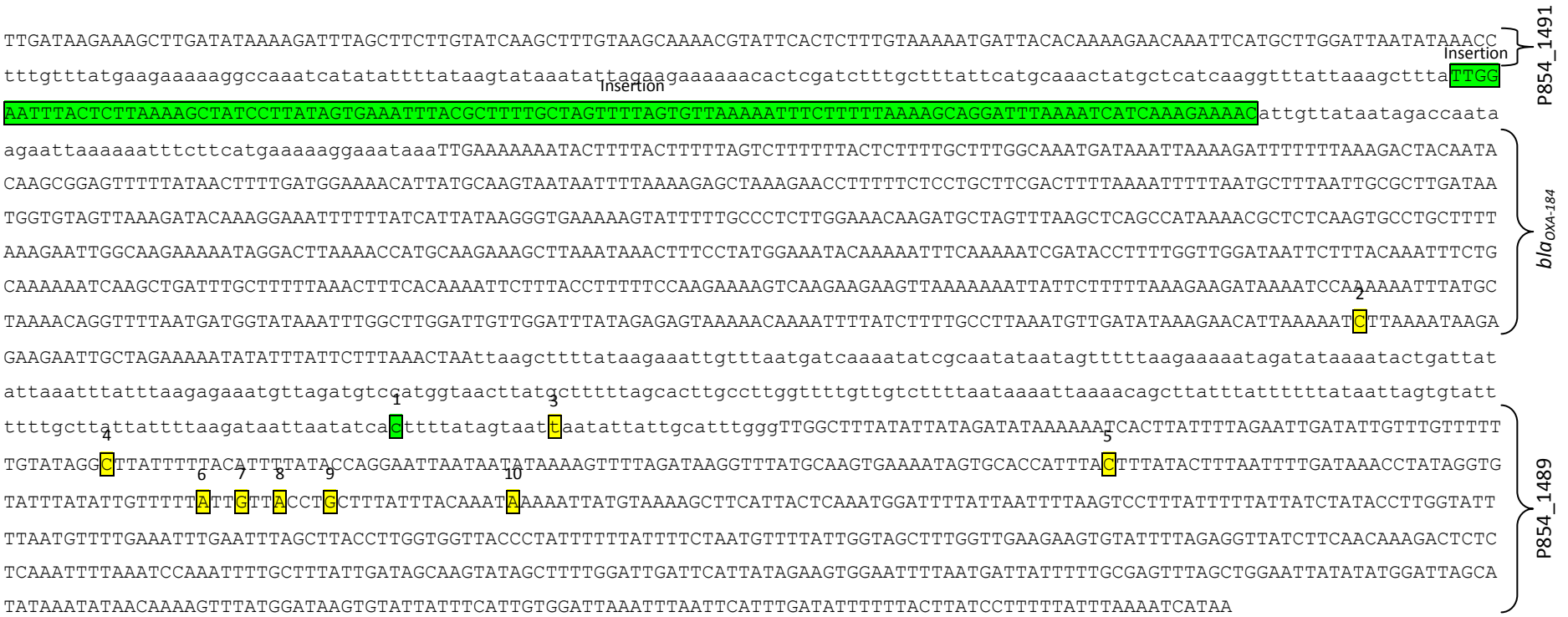
Figure 4.19 PCR amplification of a1670bp fragment including *bla*$_{OXA-184}$ and the region up and downstream from potential CjBla2 producers using external primers 1010 and 1011



HL1, HyperLadder 1.

Following PCR amplification of *bla*$_{OXA-184}$ using the external gene primers 1010 and 1011 (Materials and methods, Table 2.9) 5µl of each PCR reaction was loaded on to a 1% (w/v) agarose gel and electrophoresed at 100 V for approximately 45 minutes. The amplimer obtained for P854 and isolates P339 to P1058 is 1670 bp in size; P843 produced an amplimer that was slightly larger than the expected 1670bp and P1170, P1177 and the negative control P270 (NCTC11168) did not produce any amplimers.

Figure 4.20 Genetic variation of *bla*$_{OXA-193}$ and the surrounding region in the CjBla2 panel isolates



Group	Isolates	Description	Point Mutations		
1:P854 - like	P854, P339, P853, P859, P862, P1007	Identical to P854.	None		
2: P843	P843	Has a 108bp insertion in the intergenic region upstream of P854_1490 and a point mutation in the intergenic region downstream of P854_1490.	1. C→G		
3: P854_1490 Leu→ Ile variants	P852, P858, P888, P891, P1052, P1058	Has a point mutation in P854_1490 that leads to a coding change from Leucine to Isoleucine, has a point mutation in the intergenic region downstream of P854_1490 and seven point mutations in P854_1489.	2.C→A 5.C→T 8.A→G	3.T→C 6.A→G 9.G→A	4.C→T 7.G→A 10.A→G

Lowercase text indicates non-coding intergenic regions of sequence; uppercase text represents coding sequence (with the exception of the labeled 'insertion' highlighted in green).

in Group 3 isolates it is not possible to determine whether they will contain *bla*$_{OXA-184}$ or *bla*$_{OXA-185}$.

The three groups are unlikely to be the result of clonal expansion as examples of both Group 1 and Group 3 were found on two separate farms and were of at least three different *flaA* types (Figure 4.21). As there has only been members of Group 2 identified such conclusions cannot be drawn.

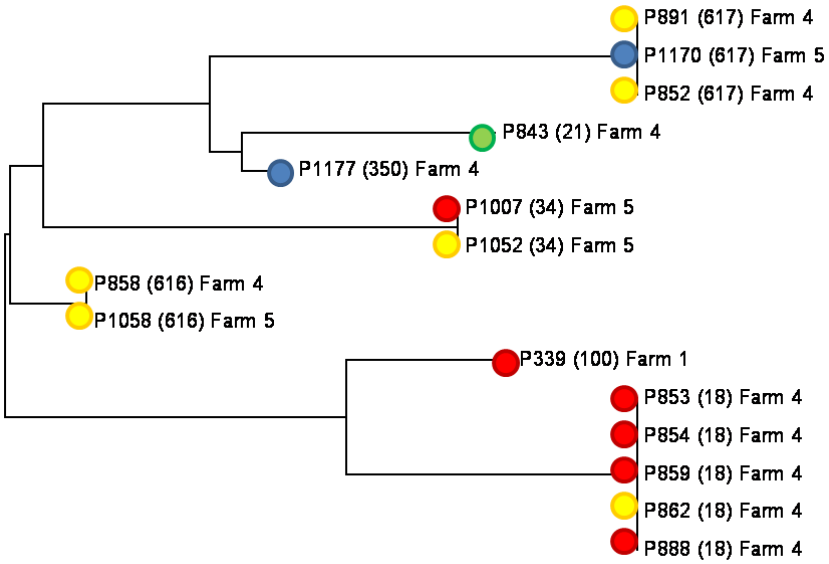
4.10 Cloning the *bla*$_{OXA-184}$ gene

In order to determine whether *bla*$_{OXA-184}$ encodes a functional beta-lactamase, the gene was cloned into a plasmid vector which was transformed into *Escherichia coli* α-Select cells (a type of DH5α cells purchased from BioLine) in order to allow expression of the gene in a susceptible host background. This strategy was chosen as *Campylobacter* have been shown to be naturally resistant to some beta-lactams (in particular the cephalosporins) and so the full substrate profile of beta-lactamase cannot be determined within this background (Pumbwe and Piddock 2002; Pumbwe, Randall et al. 2004; Guo, Lin et al. 2010).

The *bla*$_{OXA-184}$ gene was amplified using primers designed to insert an *Eco*RI restriction site before the start codon of the gene and an *Xba*I restriction site downstream of the *bla*$_{OXA-184}$ gene (Figure 4.22).

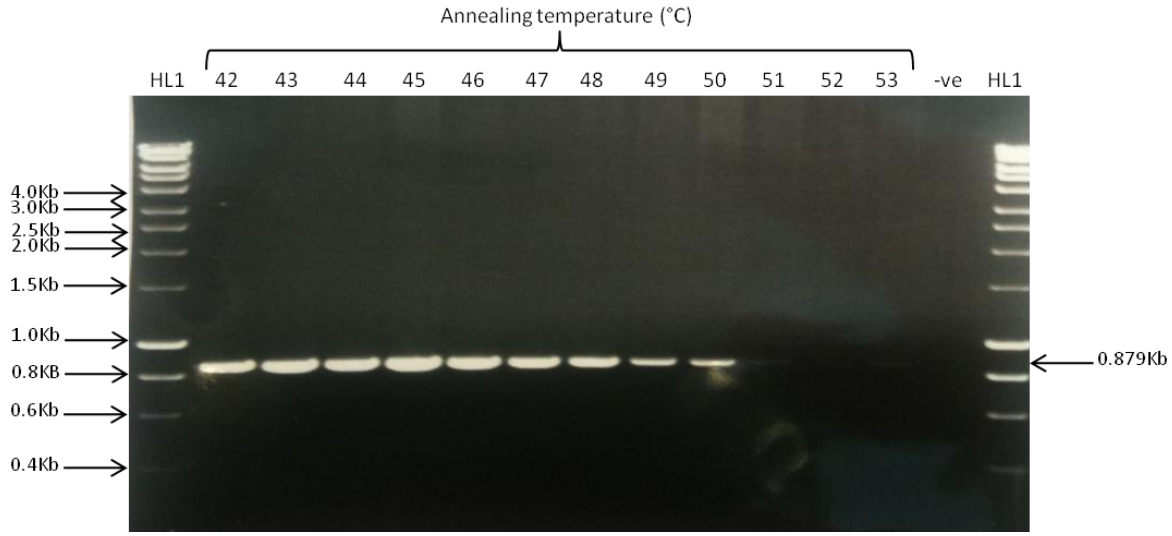
The PCR amplimer was digested with *Eco*RI and *Xba*I to create 'sticky ends' for cloning (Figure 4.23). A suitable pUC based vector, pHSG396, with a chloramphenicol resistance marker (*cat*) was selected for use and linearised with *Eco*RI and *Xba*I (Figure 4.23). The

Figure 4.21 Neighbour joining tree of CjBla2 producers based on their *flaA* gene



Red circles, Group 1 (OXA-184). Yellow circles, Group 3 (OXA-185). Green circle, Group 2 (OXA-184, but contains a 108 bp insertion upstream of $bla_{OXA\text{-}184}$). Blue circle, Group 4 (amplimer produced during internal $bla_{OXA\text{-}184}$, no amplimer produced during external $bla_{OXA\text{-}184}$ PCR).
Number in parentheses relates to the isolate's *flaA* type.
Farm number represents the farm from which the bacteria were originally isolated.

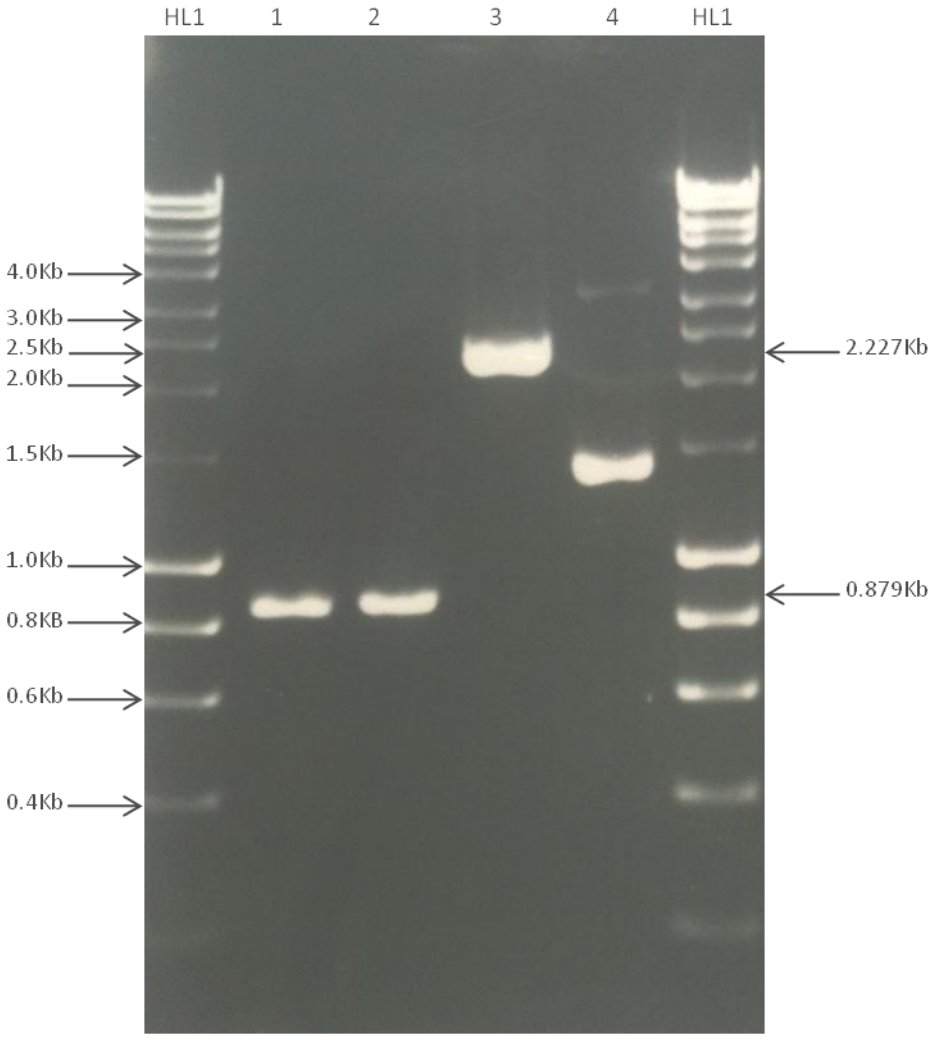
Figure 4.22 Gradient PCR amplification of an 879bp fragment covering *bla*$_{OXA-184}$ and introducing an *EcoR*I restriction site upstream of the start codon and an *Xba*I restriction site downstream of the gene



HL1, HyperLadder 1.

Following gradient PCR amplification of *bla*$_{OXA-184}$ using the cloning primers 1014 and 1014 (Materials and Methods, Table2.10) 5µl of each PCR reaction was loaded on to a 1% (w/v) agarose gel and electrophoresed at 100V for approximately 45 minutes. Annealing temperatures of 42°C to 50°C produced good amplimers of the expected size (879bp). Annealing temperatures of 53°C or higher yielded little to no amplimer.

Figure 4.23 Restriction enzyme digestion of PCR amplimer and pHSG398 vector in preparation for ligation and cloning



Lane	Description
1	PCR amplimer digested with EcoRI and XbaI
2	Undigested PCR product (control)
3	Linearised vector pHSG398 digested with EcoRI and XbaI
4	Undigested vector pHSG398 (control)

The 879bp amplimer of $bla_{OXA-184}$ (produced by PCR amplification with cloning primers 1012 and 1014) was digested with *EcoR*I and *Xba*I for one hour, 5 µl of digested amplimer and 5 µl of the undigested control were loaded onto a 1% agarose gel and electrophoresed at 100V for approximately 45 minutes. Simultaneously the vector pHSG298 was also digested for 1 hour with *EcoR*I and *Xba*I, 5 µl of digested plasmid and 5 µl of the undigested control were loaded onto a 1% agarose gel and electrophoresed at 100V for approximately 45 minutes. Both digested and un-digested amplimer are approximately 879bp. Digested plasmid has been linearised and is approximately 2.227Kb in size, undigested plasmid not linearised and is running in two circular forms, one at approximately 3.5 Kb in size (open circular form), one at just less than 1.5 Kb (covalently closed circular form).

bla$_{OXA-184}$ insert was ligated into the vector which was then transformed into α-Select cells.

When the previously characterised *bla*$_{OXA-193}$ was expressed in *E. coli* cells it was noted by the authors that the *Campylobacter* promoter sequence was poorly recognised resulting in weak transcription (Alfredson and Korolik 2005). To avoid the problem of poor transcription only the *bla*$_{OXA-184}$ gene was cloned not the promoter region or ribosome binding site, in order to allow the gene to be under the control of the *lacZ* promoter and ribosome binding site on the plasmid.

Blue-white screening was used to identify candidate colonies containing inserted DNA. Nine white colonies were sub-cultured and then subjected to PCR amplification for *bla*$_{OXA-184}$, all nine produced amplimers of the expected size (Figure 4.24).

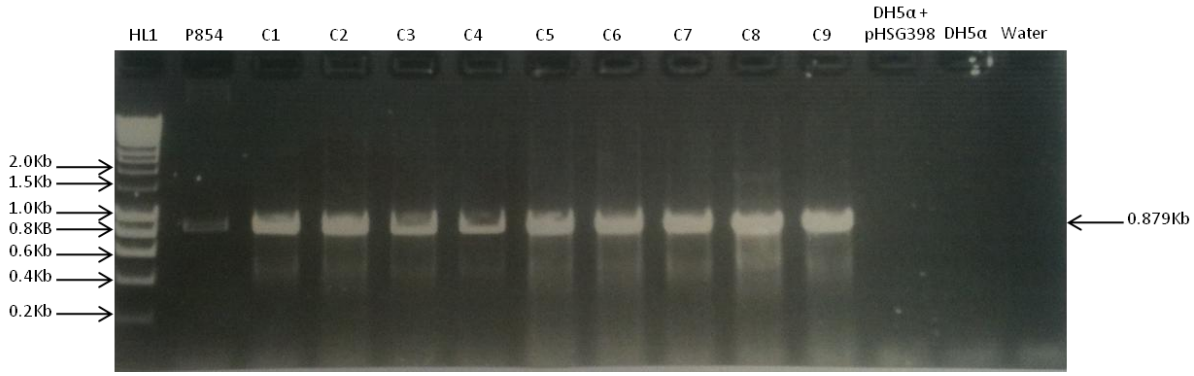
Plasmids were isolated from three candidate colonies and were shown to be larger than the original pHSG398 (Figure 4.25).

4.11 Checking the putative α-Select pHSG398 (*bla*$_{OXA-184}$) mutants

The MICs of a range of beta-lactams in *E. coli* α-Select cells and in three candidate α-Select pHSG398(*bla*$_{OXA-184}$) mutants were obtained. When the MIC values obtained for the mutant containing pHSG398(*bla*$_{OXA-184}$) were compared to those obtained for the parental strain (α-Select) there was no significant difference between them (Table 4.5).

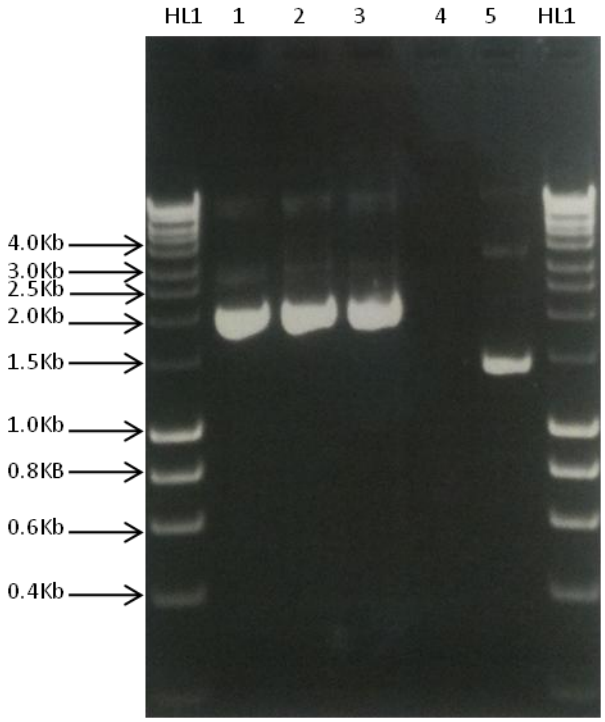
RNA was isolated from *E. coli* α-Select cells and from a putative α-Select pHSG398(*bla*$_{OXA-184}$) mutant and used to create cDNA. A control rtPCR for 16S (a gene that should be ubiquitously expressed) and a PCR for *bla*$_{OXA-184}$ were performed using cDNA as template.

Figure 4.24 PCR amplification of an 879bp fragment covering *bla*$_{OXA-184}$ from candidate colonies which may contain pHSG398 plus the *bla*$_{OXA-184}$ insert



HL1, HyperLaddder1; C1-C9, candidate 1-9
Following PCR amplification of *bla*$_{OXA-184}$ using the cloning primers 1012 and 1014 (Materials and methods, Table 2.10) 5 µl of each PCR reaction was loaded on to a 1% (w/v) agarose gel and electrophoresed at 100 V for approximately 45 minutes. The amplimer obtained for P854 and all nine candidate colonies is 879bp in size; the negative controls DH5α and DH5α + pHSG398 did not produce any amplimers.

Figure 4.25 Plasmid isolation from three colonies which may contain pHSG398(*bla*$_{OXA-184}$)



Lane	Description
1	Plasmid isolated form candidate 1
2	Plasmid isolated form candidate 2
3	Plasmid isolated form candidate 7
4	No plasmid isolated from DH5α cells
5	Plasmid isolated from DH5α cells containing pHSG398

HL1, HyperLadder1

Following isolation of plasmid DNA, 5 µl of each plasmid preparation was loaded on to a 1% (w/v) agarose gel and electrophoresed at 100V for approximately 45 minutes. The plasmid obtained for the three candidate colonies is running in three forms (approximately 10Kb, 3Kb and 2Kb) as is the plasmid obtained from DH5α cells + pHSG398 (approximately 10Kb, 4Kb and 1.5Kb). As the plasmids have not been linearised their sizes cannot be accurately estimated although the plasmid isolated from the candidates is clearly different from the pHSG398.

Table 4.5 Minimum inhibitory concentration of a range of beta-lactams for putative CjBla2 producers

Strain	Minimum Inhibitory Concentration (MIC) (μg/ml)						
	PEN	AMP	OXA	CEF	CFX	CTX	FOX
α-Select	**16**	**1**	**256**	**4**	**4**	**0.125**	**16**
Candidate 1	16	2	256	4	4	0.125	8
Candidate 1	16	1	256	4	4	0.125	16
Candidate 7	16	1	256	4	4	0.125	16

PEN, penicillin; AMP, ampicillin; OXA, oxacillin; CEF, cephalothin; CFX, cefuroxime; CTX, cefotaxime; FOX, cefoxitin.
MICs are the modal value of at least three biological repeats. MIC values obtained for the parental strain (α-Select) are shown in bold. Candidates 1, 2 and 7 are all putative α-Select pHSG398(*bla*$_{OXA-184}$) mutants.
No MIC values obtained for the candidates are significantly different (≥ two doubling dilutions) to those obtained for the parental strain.

Amplimers from the 16S PCR were obtained from all three replicates of α-Select cells and from all three replicates of a putative α-Select pHSG398(*bla*$_{OXA-184}$) mutant, therefore validating the RNA extraction and cDNA synthesis procedures (Figure 4.26). Amplimers were also obtained following *bla*$_{OXA-184}$ PCR from all three replicates of α-Select cells and from all three replicates of a putative α-Select pHSG398(*bla*$_{OXA-184}$) mutant (Figure 4.27).

4.12 Inactivation of *bla*$_{OXA-184}$ in P854

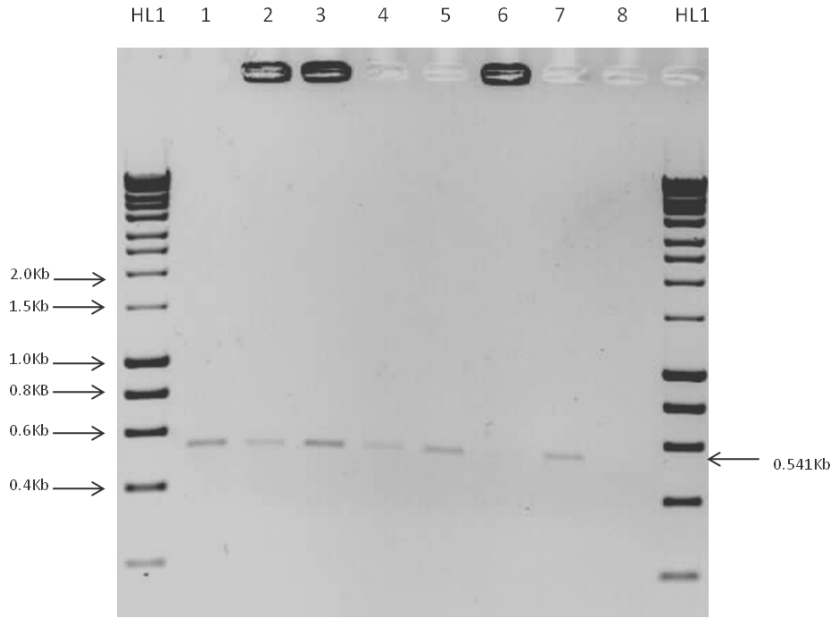
A strategy was devised to inactivate the *bla*$_{OXA-184}$ gene in P854 based on a modified Datsenko and Wanner protocol (Datsenko and Wanner 2000). The *aph* gene was amplified by PCR using primers that inserted a 40 bp region of homology to *bla*$_{OXA-184}$ both up and downstream of aph (Figure 4.28). This PCR amplimer was then used to insertionally inactivate *bla*$_{OXA-184}$ in P854 by natural transformation and homologous recombination. No *bla*$_{OXA-184}$ mutants were obtained during preliminary experiments.

4.13 Discussion

The oxacillinase of *C. jejuni* NCTC11168 (product of the *cj0299* gene) was submitted to the Lahey clinic and was assigned the number OXA-193, this has helped in the clarification of *Campylobacter* oxacillinases.

Work in this laboratory identified two poultry isolates of *C. jejuni,* P843 and P854, that produced no amplimers for *bla*$_{OXA-193}$ but produced a beta-lactamase that has a different isoelectric point and molecular mass to that of the previously characterised OXA-61 and OXA-193 oxacillinases (Alfredson and Korolik 2005; Griggs, Peake et al. 2009). The novel beta-lactamase was termed CjBla2 and preliminary mass spectrometry experiments to identify the protein suggested that PurU (annotated as a formyltetrahydrofolate

Figure 4.26 rtPCR of *16S* from α-Select cDNA and cDNA obtained from a putative α-Select pHSG398(*bla*$_{OXA-193}$) mutant

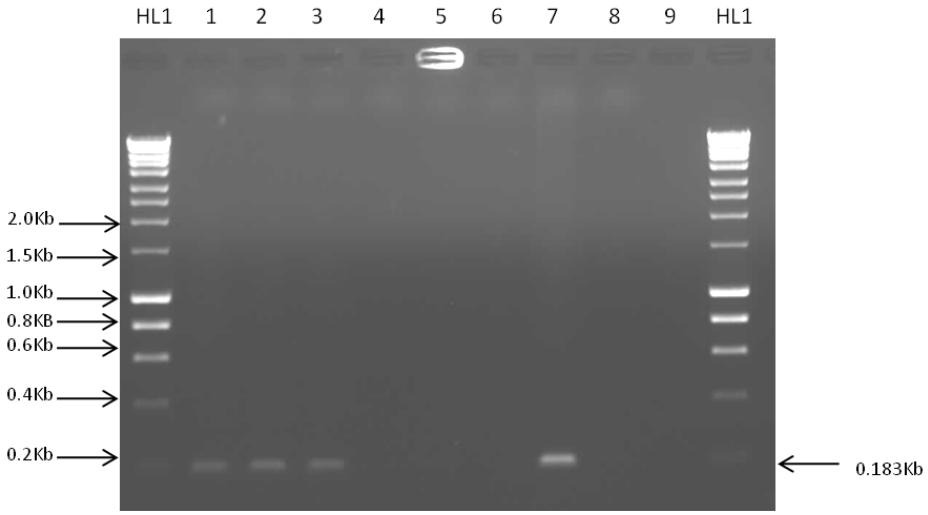


HL1, HyperLaddder1

1. α-select cDNA
2. α-select cDNA
3. α-select cDNA
4. putative α-select pHSG398(*bla*$_{OXA-184}$) mutant cDNA
5. putative α-select pHSG398(*bla*$_{OXA-184}$) mutant cDNA
6. putative α-select pHSG398(*bla*$_{OXA-184}$) mutant cDNA
7. Positive control (α-select DNA)
8. Negative control (water)

Following PCR amplification of *16s*, 5 µl of each PCR reaction was loaded on to a 1% (w/v) agarose gel and electrophoresed at 100 V for approximately 45 minutes. Correct sized amplimers (541 bp) were obtained for all samples and the positive control. The negative control did not produce any amplimers.

Figure 4.27 rtPCR of *blaOXA-184* from α-Select cDNA and cDNA obtained from a putative α-Select pHSG398(*bla*$_{OXA-193}$) mutant

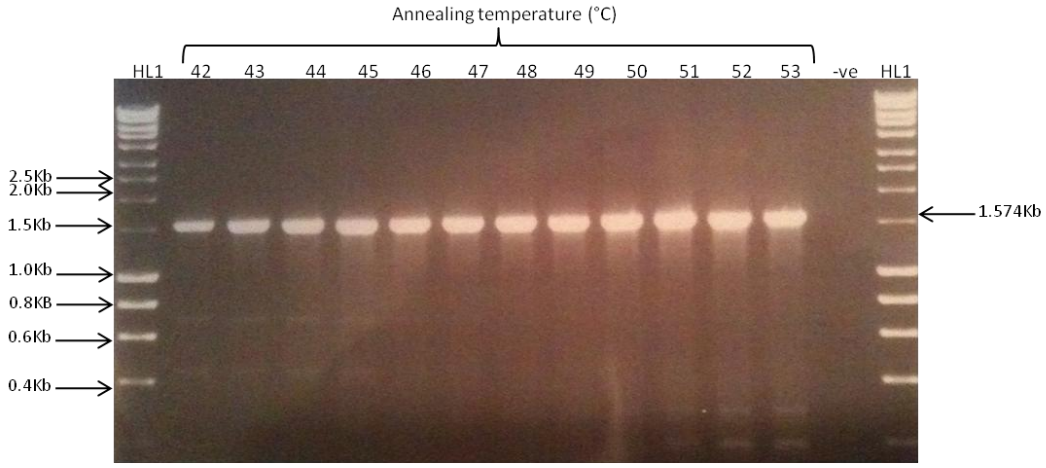


HL1, HyperLaddder1

1. putative α-select pHSG398(*bla*$_{OXA-184}$) mutant cDNA
2. putative α-select pHSG398(*bla*$_{OXA-184}$) mutant cDNA
3. putative α-select pHSG398(*bla*$_{OXA-184}$) mutant cDNA
4. α-select cDNA
5. α-select cDNA
6. α-select cDNA
7. Positive control (P854 DNA)
8. Negative control (α-Select DNA)
9. Negative control (water)

Following PCR amplification of *bla*$_{OXA-184}$, 5 µl of each PCR reaction was loaded on to a 1% (w/v) agarose gel and electrophoresed at 100 V for approximately 45 minutes. Correct sized amplimers (183 bp) were obtained for all replicates of a putative α-select pHSG398(*bla*$_{OXA-184}$) mutant and the positive control. The negative controls and the three replicates of α-select cDNA did not produce any amplimers.

Figure 4.28 Gradient PCR amplification of *aph* with primers that introduce 40bp of homology to $bla_{OXA-184}$ at either end of the amplimer



HL1, HyperLadder 1.

Following gradient PCR amplification of $bla_{OXA-184}$ using the cloning primers 1051 and 1052 (Materials and Methods, Table2.13) 5 µl of each PCR reaction was loaded on to a 1% (w/v) agarose gel and electrophoresed at 100V for approximately 45 minutes. Annealing temperatures of 42°C to 53°C produced good amplimers of the expected size (1.574 Kb).

deformylase), the product of the *cj0790* gene of *C. jejuni* NCTC11168, was the most similar to CjBla2 (Griggs, Peake et al. 2009). A PurU mutant was constructed but when tested for beta-lactamase production, it was found to have detectable beta-lactamase activity comparable to that of the parental strain P854, suggesting that *cj0790* does not encode CjBla2. Confirmation that CjBla2 of P843 and P854 was unlikely to be the product of a mutated $bla_{OXA\text{-}193}$ gene was obtained as PCR with combinations of four $bla_{OXA\text{-}193}$ primers, gave no amplimers and a labelled $bla_{OXA\text{-}193}$ DNA probe was unable to hybridise to digested genomic DNA from P843 and P854 during Southern blotting.

DNA sequencing of the P854 genome and subsequent assembly and annotation resulted in the identification of four putative beta-lactamase genes. *P854_1490* was identified as the most probable candidate gene to encode CjBla2 given that (unlike the other three genes) no homologues could be found in other sequenced *Campylobacter* genome. When BLAST searching the closest hit with the predicted P854_1490 protein was with OXA-193. However, the two proteins are not very closely related as they share only 64% identity with one another (just one amino acid substitution denotes a novel oxacillinase). The putative P854_1490 is likely to be an oxacillinase (Ambler Class D beta-lactamase) as it contains all the highly conserved and identically conserved motifs typical of an oxacillinase (Poirel, Naas et al. 2010) and clusters with other oxacillinases (forming a group with oxacillinases of *Campylobacter* origin). The amino acid sequence of P854_1490 was accepted by the Lahey clinic to be a novel beta-lactamase and was assigned the number OXA-184.

Interestingly, $bla_{OXA\text{-}184}$ was demonstrated to be in a very similar genomic context to $bla_{OXA\text{-}193}$. A *mod* operon (encoding a molybdenum transport system) was present downstream of the gene, as is the case with $bla_{OXA\text{-}193}$ in *C. jejuni* NCTC11168. Upstream however, the *pan*

operon (involved in pantothenate biosynthesis), is present in NCTC11168, but not present upstream of *bla*$_{OXA-184}$ in P854. As P854 lacks both the *pan* operon and *bla*$_{OXA-193}$ this is why *bla*$_{OXA-193}$ PCR with primers from *panB* and *bla*$_{OXA-193}$ failed to produce amplimers for P854.

As the genes flanking *bla*$_{OXA-184}$ (including a pseudo-gene immediately upstream) are not present in other sequenced genomes of *C. jejuni* it is possible that these genes were acquired at the same time as the *bla*$_{OXA-184}$ gene. Indeed, a similarity can be drawn between the genomic context of *bla*$_{OXA-184}$ and that of the *CLA0304* putative beta-lactamase gene of *C. lari* RM2100 as both are followed downstream by a metal dependent membrane permease, suggesting that perhaps this region was acquired by P854 from an unknown *C. lari* genome.

The 'CjBla2' phenotype, (i.e. ampicillin resistance, lacking amplimers following *bla*$_{OXA-193}$ PCR, inability of genomic DNA to hybridise to a *bla*$_{OXA-193}$ labelled probe and beta-lactamase detection) was found for 13 other *Campylobacter* poultry isolates. As the fifteen isolates were collected from three separate farms during 2001-2002 and represent seven *flaA* types the gene(s) responsible for CjBla2 are disseminated in the *Campylobacter* population.

Seven of the 15 isolates in the 'CjBla2' group (including P854) contained identical genes to *bla*$_{OXA-184}$ although one, P843, contained a 108bp insertion in the upstream intergenic region. Six of the other further isolates contained a putative oxacillinase with a single amino acid substitution (Leu→Ile) difference to that encoded by *bla*$_{OXA-184}$. All six of these isolates also contained numerous conserved point mutations up and down stream of the oxacillinase gene when compared to the P854 genome. The remaining two isolates from the 15 isolate 'CjBla2' group produced an amplimer during *bla*$_{OXA-184}$ PCR with internal gene primers, but upon PCR with primers external to the oxacillinase gene did not produce an amplimer. As the internal PCR did not cover the site encoding the Leu→Ile variation, it is unclear whether

these two isolates encode (i) a protein identical to OXA-184, (ii) a protein identical to OXA-185 or (iii) another oxacillinase. When a dendogram was produced based on the *flaA* type of the isolates of the group they do not cluster according to which oxacillinase they encode, suggesting that these genotypes are not simply the result of clonal expansion.

Campylobacter have innate resistance to cephalosporin type beta-lactamases, and some resistance to penicillin type beta-lactamases due to the CmeABC efflux pump (Chapter 3) (Guo, Lin et al. 2010). In order to assess the full substrate profile of the OXA-184 oxacillinase the $bla_{OXA-184}$ gene was cloned into a fully susceptible *E. coli* background (α-Select) using a plasmid vector suitable for expression under the control of the *lac* promoter. The MICs of a range of beta-lactams for the *E. coli* transformants were then determined, when compared to those of the parental strain no significant difference could be seen. RNA was extracted from an *E. coli* $bla_{OXA-184}$ containing mutant and used to generate cDNA which was the template for both a 16S rtPCR and a $bla_{OXA-184}$ rtPCR. Amplimers were produced during 16S rtPCR confirming that RNA isolation and cDNA synthesis were successful. Amplimers were also obtained following $bla_{OXA-184}$ rtPCR confirming that the *E. coli* mutant is transcribing the $bla_{OXA-184}$ gene. The $bla_{OXA-184}$ gene which in the mutants is under the control of the inducible *lacZ* promoter has been shown to be transcribed. As the *Campylobacter* ribosome binding site has not been cloned with the coding sequence of the gene, expression will rely on the *lacZ* ribosome binding site and so if the gene has been inserted out of frame with the *lacZ* gene no functional protein may be being translated. It is also possible that a LacZ-OXA-184 fusion protein is being generated however the possible production of a fusion protein may not eliminate the potential beta-lactamase activity of the protein as there are instances reported where functional beta-lactamase fusion proteins have been created successfully

(Lattemann, Maurer et al. 2000). Further experiments will need to be undertaken to determine whether the lack of beta-lactam resistance in the *E. coli* mutant is due to the mutant not translating a functional OXA-184 or whether the $bla_{OXA-184}$ does not encode a functional oxacillinase.

An attempt was made to inactivate $bla_{OXA-184}$ in P854 using natural transformation but preliminary experiments were not successful as $bla_{OXA-184}$ mutants were not obtained. It is possible that the 40bp of homology the transforming DNA had with the target gene was insufficient to allow homologous recombination despite being within the range suggested by Datsenko and Wanner (Datsenko and Wanner 2000). Techniques that have successfully allowed homologous recombination in *Campylobacter* in the past (such as allelic replacement) have much longer stretches of homologous DNA (in excess of 200bp) (Karlyshev and Wren 2005). The method developed here could be improved by using primers that create much longer stretches of homologous DNA during PCR.

Two novel, putative oxacillinases have been identified from *Campylobacter* and have been shown to be disseminated in *Campylobacter* isolated from poultry, further work needs to be performed to characterise these enzymes phenotypically.

4.14 Further Work

- Inactivate the $bla_{OXA-184/5}$ gene in P854/P852 and characterise the mutant phenotype MICs of beta-lactam antibiotics and express both $bla_{OXA-184}$ and $bla_{OXA-185}$ in a beta-lactam susceptible background to determine the activity of the oxacillinases.
- Characterise the OXA-184 and OXA-185 enzymes in terms of their molecular mass and isoelectric points.
- Screen further isolates for the $bla_{OXA-184}$ and $bla_{OXA-185}$ genes.

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Chapter 5 – Results and Discussion

Is beta-lactam resistance transferrable between *Campylobacter* isolates?

5.1 Background

The most important mechanism of beta-lactam resistance in Gram negative bacteria is the production of beta-lactamases, enzymes that are capable of hydrolysing the beta-lactam ring of beta-lactam antibiotics rendering them ineffective (Medeiros 1997; Bush and Mobashery 1998; Bush 2001). Following the introduction of penicillin in the 1960s, plasmid mediated beta-lactam resistance became a problem; by 1968 17% of surgical patients in a London hospital had ampicillin resistant enterococci in their faeces and conjugation experiments using *E. coli* K12 revealed that the majority of isolates could transfer resistance (Datta 1969). Although *Campylobacter* containing plasmids have been described containing resistance genes to tetracycline, chloramphenicol, kanamycin and streptomycin (Taylor, Gradis et al. 1981; Tenover and Elvrum 1988; Wang and Taylor 1990; Gibreel, Skold et al. 2004), plasmid encoded beta-lactam resistance in *Campylobacter* have not been described.

5.2 Aims and Hypotheses

It was hypothesised that resistance to ampicillin is transferred alongside tetracycline resistance between *Campylobacter* isolates.

The aims of this work were to:

a) Determine whether beta-lactam resistance could be transferred between isolates of *Campylobacter jejuni* and/or the closely related *Campylobacter coli.*

b) If transfer does occur, determine the gene(s) responsible and the mechanism of transfer.

5.3 Creation and selection of ampicillin resistant transconjugant *Campylobacter*

During a study on poultry isolates by this laboratory (Piddock, Griggs et al. 2008) data from unpublished experiments suggested that during conjugation resistance to ampicillin could be co-transferred with tetracycline resistance. In this PhD project these experiments were repeated to confirm these data.

Two isolates of *C. coli*, P1131 and P1931 were selected for use as 'donor' strains in these experiments. These isolates were resistant to tetracycline (MIC = 256 μg/ml and 128 μg/ml respectively) and ampicillin (MIC = 128 μg/ml and 256 μg/ml respectively), were susceptible to kanamycin (MIC = 16 μg/ml) (see Table 5.1a) and had previously been shown to contain transferrable tetracycline resistance. Although the two isolates of *C. coli* had the same phenotype, they were isolated from different farms, during different Defra funded studies and were isolated three years apart (see Table 5.1b).

P1131 and P1931 were conjugated with an ampicillin, tetracycline susceptible strain of NCTC11828 which had been manipulated to contain a chromosomal *aph* cassette making it resistant to kanamycin (P1983). Transconjugants were selected by plating the conjugation mixture onto Mueller Hinton agar containing 10μg/ml tetracycline and 50μg/ml of kanamycin. A control was set up using the same recipient strain (P1983) but with the well characterised *Campylobacter* strain 81-176 (P1984) that is ampicillin and kanamycin sensitive and contains the transferable pTet plasmid (Batchelor, Pearson et al. 2004).

Table 5.1a Known minimum inhibitory concentrations of antibiotics for isolates P1131 and P1931

Isolate	Minimum Inhibitory Concentration (µg/ml)							
	Ampicillin	Tetracycline	Kanamycin	Ciprofloxacin	Nalidixic Acid	Trimethoprim	Chloramphenicol	Erythromycin
P1131	128	256	16	32	128	512	4	8
P1931	256	128	16	0.25	16	512	2	4

Table 5.1b Information about isolates P1131 and P1931

Isolate	Species	Defra Study	Date Isolated	Origin	Treatment Phase	Sample type	*flaA* type	Reference
P1131	*C. coli*	OZO501	11/11/2002	Farm 6	2 weeks post-treatment with a fluoroquinolone	Chicken faeces	*flaA*18	(Humphrey, Jorgensen et al. 2005)
P1931	*C. coli*	VM0220	02/11/2005	Lang 012	4 weeks post-treatment with chlortetracycline	Chicken faeces	*flaA*66	(Piddock, Griggs et al. 2008)

Putative transconjugants were achieved from all experiments. Conjugation frequencies were calculated to be $1.2x10^{-8}$ for the conjugation of P1131 X P1983 and $4.03x10^{-7}$ for the conjugation of P1931 X P1983. Once confirmed to be *Campylobacter* by microscopy and culture a representative isolate from each experiment was assigned a laboratory number; P1131 X P1983 = P2241; P1931 X P1983 = P2244 and P1984 X P1983 = P2238 (Materials and Methods Table 2.3) and retained for further study.

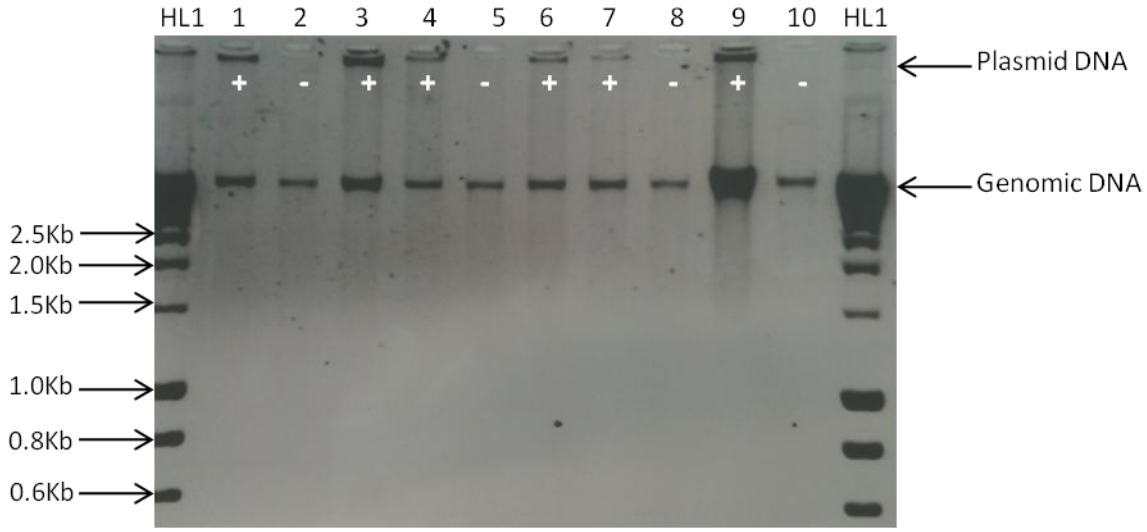
5.4 Characterisation of transconjugants

5.4.1 Presence of plasmid DNA

The donors and transconjugants contained plasmids of a similar size which can be estimated to be approximately 45kb as the sequenced pTet plasmid (present in P1984 and P2238) is 45.2Kb in size (Batchelor, Pearson et al. 2004); the recipient contained no plasmids (Figure 5.1). As plasmid isolation with the QIAprep kit yielded low concentrations of plasmid DNA (approximately 20ng/µl) and the DNA was contaminated with genomic DNA (Figure 5.1), other methods of plasmid isolation were explored in order to obtain higher quantities of pure plasmid DNA for further experimentation.

Promega's PureYield™ Plasmid Miniprep System (Figure 5.2), the alkaline lysis method of Kado and Liu (Kado and Liu 1981) (Figure 5.3), and alkaline lysis by the method of Birnboim and Doly (Birnboim and Doly 1979) followed by purification by caesium chloride density gradient centrifugation (Figure 5.4) were compared. All methods resulted in the isolation of plasmid DNA contaminated with genomic DNA.

Figure 5.1 Isolation of plasmid DNA using QIAprep kit (Qiagen)



Lane	Isolate / Strain	Donor / Recipient / Transconjugant	Plasmid DNA?
1	P1984	Donor	Present
2	P1983	Recipient	Absent
3	P2238	Transconjugant	Present
4	P1131	Donor	Present
5	P1983	Recipient	Absent
6	P2241	Transconjugant	Present
7	P1931	Donor	Present
8	P1983	Recipient	Absent
9	P2244	Transconjugant	Present
10	P270 (NCTC11168)	Plasmid-free control	Absent

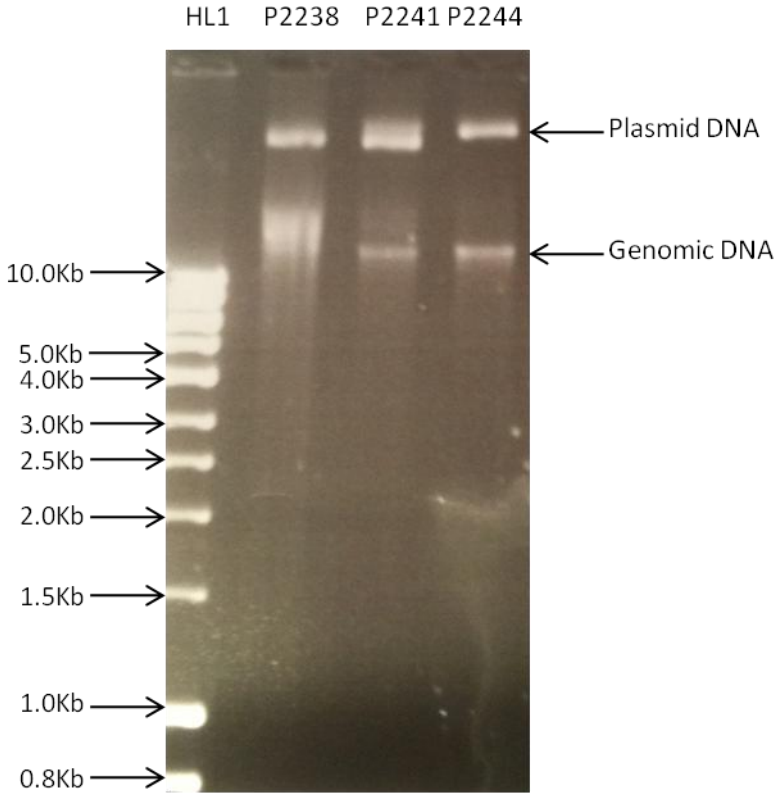
HL1, HyperLadder 1

+/- indicates presence or absence of plasmid DNA.

Five microlitres of plasmid DNA isolated from donors recipients and transconjugants was electrophoresed on a 0.8% (w/v) agarose gel at 80 V for approximately four hours.

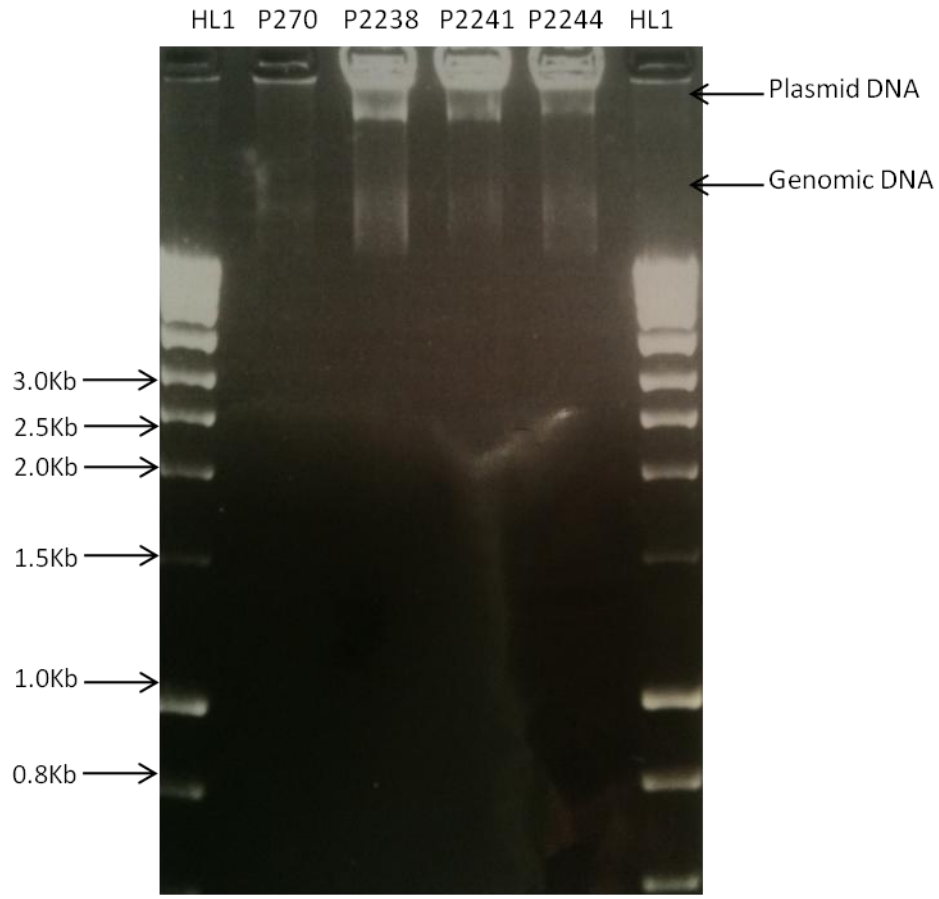
Contaminating genomic DNA is present in all samples.

Figure 5.2 Isolation of plasmid DNA using PureYield™ Plasmid Miniprep System (Promega)



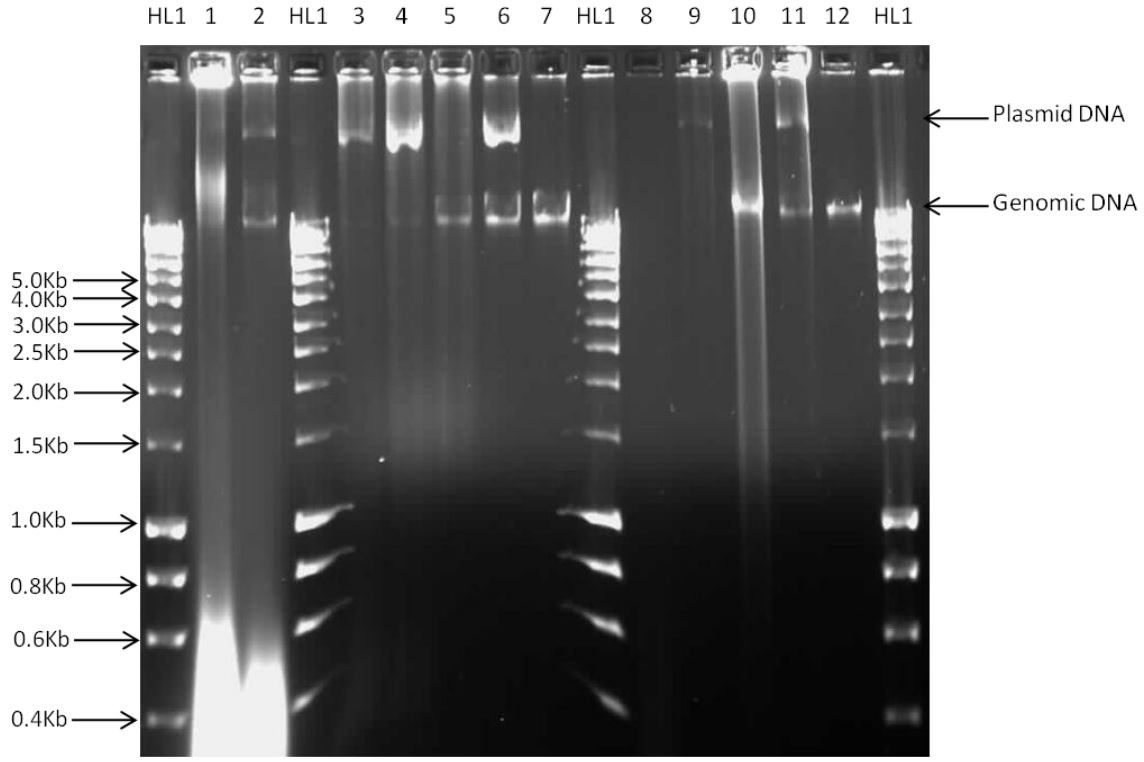
HL1, HyperLadder 1
Five microlitres of plasmid DNA isolated from the transconjugants P2238, P2241 and P2244 was electrophoresed on a 0.8% (w/v) agarose gel at 80 V for approximately four hours. Contaminating genomic DNA is present in all samples.

Figure 5.3 Isolation of plasmid DNA using the alkaline lysis method of Kado and Liu (Kado and Liu 1981)



HL1, HyperLadder 1
Five microlitres of plasmid DNA isolated from the plasmid free control P270 (NCTC11168) and the transconjugants P2238, P2241 and P2244 was electrophoresed on a 0.8% (w/v) agarose gel at 80 V for approximately four hours.
Contaminating genomic DNA is present in all samples.

Figure 5.4 Isolation of plasmid DNA by alkaline lysis using the method of Birnboim and Doly followed by purification by caesium chloride density gradient centrifugation
(Birnboim and Doly 1979)



Lane	Plasmid DNA Sample
1	P2241 before CsCl
2	P2244 before CsCl
3	P2241 following CsCl density gradient centrifugation (fraction1)
4	P2241 following CsCl density gradient centrifugation (fraction 2)
5	P2241 following CsCl density gradient centrifugation (fraction 3)
6	P2244 following CsCl density gradient centrifugation (fraction 1)
7	P2244 following CsCl density gradient centrifugation (fraction 2)
8	P2241 following CsCl density gradient centrifugation and further purification (fraction 1)
9	P2241 following CsCl density gradient centrifugation and further purification (fraction 2)
10	P2241 following CsCl density gradient centrifugation and further purification (fraction 3)
11	P2244 following CsCl density gradient centrifugation and further purification (fraction 1)
12	P2244 following CsCl density gradient centrifugation and further purification (fraction 2)

HL1, HyperLadder 1
Five microlitres of plasmid DNA isolated from the transconjugants P2241 and P2244 was electrophoresed on a 0.8% (w/v) agarose gel at 80 V for approximately four hours. Contaminating genomic DNA is present in all samples.

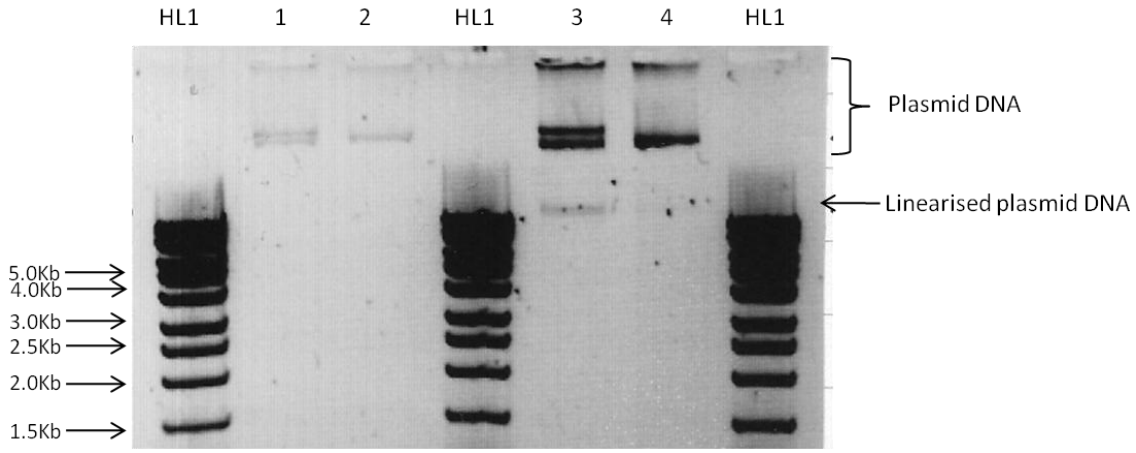
QIAGEN's large construct kit was the only method by which a large volume (1 ml) of concentrated (approximately 1 µg/ml), pure plasmid DNA could be isolated (Figure 5.5). Plasmid DNA was isolated from P2241 and P2244. From here on these plasmids are referred to as pBla1 and pBla2, respectively. Five micro litre aliquots from the 15ml of each of the plasmid samples eluted from the QIAGEN column was retained and electrophoresed at the same time as a 5 µl aliquot of the plasmid DNA samples once they had been concentrated by ethanol precipitation (Figure 5.5). DNA was clearly less concentrated when first eluted. Following ethanol precipitation samples were more concentrated and linearised plasmid DNA was seen. Three bands were seen for pBla1 and two for pBla2 (excluding linearised DNA). It cannot be determined at this stage whether the numerous bands in each samples represent different forms of one plasmid (closed circular, open circular and supercoiled) or whether the samples each contain more than one plasmid. All of the bands are likely to be plasmid DNA (not contaminating genomic DNA) as QIAGENs Large Construct Kit contains an exonuclease digestion step to remove genomic DNA as well as any nicked or damaged circular DNA.

5.4.2 Plasmid mediated antibiotic resistance

Minimum inhibitory concentrations (MICs) of a range of beta-lactam (Table 5.2) and non-beta-lactam (Table 5.3) antibiotics for donor, recipient and transconjugant strains were determined using the agar doubling-dilution method recommended by the CLSI Campylobacter Working Group (McDermott, Bodeis et al. 2004).

Both donors (P1131 and P1931) were resistant to tetracycline (MIC = 256 µg/ml and 128 µg/ml) and sensitive to kanamycin (MIC = 16 µg/ml); the recipient was sensitive to

Figure 5.5 Isolation of plasmid DNA using Large construct kit (Qiagen)



Lane	Sample
1	pBla1 following elution from column
2	pBla2 following elution from column
3	pBla1 following concentration by ethanol precipitation
4	pBla2 following concentration by ethanol precipitation

HL1, HyperLadder 1

Five microlitres of plasmid DNA isolated from the transconjugants, P2241 (pBla1) and P2244 (pBla2) was electrophoresed on a 0.8% (w/v) agarose gel at 80 V for approximately four hours.

Linearised plasmid DNA can be seen in the pBla1 preparation following ethanol precipitation that wasn't present before ethanol precipitation (Lanes 1 and 3).

No contaminating genomic DNA is present in any of the samples.

Table 5.2 Minimum inhibitory concentration (MIC) of beta-lactam antibiotics for donor, recipient and transconjugant strains

Laboratory Code	Description	MIC (µg/ml)							
		PEN	AMP	OXA	CEF	CXM	CTX	FOX	MER
P1131	pBla1 donor	128	128	512	1024	256	16	256	0.25
P1931	pBla2 donor	256	256	512	256	128	4	128	0.12
P1983	Conjugation recipient	16	4	128	256	128	4	128	0.06
P2241	pBla1 transconjugant	**128**	**128**	**512**	**1024**	256	8	256	0.12
P2244	pBla2 transconjugant	**128**	**256**	256	256	128	8	128	0.12

PEN, penicillin; AMP, ampicillin; OXA, oxacillin; CEF, cephalothin; CXM, cefuroxime, CTM, cefotaxime; CXM, cefoxitin; MER, meropenem. **Bold, red text** indicates a significant increase (two or more doubling dilutions) in MIC of an antibiotic in the transconjugants when compared to the recipient. Values given are the median value of at least three biological replicates.

Table 5.3 Minimum inhibitory concentration (MIC) of non beta-lactam antibiotics for donor, recipient and transconjugant strains

Laboratory code	Description	MIC (µg/ml)											
		KAN	TET	NAL	CHL	CIP	ERY	STR	TRM	ETB	ACO	SDS	TRIC
P1131	pBla1 donor	16	256	128	4	32	8	1	512	4	128	256	32
P1931	pBla2 donor	16	128	16	2	0.25	4	1	512	4	64	256	32
P1983	Conjugation recipient	256	0.25	8	2	0.12	8	1	256	2	32	256	32
P2241	pBla1 transconjugant	256	**256**	**128**	4	**32**	8	1	512	4	64	256	32
P2244	pBla2 transconjugant	256	**128**	8	2	0.25	8	1	512	2	32	256	32

KAN, kanamycin; TET, tetracycline; NAL, nalidixic acid; CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; STR, streptomycin; TRM, trimethoprim; ETB, ethidium bromide; ACO, acridine orange; SDS, sodium dodecyl sulphate; TRIC, triclosan.
Bold, red text indicates a significant increase (two or more doubling dilutions) in MIC of an antibiotic in the transconjugants when compared to the recipient. Values given are the median value of at least three biological replicates.

tetracycline (MIC = 0.25 μg/ml) and resistant to kanamycin (MIC = 256 μg/ml). All transconjugants were resistant to both agents. P1131 and P1931 were resistant to ampicillin (MIC = 128 μg/ml and MIC = 256 μg/ml) whereas the recipient was susceptible to the antimicrobial (MIC = 4 μg/ml). The transconjugants P2241 and P2244 have similar levels of resistance as their respective donor strains for ampicillin, penicillin and in the case of P2241 oxacillin and cephalothin, the MICs of which are all more than two doubling dilutions higher than those of the recipient (differences of two or more doubling dilutions are considered to be significant) suggesting that resistance to these agents is plasmid associated.

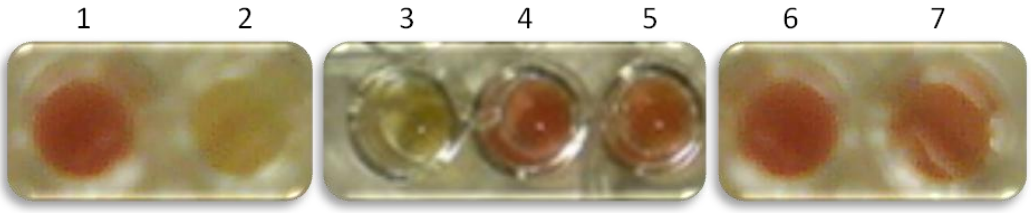
The MICs of nalidixic acid and ciprofloxacin suggested that plasmid pBla1 may contain resistance determinant(s) to these agents as the MIC of nalidixic acid and ciprofloxacin in P2241 were four and eight doubling dilutions higher than in the recipient strain; this takes the MICs to a similar level as found in the donor strain, P1131.

5.4.3 Beta-lactamase activity

Beta-lactamase activity can be determined by use of a chromogenic cephalosporin, nitrocefin, which when hydrolysed turns from yellow to red. Sonicated samples of the recipient strain (P1983), the two donor strains (P1131 and P1931) and the two transconjugants (P2241 and P2244) were tested for beta-lactamase production by the addition of nitrocefin.

The donor and transconjugant strains (P1131, P1931, P2241 and P2244) produced a beta-lactamase as there was a colour change from yellow to red upon addition of nitrocefin. No colour change was seen for recipient strain P1983 suggesting that it has no detectable beta-lactamase activity (Figure 5.6).

Figure 5.6 Detection of beta-lactamase activity using nitrocefin



Well	Sample	Beta-lactamase activity
1	P854 (+ve control)	+
2	P270 (-ve control)	-
3	P1983	-
4	P1131 (contains pBla1)	+
5	P1931 (contains pBla2)	+
6	P2241 (contains pBla1)	+
7	P2244 (contains pBla2)	+

Sonicated bacterial cell suspensions were mixed with nitrocefin and viewed after 30 minutes incubation at 37°C. A change of colour from yellow to red indicates a nitrocefin positive result, i.e. beta-lactamase activity. If the yellow colour of the nitrocefin remains unchanged the result was negative, i.e. no beta-lactamase activity detected.

5.4.4 *flaA* typing

Typing using the *flaA* SVR region was performed on all strains to confirm that any DNA transfer was the result of conjugation rather than natural transformation. Donor, recipient and transconjugant strains were *flaA* typed, the short variable region of the *flaA* gene was PCR amplified and sequenced then compared to the *flaA* database to determine the allele (Table 5.4). All transconjugants possessed the *flaA*239 allele, the same allele as the recipient strain.

5.4.5 Growth kinetics

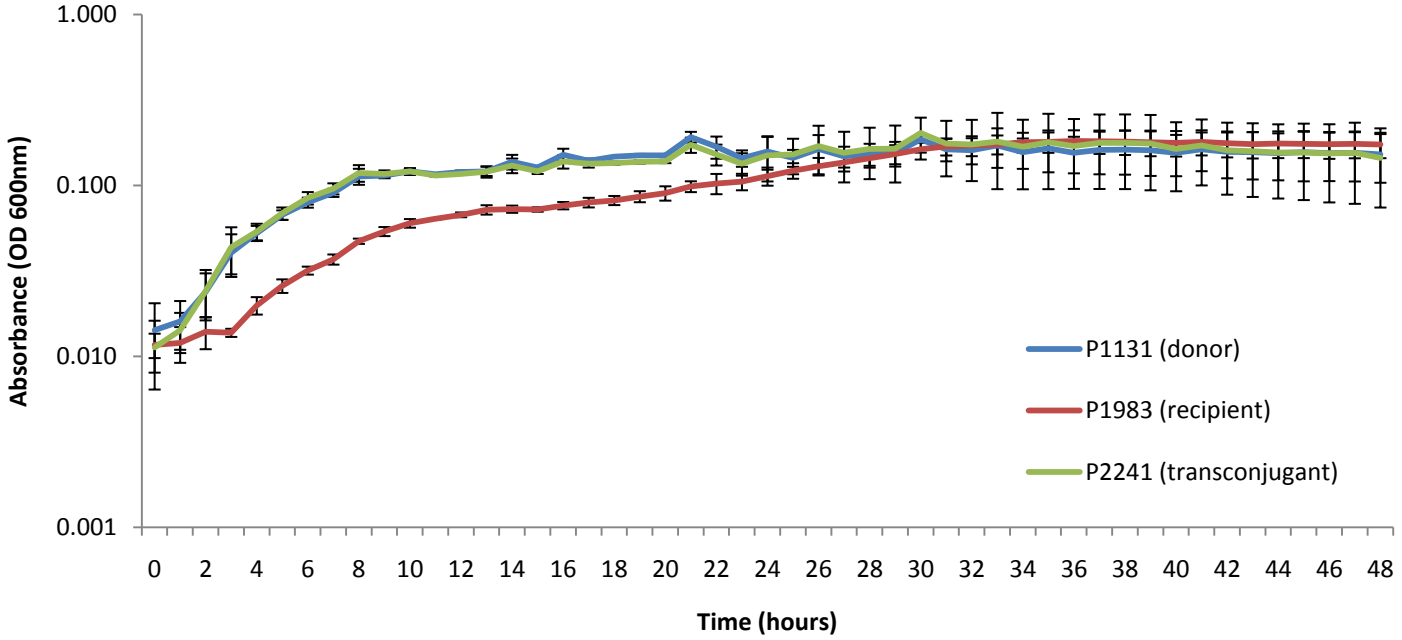
Following an observation that the transconjugant and donor strains often grew better than the recipient strain it was decided that the growth kinetics of these should be investigated. The growth kinetics of donor, recipient and transconjugant strains were investigated by measuring the change in absorbance (at an optical density of 600nm) of broth cultures over 48 hours using a Bioscan spectrophotometer. Data was gathered for each strain on three separate occasions and on each occasion ten replicates were used to calculate mean values. In order to determine whether the plasmid pBla1 affects the growth of the recipient strain, data for the transconjugant P2241 was compared with that of the recipient strain P1983 (Figure 5.7). The final absorbance of P1983 at OD_{600nm} was 0.17 ± 0.03 whereas P2241 had a final absorbance of 0.15 ± 0.07. When these two values were compared using a student's two-tailed T-test P=0.726, there was no significant difference between the final absorbance achieved. The generation time was calculated during log phase (3-7hours) and was found to be 213 minutes $\pm$ 38.8 for P1131 and for P2241 was found to be 212 minutes $\pm$ 73.3. There was no significant difference between these generation times (P = 0.989).

Table 5.4 *flaA* typing of donor, recipient and transconjugant strains

Strain	Description	*flaA* allele	FlaA peptide
P1984	Control (pTet) donor	359	9
P1131	pBla1 donor	18	20
P1931	pBla2 donor	66	1
P1983	Conjugation recipient	239	9
P2238	Control (pTet) transconjugant	239	9
P2241	pBla1 transconjugant	239	9
P2244	pBl2 transconjugant	239	9

flaA typing was performed on donor recipient and transconjugants by sequencing the short variable region, sequencing data was compared to the *flaA* database to determine the *flaA* type and the FlaA peptide.
This work made use of the *Campylobacter jejuni* Multi Locus Sequence Typing website (http://pubmlst.org/ campylobacter/) developed by Keith Jolley and Man-Suen Chan and sited at the University of Oxford (Jolley et al. 2004, *BMC Bioinformatics*, **5**:86). The development of this site has been funded by the Wellcome Trust.

Figure 5.7 Growth kinetics of pBla1 donor, recipient and transconjugant



Strain	Final OD_{600}	*P* value	Generation time (minutes)	*P* value
P1131	0.15 ± 0.05	N/A	231.1 ± 19.9	N/A
P1983	0.17 ± 0.03	N/A	213.3 ± 38.8	N/A
P2241	0.15 ± 0.07	0.73	212.2 ± 73.7	0.989

Data shown is the average of three biological repeats each consisting of ten technical replicates.

The T-tests performed were two-tailed and compared the growth of the transconjugant to that of the recipient during log phase (3-7 hours) to determine whether the presence of plasmid pBla1 had an effect on growth.

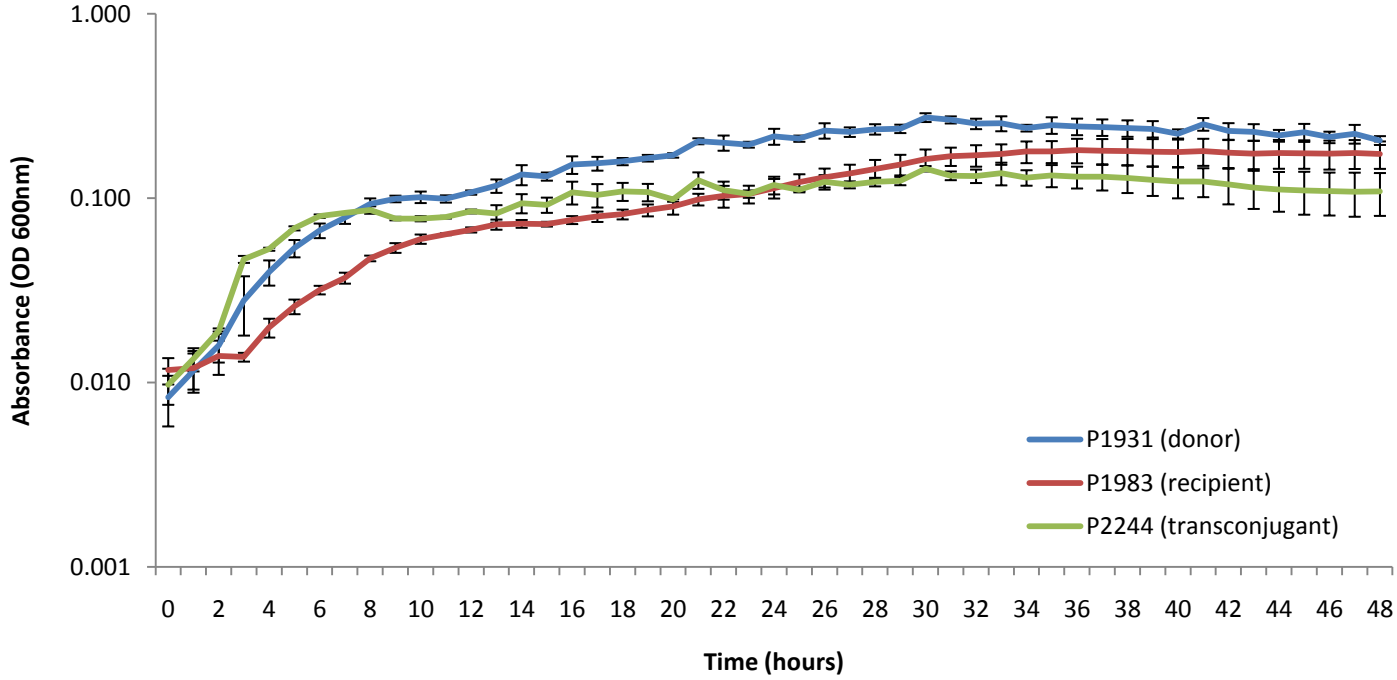
There was also no significant difference between the final absorbance of the pBla2 transconjugant P2244 and the recipient P1983 (P = 0.115); neither was there any difference in generation time during log phase (P = 0.191) (Figure 5.8). However, it is also clear by examination of these graphs that the log phase of the recipient strain is delayed by approximately four hours when compared to the donor and transconjugant strains (Figure 5.7 and 5.8).

5.5 Discussion

Preliminary experiments performed previously in this laboratory (unpublished data) suggested that some strains of *Campylobacter* may be able to transfer ampicillin resistance alongside tetracycline resistance during conjugation. Two plasmids; pBla1 from *C. coli* P1131 and pBla2 from *C. coli* P1931 have been successfully transferred to an ampicillin and tetracycline sensitive plasmid free strain of *C. jejuni,* P1983. The conjugation frequency of the plasmids was found to be $1.2X10^{-8}$ and $4.03X10^{-7}$ respectively, this transfer frequency is much less (approximately 100 to 1000 times less) than the conjugation frequency of the control plasmid, pTet, which was found to be $3X10^{-4}$, the same level as found in other studies (Batchelor, Pearson et al. 2004). The lower frequency of transfer may be plasmid specific or due to the fact that the transfer of pBla1/2 was from a *C. coli* donor to a *C. jejuni* recipient. Further experiments would be required to determine which is the case and would include transferring the plasmids from a *C. jejuni* donor to a *C. jejuni* recipient and from a *C. coli* host to a *C. coli* recipient.

An acknowledged problem of assessing conjugation in *Campylobacter* is the fact that the bacteria are naturally competent and so able to acquire DNA without conjugation taking

Figure 5.8 Growth kinetics of pBla2 donor, recipient and transconjugant



Strain	Final OD_{600}	*P* value	Generation time (minutes)	*P value*
P1931	0.21 ± 0.01	N/A	186.08 ± 40.62	N/A
P1983	0.17 ± 0.03	N/A	213.32 ± 67.2	N/A
P2244	0.11 ± 0.03	0.115	277.34 ± 21.9	0.191

Data shown is the average of three biological repeats each consisting of ten technical replicates.

The T-tests performed were two-tailed and compared the growth of the transconjugant to that of the recipient during log phase (3-7 hours) to determine whether the presence of plasmid pBla2 had an effect on growth.

place. In order to ensure that effects seen are the result of conjugation a method of distinguishing 'donor' and 'recipient' strains must be established (Bacon, Alm et al. 2002).

In this study *flaA* typing was use to distinguish between the plasmid donors P1131 (*flaA* 18) and P1931 (*flaA* 66) and the plasmid free recipient strain, P1983 (*flaA* 239). All putative transconjugants were the same *flaA* type as the recipient strain (*flaA* 239) therefore conjugation was confirmed as the mechanism of DNA transfer.

The MIC data obtained confirm that the preliminary data which suggested that ampicillin resistance had co-transferred with tetracycline resistance was correct. Plasmids pBla1 and pBla2 appear to transfer resistance to tetracycline and the beta-lactams penicillin, ampicillin and in the case of pBla1, oxacillin. The level of resistance in the transconjugants was equivalent to that of the donor stains.

Data from the experiments with nitrocefin suggest that the transferrable beta-lactam resistance determinant is a beta-lactamase as the recipient strain had no beta-lactamase activity whereas the transconjugants containing pBla1 and pBla2 did.

The plasmid Bla1 also appears to confer resistance to nalidixic acid and ciprofloxacin. This was unexpected as resistance to fluoroquinolone antibiotics in *Campylobacter* is usually through mutation of DNA gyrase (*gyrA*) (Power, Munoz-Bellido et al. 1992; Smith and Fratamico 2010). Natural transformation of the *gyrA* gene from the 'conjugation recipient' to the 'conjugation donor' was originally thought to be the cause of the fluoroquinolone resistance in the transconjugant strains but as *flaA* typing confirmed that the mutants were created by conjugation, this appears not to be the case. However unlikely, it is not impossible that both conjugation and natural transformation have taken place between the

same bacteria. Further experimentation including the sequencing of the *flaA* gene and the pBla1 plasmid would be necessary to identify the source of the fluoroquinolone resistance.

Growth kinetics data generated using the Bioscan spectrophotometer indicates that acquisition of either plasmid Bla1 or Bla2 had no affect on generation time or final absorbance suggesting that presence of these plasmids does not adversely affect the growth of the host bacterium; strains containing either plasmid pBla1 or pBla2 actually appeared to enter a logarithmic phase of growth approximately four hours before the equivalent strain lacking any plasmids. Having no detrimental effect on their host bacterium's growth may aid the persistence of theses plasmids.

The Bioscan spectrophotometer which was used to generate the growth kinetics data in this study does not regulate the atmosphere within the unit therefore growth is dependent on the atmosphere within the microtitre tray remaining constant and is unlikely to result in optimal growth.

Future Work

- Sequence the plasmids pBla1 and pBla2. Once assembled and annotated the sequence of these plasmids would be analysed to identify the gene(s) responsible for the transferrable beta-lactam resistance.
- Following the sequencing of pBla1 and pBla2 assess how similar the two plasmids are, determine whether they share a common ancestor or whether two distinct beta-lactam resistance plasmids are circulating within *Campylobacter* in UK poultry flocks.
- Screen the panel of *Campylobacter* isolates gathered from Defra funded studies VM0220 and OZO501 for the presence of pBla1 and pBla2 in order to determine how prevalent

the plasmids are within *Campylobacter* isolated from UK chicken flocks. Also acquire and screen *Campylobacter* isolated from other sources.

- Conjugate pBla1 and pBla2 from between two isolates of *C. jejuni* and between two isolates of *C. coli* to determine whether *Campylobacter* species is a restriction barrier for conjugation of these plasmids.
- Sequence the *gyrA* genes of donor, recipient and transconjugant strains to determine the source of the fluoroquinolone resistance.

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Chapter 6 – Results and Discussion

The oxacillinases of *Campylobacter*

6.1 Background

For the last twenty five years beta-lactamases have been thought to be the main mechanism responsible for beta-lactam resistance in *Campylobacter* (Fliegelman, Petrak et al. 1985; Lucain, Goosses et al. 1985). Beta-lactamases have been consistently found to be produced by a large proportion of *Campylobacter* isolates, often around 90% of ampicillin or amoxicillin resistant isolates test positive for beta-lactamase production (Fliegelman, Petrak et al. 1985; Lariviere, Gaudreau et al. 1986; Tajada, Gomez-Garces et al. 1996; Elviss, Williams et al. 2009; Griggs, Peake et al. 2009). When the activity profile of *Campylobacter* beta-lactams is investigated they are found to be active against the penicillin type beta-lactamases but show weak to no hydrolysis of the cephalosporins and imipenem (Lucain, Goosses et al. 1985; Lachance, Gaudreau et al. 1991; Alfredson and Korolik 2005; Griggs, Peake et al. 2009).

The first *Campylobacter* beta-lactamase protein to be identified was an Ambler Class D beta-lactamase and was assigned the classification of OXA-61 from the Lahey clinic (http://www.lahey.org/studies/)(Alfredson and Korolik 2005). OXA-61 was found to vary by only one amino acid substitution, Glu→Gly at position 202, when compared to the predicted putative Class D beta-lactamase encoded by the *cj0299* gene of *Campylobacter jejuni* NCTC11168, now known as OXA-193 (Alfredson and Korolik 2005; Griggs, Peake et al. 2009).

With the whole genome sequences of twenty one *Campylobacter jejuni* isolates, two *Campylobacter coli* isolates and three *Campylobacter lari* isolates now available it is now possible to determine how common it is for these isolates to carry an oxacillinase.

6.2 Aims and Hypotheses

It was hypothesised that most *Campylobacter jejuni* would encode an oxacillinase on their genome.

The aims of this work were to:

a) Determine which sequenced isolates encode an oxacillinase.

b) Determine how varied these *Campylobacter* oxacillinases are from each other and from other known beta-lactamases.

c) Determine whether the genes encoding these *Campylobacter* oxacillinases are in a similar genomic context to each other.

d) Determine whether the presence of *Campylobacter* oxacillinases is always linked to resistance to penicillin-type beta-lactams.

6.3 Do all sequenced *Campylobacter* isolates encode an oxacillinase?

Of the twenty four *Campylobacter jejuni, coli* and *lari* genome sequences listed in GenBank, only eighteen could be used when BLAST searching, the remaining six were excluded from further study as their beta-lactamase genes were not accessible for analysis. *C. jejuni* strains GC015, P854, P852 were also included for comparison as they encode the oxacillinases OXA-61, OXA-184 and OXA-185 (Chapter 4)(Alfredson and Korolik 2005).

The *C. jejuni* NCTC11168 oxacillinase protein, OXA-193, was used to query the seventeen *Campylobacter* genomes (using protein BLAST) to determine whether they encoded an oxacillinase protein.

Of the *C. jejuni, C. coli and C. lari* genome sequences submitted to GenBank available for BLAST searching and including *C. jejuni* NCTC11168, GC015, P854 and P852, the majority, 66% (14/21) encode an oxacillinase (Table 6.1). The oxacillinase encoded by *C. jejuni* NCTC11168, OXA-193, was the most common *Campylobacter* oxacillinase, 50% (7/14) of isolates with an oxacillinase gene encode an oxacillinase protein indistinguishable from this one (Table 6.1 and Table 6.2). The strains which encode OXA-193 include both *C. jejuni* and *C. coli* and were isolated from both human and ovine sources showing that it is not found in only a single *Campylobacter* species or animal host (Table 6.2)

There are seven other distinct oxacillinases encoded by *Campylobacter,* these vary in size from 248 to 257 amino acids in length and when compared to OXA-193 share between 52% and 99% identity with the protein (Table 6.3). The oxacillinase of *C. jejuni* GC015 and OXA-61 have only one amino acid difference when compared to OXA-193, although the substituted amino acid is different in each case, both of these therefore share 99% identity with OXA-193 (Table 6.3 and Figure 6.1). The oxacillinase of *C. jejuni* RM1221 also shares 99% identity with OXA-193, in this instance however the protein is four amino acids shorter than OXA-193 and there is an amino acid substitution in the final residue of the RM1221 oxacillinase when compared to the amino acid at position 253 of OXA-193 (Table 6.3 and Figure 6.1). The remaining three oxacillinases from *C. jejuni* (1336, P854 and P852) are 248-249 amino acids in length and share only 65% (C1336_000060032) and 64% (OXA-184 and OXA-185) identity with OXA-193; similarly the oxacillinase from *C. lari*

Table 6.1 All *Campylobacter jejuni*, *coli* and *lari* genomes listed in GenBank and whether or not they encode an oxacillinase

Campylobacter strain	Putative oxacillinase?	GenBank Accession No.	Similarity to Cj0299 (Identity)
Campylobacter jejuni GC015	Yes (OXA-61)	*	99%
Campylobacter jejuni P854	Yes (OXA-184)	*	64%
Campylobacter jejuni P852	Yes (OXA-185)	*	64%
Campylobacter jejuni subsp. jejuni NCTC 11168	Yes (OXA-193)	YP_002343737	100% **
Campylobacter jejuni subsp. jejuni IA3902	Yes	ADC27923	100%
Campylobacter jejuni subsp. jejuni 81116	Yes	YP_001481852	100%
Campylobacter jejuni subsp. jejuni 84-25	Yes	ZP_01100440	100%
Campylobacter jejuni subsp. jejuni CF93-6	Yes	ZP_01067390	100%
Campylobacter jejuni subsp. jejuni CG8486	Yes	ZP_01810676	100%
Campylobacter coli JV20	Yes	ZP_07402011	100%
Campylobacter jejuni subsp. jejuni HB93-13	Yes	ZP_01072081	99%
Campylobacter jejuni RM1221	Yes	YP_178363	99%
Campylobacter jejuni subsp. jejuni 1336	Yes	ZP_06373212	65%
Campylobacter lari RM2100	Yes	YP_002575950	52%
Campylobacter jejuni subsp. jejuni 260.94	No	N/A	N/A
Campylobacter jejuni subsp. jejuni BH-01-0142	No	N/A	N/A
Campylobacter jejuni subsp. jejuni CG8421	No	N/A	N/A
Campylobacter jejuni subsp. jejuni 414	No	N/A	N/A
Campylobacter jejuni subsp. jejuni 81-176	No	N/A	N/A
Campylobacter jejuni subsp. doylei 269.97	No	N/A	N/A
Campylobacter coli RM2228	No	N/A	N/A
Campylobacter jejuni subsp. jejuni 305	***	N/A	N/A
Campylobacter jejuni subsp. jejuni 327	***	N/A	N/A
Campylobacter jejuni subsp. jejuni DFVF1099	***	N/A	N/A
Campylobacter jejuni subsp. jejuni ICDCCJ07001	***	N/A	N/A
Campylobacter jejuni subsp. jejuni M1	***	N/A	N/A
Campylobacter jejuni subsp. jejuni S3	***	N/A	N/A

* Genome not submitted to GenBank

** Is OXA-193

*** Genome listed in GenBank but not available to be searched using the Basic Local Alignment Search Tool (BLAST)

The presence or absence of an oxacillinase was determined by using the sequence of the *C. jejuni* NCTC11168 beta-lactamase (OXA-193) to interrogate the test genome using Protein BLAST. All data from GenBank (http://www.ncbi.nlm.nih.gov/) last accessed 06/09/2010.

Table 6.2 *Campylobacter* oxacillinases identical to OXA-193

GenBank Accession No.	Organism	Strain	Origin	Locus Tag	Description of protein	Reference
YP_002343737	*C. jejuni*	NCTC 11168	Human	Cj0299	putative periplasmic beta-lactamase	(Parkhill, Wren et al. 2000; Gundogdu, Bentley et al. 2007)
ADC27923	*C. jejuni*	IA3902	Sheep , aborted placenta	CJSA_0273	putative periplasmic beta-lactamase	
YP_001481852	*C. jejuni*	81116	Human	C8J_0276	putative periplasmic beta-lactamase	(Pearson, Gaskin et al. 2007)
ZP_01810676	*C. jejuni*	CG8486	Human	Cj8486_0288	putative periplasmic beta-lactamase	(Poly, Read et al. 2007)
ZP_01100440	*C. jejuni*	84-25	Unknown	CJJ8425_0323	beta-lactamase	
ZP_01067390	*C. jejuni*	CF93-6	Unknown	CJJCF936_0321	beta-lactamase	
EFM36662	*C. coli*	JV20	Human	HMPREF9399_1369	possible beta-lactamase	

All of the above have the following protein sequence (257 aa):

MKKITLFLLFLNLVFGQDKILNNWFKEYNTSGTFVFYDGKTWASNDFSRAMETFSPASTFKIFNALIALDSGVIKTKKEIFYHYRGEKVFLSSWAQDMNLSSAIKYSNVLA
FKEVARRIGIKTMQEYLNKLHYGNAKISKIDTFWLDNSLKISAKEQAILLFRLSQNSLPFSQEAMNSVKEMIYLKNMENLELFGKTGFNDEQKIAWIVGFVYLKDENKYKA
FALNLDIDKFEDLYKREKILEKYLDELVKKVKNDG

All data from GenBank (http://www.ncbi.nlm.nih.gov/) last accessed 06/09/2010.

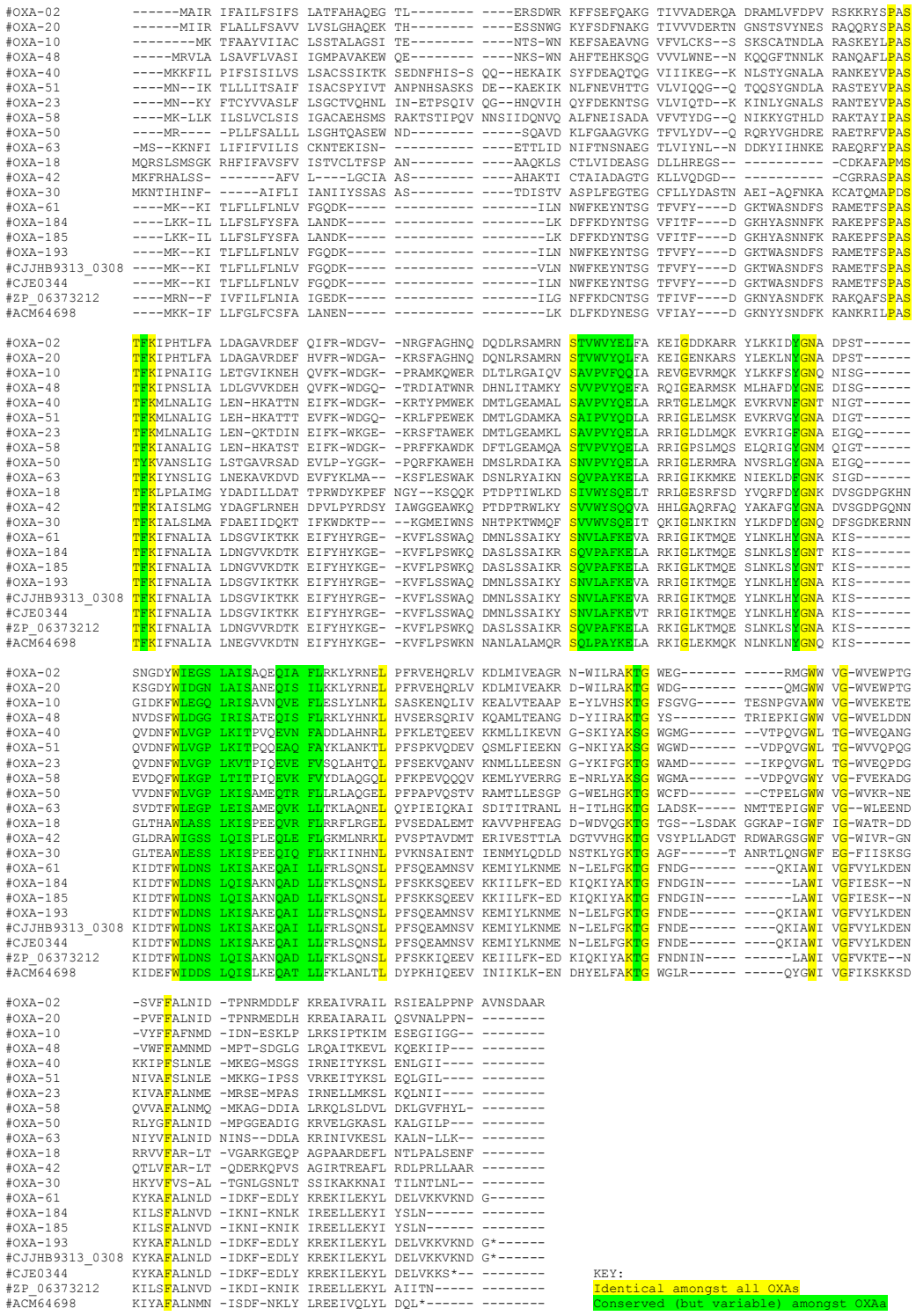
Table 6.3 *Campylobacter* oxacillinases not identical to OXA-193

GenBank Accession No.	Organism	Strain	Locus Tag	Description of protein	Number of amino acids	Similarity to Cj0299 (Identity)	Reference
AAT01092	*C. jejuni*	GC015	*	OXA-61	257	99%	(Alfredson and Korolik 2005)
ZP_01072081	*C. jejuni*	HB93-13	CJJHB9313_0308	class D beta-lactamase	257	99%	
YP_178363	*C. jejuni*	RM1221	CJE0344	beta-lactamase	253	99%	(Fouts, Mongodin et al. 2005)
ZP_06373212	*C. jejuni*	1336	C1336_000060032	hypothetical protein	249	65%	
**	*C. jejuni*	P854	P854_1490	OXA-184	248	64%	This study
**	*C. jejuni*	P852	*	OXA-185	248	64%	This study
ACM64698	*C. lari*	RM2100	Cla_1383 / CLA0304	conserved hypothetical protein, possible beta-lactamase	248	52%	

* Full genome not available therefore no locus tag has been described

**Sequence not yet submitted to GenBank

Figure 6.1 An alignment of oxacillinases including the putative *Campylobacter* oxacillinases



All the putative *Campylobacter* oxacillinases align with known oxacillinases and contain all the identical and conserved/variable regions associated with an oxacillinase (Poirel, Naas et al. 2010).

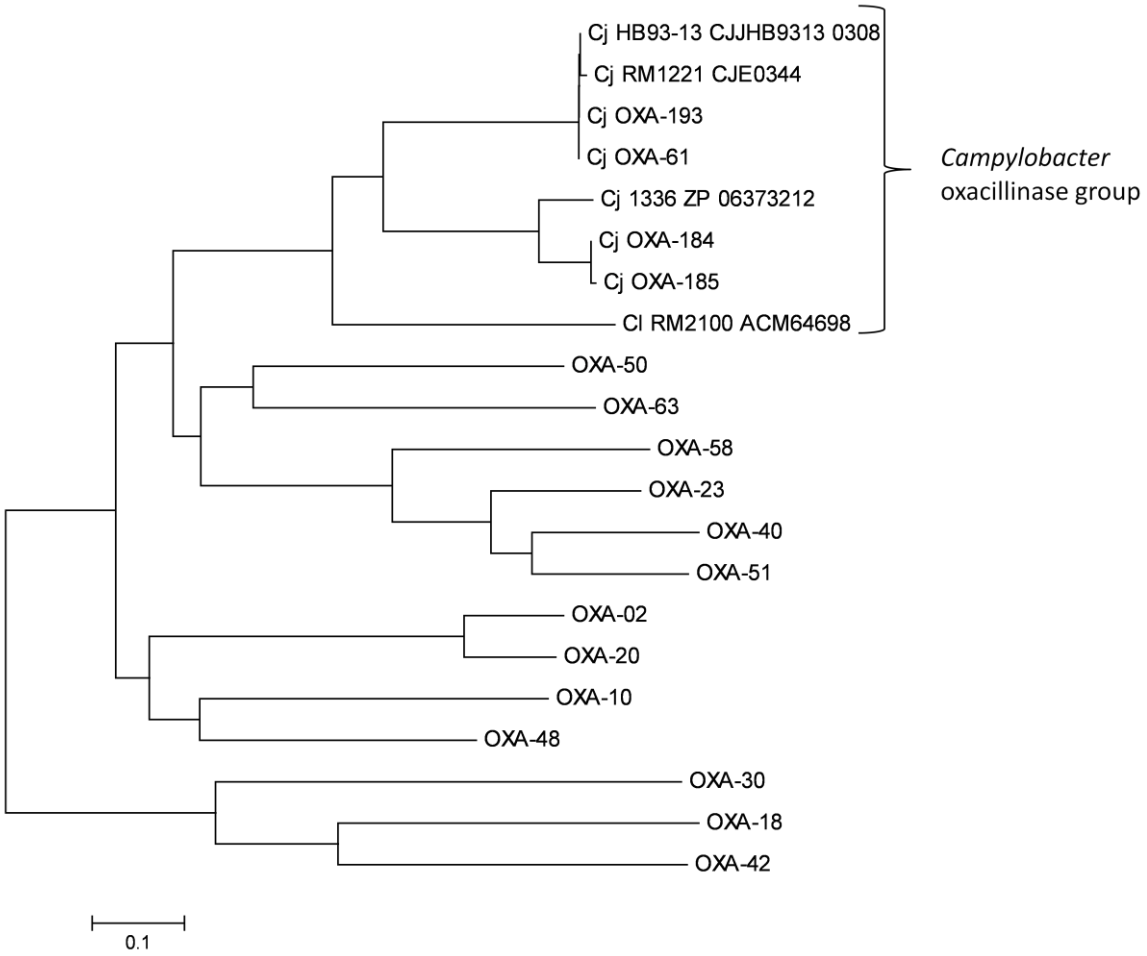
RM2100 is 248 amino acids in length and shares 52% identity with OXA-193 (Table 6.3 and Figure 6.1). The identified *Campylobacter* oxacillinases were aligned by their amino acid sequences to a selection of oxacillinases (covering all the evolutionary groups of oxacillinases). When aligned, the *Campylobacter* oxacillinases contained all the identical and conserved/variable regions of an oxacillinase, including the active site tetrad STFK (Figure 6.1).

A neighbour-joining tree was created based on the amino acid sequences of the oxacillinases (Figure 6.2). The *Campylobacter* beta-lactamases formed a group distinct from other oxacillinases of different origin. The *C. jejuni* 1136 oxacillinase, OXA-184 and OXA-185, which share 64-65% homology to OXA-193, are grouped together as a separate sub-group to the oxacillinases which share 99% homology to OXA-193 (Figure 6.2). The *C. lari* oxacillinase shares a similar level of homology to OXA-193 (52%) as the oxacillinases of *C. jejuni* 1336, OXA-184 and OXA-185. This oxacillinase however is evolutionally distinct and does not cluster directly with them (Figure 6.2). Despite this the *Campylobacter* oxacillinases as a group are still very much distinct from oxacillinases isolated from other organisms.

6.4 Are all *Campylobacter* oxacillinase genes within the same genomic context?

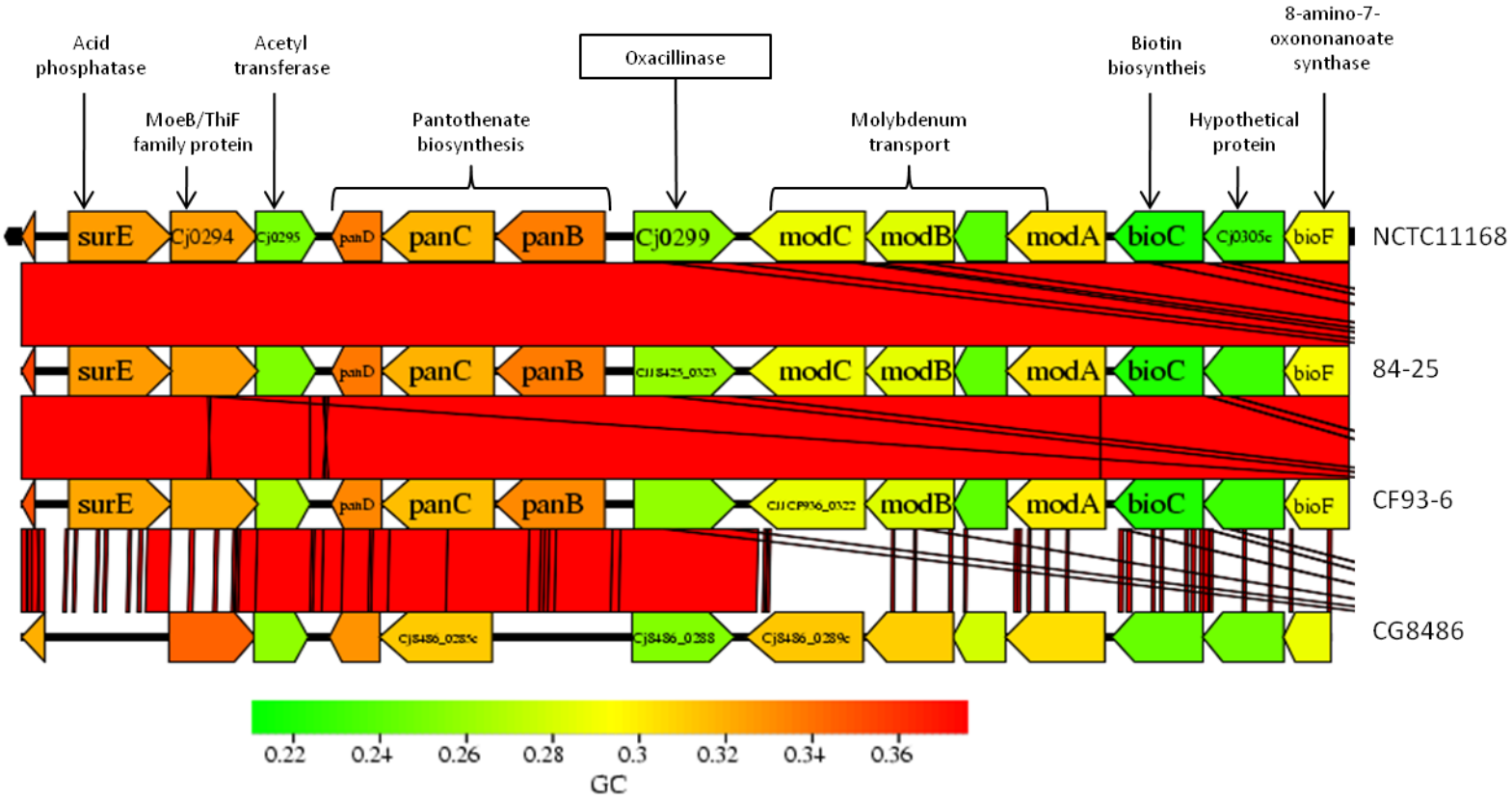
When the oxacillinase gene containing region of *C. jejuni* NCTC11168 was aligned with the corresponding region of the genomes of some *C. jejuni* strains which encode identical oxacillinases, the beta-lactamase gene was located in approximately the same region on each genome (Figure 6.3). However, there were some differences between the regions; the

Figure 6.2 The evolutionary relationship between *Campylobacter* oxacillinases and those derived from other species



Neighbour-Joining tree of oxacillinase proteins produced using MEGA 4(Tamura, Dudley et al. 2007).

Figure 6.3 The genomic context of genes encoding *Campylobacter* oxacillinases identical to OXA-193



An alignment of the oxacillinase containing region of *C. jejuni* NCTC11168 (269064:279358) with three other *C. jejuni* genomes, each of which encodes an oxacillinase identical to Cj0299 of *C. jejuni* NCTC11168. Figure produced using xBase (http://xbase.bham.ac.uk).

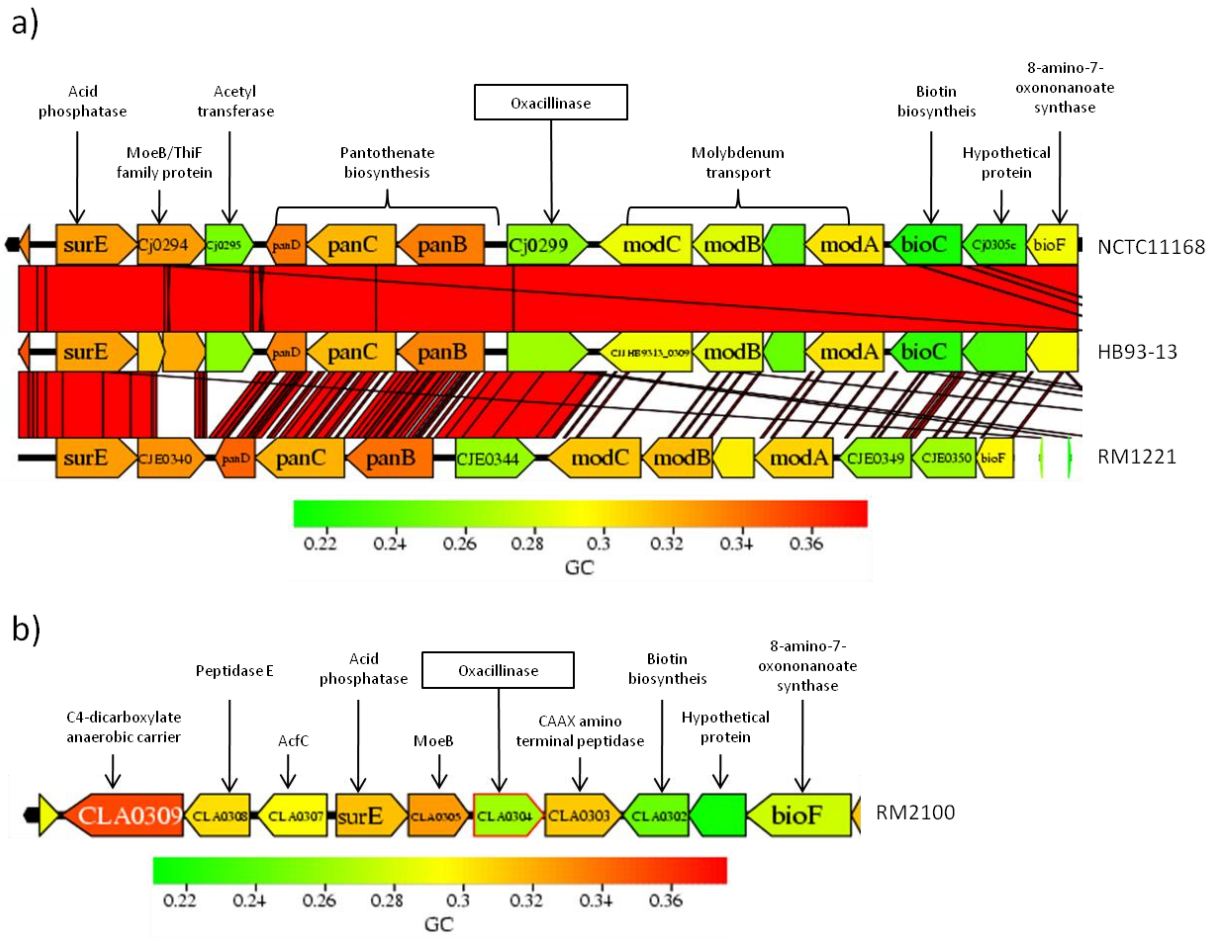
alignment *C. jejuni* CG8486 revealed that it lacks the *panB* gene (upstream of $bla_{OXA-193}$ in *C. jejuni* NCTC11168) from the pantethonate biosynthesis operon *panBCD* (Figure 6.3).

Although *panB* is not annotated on the genome of CG8486, BLAST searching revealed it to be present in the correct region of the genome (GenBank accession number AASY01000000). When the genomes from strains which produce different oxacillinases were compared the beta-lactamase genes were seen in approximately the same position within their respective genomes, however there were some differences in the region as a whole. *C. jejuni* HB93-13 contains two truncated *moeB/thiF* family genes rather than one (Figure 6.4a). The GC content of the *modB* and *modC* genes (GC=32%) of *C. jejuni* RM1221 was higher than the corresponding genes in *C. jejuni* NCTC1168 (GC=29%) and HB93-13 (GC=29%), although the genes were in the same location (Figure 6.4a). *C. jejuni* RM1221 also lacks the acetyl transferase gene upstream of the beta-lactamase gene which is conserved within the other *C. jejuni* genomes (Figure 6.3 and Figure 6.4a).

The structure of the region containing the beta-lactamase gene was different in the *C. lari* RM2100 isolate. Nonetheless some of the surrounding genes echoed those present in the corresponding region of *C. jejuni* NCTC11168; *surE, moeB* and a hypothetical protein between a *bioC* and a *bioF* gene were common to both *C. lari* and *C. jejuni* (Figure 6.4b).

One example of an isolate which lacked an oxacillinase gene was aligned with the oxacillinase containing region of *C. jejuni* NCTC11168; as well as lacking an oxacillinase gene, *C. jejuni* 260.94 also lacked an acetyl transferase gene upstream of the usual oxacillinase site, and so this region is similar to that in *C. jejuni* RM1221 (Figure 6.4a and Figure 6.5).

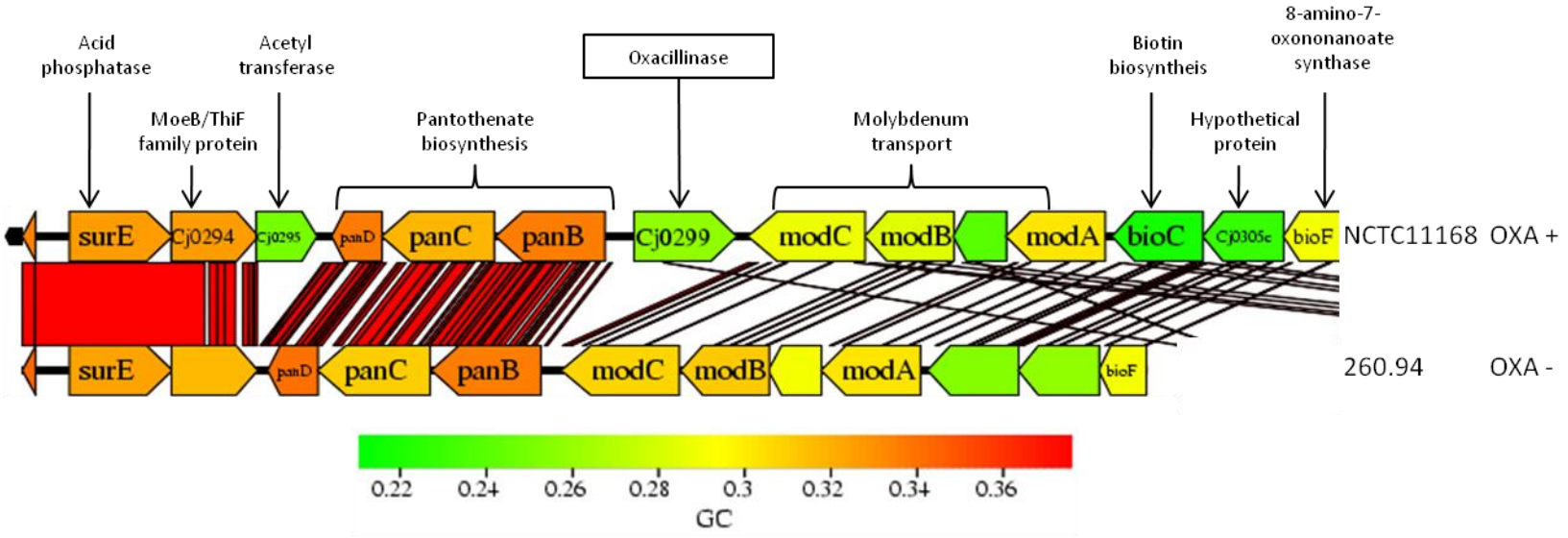
Figure 6.4 The genomic context of *Campylobacter* oxacillinases genes encoding oxacillinases distinct from OXA-193



a) An alignment of the oxacillinase containing region of *C. jejuni* NCTC11168 (269064:279358) with two other *C. jejuni* genomes, each of which encodes an oxacillinase not identical to $bla_{OXA\text{-}193}$ of *C. jejuni* NCTC11168.

b) The oxacillinase containing region of *C. lari* RM2100 (147486:155891).
Figure produced using xBase (http://xbase.bham.ac.uk).

Figure 6.5 The oxacillinase containing region of *C. jejuni* NCTC11168 aligned with the corresponding region of a strain that does not encode an oxacillinase



An alignment of the oxacillinase containing region of *C. jejuni* NCTC11168 (269064:279358) with *C. jejuni* 260.94 an isolate that does not encode an oxacillinase.
Figure produced using xBase (http://xbase.bham.ac.uk).

6.5 Is the presence of *Campylobacter* oxacillinases always associated with resistance to penicillin type beta-lactams?

A database containing 4678 poultry isolates of *Campylobacter* from two Defra funded studies was used to select *Campylobacter* isolates for further study (Griggs, Johnson et al. 2005; Humphrey, Jorgensen et al. 2005; Piddock, Griggs et al. 2008). Isolates that were recorded to (i) contain an oxacillinase gene (detectable by PCR using primers for $bla_{OXA-193}$), (ii) lack beta-lactamase activity detectable by a spot test with nitrocefin and also (iii) ampicillin sensitive (minimum inhibitory concentration ≥16 μg/ml) were identified. Eight isolates contained an oxacillinase gene by DNA sequencing, lacked beta-lactamase activity and were ampicillin sensitive (Table 6.4) (McDermott, Bodeis et al. 2004).

6.6 Discussion

The production of beta-lactamases by *Campylobacter* has long been linked to resistance to penicillin type beta-lactams (Lucain, Goosses et al. 1985; Lachance, Gaudreau et al. 1991; Alfredson and Korolik 2005; Griggs, Peake et al. 2009). Following the identification of an oxacillinase type beta-lactamase (OXA-61) from a clinical isolate of *C. jejuni* and genome sequencing of over 20 strains of *Campylobacter* a better understanding of the diversity and context of genomically encoded *Campylobacter* oxacillinases can be obtained (Parkhill, Wren et al. 2000; Alfredson and Korolik 2005; Fouts, Mongodin et al. 2005; Pearson, Gaskin et al. 2007; Poly, Read et al. 2007).

The majority of *Campylobacter* (12/19) were found to contain a gene which encoded a putative oxacillinase. The most prevalent was the OXA-193 oxacillinase encoded by *C. jejuni* NCTC11168, 58% of those *Campylobacter* which contained a beta-lactamase gene encoded a

Table 6.4 *Campylobacter* isolates found to contain an oxacillinase gene and be sensitive to ampicillin

Isolate	Species	Sequenced beta-lactamase gene	Beta-lactamase activity	Minimum Inhibitory Concentration (μg/ml)		
				ampicillin	penicillin	oxacillin
NCTC11168	*C. jejuni*	Yes, $bla_{OXA-193}$	Negative	4	8	64
P323	*C. coli*	Yes, identical to $bla_{OXA-193}$	Negative	8	32	128
P324	*C. coli*	Yes, identical to $bla_{OXA-193}$	Negative	8	32	512
P851	*C. jejuni*	Yes, identical to $bla_{OXA-193}$	Negative	4	16	512
P1102	*C. jejuni*	Yes, identical to $bla_{OXA-193}$	Negative	8	16	512
P1154	*C. jejuni*	Yes, identical to $bla_{OXA-193}$	Negative	4	16	512
P1197	*C. jejuni*	Yes, identical to $bla_{OXA-193}$	Negative	4	16	512
P1199	*C. jejuni*	Yes, identical to $bla_{OXA-193}$	Negative	8	16	512

Ampicillin break point (assigned by laboratory); Minimum inhibitory concentration ≥16 μg/ml. Minimum inhibitory concentrations represent the modal average from three biological replicates. Beta-lactamase activity was confirmed by three positive biological replicates of nitrocefin spot test.

protein identical to this one. Other *Campylobacter* oxacillinases shared 52-99% homology with OXA-193.

When the oxacillinases of *Campylobacter* were compared with other known oxacillinases, they form a distinct '*Campylobacter* oxacillinase group' distinct from the other oxacillinases. The proteins with 99% homology to OXA-193 unsurprisingly form a cluster with OXA-193. The oxacillinases from *C. jejuni* P854 and 1336 form a cluster of their own, the *C. lari* RM2100 oxacillinase, although of a similar size and homology to OXA-193 appears to have evolved separately from the *C. jejuni* oxacillinases, perhaps as the species themselves have diverged.

The oxacillinases of *Campylobacter* are quite varied, sharing as little as 64% homology with one another. As a single amino acid change denotes a new enzyme and would be entitled to an OXA number from the Lahey clinic (Jacoby and Bush 2010), a difference of 64% is striking. Despite the breadth of oxacillinase protein sequences encoded by *Campylobacter*, there was a dominance of OXA-193 and OXA-193-like beta-lactamases. This could be due to these enzymes having an improved spectrum or rate of activity, alternatively this could simply reflect an unknown sequencing bias in terms of the isolates which are being sequenced.

All of the oxacillinase genes are present within approximately the same genomic context, although this region was flexible. As well as maintaining a range of possible beta-lactamase genes or lack of a beta-lactamase gene, other genes varied in their GC content such as the *modABC operon*, whereas others such as the acetyl transferase (in *C. jejuni* RM1221) and the *panABC* operon (in P854 and 1336) were not always present. This region also appears to be able to gain potentially useful genes; *C. jejuni* 1336 has acquired an extra *cdt* operon (GenBank accession number C1336_000060027-9) which codes for the known

Campylobacter virulence factor, cytolethal distending toxin (Frisan, Cortes-Bratti et al. 2002; Biswas, Fernando et al. 2006; Lindmark, Rompikuntal et al. 2009). *C. jejuni* 1336 also contains a *cdt* operon in its usual position within the genome, which is distinct in sequence to the extra copy, i.e. the operon is not simply duplicated suggesting that the 'extra' *cdt* could have been acquired from a different source. The *cdtA* gene in the usual position appears to be truncated so it is hypothesised that the protein encoded by this gene is not fully functional. If this is indeed the case, then the presence of another copy of this gene would be an advantage to the bacterium.

It has also been noted that presence of an oxacillinase gene does not necessarily lead to ampicillin resistance. Seven *Campylobacter* poultry isolates and the reference strain *C. jejuni* NCTC1168 were all found to contain oxacillinase encoding genes, yet the MIC values of ampicillin were below the laboratory breakpoint (16µg/ml) meaning these isolates were all susceptible to the drug. It may be the case that these isolates show little or no expression of their oxacillinase genes, further experiments would be able to clarify this point.

The flexibility of this region has been noted previously and may allow the bacterium to acquire advantageous genes from other bacteria (Parker, Quinones et al. 2006). *Campylobacter* are naturally competent, it has been demonstrated that they show a preference for the uptake DNA of *Campylobacter* origin (Wang and Taylor 1990) however if the bacteria are acquiring these oxacillinases from another species they have yet to be identified. Obtaining beta-lactamases from other species is a well described phenomenon, a good example of which is the mobilization of CTX-M type beta-lactamases from the genomes of three species of the soil dwelling bacterium *Kluyvera* (*K. georgiana, K. ascorbata* and *K.*

cryocrescens) onto plasmids which have been able to widely disseminate through the enterobacteriaceae (Hawkey 2008).

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Chapter 7 –Overall Discussion and Conclusions

The aim of this study was to better understand mechanisms of beta-lactam resistance in *Campylobacter.*

The original hypotheses to be investigated during this study included the following:

- beta-lactam resistance can be conferred by the efflux pumps CmeABC and/or CmeDEF
- a novel beta-lactamase(s), CjBla2, which is distinct from the characterised OXA-61 and OXA-193 of *C. jejuni,* are responsible for beta-lactam resistance in *Campylobacter.*
- the *purU* gene encodes the novel beta-lactamase CjBla2
- beta-lactam resistance may be transferred alongside tetracycline resistance in *Campylobacter*
- penicillin binding proteins may play a role in beta-lactam resistance in *Campylobacter.*

In other bacterial species functioning RND type efflux pumps have been shown to be necessary for resistant bacteria to be selected and for "full" bacterial resistance to be achieved (Ricci, Tzakas et al. 2006; Li and Nikaido 2009). In *Campylobacter* two multidrug efflux pumps CmeABC and CmeDEF have been identified and partially characterised in terms of their ability to efflux beta-lactams (Lin, Michel et al. 2002; Pumbwe and Piddock 2002; Pumbwe, Randall et al. 2004; Pumbwe, Randall et al. 2005). During my study I determined that not only does the efflux pump CmeABC play a role in the efflux of ampicillin (as had been previously described), but it is also responsible for the innate resistance of *Campylobacter* to the cephalosporin group of antibiotics. Similar findings were published in parallel to my work (Guo, Lin et al. 2010). To elucidate whether efflux is required for 'full'

beta-lactam resistance in *Campylobacter*, as in other species, (Ricci and Piddock 2009) multiple attempts were made to inactivate the *cmeB* gene in two, beta-lactamase producing *C. jejuni* isolates. However, no mutants were obtained.

Although efflux plays a role in innate beta-lactam resistance in *Campylobacter*, beta-lactamase production has been associated with ampicillin resistance in these bacteria for over 25 years (Fliegelman, Petrak et al. 1985; Lucain, Goosses et al. 1985). Studies have consistently shown that the majority of *Campylobacter* isolates (83-95%) produce beta-lactamases (Fliegelman, Petrak et al. 1985; Lariviere, Gaudreau et al. 1986; Tajada, Gomez-Garces et al. 1996) and that production of a beta-lactamase is associated with resistance to penicillin type beta-lactamases (Lachance, Gaudreau et al. 1991). In 2005 an oxacillinase, OXA-61, was identified in a human clinical isolate of *C. jejuni* and shown to have activity against penicillin, ampicillin and carbenicillin (Alfredson and Korolik 2005). It was then shown that an enzyme with a single amino acid substitution to OXA-61 (G→E at position 202) was encoded by the *cj0299* gene of *C. jejuni* NCTC11168 and was shown to be present in 91% (347/380) of ampicillin resistant poultry isolates (Griggs, Peake et al. 2009). During this study the number OXA-193 was assigned to Cj0299 by the Lahey clinic (http://www.lahey.org/studies/) upon my request (Jacoby and Bush 2010).

In the experiments completed preceding my study another beta-lactamase was identified from two poultry isolates of *C. jejuni,* P843 and P854, which had a different isoelectric point and molecular mass to that of OXA-61 or OXA-193 (both P843 and P854 had been shown to lack $bla_{OXA-193}$ by PCR), this novel beta-lactamase was termed CJBla2. Preliminary mass spectrometry of the protein had identified the predicted product of the *purU* gene (*cj0790*) as most likely to be CjBla2 (Griggs, Peake et al. 2009). During my study inactivation of *purU*

(*cj0790*) in one of the CjBla2 producing strains suggested that the gene was unlikely to encode CjBla2, as the *purU* mutant showed the same level of beta-lactamase activity as the parental strain. Southern blot analysis and further PCR for $bla_{OXA\text{-}193}$ determined that CjBla2 was not the product of a mutated $bla_{OXA\text{-}193}$ gene.

Following the full genome sequencing of CjBla2 producing strain P854, four putative beta-lactamase genes were identified. Only one of these, *P854_1490,* was novel. It was subsequently determined that the product of this gene was likely to be an oxacillinase and it was awarded the number OXA-184 from the Lahey clinic (http://www.lahey.org/studies/) (Jacoby and Bush, personal communication). The closest relative of OXA-184 was determined to be Cj0299 of *C. jejuni* NCTC11168; however the two proteins share just 64% identity. Screening a panel of fourteen isolates with the 'CjBla2 phenotype' (i.e. ampicillin resistant, detectable beta-lactamase activity but lacks amplimers following PCR of *cj0299*) revealed six of these (including P843) also encode OXA-184. A further six isolates encode a variant of OXA-184 which has a single leucine to isoleucine amino acid substitution, this variant was assigned the number OXA-185 from the Lahey clinic (Jacoby and Bush, personal communication). The remaining two isolates of the 'CjBla2' panel may encode either OXA-184 or OXA-185 but within a different genomic context as external gene primers based upon amplifying the genomic context of P854 did not produce amplimers following PCR.

The genes encoding OXA-184 and OXA-185 were within a similar genomic location to OXA-193. This region of the *Campylobacter* genome appears to tolerate a variety of oxacillinase genes or not contain any at all, this fits with previous findings that this region of the genome is hypervariable (Parker, Quinones et al. 2006).

As well as encoding an oxacillinase gene on their genome, data produced in my study suggest that beta-lactamase resistance may also be transferrable between isolates of *Campylobacter*. Two plasmids, pBla1 and pBla2, were identified from two poultry isolates of *C. coli*, P1131 and P1931, respectively. Conjugation experiments revealed that pBla1 and pBla2 were able to transfer resistance to tetracycline and the beta-lactams penicillin, ampicillin, oxacillin and cephalothin. Transconjugants were shown to have gained beta-lactamase activity suggesting that this beta-lactam resistance determinant is a gene encoding a beta-lactamase. Interestingly, experimental results also suggested that pBla1 transconjugants had acquired fluoroquinolone resistance, usually determined by a mutation in the genomically encoded *gyrA* gene. This is an intriguing result and warrants further investigation but was beyond the time-frame of this thesis. Sequencing of the plasmids pBla1 and pBla2 is now required to determine the gene(s) responsible for transferrable beta-lactam resistance.

Time limitations also did not allow for the study of the role of *Campylobacter* penicillin binding proteins (PBPs) in beta-lactam resistance.

Campylobacter is known to be the leading cause of bacterial gastroenteritis in the world. During the first quarter of 2010 the incidence of bacteriologically confirmed cases of Campylobacter disease in England and Wales had risen from 2158 cases (in 2009) to 2777 cases (HPA 2010). Recommended treatment for such cases is with the antibiotics erythromycin or ciprofloxacin (BNF 2009), although empirical treatment with ciprofloxacin is the most often administered despite some reports of 15% of isolates in England and Wales being resistant to ciprofloxacin and in some countries being as high as 90% (ECDC 2009). This study has highlighted the major role of oxacillinases in beta-lactam resistance in

Campylobacter. As 95% of *C. jejuni* isolates are susceptible to augmentin (a combination beta-lactam and beta-lactamase inhibitor) (ECDC 2009), augmentin may be useful in the treatment of *Campylobacter* infection.

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Publications resulting from this study

Original Articles

Deborah Griggs, **Leanne Peake**, Margaret Johnson, Saba Ghori, Alex Mott

Beta-lactamase-mediated beta-lactam resistance in *Campylobacter* species: prevalence of Cj0299 (*bla*$_{OXA-61}$) and evidence for a novel beta-lactamase in *C. jejuni*.

Antimicrobial Agents and Chemotherapy, August 2009, p.3357-64. Vol. 53, No. 8

Conference Presentations

Invited oral presentation: "β-lactam resistance in *Campylobacter jejuni*" **Leanne Stones**. British Society of Antimicrobial Chemotherapy, Antimicrobial Research Mechanisms workshop, November 2009.

Leanne Peake, Deborah J. Griggs, Margaret N. Johnson, Saba Ghori, Alexander Mott and Laura J.V. Piddock. B-lactamase mediated β-lactam resistance in *Campylobacter* species: Evidence for a novel β-lactamase in *Campylobacter jejuni*. Society of General Microbiology, Spring Meeting, 2009. Poster number HAR30/06.

Appendix A

Functional Genomics (University of Birmingham) protocol for setting up sequencing reactions

- Prepare your DNA using a recommended isolation kit in the normal way.
- Quantify the DNA to check the concentration is correct using the table below as a guide.
- Add your Primer to the DNA template and make up to a total volume of 10ul.

Template	Quantity
PCR product	
100-200bp	1-3ng
200-500bp	3-10ng
500-1000bp	5-20ng
1000-2000bp	10-40ng
>2000bp	40-100ng
Single Stranded DNA	50-100ng
Double Stranded DNA	200-500ng
Cosmid, BAC	0.5-1.0ug
Bacterial Genomic	2-3ug

Example:

Double stranded DNA	200 - 500 ng
Primer	3.2 pmol
Sterile water	q.s
Total Volume	10ul

PCR Conditions Used.

96°C	10 secs	X25
50°C	5 secs	
60°C	4 mins	

Primer Annealing Temperature = 55°C – 60°C

Check your primers annealing temperature. To use this service your primer annealing temperature needs to be between 50°C to 65°c

Appendix B

Southern Blotting Recipes

Depurination solution

Distilled water	987.5 mls
10M HC1	12.5 mls

Denaturing solution

NaCl	87.7g
NaOH	20.0g
Water to	1 litre

Neutralizing solution

NaC1	87.7g
Tris	60.6g
EDTA (Na_2 salt)	0.37g

Adjust to pH 7.2 with 10M HCL

Water to	1 litre

20X SSC

NaCl	175.3g
Sodium Citrate	88.2g

Adjust pH to 7.0 with 10M NaOH

Adjust volume to 1L

Hybridisation buffer (Supplied with kit)

Add NaCl to a final concentration of 0.5M

Add 4% (w/v) blocking reagent

Add reagents and mix at room temperature for 1h (can aliquot and store at –20 C)

Primary wash buffer (1L)

Urea	120g
SDS	1g
0.5M NaP04*	100ml
NaCl	8.7g
1.0M MgCl2	1ml
Blocking reagent	2g

*can make by adjusting pH of sodium dihydrogen phosphate to 7 with NaOH

Secondary wash buffer (20X stock)

Tris	121g
NaCl	112g

For (1X) secondary wash buffer:

Dilute stock 1:20 and add 2ml/L of 1M MgCl2

Appendix C

Antibiotic concentrations for agar doubling dilutions

The table below shows how much of a particular stock should be added to 20ml of molten agar in order to achieve the required final concentration of antibiotic in the agar plate.

Final concentration required (µg/ml)	1024	512	256	128	64	32
Volume of 10,000µg/ml stock to add	2048	1024	512	256	128	64
Final concentration required (µg/ml)	16	8	4	2	1	
Volume of 1000µg/ml stock to add	320	160	80	40	20	
Final concentration required (µg/ml)	0.5	0.25	0.12	0.06		
Volume of 100µg/ml stock to add	100	50	25	13		
Final concentration required (µg/ml)	0.03	0.015	0.008			
Volume of 10µg/ml stock to add	60	30	15			

Appendix D

Recipe for SOC

1. Add the following to 900ml of distilled H_2O
    - 20g Bacto Tryptone
    - 5g Bacto Yeast Extract
    - 2ml of 5M NaCl.
    - 2.5ml of 1M KCl.
    - 10ml of 1M $MgCl_2$
    - 10ml of 1M $MgSO_4$
    - 20ml of 1M glucose
2. Adjust to 1L with distilled H_2O
3. Sterilize by autoclaving

Recipe obtained from http://www.thelabrat.com/protocols/15.shtml

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