Identification of *Bacteroides* Genes Involved in Metronidazole Resistance

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CONTENTS

ABSTRACT

Bacteroides species are Gram-negative obligate anaerobes that live in the gastrointestinal tract of mammals and are thought to account for approximately 30% of the colonic microbiota. Certain *Bacteroides* species, such as *B. fragilis* and to a lesser extent *B. thetaiotaomicron*, can become opportunistic pathogens and cause severe infection. The antibiotic of choice for treating such infections is metronidazole, a DNA damaging agent. Metronidazole enters the bacterial cell as an inert prodrug, and is activated by cellular reduction into a cytotoxic compound which is thought to cause DNA strand breaks. Certain metronidazole resistant *B. fragilis* strains have been described, where the drug was not reduced inside the cell due to decreased activity of the metabolic enzymes which are involved in this process. Little is known about the mechanisms involved in repair of metronidazole damage and the potential for resistance. In this study, two different approaches were used to isolate and analyse *Bacteroides* genes involved in metronidazole resistance, with emphasis on DNA repair genes. These methods were transposon mutagenesis of *Bacteroides*, and functional complementation of *E. coli* metronidazole sensitive mutants with genes from *B. fragilis*.

Construction of *B. fragilis* and *B. thetaiotaomicron* transposon mutant banks was performed. Screening of the *B. fragilis* mutant bank did not identify any metronidazole mutants. However, four *B. thetaiotaomicron* metronidazole mutants were isolated, Mutants A and B with increased sensitivity to metronidazole, and Mutants C and D with increased resistance to the drug. Mutant B contained a disruption within a glycosyltransferase gene located in one of the capsular polysaccharide loci, and whose product is involved in catalysing the first step of the pathway. This suggests the involvement of the *Bacteroides* capsule in entry or uptake of metronidazole. Mutant A was a double integration mutant derived from Mutant B, containing a second integration, a cointegrate, in a different unidentified chromosomal locus. Metronidazole resistant Mutant C contained a disrupted two-component histidine kinase, with a putative function related to cytosine-specific methyltransferases. The tranposon insertion in Mutant D was within an intergenic region between the *fucO* and the transcriptional regulator of the

putative rhamnose dissimilation gene cluster that is linked to pyruvate metabolism, suggesting decreased activation of the drug as a possible resistance mechanism.

Suppression of *E. coli* DNA repair mutants with a *B. fragilis* bf1 gene library was used for the isolation of *B. fragilis* genes involved in the DNA repair of metronidazole damage. The presence of an AraC family transcriptional regulator, ORF2, was sufficient to confer increased metronidazole and mitomycin C resistance to the metronidazole sensitive *E. coli uvrA* and *uvrB* DNA repair mutants, but had no effect on the resistance of *E. coli uvrC* or *recA* DNA repair mutants. It is possible that ORF2 might be regulating *E. coli* genes to exert a heterologous effect. A gene, ORF1, whose product had high amino acid sequence identity to the RecQ DNA helicases, was located upstream of ORF2. These proteins are involved in DNA repair, replication and stabilization of the genetic material. Although this gene was not involved in the resistance phenotype of the *E. coli uvrA* and *uvrB* mutants, the presence of such gene, upstream of ORF2, is of interest.

The transcriptional regulation of ORF2 in *B. fragilis* was examined by primer extension and northern blot analysis. The promoter study revealed a single transcriptional start site, an adenine, 59 bp upstream of the putative ORF2 start codon, as well as putative promoter consensus sequences. ORF2 was expressed as a single transcript of 600 nucleotides. There was no significant difference in expression of ORF2 following metronidazole exposure of the cells. Expression of ORF1, however, was dramatically decreased when cells were exposed to the drug. Further work is needed in order to elucidate the function of ORF1 and ORF2 in *B. fragilis*, as well as the mechanism of resistance of ORF1 in *E. coli*.

ABBREVIATIONS

A	adenosine	OD_x	optical density at x nm
aa	amino acids	ORF	open reading frame
Ap	ampicillin	ori	origin of replication
ATCC	American Type Culture Collection	p	plasmid
ATP	adenosine 5'-triphosphate	PCR	polymerase chain reaction
bp	base pair(s)	PolI	DNA polymerase I
C	cytosine	r	(superscript) resistant
C-	carboxy-(terminal)	RNA	ribonucleic acid
CFE	cell free extract	RNase	ribonuclease
CFU	Colonies Forming Units	rpm	revolutions per minute
Da	Dalton	rRNA	ribosomal RNA
DIG	digoxygenin	s	(superscript) sensitive
DNA	deoxyribonucleic acid	T	thymidine
DNAse	deoxyribonuclease	TAE	Tris-acetate-EDTA
dNTP	deoxynucleotide triphosphate		electrophoresis buffer
EDTA	ethylenediaminetetraacetic acid	UV	ultraviolet light
G	guanosine	w/v	weight per volume
GDH	glutamate dehydrogenase	WT	wild type
GOGAT	glutamate synthase	YT	yeast tryptone broth
GS	glutamine synthetase	α	alpha
h	hour	β	beta
kb	kilobase pairs	λ	lambda
kDa	kilodalton	µg	microgram
LB	Luria-Bertani medium	µl	microlitre
Mb	Megabase pair	µM	micromolar
Met	Metronidazole		
MIC	Minimum Inhibitory Concentration		
min	minute(s)		
mM	millimolar		
mRNA	messenger RNA		
MTC	mitomycin C		
MW	molecular weight		
ng	nanogram		
nm	nanometer		
N-	amino-terminal		
NCTC	National Collection of Type Cultures		
nt	nucleotide(s)		
NCBI	National Centre for Biotechnology Information		

CHAPTER 1

General Introduction

1.1 *Bacteroides* species

Members of the genus *Bacteroides* are normal commensals of the gut. However, under certain circumstances, some species, especially *B. fragilis*, can become opportunistic pathogens and even cause death. Treatment of *B. fragilis* infections involves the use of metronidazole, a drug specific for anaerobic cells causing bacterial death through DNA damage. The objectives of this research were to identify *Bacteroides* genes involved in metronidazole resistance, with particular emphasis on genes involved in DNA repair. This introduction, therefore, comprises three main topics, including an introduction to the *Bacteroides* (Section 1.1), the mode of action of metronidazole (Section 1.2) and the mechanisms used by prokaryotic cells for DNA repair (Section 1.3).

1.1.1 Introduction to *Bacteroides* species

Humans are part of a microbial world, and it has been estimated that the number of microbes in the mucosal surfaces of a human adult is larger than the number of somatic and germ cells (Savage *et al*, 1977). Of all the organs in the human body, the intestine is the most densely populated with microbes, and since it has a relatively low level of oxygen, these microbes are mainly anaerobes (Moore *et al*., 1978). One of the most abundant members of this gut microbiota is the genus *Bacteroides*.

Members of the genus *Bacteroides* are described as Gram negative, saccharolytic, nonpigmenting, obligately anaerobic, non-spore forming rod-shaped bacteria (Shah and Collins, 1989). Their DNA G+C content varies from 39 to 48%. The most medically important member of the *Bacteroides* genus is *Bacteroides fragilis*, which accounts for more than half of the anaerobes found in clinical specimens (Frank and Dennis, 1977). *Bacteroides distasonis*, *B. thetaiotaomicron* and *B. ovatus* are also opportunistic pathogens, but to a lesser extent (Salyers, 1984). The *Bacteroides* species have very similar biochemical characteristics and were originally clustered together as the same species (Shah and Collins, 1983). However, it has been shown that they do not share more than 30% DNA homology using DNA-DNA hybridisation studies (Robert *et al*., 1987). *B. fragilis* itself is divided into two divisions based on conserved differences on the sequence of the glutamine synthetase encoding gene, *glnA* and the *recA* gene

(Gutacker *et al.*, 2002). Division I is also associated with the presence of the *cepA* gene, coding for a serine-β-lactamase, while Division II contains the *cfiA* gene, coding for a metallo-β-lactamase. The *Bacteroides* form part of the *Bacteroidaceae* family. This family has been divided into three clusters according to rRNA gene sequence analysis: the *Prevotella* cluster including species found in the rumen, the *Porphyromonas* cluster having species present in the oral cavity and the *Bacteroides* cluster described above (Paster *et al.*, 1994). The *Bacteroidaceae* family is grouped together with the *Cytophaga* and the *Flavobacterium* according to both 16S and 5S rRNA analysis (Weisburg *et al.*, 1985; Van den Eynde *et al.*, 1989). This group, called the *Bacteroidetes*, forms one of the 10 phyla of the Eubacteria. These are more closely related to each other than to other groups, and share many characteristics in both rRNA sequence and other structures (Weisburg *et al.*, 1985). This phylum is thought to have branched off from the main eubacterial group before the divergence of Gram-negative and Gram-positive bacteria (Woese, 1987; Weisburg *et al.*, 1985). The *Fibrobacteres*, *Chlorobi* and *Bacteroidetes* phyla form a superphylum called the FCB group. These three phyla are thought to have diverged from a common ancestor, as shown by signatures in specific proteins such as DNA Gyrase B, the ATPase-α-subunit, the FtsK protein and the UvrB protein, present in only these three groups (Gupta, 2004).

1.1.2 *Bacteroides*, commensals or symbionts?

The human intestinal tract contains a large number of bacteria, with the majority in the proximal colon having 500 to 1000 different bacterial species (Savage, 1977). It is difficult to assign numerical values due to the fact that a large number of bacterial species are non-culturable. The intestine is largely devoid of oxygen explaining the fact that 99.9% of the cultivable bacteria in the intestine are obligate anaerobes (Moore and Holdeman, 1974). By comparing 16S rRNA analysis and culture methods, it was predicted that 60 to 70% of the bacteria in the human gut could not be cultured (Hayashi *et al.*, 2002; Suau *et al.*, 1999). Of those that could be cultured, the predominant genera included *Bacteroides*, *Eubacterium*, *Clostridium*, *Ruminococcus*, *Peptococcus*, *Peptostreptococcus*, *Bifidobacterium* and *Fusobacterium* (Suau *et al.*, 1999), while the enteric bacteria accounted for less than 1% (Sghir *et al.*, 2000). Despite this, 95% of the

culturable detected species were within the *Bacteroides* group, the *Clostridium coccoides* group and the *Clostridium leptum* subgroup. *Bacteroides* are thought to account for approximately 30% of the colonic bacteria (Salyers, 1984; Sghir *et al.*, 2000; Marteau *et al.*, 2001). The most numerous *Bacteroides* species found in the gut include *B. vulgatus*, *B. distasonis* and *B. thetaiotaomicron*, with smaller numbers of *B. fragilis*, *B. ovatus* and *B. uniformis* (Salyers, 1984).

These bacterial species are known as commensals of the human gut, where neither of the two members of the relationship is harmed. The gut microbiota do, however, also appear to benefit the host in various ways and this includes the *Bacteroides*. For example, *B. thetaiotaomicron* colonization of the gut of germ free mice improved the host's nutrient absorption by increased expression of genes involved in lipid and micronutrient absorption (Hooper *et al.*, 2001). It was also involved in reinforcing the hosts' mucosal barrier as well as increasing the submucosal capillary network. Colonization also affected the enteric nervous system and intestinal motility. *B. thetaiotaomicron* is also able to degrade plant polysaccharides which are indigestible to the host (Hooper *et al.*, 2002). In exchange, the host supplies *B. thetaiotaomicron* with nutrient sources. *B. thetaiotaomicron* is able to instruct the host to produce hydrolysable fucosylated glycans on the epithelial cell surface which are used as carbon source by the bacterium (Hooper *et al.*, 1999). The successful adaptation of *B. thetaiotaomicron* to this ever-changing environment is probably due to its complex sensory and glycolytic systems identified with the sequencing of its genome (Xu *et al.*, 2003). *B. fragilis* is a smaller component of the human intestinal microbiota. However it also has an intricate sensory and glycolytic structure allowing it to be competitive in this harsh environment.

1.1.3 *Bacteroides* physiology and metabolism

The *Bacteroides*, including *B. fragilis*, *B. thetaiotaomicron*, *B. vulgatus* and *B. distasonis* are able to utilize simple sugars, such as glucose, as well as haeme, vitamin B12, minerals and ammonium chloride (Varel and Bryant, 1974). However, the majority of simple sugars are absorbed in the small intestine and are seldom available as carbon sources. For this reason, *Bacteroides* have had to adapt and are able to utilize complex

polysaccharides, such as plant polysaccharides that the host is unable to digest, or host-derived polysaccharide products (Salyers *et al.*, 1977a; Salyers *et al.*, 1977b). A large number of polysaccharide degrading enzymes have been identified from *Bacteroides*, such as amylases from *B. thetaiotaomicron* and *B. ovatus* (Anderson and Salyers, 1989; Degnan *et al.*, 1997), pullulanases from *B. thetaiotaomicron* (Smith and Salyers, 1989; Smith and Salyers, 1991), and xylanase, xylosidase and arabinosidase from *B. ovatus* (Weaver *et al.*, 1992).

Most studies on *Bacteroides* polysaccharide utilization have been done on *B. thetaiotaomicron* and the genome analysis of this bacterium provides an insight into the incredible repertoire of enzymes involved in this process. The *B. thetaiotaomicron* proteome contains 172 glycosylhydrolases, more than any other sequenced prokaryote (Xu and Gordon, 2003). It also possesses at least 11 enzymes involved in degradation of host-derived products (Comstock and Coyne, 2003), including mucin-degrading hydrolases and chondroitin sulphatases (Xu and Gordon, 2003; Linn *et al.*, 1983). The capacity to utilize such mucopolysaccharides must confer a distinctive competitive advantage over the rest of the microflora in the gut. The proteome also includes 163 outer membrane polysaccharide-binding proteins and 20 sugar-specific transporters (Comstock and Coyne, 2003). The best studied of these is the starch utilization pathway, the *sus* operon, which binds starch on the cell surface and transports it into the periplasm for further degradation (Reeves *et al.*, 1997; Shipman *et al.*, 2000). Many of the outer membrane-binding proteins are paralogous to the *sus* enzymes and probably serve a similar function with other polysaccharides (Comstock and Coyne, 2003).

Apart from polysaccharide utilization, bacteria are thought to be actively involved in metabolism of nitrogen compounds in the intestine (Macfarlane *et al.*, 1988). *Bacteroides* is thought to play an especially important role in this. The proteolytic activity of *B. fragilis* is intracellular during exponential growth, while it becomes extracellular as the bacterium reaches stationary phase (Gibson and Macfarlane, 1988a). The proteolytic activity is due to three main proteases, one exopeptidase and two endopeptidases (Gibson and Macfarlane, 1988b). These seem to be constitutively expressed, but are upregulated following nitrogen-limiting conditions (Gibson and Macfarlane, 1988b; Macfarlane *et al.*,

1992). A fibrinogenolytic protease has also been identified in having a much higher activity in *B. fragilis* than the other non-pathogenic *Bacteroides*, suggesting a possible role in pathogenesis (Chen *et al.*, 1995). *Bacteroides* is unable to use peptides or amino acids as a sole carbon source. Ammonia and peptides are used as a nitrogen source but not free amino acids (Varel and Bryant, 1974). The primary metabolic pathway for assimilation of ammonia in *Bacteroides* is thought to be via the use of glutamate dehydrogenases (GDH) (Yamamoto *et al.*, 1984; Baggio and Morrison, 1996). Both *B. fragilis* and *B. thetaiotaomicron* have two different GDH proteins, GdhA and GdhB, that function under low and high nitrogen conditions respectively (Abrahams and Abratt, 1998; Baggio and Morrison, 1996). There seems to be low or no GOGAT activity (Yamamoto *et al.*, 1984; 1987). The *B. fragilis* glutamine synthetase, GS, encoded by the *glnA* gene, is a novel GSIII protein differing from the other two forms of GS found in eukaryotes and prokaryotes (Southern *et al.*, 1986; Hill *et al.*, 1989). This, once again, suggests the early divergence of the *Bacteroides*.

1.1.4 *Bacteroides* pathogenicity and virulence

As mentioned earlier, *B. fragilis* is an opportunistic pathogen, with other *Bacteroides* species such as *B. thetaiotaomicron* acting as pathogens to a lesser extent. When a rupture occurs in the wall of the intestine, a mixture of colonic bacterial species enter the abdomen causing infection. *B. fragilis* is one of the most important organisms in these infections (Frank and Dennis, 1977). The *Bacteroides*, especially *B. fragilis* are thought to possess an array of virulence factors involved in infection.

1.1.4.1 *Bacteroides* capsule as a virulence factor

The *B. fragilis* capsule is thought to be one of the main virulence factors of this bacterium. The capsular polysaccharide on its own is able to promote the formation of intra-abdominal abscesses, while the lipopolysaccharide is not (Onderdonk *et al.*, 1977; Kasper *et al.*, 1984). The capsular polysaccharide is also thought to facilitate adhesion of *B. fragilis* to the epithelial cells (Gibson *et al.*, 1998) and possibly interfere with phagocytic killing of cells (Onderdonk *et al.*, 1990). The capsule might also be involved in aerotolerance. Patrick *et al.* (1984) demonstrated that capsulate strains of *B. fragilis*

survived better aerobically than non-capsulate strains, while there was a negligible difference in survival between the two types anaerobically. *B. fragilis* has a complex capsular variation as shown by different populations containing large capsules, small capsules or an electron-dense layer. Studies done in a mouse model showed that these different capsular populations were able to vary their capsular status, where a large capsule population became non-capsulate within 24 hours (Patrick *et al.*, 1995).

A large number of *B. fragilis* strains were examined and found to have complex capsules consisting of at least two different polysaccharides and these were found to be antigenically diverse among the strains (Pantosti *et al.*, 1993). *B. fragilis* NCTC 9343 was shown to possess two distinct capsule polysaccharides, PS A and PS B. These were surface exposed and had different physiochemical properties (Pantosti *et al.*, 1991). Polysaccharide A was a neutral zwitterionic polysaccharide, with tetrasaccharide repeating units possessing a positively charged free amino group and a negatively charged pyruvate substituent (Tzianabos *et al.*, 1992). Polysaccharide B had an overall negative charge, and contained hexasaccharide repeat units with negatively charged galacturonic acid and a substituent with both a negative and positive charge. These two polysaccharides were held together by ionic interactions in a PS A: PS B ratio of 1:3 and were co-expressed on the cell surface. Polysaccharides possessing oppositely charged groups have potent abscess-inducing properties, as is the case with *B. fragilis* (Tzianabos *et al.*, 1994). These zwitterionic polysaccharides elicit a potent CD4+ T-cell response, which is dependent on the free amino and carboxyl groups on the repeating units (Tzianabos *et al.*, 2000). One of the capsule polysaccharides from *B. fragilis* strain 638R, PS A2, was found to have zwitterionic pentasaccharide repeats and was also able to cause abscesses (Wang *et al.*, 2000). The capsular polysaccharides of *B. fragilis* 638R are known to be immunologically distinct from those of *B. fragilis* 9343 (Comstock *et al.*, 1999a).

It was only in 1999 that the genes regulating the formation of these polysaccharides started being revealed, and it has turned out to be a fascinating and complex story. Using transposon mutagenesis of *B. fragilis* 638R and 9343, Comstock *et al.* (1999b) identified an operon in *B. fragilis* 9343 containing 16 open reading frames coding for proteins involved in polysaccharide biosynthesis. This locus was found to form a new polysaccharide, PS C1. Inactivation of the operon still allowed *B. fragilis* 9343 to cause

abscesses. The equivalent locus was identified in *B. fragilis* 638R, PS C2 (Comstock *et al.*, 1999a). It contained genes involved in polysaccharide biosynthesis and conferring charged groups to polysaccharides, but it was distinct from the PS C1 locus of *B. fragilis* 9343. The PS A1 and PS B1 loci from *B. fragilis* 9343 were isolated and shown to contain open reading frames with homologues of polysaccharide biosynthesis proteins (Coyne *et al.*, 2000 and 2001). Deletion of PS A1 decreased the ability of *B. fragilis* 9343 to cause abscesses, suggesting that polysaccharide A is an important virulence determinant (Coyne *et al.*, 2001). The incomplete sequence of the *B. fragilis* 9343 genome allowed Krinos *et al.* (2001) to identify a total of 8 loci involved in capsular polysaccharide biosynthesis, PS A-PS H. The first two genes of these operons were extremely conserved, *upxY* (where x represents the PS locus) and *upxZ* and were thought to be involved in transcriptional regulation of their corresponding locus. Variable expression of polysaccharides in the capsule of *B. fragilis* is thought to involve regulation of these different loci. Complete sequencing of the *B. fragilis* 9343 genome (www.sanger.ac.uk/projects/b_fragilis) and the published genome of *B. fragilis* YCH46 (Kuwahara *et al.*, 2004) has revealed a ninth operon, PS I or PS-9, respectively. These loci seem to undergo phase variation resulting in a reversible on-off phenotype. An invertible region of DNA, *fin*, upstream of *upxY* in polysaccharide biosynthesis loci PS 1-7 (A, B, D-H) was shown to have inverted repeats at each end (Patrick *et al.*, 2003). These inverted repeats, designated *fixL* and *fixR*, were AT-rich and 30 to 32 bp in length. The region between *fixL* and *fixR* contained a functional promoter (Krinos *et al.*, 2001; Patrick *et al.*, 2003). Inversion of this region would position the promoter in the correct orientation for transcription of the operon or not. A chromosomally-encoded serine site-specific recombinase, Mpi, was shown to be involved in inverting these 7 polysaccharide biosynthesis loci as well as other promoters distributed throughout the genome (Coyne *et al.*, 2003). A *B. fragilis* 9343 plasmid-borne Hin invertase homologue, FinB, has also been shown to bind to those regions (Patrick *et al.*, 2003). Polysaccharide biosynthesis locus C and I, also named PS-2 and PS-7 (Kuwahara *et al.*, 2004), did not have the invertible region (Patrick *et al.*, 2003; www.sanger.ac.uk/projects/b_fragilis, Kuwahara *et al.*, 2004).

Capsular variation has also been reported in *B. thetaiotaomicron*, as identified by populations forming non encapsulated or encapsulated cells (Burt *et al.*, 1978). Sequencing of the *B. thetaiotaomicron* genome identified 7 capsule polysaccharide biosynthesis loci, each having 1 or 2 regulatory *upxY* and *upxZ* homologues (Xu *et al.*, 2003). An invertible promoter was found in four of these loci as well as the presence of an integrase upstream of the 4 operons (Xu *et al.*, 2003, Kuwahara *et al.*, 2004). Gene BT4624 was not linked to any of the polysaccharide loci, but coded for a putative resolvase having high similarity to the *B. fragilis* Mpi recombinase. This suggests that *B. thetaiotaomicron* is also able to alter its capsular composition. This system might allow *B. fragilis* and *B. thetaiotaomicron* to camouflage themselves from other members of the intestinal flora as well as from the host to evade the immune system.

1.1.4.2 Other *Bacteroides* virulence factors

The oxygen tolerance of *Bacteroides* is thought to be an important virulence factor, since the abdominal cavity is aerobic. *B. fragilis* has a complex redox stress response involving a redox regulator, OxyR (Rocha *et al.*, 2000). This regulator controls various redox genes such as *katB*, encoding for a catalase (Rocha and Smith, 1995), *dps*, *ahpCF*, encoding for a hydroperoxide reductase, *tpx* and *ccp*, encoding for peroxidases (Herren *et al.*, 2003), as well as *ftnA*, the ferritin gene which is upergulated in response to oxygen and iron levels (Rocha and Smith, 2004). Tang *et al.* (1999) also identified the *BatI* operon with unknown function, involved in aerotolerance. *B. thetaiotaomicron* is also aerotolerant. Under aerobic conditions, fumarase and pyruvate:ferredoxin oxidoreductase, involved in central metabolism, are inactivated but remain stable for long periods (Pan and Imlay, 2001). Once anaerobic conditions are restored, these enzymes are rapidly repaired allowing *B. thetaiotaomicron* to recover from oxygen exposure.

Bacteroides produce enzymes which affect the host and are, therefore, considered virulence factors. *B. fragilis* is known to produce a protease when heme is limited, which degrades the host's hemopexin providing a heme source (Rocha *et al.*, 2001). *B. fragilis* also produces a neuraminidase (Goddoy *et al.*, 1993; Russo *et al.*, 1990), while *B. thetaiotaomicron* produces a chondroitin sulphatase (Salyers and Kotarski, 1980; Cheng *et al.*, 1995). A heparinase from *B. stearicus* has also been isolated (Kim *et al.*, 2000).

These degrade the host's cell surface components. *B. fragilis* has even been shown to induce cytokine expression in the host, a signal which leads to inflammation and abscess formation (Kim *et al.*, 2000).

Certain *B. fragilis* strains produce an extracellular zinc metalloprotease known as fragilysin or BFT, which cleaves the host's intercellular proteins between the epithelium resulting in fluid secretion (Vines *et al.*, 2000). The *B. fragilis* strains secreting BFT are known as enterotoxigenic (ETBF). Three isotypes of BFT have been identified and these are encoded by distinct loci within the *B. fragilis* pathogenicity island present in ETBF only (Sears, 2001). The *bft* gene is located within a novel conjugative transposon, which might allow transfer of this virulence factor (Franco, 2004).

1.1.5 *Bacteroides* Genetics

Bacteroides species are in contact with a large number of other organisms and this may account for the fact that they contain an array of genetic elements from diverse sources (Smith *et al.*, 1998). *Bacteroides* contains a large number of compatible small cryptic plasmids (Odelson *et al.*, 1987) that possess *oriT* and *trans*-acting mobilization genes, and they are thought to be transferred by conjugation. These do not seem to be involved in antibiotic or heavy metal ion resistance. However, plasmids conferring resistance to a large number of antibiotics have been identified and will be discussed below, as well as transposons and IS elements.

Plasmid construction seems to be modular in *Bacteroides* (Smith *et al.*, 1998). Plasmids share replication cassettes, mobilisation cassettes and IS elements. This suggests that *Bacteroides* genetic elements are from diverse sources. Another observation supporting the wide genetic diversity of *Bacteroides* is the fact that different plasmids have different replication mechanisms as well as different G+C content, implying different origins.

The presence of plasmids allows transfer of genetic material, which can be exploited in the laboratory. However, genetic manipulation of *Bacteroides* is problematic. Due to its early divergence in evolution, it has developed a unique system of gene expression

control which seems to be different from that in Gram positive and Gram negative bacteria (Smith *et al.*, 1992). This is supported by the fact that *Bacteroides* possess unusual promoter recognition sequences (Bayley *et al.*, 2000). Using transposable elements, several *E. coli-Bacteroides* shuttle vectors have been designed that allow gene expression in *Bacteroides*. However, transformation is rare in *Bacteroides*, especially in clinical strains of *B. fragilis* (Smith, 1995), and it is thought that DNA is degraded due to secretion of DNases and due to restriction modification systems. Electroporation has been used to transform into *B. fragilis*, as described by Smith *et al.* (1990), but plasmid DNA from *E. coli* seems to be inefficient. Apart from their importance as experimental tools, *Bacteroides* genetic elements are involved in conferring resistance to a wide range of antibiotics.

1.1.6 Antibiotic resistance

Bacteroides are resistant to a large number of antibiotics and many of the genes responsible for this are present on mobilizable elements.

The enzyme involved in penicillin G resistance is a metallo-β-lactamase, encoded by the *ccrA* gene (Rasmussen *et al.*, 1990; 1991). This enzyme is able to inactivate nearly every class of β-lactam antibiotic and it is also insensitive to β-lactamase blocking agents. Macrolide (erythromycin)-Lincosamide (clindamycin)-Streptogramin (MLS) resistance is encoded by the *erm* genes. These code for an rRNA methylase that acts on adenine residues in the 23s rRNA subunit (Arthur et al., 1987), reducing the affinity between the antibiotic and the 50S ribosomal subunit. In the 1960's nearly all *B. fragilis* strains were sensitive to tetracycline, but by the early 1980's resistance had increased to 60% (Tally and Malamy, 1982). Resistance to tetracycline is divided into different classes. Classes A to E, as well as F and L, are due to decreased accumulation of tetracycline by resistant cells (Speer *et al.*, 1991). Resistance of classes M to O and Q are ribosomal protection type resistances. Class X causes detoxification of tetracycline. Genes *tetF* and *tetX* only function aerobically.

The *ermF(S)* gene for MLS resistance is found on the closely related compound transposons Tn*4351*, Tn*4400* and Tn*4551* (Smith *et al.*, 1998). Tn*4351* and Tn*4551* are flanked by the insertion element IS*4351*, while Tn*4400* contains IS*4400*, which is almost identical to IS*4351*. In addition to conferring MLS resistance, these transposons seem to confer tetracycline resistance on *E. coli* when grown aerobically, while anaerobic *E. coli* or *B. fragilis* remain tetracycline sensitive. Under anaerobic conditions, the gene involved in resistance is transcribed but the product is non-functional (Speer *et al.*, 1991). These transposons are carried on plasmids such as pBF4, pBFTM10 and pBI136. Plasmid pBFTM10 encodes an additional tetracycline resistance gene, *tetF*, which is only expressed aerobically (Robillard *et al.*, 1985; Park and Levy, 1988).

Transfer of tetracycline resistance can sometimes occur together with the transfer of MLS resistance. This transfer is not plasmid encoded and the conjugal elements involved in transfer are believed to be chromosomal (Salyers and Shoemaker, 1995). The frequency of transfer increases when the donor is exposed to low concentrations of tetracycline. The conjugal elements involved in this transfer are called Tc^r elements. These are conjugative transposons that integrate into the chromosome. Tetracycline resistance is mediated by the *tetQ* gene that confers resistance by a ribosome protection mechanism. Tc^r elements are not only able to self-transfer but they also mediate transfer of elements that are not self-transmissible and need to be mobilised.

All these genetic elements make *B. fragilis* infections very difficult to treat using the commonly used antibiotics. One of the few drugs which still has an effect on *B. fragilis* is metronidazole, although resistance is already starting to appear.

1.2 Metronidazole

1.2.1 Introduction

Metronidazole, 1-2'-hydroxyethyl-2-methyl-5-nitroimidazole (Appendix 1), has had an incredible impact on the treatment of anaerobic protozoan and bacterial infections. Its discovery, development and use as a broad-spectrum antibiotic took many years and was surrounded by controversy that persists to this day in certain countries.

In 1955, crude extracts of *Streptomyces* were found to kill the pathogen *Trichomonas vaginalis*, due to the compound azomycin (Despois *et al.*, 1956). Two years later, metronidazole, related to azomycin, was chemically synthesised. This was followed by a series of synthetic relatives referred to as 5-nitroimidazoles. In 1959, it was introduced clinically for treatment of *Trichomonas vaginalis* infections (Ingham *et al.*, 1980). It was only years later, in 1962, that Shinn reported its effectiveness in Vincent's disease, acute ulcerative gingivitis caused by *Fusobacterium necrophorum*. In the early 1970's, it was found to have antibacterial activity against anaerobic bacteria, including all strains of *B. fragilis* (Noble and Tally, 1984). Following those findings, metronidazole was routinely used to treat anaerobic infections and as a prophylactic agent in the prevention of postoperative infections following gastrointestinal and gynecological surgery (Willis *et al.*, 1976; Willis *et al.*, 1977). Overall, it has been recognised to have a broad spectrum of activity against Gram positive and Gram negative bacteria, protozoa, helminths and even hypoxic tumours (Edwards, 1980).

In 1975, the use of the drug was questioned by Dykers following reports that metronidazole caused mutagenicity in bacteria and carcinogenicity in mice (Voogd *et al.*, 1974; Rustia and Shubik, 1972). The Ames test and the SOS chromotest found it to have mutagenic and genotoxic activity (De Meo *et al.*, 1992). However, the benefits of the drug seem to outweigh its risk factors and metronidazole is still used in the treatment and prophylaxis of anaerobic infections.

Metronidazole is an inert prodrug which, upon reduction of its nitro group, forms an active intermediate with bactericidal activity (Ings *et al.*, 1974). This activation of the drug influences its mode of action of the drug, including its mechanism of entry into the cell, its activation, its cellular targets as well as its selective toxicity towards anaerobic organisms and the incidence of resistance.

1.2.2 Entry of metronidazole

Indirect evidence suggests that metronidazole enters cells by passive diffusion (Ings *et al.*, 1974). Reduction of the drug into its cytotoxic intermediate resulted in a decrease of

drug concentration intracellularly and this increased the transmembrane concentration gradient allowing metronidazole to keep entering the cell. This was supported by studies done by Müller and Lindmark (1976) showing a linear relationship of accumulation inside the cell to the extracellular drug concentration in Trichomonads. Tally *et al.* (1978) showed metronidazole uptake in both resistant and susceptible bacteria, however, the rate of uptake and accumulation was higher in the susceptible ones. Metronidazole is of low molecular weight and is thought to penetrate all cell membranes (Noble and Tally, 1984). A number of *B. fragilis* strains resistant to metronidazole have shown decreased uptake of the drug (Britz and Wilkinson, 1979; Ingham *et al.*, 1978; Willis *et al.*, 1978). However, this was believed to be due to decreased activity of nitroreductases involved in activating metronidazole.

Since there is no direct evidence that metronidazole enters the cell by diffusion, the possibility of entry due to active transport must not be discarded. Moore *et al.* (1995) showed that a number of related imidazole compounds inhibited metronidazole uptake in *Helicobacter pylori*, suggesting the possibility for a transport system specific for that group of molecules. However, no further work has been done and there is, as yet, no evidence of any such system.

1.2.3 Activation of metronidazole

As mentioned previously, metronidazole itself has no cytotoxic activity, and its nitro group must be reduced to an active intermediate (Ings *et al.*, 1974). It was believed to have selective toxicity specific to anaerobic organisms only, because only they would have a sufficiently low redox potential to reduce the nitro group (Rowley *et al.*, 1976). Following various contradictory reports on the cytotoxicity of metronidazole on facultative anaerobes, Ingham *et al.* (1980) showed that metronidazole had activity against these but only under strict anaerobic conditions. The lowest redox potential of aerobic cells is –350 mV (Edwards *et al.*, 1973) while that of metronidazole is –486 mV (Smith and Edwards, 1995). For this reason metronidazole cannot be reduced in aerobic cells explaining the selective toxicity to anaerobic cells.

The reduction process is not fully understood and seems to be slightly different in anaerobes, facultative anaerobes and microaerophiles. However, in all these organisms, the reduced toxic derivative is short-lived (Müller, 1983) and converted into inactive end products, acetamide (Koch *et al.*, 1979) and N-(2-Hydroxyethyl)-oxamic acid (Koch and Goldman, 1979).

Most studies on reduction of metronidazole have been done on anaerobes. Reduction is thought to occur in one-electron steps where the nitro moiety of metronidazole (RNO_2) is reduced to form a free radical anion ($RNO_2^{\bullet -}$) (Müller, 1983). This reaction is reversible in the presence of O_2 (Perez-Reyes *et al.*, 1980). The subsequent steps are irreversible and form a nitroso derivative (RNO), a nitroso free radical ($RNO^{\bullet -}$) and a hydroxylamine derivative (RNHOH). Any of these derivatives could be the cytotoxic species. The first hint on how metronidazole was reduced inside the cell came from Edwards and Mathison (1970). *T. vaginalis* normally produces H_2 and CO_2 gas, but in the presence of metronidazole, only CO_2 was emitted. This was also observed in *Clostridium pasteurianum* (Lockerby *et al.*, 1985). These gases are by-products of the pyruvate phosphoroclastic system in anaerobes (Mortenson *et al.*, 1963) where pyruvate is enzymatically oxidized to acetyl phosphate and CO_2 causing reduction of ferredoxin. Reduced ferredoxin is then oxidized releasing H_2. In the presence of metronidazole, reduced ferredoxin was found to donate its electrons to metronidazole, reducing it (Edwards *et al.*, 1973; Lindmark and Müller, 1976). Lindmark and Müller (1976) showed that metronidazole accepted electrons from reduced ferredoxin in the absence of any enzymes *in vitro* allowing for the possibility that electron transport proteins with a low redox potential, such as ferredoxin, were involved in reducing metronidazole. Evidence also suggests that enzymes, termed nitroreductases, are involved in reduction of metronidazole inside the anaerobic cell. In *C. pasteurianum* hydrogenase 1, the enzyme involved in oxidation of reduced ferredoxin releasing H_2, has been shown to reduce metronidazole by oxidizing ferredoxin without the release of H_2 (Lockerby *et al.*, 1985; Church *et al.*, 1988). The hydrogenase enzyme in *T. vaginalis* and *Tritrichomonas foetus* plays a role in metronidazole reduction as shown by resistant strains lacking hydrogenase activity (Kulda *et al.*, 1993; Cerkasovova *et al.*, 1984). Pyruvate dehydrogenase, or

pyruvate:ferredoxin oxidoreductase, also seems to be involved in reduction of metronidazole since *B. fragilis* and *Clostridium perfringens* strains resistant to the drug had a decreased activity of the enzyme (Britz and Wilkinson, 1979; Diniz *et al.*, 2004; Sindar *et al.*, 1982). This enzyme is a pyruvate dehydrogenase that uses ferredoxin as an electron acceptor and is also involved in metronidazole resistance in *T. vaginalis* and *T. foetus* (Kulda *et al.*, 1993; Cerkasovova *et al.*, 1984). Narikawa (1986) tested 41 different species, spanning 19 genera, and found that the distribution of metronidazole susceptibility was the same as that of pyruvate:ferredoxin oxidoreductase activity.

As mentioned previously, facultative anaerobes are susceptible to metronidazole but only in the absence of O_2 (Ingham *et al.*, 1980). In the presence of O_2, the radical anion ($RNO_2^{\bullet-}$) is re-oxidised to metronidazole (RNO2) and the superoxide anion ($O_2^{\bullet-}$) is produced in a process known as "futile cycling" (Perez-Reyes *et al.*, 1980). Superoxide can be changed by superoxide dismutase into hydrogen peroxide and O_2. In the presence of iron or other metals hydrogen peroxide and superoxide can react to form the highly reactive hydroxyl free radicals, that can indirectly also cause DNA damage.

The mechanism of reduction of metronidazole in facultative anaerobes has not been completely characterized. Studies done on *Actinobacillus actinomycetemcomitans* showed the absence of ferredoxin-linked pyruvate oxidoreductase activity, however there was nitroreductase activity which appeared to be responsible for the susceptibility of the bacterium to metronidazole under anaerobic conditions (Pavicic *et al.*, 1995). Yeung *et al.* (1984) found that *E. coli* mutants that were unable to reduce nitrate and chlorate and had DNA repair mutations were more resistant to metronidazole than the strains containing the DNA repair mutations alone. *E. coli* has two types of nitroreductases, type I and type II (Peterson *et al.*, 1979). Type I nitroreductases are oxygen-insensitive because they convert organic nitro compounds into stable products by a 2-electron step reduction. This does not allow futile cycling and production of superoxide. Type II nitroreductases are oxygen-sensitive because they catalyse univalent reduction resulting in nitro radical anions which can be re-oxidised in the presence of oxygen to form superoxide. Metabolism via the 2-electron reduction of the nitro moiety produces nitroso and hydroxylamine intermediates which can interfere with DNA and proteins. Two

oxygen-insensitive nitroreductases have been characterized in *E. coli*, NfsA, the major nitroreductase and NfsB, the minor one. NfsA is a flavin mononucleotide containing protein and uses NADPH as an electron donor (Zenno *et al.*, 1996). It has a broad specificity for nitro-substituted compounds. NfsB is a flavin mononucleotide flavoprotein which can use either NADH or NADPH (Michael *et al.*, 1994) and has been linked to the reduction of metronidazole (Goodwin *et al.*, 1998; Verdijk *et al.*, 2004). Interestingly, NfsB has amino acid sequence similarity to the *H. pylori* RdxA nitroreductase (Whiteway *et al.*, 1998), the nitroreductase involved in reduction of metronidazole in *H. pylori*.

Unlike obligate or facultative anaerobes, microaerophiles such as *H. pylori*, require 2-17% oxygen for growth. Microaerophiles are susceptible to metronidazole (Hoff and Sticht-Groh, 1984), an unexpected result since the amount of oxygen in that environment should interfere with metronidazole reduction. The mechanism of action of metronidazole was thought to be due to futile cycling, where reactive oxygen species accumulated and were responsible for cell killing (Lacey *et al.*, 1993; van Zwet *et al.*, 1995). This hypothesis was discarded following reports showing that the presence or absence of catalase did not affect the efficacy of metronidazole in *H. pylori*, and sensitive strains were able to reduce metronidazole (Jorgensen *et al.*, 1998). There are various nitroreductases involved in reduction of metronidazole in *H. pylori*. The main nitroreductase is RdxA, an oxygen-insensitive chromosomally encoded NADPH nitroreductase (Goodwin *et al.*, 1998). It reduces metronidazole in a 2-electron step reduction into hydroxylamine, preventing futile cycling from occurring. Other nitroreductases involved in metronidazole reduction are FrxA, a NAD(P)H flavin oxidoreductase as well as FdxB, a ferredoxin-like protein (Kwon *et al.*, 2000). Metronidazole resistant *H. pylori* becomes sensitive again anaerobically (Smith and Edwards, 1995). This suggests the presence of other nitroreductases that are involved in metronidazole reduction anaerobically. Goodwin *et al.* (1998) proposed that pyruvate oxidoreductase could be such an enzyme. Whether reduced in a 1-electron step in anaerobic environments, or 2-electron reduction in microaerophiles, the cytotoxic intermediates of metronidazole and their effect on cellular components remain the same.

1.2.4 Metronidazole targets and cytotoxicity

It is now widely accepted that the main target of reduced metronidazole is DNA. Reports showing that metronidazole caused mutagenicity in bacteria and carcinogenicity in mice (Voogd *et al.*, 1974; Rustia and Shubik, 1972) hinted at the possibility that the main target of metronidazole was DNA. Plant and Edwards (1976) showed that metronidazole caused *in vivo* degradation of DNA in *Clostridium bifermentas*, and DNA synthesis was inhibited. RNA synthesis was not affected by the presence of metronidazole. Various *in vitro* studies demonstrated that reduced metronidazole caused single strand breaks in DNA, while unreduced metronidazole did not (Edwards, 1977; Knight *et al.*, 1978; Rowley *et al.*, 1979). The reactive intermediate did not act as an intercalator or cross-linking agent (Edwards *et al.*, 1977), and organisms with a high A-T % seemed to be more susceptible to metronidazole (Rowley *et al.*, 1980). Zahoor *et al.* (1987) proposed that the protonated 1-electron nitro radical anion would act as an electron acceptor from DNA in regions of thymidine residues causing strand breaks and release of thymidine. Hydroxylamine is known to cleave sites with thymidine residues (Yamamoto *et al.*, 1993), so the hydroxylamine derivative of metronidazole cannot be ruled out as the possible cytotoxic derivative. Diniz *et al.* (2000) and Sisson *et* al. (2000) reported high level of DNA breakage and fragmentation in *B. fragilis* and *H. pylori*, respectively.

Indirect evidence suggesting that DNA is the main target of metronidazole came from *E. coli* and *H. pylori* DNA repair mutants. *E. coli* strains with mutations in the DNA repair gene *uvrB* and *recA* were more susceptible to metronidazole than the WT (Jackson *et al.*, 1984; Yeung *et al.*, 1984). Inactivation of the *recA* gene in *H. pylori* greatly enhanced the sensitivity of that strain to metronidazole (Thompson and Blaser, 1995). Metronidazole also caused morphological changes in *E. coli* and *B. fragilis* cells which were consistent with DNA damage and the SOS response: sharp growth end-point and cell elongation due to the inhibition of cell division (Jackson *et al.*, 1984; Skarin and Mårdh, 1981). Interestingly, an *E. coli tag* mutant, containing a deficient 3-methyladenine-DNA-glycosylase gene involved in repair of DNA damage due to alkylating agents, was not found to be more sensitive to metronidazole than its WT strain (Yeung *et al.*, 1984). This suggests that the DNA damage caused by metronidazole is specifically repaired, and repair involves the UvrB protein of the Excision Nucleotide repair system, and the RecA

protein involved in general DNA repair and recombination, but not 3-methyladenine-DNA-glycosylase.

Other cellular components also seem to be affected by metronidazole. Müller and Lindmark (1976) suggested that metronidazole also interacted with proteins. In *C. pasteurianum*, the rapid killing of cells following exposure to high metronidazole concentrations could not be due to DNA damage (Church *et al.*, 1991). It was proposed that metronidazole interacted with the cell membrane causing cell lysis.

Even though DNA seems to be the major target of metronidazole, the drug is able to interact with other cellular macromolecules and affect cells in different ways.

1.2.5 Metronidazole resistance

High-level metronidazole resistance is rare in anaerobes but surprisingly high in *H. pylori*. In developing countries, more than half of *H. pylori* strains are resistant to the drug and in developed countries 10-30% (Goodwin *et al.*, 1998).

The major cause of metronidazole resistance is lack of activation of the drug due to decreased activity of cellular enzymes involved in reducing metronidazole into its active intermediate.

In *T. vaginalis*, resistance was caused by abolition of the activity of the pyruvate ferredoxin oxidoreductase system (Cerkasovova *et al.*, 1988). Under normal metabolic conditions in *Trichomonas*, one third of the pyruvate is oxidized in the cytosol to lactate and ethanol while the rest enters the hydrogenosome to be oxidized to acetate. In resistant *Trichomonas*, 97% of the pyruvate was oxidized to lactate in the cytosol by lactate dehydrogenase, to compensate for the decreased activity of pyruvate ferredoxin oxidoreductase. While there have been no metronidazole resistant *Clostridia* reported clinically (Sindar *et al.*, 1982), a strain of *C. perfringens* was made resistant by mutation. It had decreased pyruvate:ferredoxin oxidoreductase activity.

The first reported case of *Bacteroides* metronidazole resistance appeared in 1977 (Chardon *et al.*, 1977). *In vitro* studies showed decreased uptake of the drug due to decreased activity of ferredoxin-linked pyruvate oxidoreductase in a *B. fragilis* resistant strain, now called NCTC 11295 (Britz and Wilkinson, 1979). This caused an alteration in the end products of glucose metabolism, which in turn caused slower growth of the resistant strain. In order to compensate for the decreased activity of the oxidoreductase and to generate ATP for cell survival, it had increased levels of lactate dehydrogenase activity and increased levels of lactate, as seen in *Trichomonas* (Narikawa *et al.*, 1991).
Capsules of highly resistant *B. fragilis* mutants, with MICs higher than 100 µg/ml, were larger than the parent or than the moderately resistant mutants (MICs of 25 µg/ml) (Britz and Wilkinson, 1979). Both the highly resistant and moderately resistant mutants had impaired metronidazole reduction. This could imply that the larger capsules prevented entry of metronidazole into the cell by acting as a barrier, and this would explain the difference in metronidazole resistance.
Metronidazole resistant *B. fragilis* strains were selected for in the laboratory by passage in the presence of metronidazole (Diniz *et al.*, 2004). These mutant strains contained changes, such as upregulation of lactate dehydrogenase, downregulation of flavodoxin, and impaired pyruvate ferredoxin oxidoreductase activity.

The *metA* gene of *B. fragilis* was found to confer resistance to metronidazole (Dachs *et al.*, 1995). The MetA protein reduced DNA breakage caused by metronidazole, but it did not inactivate metronidazole. This suggested that its mode of action could be by repairing the damage. A *recA* mutant of *B. thetaiotaomicron* was found to be more sensitive to metronidazole than the wild type (Cooper *et al.*, 1997), once again suggesting the involvement of DNA repair systems.

Genes involved in moderate levels of nitroimidazole resistance, *nim* genes, have been identified in *Bacteroides* species (Haggoud *et al.*, 1994; Trinh *et al.*, 1995; Trinh and Reysset, 1996). Three of the four genes are plasmid-borne, *nimA* in pIP417 from *B. vulgatus* BV-17, *nimC* in pIP419 from *B. thetaiotaomicron* BT-13 and *nimD* in pIP421 from *B. fragilis* BF-F239, while *nimB* was found in the chromosome of *B. fragilis* BF-8. These genes are around 500 bp in length and show high sequence identity and similarity

to each other, suggesting a common ancestral gene. They contain insertion sequences (IS) upstream of their start codon, and transcription is thought to be directed by promoters on the right ends of these IS elements. All four genes are transferable by conjugation (Trinh and Reysset, 1996). Haggoud *et al.* (1994) reported a similar uptake of metronidazole in *B. fragilis* 638R, with or without the presence of *nimA* and *nimB*, indicating that these genes were not involved in preventing entry of the drug. Carlier *et al.* (1997) have suggested that the *nim* genes code for a nitroimidazole reductase that converts 4- or 5-nitroimidazoles into 4- or 5-aminoimidazole and not forming the cytotoxic nitroso radicals.

Metronidazole resistance is often found in *H. pylori*. Inactivationof the *rdxA* gene, coding for a nitroreductase, by missense or nonsense point mutations increased the MIC of susceptible strains from 8 to 32 µg/ml (Kwon *et al.*, 2000a). Further inactivation of *fdxB*, a gene coding for ferredoxin-linked protein, doubled the MIC to 64 µg/ml. Inactivation of another nitroreductase alone, *frxA*, increased the MIC to 32 µg/ml, while double inactivation of *rdxA* and *frxA* produced high level resistance, with MIC larger than 100 µg/ml. This would explain the different levels of resistance found in *H. pylori* isolates.
Growth of *H. pylori* in metronidazole-containing medium caused decreased expression of ferredoxin oxidoreductase gene and *rdxA*, moderate decreased expression of pyruvate oxidoreductase and *fdxB*, and unchanged expression of ferredoxin and flavodoxin genes (Kwon *et al.*, 2000b). This implies that metronidazole resistance could be acquired by regulation of gene expression.
Metronidazole resistance has also been related to efficient DNA repair and the involvement of the RecA protein (Chang *et al.*, 1997).

The involvement of DNA repair genes in metronidazole resistance in *B. fragilis* and *H. pylori* indicates the importance of DNA repair in resistance. Repair mechanisms induced by other DNA damaging agents have been widely studied in *E. coli.* Some of these will now be discussed with a view to understanding how the strand breaks caused by metronidazole might be repaired.

1.3 DNA repair

Preservation of its genomic material is vital for the survival of the individual cell. DNA is constantly under attack from environmental stresses, as well as from intrinsic mistakes by the cellular machinery. Despite all this, mutations are rare in a population. This is achieved through the constant surveillance of the genome and the immediate response to damage of the DNA.

Very little is known about the DNA repair systems of *Bacteroides* species. The recent publication of the *B. thetaiotaomicron* genome (Xu *et al.*, 2003) and the *B. fragilis* YCH46 genome (Kuwahara *et al.*, 2004) will give a better insight on the possible mechanisms that these organisms use to protect and repair their genomic material. Due to the fact that DNA repair systems in obligate anaerobes are virtually unknown, some of the well-studied *E. coli* DNA repair pathways will be discussed, with the emphasis on those possibly having a role in repairing metronidazole induced DNA strand break damage.

1.3.1 Recombinational Repair

1.3.1.1 RecA

The RecA protein catalyses the central reaction in all types of recombination, whether it is for genetic diversity or for repair. It is also the central protein involved in induction of the SOS response (Radman, 1974; Kuzminov, 1999). The SOS system controls the induction of a number of cellular responses, including DNA repair, damage-induced mutation (Witkin, 1969) and filamentation of cells (Green *et al.*, 1969), following DNA damage or disruption during replication. The system involves a network of 31 known genes and 38 putative genes, under the control of two key proteins, RecA and LexA (Fernandez de Henestrosa *et al.*, 2000). Under normal growth conditions, the LexA repressor binds to the SOS box in promoters of the SOS genes, preventing transcription (reviewed by Kuzminov, 1999). When replication is inhibited, the SOS response is induced. Single stranded DNA accumulates when replication is blocked, and the RecA protein forms filaments on the DNA in the presence of ATP and becomes activated. Activated RecA acts as a co-protease and catalyses the autocleavage of LexA (Little *et*

al., 1980). This decrease in the level of LexA bound to the SOS boxes allows transcription of the SOS genes.

The fact that *B. thetaiotaomicron* and *H. pylori recA* mutants are sensitive to metronidazole suggests an involvement of RecA in reducing the effect of the drug (Cooper *et al.*, 1997;Chang *et al.*, 1997).

In recombination, the ssDNA-bound RecA filament is involved in finding a DNA duplex which is homologous to the ssDNA. It is not known how RecA is able to find homologous DNA. It is thought to have more than one DNA binding site to accommodate the ssDNA and its homologous duplex DNA (Cox, 1995).

Once it has found a homologous duplex DNA, it catalyses the exchange of strands. The ssDNA forms hydrogen bonds with the complementary strand of the duplex DNA. This displaces the other strand of the DNA duplex. RecA is assisted at all times by Single Strand Binding protein (SSB). Whenever single stranded DNA is present, SSB quickly complexes with it, covering it (Kuzminov, 1999). It is thought to help RecA to form filaments on the ssDNA by preventing formation of secondary structures. Once strand exchange has occurred and the other strand of the duplex is displaced, SSB is likely to bind to it. This pairing of DNA strands causes the formation of Holliday junctions which must be removed. These are removed by RuvABC resolvase or by RecG helicase (Kuzminov, 1999).

RecA is often targeted to sites of recombinational repair by different protein complexes. According to the type of repair needed, there are different pathways involved, such as the RecF, involved in repair of daughter strand gaps, or the RecBCD pathway, involved in the repair of single and double strand breaks and a possible system involved in repair of metronidazole damage.

1.3.1.2 The RecF Pathway

When the replication machinery encounters a non-coding lesion, the replication fork is blocked. Replication is known to reinitiate downstream of the lesion causing a single stranded gap in the daughter strand (Kuzminov, 1999). This gap is thought to be repaired

by the RecF pathway in a RecA-dependent manner. The RecF pathway consists of three repair proteins, RecF, RecR and RecO.

RecF has been shown to bind linear single stranded DNA with a preference for the ends (Griffin and Kolodner, 1990). In the presence of ATP and Mg^{2+}, it is able to bind both single and double stranded DNA (Madiraju and Clark, 1992). RecO binds both single and double stranded DNA in the presence of Mg^{2+} and is able to promote renaturation of complementary DNA strands. Very little is known about the biochemistry of RecR. The genes coding for these proteins are on different loci in the chromosome, however each of these genes is located adjacent to genes coding for subunits of the replication machinery (Kuzminov, 1999).

When a single stranded gap is formed on the daughter strand, SSB binds to it immediately. RecF and RecR form a complex which is thought to be involved in sensing for a stalled replication fork (Kuzminov, 1999). The RecFR complex brings RecO to the SSB-covered single strand gap. The RecOR complex is thought to bind the SSB-covered DNA at the junction between the single strand and duplex DNA (Sandler and Clark, 1994). This allows RecA to form filaments on the single stranded gap. The RecA filament then finds an intact duplex homologous to the gap and pairs them. Recombination of these strands then takes place.

The function of RecF is thought to be that of directing the repair to the daughter strand. The RecR protein seems to act as a mediator between RecF and RecO, while RecO is then involved in recruitment of RecA for recombination to take place (Kuzminov, 1999). RecF, RecO and RecR are required to stabilize the replication fork. RecQ, a DNA helicase and RecJ, an ssDNA exonuclease, are involved in degradation of the nascent lagging strand (Courcelle and Hanawalt, 1999). This is thought to increase the ssDNA substrate for RecA binding and prevent illegitimate recombination. In the absence of RecFOR, RecJ and RecQ are involved in degradation of the replication fork and RecQ is involved in recombination together with RecA and SSB (Chow and Courcelle, 2004).

1.3.1.3 The RecBCD Pathway

When a replication fork encounters a single stranded break, it collapses and this forms double stranded interruptions (Kuzminov, 1999). This suggests that unrepaired single stranded breaks lead to double stranded breaks. Repair of double stranded breaks is achieved by the RecBCD recombinational repair pathway. Metronidazole is thought to cause single stranded DNA breaks and possibly double stranded breaks. It is possible then, that the RecBCD pathway is involved in repair of metronidazole DNA damage.

As an overview of the pathway, double stranded DNA (dsDNA) ends are degraded by ExoV, also known as RecBCD (Kuzminov, 1999). Once RecBCD encounters a Chi site in the DNA, it continues degrading the DNA, but only the 5' ending strand, leaving a 3' single stranded overhang. This is the target for SSB. RecBCD promotes RecA binding to the SSB-covered ssDNA. RecA is then involved homologous recombination.

ExoV (RecBCD) is a heterotrimer of RecB, RecC and RecD held together by Mg^{2+} (Taylor and Smith, 1995a; Wright *et al.*, 1971). The *recB* and *recD* genes form an operon, with the nearby *recC* gene having its own promoter (Kuzminov, 1999). None of these genes are SOS inducible. RecBCD has potent DNA helicase activity, dsDNA exonuclease and ssDNA exonuclease activity. Unlike most other exonucleases, it requires ATP hydrolysis and Mg^{2+} for degradation of DNA. Both exonuclease activities degrade the DNA to oligonucleotides, but the dsDNA exonuclease activity does so much faster. RecB and RecD are DNA-dependent ATPases containing ATP-binding sites (Masterson *et al.*, 1992; Chen *et al.*, 1997). RecB alone is also a weak helicase, unwinding DNA in a 3'-5' direction. RecB controls the hydrolysis of the 3'end strand, while RecD is involved in degradation of the 5'end strand.

When encountering a dsDNA break, RecB binds to the 3'end strand while the RecC and RecD subunits sit on the 5'end strand (Taylor and Smith, 1995b). The RecBCD enzyme moves along the DNA, unwinding and degrading both strands. The DNA is degraded unevenly with the 3'end strand receiving most of the cuts due to the positioning of the enzyme. As the dsDNA is degraded, the 3'end is thought to form a single stranded loop held together by the enzyme, while the 5'end strand hangs behind the enzyme and is cut

more slowly (Telander-Muskavitch and Linn, 1982). The reason for the loop formation is to prevent ExoI, an ssDNA-specific nuclease which degrades in a 3' to 5' direction, from degrading the strand. As the enzyme moves along, it comes across a Chi site on the DNA. In *E. coli*, Chi sites are 8 nucleotides regions with the sequence 5'-GCTGGTGG-3' (Smith *et al.*, 1981). In other bacteria, the Chi sequence is different but also short without repeats (Kuzminov, 1999). The Chi site is only recognised by RecBCD from the 3' end. Recognition of the Chi site stops the dsDNA exonuclease activity and turns the enzyme into a recombinase. This is thought to be done by ejection of the RecD subunit, however it has not been established whether the subunit is completely lost. This allows RecBCD to degrade the 5' end strand only. Degradation of only one strand causes the formation of ssDNA which is immediately bound by SSB. RecBCD interacts with RecA and promotes RecA filamentation on the SSB-covered ssDNA.

The *recBC* mutants are deficient in homologous recombination and sensitive to DNA damaging agents. However, additional suppressor mutations are able to switch on alternative pathways for double strand end repair via the RecE pathway and a modified RecF pathway.

1.3.1.4 Alternative double strand end repair via the RecE and RecF Pathways

Two suppressor mutations *sbc* (for suppressor of *recB* and *recC)* are able to compensate for *recBC* mutants that are unable to achieve double strand break repair. The first mutation, named *sbcA*, activates part of a cryptic Rac prophage in *E. coli* (Campbell, 1994). Two phage proteins are produced, the RecE and the RecT proteins. This pathway, termed the RecE pathway, is dependent on RecA, RecE, RecF, RecO, RecR, RecJ and RecT. The mechanism by which double strand breaks are repaired is still unknown. The RecE protein is a dsDNA-specific exonuclease which is able to degrade duplex DNA from the 5'end strand causing formation of 3' overhangs (Joseph and Kolodner, 1983). RecE can act on blunt ends or ends with a long 3'overhang. It is also able to act on short 5' overhangs, but ends with long 5' overhang are not substrates for RecE. Removal of the long 5' overhangs is thought to be achieved by RecJ, an ssDNA-specific exonuclease with 5' – 3' directionality (Kuzminov, 1999). Once RecJ has blunted the DNA, RecE is

able to form the 3' overhang. RecT then binds to the overhang and promotes annealing of complementary DNA, as well as protecting it from ExoI degradation.

The second mutation *sbcB* allows the RecF pathway, described previously, to handle double strand breaks (Kuzminov, 1999). This is achieved by inactivation of two cellular nucleases, ExoI which is able to degrade ssDNA 3'ends (Lehman and Nussbaum, 1964) and SbcCD, and ATP-dependent dsDNA exonuclease related to RecBCD (ExoV) (Lloyd and Buckman, 1985). This pathway involves a large number of proteins, RecA, RecF, RecO, RecR, RecJ, RecN. The RecQ, UvrD and HelD proteins are DNA helicases which are also involved. In this alternative pathway, the combined action of RecQ helicase and RecJ ssDNA exonuclease is thought to generate 3' overhangs (Kuzminov, 1999). The ExoI and SbcCD nucleases would probably degrade these ends preventing recombinational repair. These two alternative pathways are thought to interact and have joint elements.

The RecF pathway and the Nucleotide Excision Repair pathway (discussed below) are thought to work together in recovery of replication (Courcelle *et al.*, 1999) The RecF is thought to push the replication machinery past the lesion, allow filling in of the daughter strand gap by recombination, while the Nucleotide Excision Repair is then involved in removing the lesion. It has also been proposed that RecFOR, RecA, RecQ and RecJ together with Nucleotide Excision Repair are involved in repair and recovery of replication without recombination (Courcelle and Hanawalt, 2001).

1.3.2 Nucleotide Excision Repair

E. coli strains deficient in nucleotide excision repair (NER) have been shown to be more sensitive to metronidazole (Yeung *et al.*, 1984; Jackson *et al.*, 1984). This suggests an involvement of the nucleotide excision repair in dealing with metronidazole damage of DNA.

Nucleotide excision repair occurs when the DNA is damaged in a double stranded, non-replicating region of the chromosome. An oligonucleotide containing the DNA damage is

excised and the gap is filled in by using the complementary strand of the DNA. This occurs by the joint action of the UvrA, UvrB, UvrC and UvrD proteins, followed by PolI and DNA ligase. The type of damage recognised by NER is usually that which causes distortion of the DNA helix (Friedberg *et al.*, 1995).

The *uvrA* gene is constitutively expressed at low levels and is inducible by the SOS response (Friedberg *et al.*, 1995). The UvrA protein contains both ATPase and DNA binding motifs. In the absence of ATP, it is found as a monomer, however it dimerises when ATP is present causing a conformational change. Dimeric UvrA is able to bind DNA containing various types of damages, and the N-terminus is then able to bind UvrB.

The *uvrB* gene is unlinked to *uvrA* (Friedberg *et al.*, 1995). It is also SOS inducible, however it also possesses a SOS-independent promoter (Friedberg *et al.*, 1995). The UvrB protein contains 5 different domains (Theis *et al.*, 2000). Domains 1a and 3 are involved in binding ATP and have high structural similarity to helicases of superfamily I and II. Domains 2 and 4 are distinct from helicases and are thought to be involved in interactions with UvrA and UvrC. Domain 1 is involved in DNA binding. ATP binding and hydrolysis is thought to move the domains. In the $(UvrA)_2UvrB$ complex which will be discussed later, the domain motions lead to translocation of the complex along the DNA to probe for damage. In the tight UvrB-DNA preincision complex, the motions lead to distortion of the 3'incision site. This preincision complex is formed by the insertion of flexible β-hairpins between the two DNA strands (Theis *et al.*, 1999) and bound by UvrC.

The *uvrC* gene is not inducible by DNA damage and is not part of the SOS regulon (Friedberg *et al.*, 1995). UvrC is unable to associate with UvrA or UvrB in solution, however it has a high affinity for the UvrB-DNA preincision complex. The N-terminus contains the catalytic site for the 3' incision (Moolenar *et al.*, 2002). The C-terminus of UvrC is involved in the 5' incision.

The mechanism of excision repair has been extensively studied and is well known. The UvrA dimers bind to UvrB in solution (Friedberg *et al.*, 1995). This $(UvrA)_2UvrB$ complex binds to DNA nonspecifically at a site with no DNA damage. The complex

moves along the DNA using the UvrB helicase activity. When damage is encountered, UvrA dissociates leaving a stable UvrB-DNA complex. This complex causes bending of the DNA. UvrC which has high affinity for the UvrB-DNA complex binds to UvrB and hydrolyses the 5th or 4th phosphodiester bond 3' to the lesion. This is followed by the hydrolysis of the 8th phosphodiester bond 5' to the DNA lesion. The UvrD protein, also known as Helicase II, which gene is SOS-controlled, is required for excision of the oligonucleotide fragment containing the DNA damage. This also displaces the UvrC protein, while the UvrB remains bound and is released during the synthesis of the missing nucleotides by PolI. DNA ligase completes the reaction.

It is interesting to note that a protein, Cho, with homology to the N-terminus of UvrC has been identified in *E. coli* (Moolenaar *et al.*, 2002). The gene coding for it, *ydjQ*, is SOS inducible, unlike *uvrC*. Cho can incise DNA complexed with UvrB at 3' of lesion, however the incision is 4 nucleotides further away from the UvrC 3' lesion. This is most likely due to the fact that Cho and UvrC bind to different domains of uvrB. Cho is able to incise certain damaged substrates which are poorly incised by UvrC.

The UvrA, UvrB and UvrD proteins have also been involved in a replication backup sytem that replaces PolI (Moolenaar *et al.*, 2000). UvrA and UvrB are also thought to be involved in suppression of illegitimate recombination, in a pathway shared with RecQ (Hanada *et al.*, 2000).

1.3.3 DNA Repair in *Bacteroides*

DNA repair systems of obligate anaerobes have been given little attention and very little is known about the DNA repair capabilities of *Bacteroides*. With the recent publications of the *B. thetaiotaomicron* VPI-5482 genome (Xu *et al.*, 2003) and the *B. fragilis* YCH46 genome (Kuwahara *et al.*, 2004), the presence of certain DNA repair genes may in the future assist in elucidating the DNA repair systems present in these organisms. Both *B. fragilis* and *B. thetaiotaomicron* have a RecA protein (Goodman *et al.*, 1987; Cooper *et al.*, 1997). This protein is likely to be involved in DNA repair since *B. thetaiotaomicron recA* mutants are more sensitive to DNA damaging agents, including metronidazole

(Cooper *et al.*, 1997). This mutant is also more sensitive to oxygen, suggesting that DNA repair might be involved in aerotolerance of both *Bacteroides* species. *Bacteroides* is not likely to have an *E. coli*-like SOS response, since UV damage did not induce expression of RecA in *B. fragilis* (Goodman *et al.*, 1987), a characteristic which is also evident with the *Porphyromonas gingivalis* RecA, a close relative to the *Bacteroides* (Liu and Fletcher, 2001).

B. fragilis has a constitutive thymine dimer excision system which operates under both anaerobic and aerobic conditions (Abratt *et al.*, 1986). This excision repair system involves strand breakage as well as postincision replication (Abratt *et al.*, 1990). Physiological evidence suggests that this system might not be the only excision repair system in *B. fragilis* (Abratt *et al.*, 1985). It seems that the excision repair systems that recognise UV and Mitomycin C damage are linked but are not the same pathway.

1.4 Aims and Objectives

Bacteroides are a predominant bacterial genus found in the gut, and *B. fragilis*, and to a lesser extent *B thetaiotaomicron*, are medically important opportunistic pathogens involved in anaerobic infections. The high incidence of antibiotic resistance in the *Bacteroides* makes it impossible to treat such infections with conventional antibiotics. Metronidazole, a DNA damaging agent active under anaerobic conditions, is the drug of choice at the moment due to minimal resistance. *B. fragilis* metronidazole resistance has been identified involving a lack of activation of the drug. However, very little research has been done on the mechanism of repair of metronidazole damage and its possible role in resistance. The published genome sequences of *B. thetaiotaomicron* and *B. fragilis* as well as the incomplete genome sequence of *B. fragilis* NCTC 9343 were only available at the last stages of this work. For this reason, indirect approaches were used for identification of target genes at the initiation of this study.

The aims of this study were, therefore, to identify *B. fragilis* and *B. thetaiotaomicron* genes involved in the metronidazole resistance, with particular emphasis on genes

involved in repair of metronidazole DNA damage. Two approaches were used to achieve these objectives. The first approach involved the construction of *Bacteroides* transposon mutant libraries and the screening of these libraries for isolation of metronidazole sensitive or resistant mutants, with a view to identifying the disrupted genes. The second method involved functional complementation of *E. coli* nucleotide excision repair mutants, which are sensitive to metronidazole, using a *B. fragilis* gene library, to isolate and analyse *B. fragilis* genes conferring increased metronidazole resistance to the *E. coli* mutants.

CHAPTER 2

Construction of *B. fragilis* and *B. thetaiotaomicron* Transposon Mutant Libraries and Isolation of *B. thetaiotaomicron* Metronidazole Mutants

2.1 Abstract

Bacteroides species are numerous in the gastrointestinal tract and they account for approximately 30% of colonic bacteria. The best studied *Bacteroides* species are *B. fragilis*, an opportunistic pathogen and *B. thetaiotaomicron*, a commensal of the gut. *B. fragilis* infections are treated using metronidazole, however the potential for resistance is increasing. In order to isolate *Bacteroides* genes involved in metronidazole resistance, construction of transposon mutant banks of both *B. fragilis* and *B. thetaiotaomicron* was undertaken. Attempts to generate a *B. fragilis* representative mutant bank were not very successful due to the reluctance of this organism to take up foreign DNA, and no metronidazole resistant or sensitive mutants were isolated. However, a significant number of *B. thetaiotaomicron* transposon mutants were generated. Screening of the *B. thetaiotaomicron* mutant bank identified two mutants, Mutant A and B, with increased sensitivity to metronidazole. Both mutants had the same disruption within a capsular polysaccharide locus suggesting the involvement of the *Bacteroides* capsule in metronidazole resistance. Mutant A contained a second insertion of the cointegrate plasmid in a different locus of the chromosome. This insertion caused a further increased sensitivity to mitomycin C. Attempts to identify the insertion of the cointegrate were, however, unsuccessful. Two *B. thetaiotaomicron* metronidazole resistant mutants were also isolated, termed Mutants C and D. Mutant C contained a disruption within a putative two-component histidine kinase with a possible link to methyltransferases and mismatch repair. Insertion of the transposon in Mutant D was intergenic between the *fucO* gene and the downstream transcriptional regulator of the putative rhamnose metabolism gene cluster. The *fucO* gene codes for lactaldehyde reductase, an essential enzyme in the metabolism of rhamnose and fucose, and also involved in energy relations within the cell. Further studies are needed to analyse these mutants more closely and determine how the mutations may be affecting sensitivity or resistance to metronidazole.

2.2 Introduction

Bacteroides fragilis is a normal commensal of the gut. However, it is also an opportunistic pathogen, and it is the predominant anaerobe found in abdominal infections (Frank and Dennis, 1977). The most effective drug used to combat *B. fragilis* infections is metronidazole (reviewed in Section 1.2, Chapter 1). There are three possible stages at which a cell could become metronidazole resistant: Firstly, at the cell entry stage, whereby the drug could either be prevented from entering the cell, or pumped out once it entered. Secondly, at the activation stage, where the inert metronidazole would not be changed into the active form of the drug. Thirdly, at the DNA repair stage, where the DNA damage caused by metronidazole could be repaired at a faster rate.

The construction of *B. fragilis* metronidazole resistant mutants would be useful in studying the possible mechanisms of metronidazole resistance. Transposon mutagenesis allows a single gene disruption and provides the presence of a selectable marker for isolation of the disrupted gene. *B. fragilis* is not readily amenable to genetic manipulations or introduction of foreign DNA (Salyers *et al.*, 1999), but protocols have been published for transposon and site directed mutagenesis (Shoemaker *et al.*, 1986; Tang and Malamy, 2000; Baughn and Malamy, 2003). In order to circumvent the problems associated with introducing transposons into *B. fragilis*, an alternative route is the use of *B. thetaiotaomicron*, a close relative of *B. fragilis* for construction of mutants. *B. thetaiotaomicron* is more easily manipulated and foreign DNA is able to enter the cell and integrate at a higher rate than *B. fragilis* (Salyers *et al.*, 2000; Hooper *et al.*, 2001). The recent publication of the *B. thetaiotaomicron* genome (Xu *et al.*, 2003) would allow easy identification of the interrupted genes. This would then allow searching for *B. fragilis* homologues of the putative genes and further characterisation of their mode of action in metronidazole resistance in *B. fragilis*. A possible shortcoming of this approach is that although these *Bacteroides* species are closely related, there is evidence of divergence of the genomes and suitable homologues may not be present.

A modified Tn*4400* transposition system, described by Tang and Malamy (2000), was chosen due to its recent publication at the time of initiation of this study. The Tn*4400* compound transposon, which confers erythromycin (Erm) resistance to *Bacteroides*, was cloned into a plasmid containing a replicon, *oriT* and *bla* gene functional in *E. coli* only,

together with a *tetQ* gene cassette, which confers tetracycline (Tet) resistance to *Bacteroides* but no *E. coli*. Conjugation of this plasmid, pYT646, into *Bacteroides* could cause three possible transposition events to occur: tranposition of the Tn*4400* conferring Erm resistance, or transposition of the inverse transposon named Tn*4400'*, which included the IS4400 sequences with the *tetQ* gene, conferring Tet resistance, or finally, cointegrate formation where the whole pYT646 plasmid would integrate into the chromosome conferring both Erm and Tet resistance. Inverse transposition was found to occur more frequently than the other two events (Tang and Malamy, 2000).

This chapter describes the construction of *B. fragilis* and *B. thetaiotaomicron* transposon mutants using the inverse transposition system described by Tang and Malamy (2000). These mutant libraries were then screened for metronidazole sensitivity or resistance to identify putative genes involved in metronidazole response of the *Bacteroides*.

2.3 Materials and Methods

2.3.1 Bacterial strains, plasmids and growth conditions

B. thetaiotaomicron VPI-5482 (ATCC 29148) and *B. fragilis* 638R (Privitera *et al.*, 1979) were grown anaerobically at 37°C using Difco brain heart infusion broth or agar (1.5%), supplemented with haemin, menadione and cysteine (BHIS) (Holdeman and Moore, 1972). Gentamicin, tetracycline or erythromycin were added to the medium at final concentrations of 200 μg/ml, 2 μg/ml and 2 μg/ml, respectively when necessary. Cultures were grown in an anaerobic chamber (Model 1024, Forma Scientific Inc., Marietta, Ohio) containing an atmosphere of oxygen-free N_2, CO_2 and H_2 (85:10:5 by volume). *Escherichia coli* JM109 (Yanisch-Perron *et al.*, 1985) and HB101 (Sambrook *et al.*, 1989) were used for mating experiments. The transposon delivery vector pYT646B was described by Tang and Malamy (2000). The broad-host-range mobilising IncP plasmid RK231 (Guiney *et al.,* 1984) was used to transfer pYT646B. *E. coli* strains were grown aerobically at 37°C on Luria-Bertani (LB) broth or 1.5% (w/v) agar (Sambrook *et al.*, 1989) supplemented with ampicillin (100 μg/ml) when transformed with pYT646B, or tetracycline (10 μg/ml) when carrying pRK231.

2.3.2 General recombinant DNA procedures

E. coli plasmid DNA was prepared by the alkali-hydrolysis method of Ish-Horowicz and Burke (1981) or using the QIAprep® Spin Miniprep Kit (50) and QIAGEN® Plasmid Midi Kit (25) (QIAGEN). *B. fragilis* and *B. thetaiotaomicron* genomic DNA was prepared according to the method described by Wehnert *et al.* (1992). All DNA modifications were performed according to standard procedures (Sambrook *et al.*, 1989).

2.3.3 *E. coli-Bacteroides* conjugation

The transposon delivery vector pYT646B was transferred into *Bacteroides* by conjugal transfer from *E. coli* to *Bacteroides* as described by Tang and Malamy (2000). The *E. coli* donor strain containing pYT646B and *E. coli* HB101 bearing the mobilizing plasmid RK231 were grown aerobically in LB broth to an optical density (OD) (600 nm) of 0.2. *Bacteroides* recipients were grown anaerobically in BHIS broth to an OD_{600} of 0.2. The *E. coli* donors and *Bacteroides* recipients were mixed in a 2:2:5 ratio and concentrated by

centrifugation. The mixture was resuspended in 0.2 ml BHIS broth and placed on a sterile 0.45 μm filter (HAWP02500, Millipore Corp.) on a BHIS agar plate without cysteine. The conjugation mixture was incubated aerobically at 37°C for 16 hours. The cells were resuspended in 3 ml BHIS broth and incubated aerobically at room temperature for 45 min. Selection of transconjugant colonies was performed anaerobically by plating the conjugation mixture on BHIS agar plates containing gentamicin (200 μg/ml) and tetracycline (2 μg/ml) or erythromycin (2 μg/ml).

2.3.4 Construction of the *B. thetaiotaomicron* and *B. fragilis* 638R Tn*4400'* transposon mutant libraries

Construction of the *B. thetaiotaomicron* and *B. fragilis* 638R transposon mutant libraries involved two *E. coli-B. thetaiotaomicron* and ten *E. coli-B. fragilis* conjugation experiments for each library, as described in section 2.3.3. Tetracycline resistant transconjugants were replica-plated onto BHIS agar plates containing erythromycin (2 μg/ml) in order to calculate the percentage of cointegrate formation. Only tetracycline resistant (Tet^r) erythromycin sensitive (Erm^s) transconjugants were selected as Tn4400' transposon mutants and these were divided into 5 pools and stored in BHIS broth at -70°C.

2.3.5 Southern hybridization

Genomic DNA from *B. thetaiotaomicron* and *B. fragilis* 638R (20 μg each), as well as pYT646 plasmid DNA (20 ng) was digested to completion with the appropriate restriction endonucleases. The DNA was fractionated by electrophoresis on a 0.8% agarose gel in TRIS-Acetate-EDTA buffer and transferred to a Hybond N^+ nylon membrane (Amersham Pharmacia Biotech) according to the manufacturer's instructions. DNA probes were purified on 0.8% agarose gel and random primed labeled with Digoxigenin-11-dUTP (DIG) using the Digoxigenin Labeling and Detection Kit (Roche Diagnostics). DNA hybridization with the DIG-labeled probe was performed overnight at 68°C using a standard hybridization buffer as stated in the Roche Diagnostics DIG System User's Guide. Signals were detected using chemiluminescent detection with CSPD® (Roche Diagnostics), according to the manufacturer's instructions.

Stripping of the membrane to remove previously bound probe was done by washing twice with 0.2 N NaOH and 0.1 % SDS at 37°C. The membrane was then hybridized to the new probe and detection took place as described above.

2.3.6 Screening of the *Bacteroides* transposon mutant libraries for metronidazole and mitomycin C resistant or sensitive mutants.

B. thetaiotaomicron and *B. fragilis* 638R transposon mutants from all 5 pools were diluted in BHIS and plated onto BHIS agar plates to obtain single colonies. First-level screening involved replica-plating of these individual colonies onto BHIS plates containing 0.2 μg/ml metronidazole (for sensitivity) or 1.0 μg/ml metronidazole (for resistance), or 0.05 μg/ml Mitomycin C (MTC) (for sensitivity) or 0.5 μg/ml MTC (for resistance). For second-level screening, stationary phase putative metronidazole and MTC *B. thetaiotaomicron* mutants from the first-level screening were diluted appropriately and plated onto BHIS agar containing either mitomycin C or metronidazole. The minimum inhibitory concentration (MIC) was determined after 48 hours incubation at 37°C, anaerobically.

2.3.7 Metronidazole broth assays

Stationary phase *B. thetaiotaomicron* mutants were diluted to an OD_{600} of 0.1 and inoculated into 10 ml BHIS broth containing increasing concentrations of metronidazole (0-3 μg/ml). The OD_{600} was measured after 24 hours incubation at 37°C anaerobically and the MIC was determined. These experiments were repeated 6 times.

2.3.8 Determination of the site of transposon insertion by plasmid rescue

Chromosomal DNA from each mutant was digested to completion using *Hin*dIII and the chromosomal fragment adjacent to the transposon was isolated by plasmid rescue as described by Tang and Malamy (2000). The resulting ampicillin resistant plasmids (Fig 2.1) were sequenced either by cloning the junction fragment into pBluescript II SK(+)

(Stratagene, La Jolla), or by using an internal primer modified from primer L78 (Chen *et al.*, 2000) (5'-CAA TAA TCG ACC TCG TAA AAG-3') for sequencing.

Figure 2.1 Diagrammatic representation of a plasmid resulting from the rescue of the chromosomal DNA of the *B. thetatiotaomicron* mutant adjacent to the inverse transposon using *Hind*III. The black line represents the part of the inverse transposon containing the *E. coli ori*. The ampicillin resistance (*bla*) gene is shown in green. The bold blue line represents the chromosomal DNA from the *B. thetaiotaomicron* mutant. The black arrow represents the internal primer modified from primer L78.

2.3.9 Nucleotide Sequencing

Sequencing reactions were performed using the DYEnamic ET Dye terminator Cycle sequencing Kit for MegaBACE (Molecular Dynamics), which is based on traditional dideoxynucleotide chain termination chemistry (Sanger *et al.*, 1977). All reactions were performed according to the manufacturer's instructions and cycle sequenced on a GeneAmp PCR System 9700 (Perkin Elmer, Applied Biosystems) using the universal M13 sequencing forward and reverse primers or the modified L78 primer (Chen *et al.*, 2000) at concentrations of 5 μM each and hybridization temperature of 68°C. The sequencing reaction products were analysed on the MegaBACE 500 sequencer (Molecular Dynamics, Amersham Pharmacia Biotech, Amersham Biosciences), which is an automated capillary DNA sequencing system. The nucleotide sequences were analysed using the MegaBACE 500 Sequence Analyser v2.4 software and the software program DNAMAN, version 4.13 (Lynnon BioSoft). Sequence similarity searches were done using the NCBI BLAST program (Altschul et al., 1997). The *B. thetaiotaomicron* genome was used for analysis (Xu *et al.*, 2003) and the preliminary *B. fragilis* 9343 genome sequence produced by the Sanger Centre Sequencing group (www.sanger.ac.uk/projects/b_fragilis).

2.4 Results and Discussion

2.4.1 Construction and analysis of the *B. thetaiotaomicron* and *B. fragilis* 638R transposon mutant libraries

2.4.1.1 Enumeration of transposition frequencies

Transposon mutagenesis of *Bacteroides* was attempted using the delivery vector pYT646 containing the inverse transposon Tn*4400'* constructed by Tang and Malamy (2000). Two separate *E. coli-B. thetaiotaomicron* conjugation experiments were performed with transconjugation frequencies ranging from 2×10^{-6} to 4×10^{-5} per *E. coli* donor cell. The frequencies of inverse transposition of Tn*4400'* ranged from 2.5×10^{-6} to 2.5×10^{-5} per donor cell, while the frequencies of Tn*4400* normal transposition were between 3×10^{-6} and 2×10^{-5} per donor cell. Table 2.1 shows the transposition results from the most efficient conjugation experiment in *B. thetaiotaomicron*.

Table 2.1 Transposition results from a single conjugation of pYT646 into *B. thetaiotaomicron.*

	Tet^r phenotype[a] (transconjugants)	**Tet^r/Erm^s phenotype[b] (Tn*4400'* mutants)**	**Erm^r phenotype[c] (transconjugants)**
Number of colonies	**1143**	**698**	**800**
Transfer frequency (per *E. coli* donor cell)[d]	**4×10^{-5}**	**2.3×10^{-5}**	**2.5×10^{-5}**

a The full conjugation mix was plated on BHIS with tetracycline (2 μg/ml) and gentamicin (200 μg/ml) and the total number of colonies counted.

b All Tet^r colonies from the conjugation experiment were replica plated onto BHIS erythromycin plates (2 μg/ml).

c A 100 μl sample from the conjugation mix was plated on BHIS with erythromycin and gentamicin and the total number of colonies counted, to calculate the frequency of Erm^r transconjugants.

d The number of *E. coli* donor cells was scored by plating the donor mix on BHIS plates containing Tet (10μg/ml) and Erm (10μg/ml).

Ten *E. coli-B. fragilis* 638R conjugation experiments were performed but these resulted in very low transconjugation frequencies ranging from 7×10^{-9} to 1×10^{-7} per *E. coli* donor cell. The frequencies of inverse transposition of Tn*4400'* ranged from 7×10^{-10} to 7×10^{-8} per donor cell, while the frequencies of cointegrate formation were between 6×10^{-10} and 7×10^{-8} per donor cell (Table 2.2). Cointegrate formation occurs when the entire pYT646 plasmid integrates into the chromosome, carrying both Erm^r and Tet^r markers. An average of 40% of *B. thetaiotaomicron* and *B. fragilis* 638R Tet^r transconjugants were cointegrates, a much higher figure compared to the 4% cointegrate formation in *B. fragilis* achieved by Tang and Malamy (2000) and the 10% cointegrate formation in *Porphyromonas gingivalis* (Chen *et al.*, 2000), using the same Tn*4400'* mutagenesis system. This figure resembles the 50% cointegrate formation reported by Shoemaker *et al.* (1986), using a transposon vector containing the Tn*4351* transposon.

Table 2.2 Transposition results from total of 10 conjugation experiments of pYT646 into *B. fragilis* 638R.

	Tet^r phenotype[a] (transconjugants)	**Tet^r/Erm^s phenotype[b] (Tn*4400'* mutants)**	**Tet^r/Erm^r phenotype[b] (cointegrates)**
Number of colonies	**1043**	**513**	**530**
Transfer frequency (per *E. coli* donor cell)[c]	**1×10^{-7}**	**7×10^{-8}**	**7×10^{-8}**
% of transconjugants	**100**	**50**	**50**

a The full conjugation mix was plated on BHIS with tetracycline (2 µg/ml) and gentamicin (200 µg/ml) and the total number of colonies counted.

b All Tet^r colonies from the conjugation experiment were replica plated onto BHIS erythromycin plates (2 µg/ml).

c The number of *E. coli* donor cells was scored by plating 100 µl of the donor mix on BHIS plates containing Tet (10µg/ml) and Erm (10µg/ml).

A total of 1500 Tn*4400' B. thetaiotaomicron* transposon mutants were accumulated and these were divided into 5 pools of 300 mutants each. Due to the low frequency of inverse transposition, a total of only 600 Tn*4400' B. fragilis* 638R transposon mutants were obtained. The *B. fragilis* 638R mutants were divided into 5 pools of 120 mutants each. The Tet^r Erm^r colonies could only be due to a cointegrate inserted in the chromosome, since pYT646 is unable to replicate in *Bacteroides* (Tang and Malamy, 2000). A total of 1000 *B. thetaiotaomicron* and 500 *B. fragilis* 638R cointegrates were also divided into pools and stored.

2.4.1.2 Analysis of integration events within random Tn*4400' B. fragilis* mutants

Southern hybridization analysis was used in order to investigate the number of copies of Tn*4400'* inserted in the *B. fragilis* 638R chromosome and whether this had occurred in a random way. The nature of the *B. fragilis* putative cointegrate mutants was also analysed.

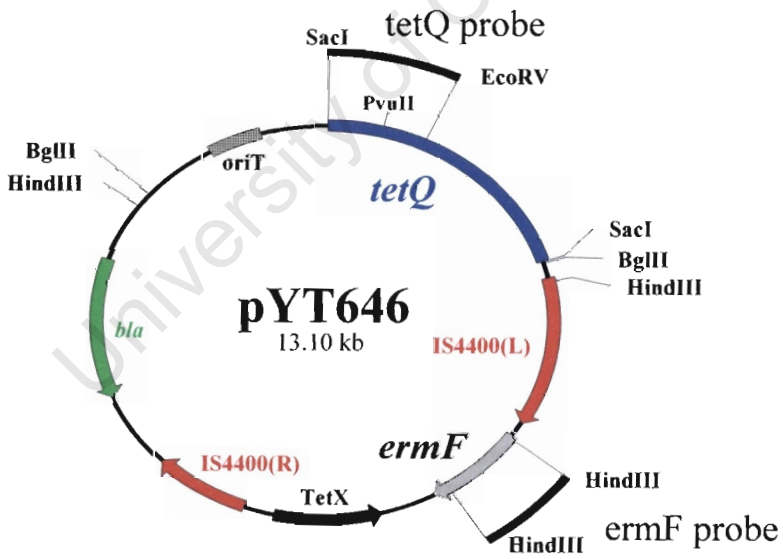

Figure 2.2 Partial restriction map of pYT646B (Tang and Malamy, 2000). The *Pvu*II restriction site was located by double restriction enzyme digests of pYT646B. The bold black lines represent the *tetQ* and *ermF* probes used for Southern hybridization analysis.

Five Tn*4400' B. fragilis* Tn*4400*' mutants, 1a to 1e, were chosen at random, as well as five *B. fragilis* cointegrate mutants, 2a to 2e. Their chromosomal DNA was digested with *Pvu*II, which has one restriction enzyme site in pYT646B, within the *tetQ* gene (Fig 2.2). The presence of the *tetQ* gene (Fig 2.3A) and *ermF* gene (Fig 2.3B) in the selected mutants was investigated in order to ensure that the Tn*4400'* mutants were in fact true inverse transposons containing the *tetQ* gene and lacking the *ermF* gene, while the suspected cointegrate mutants contained both antibiotic markers.

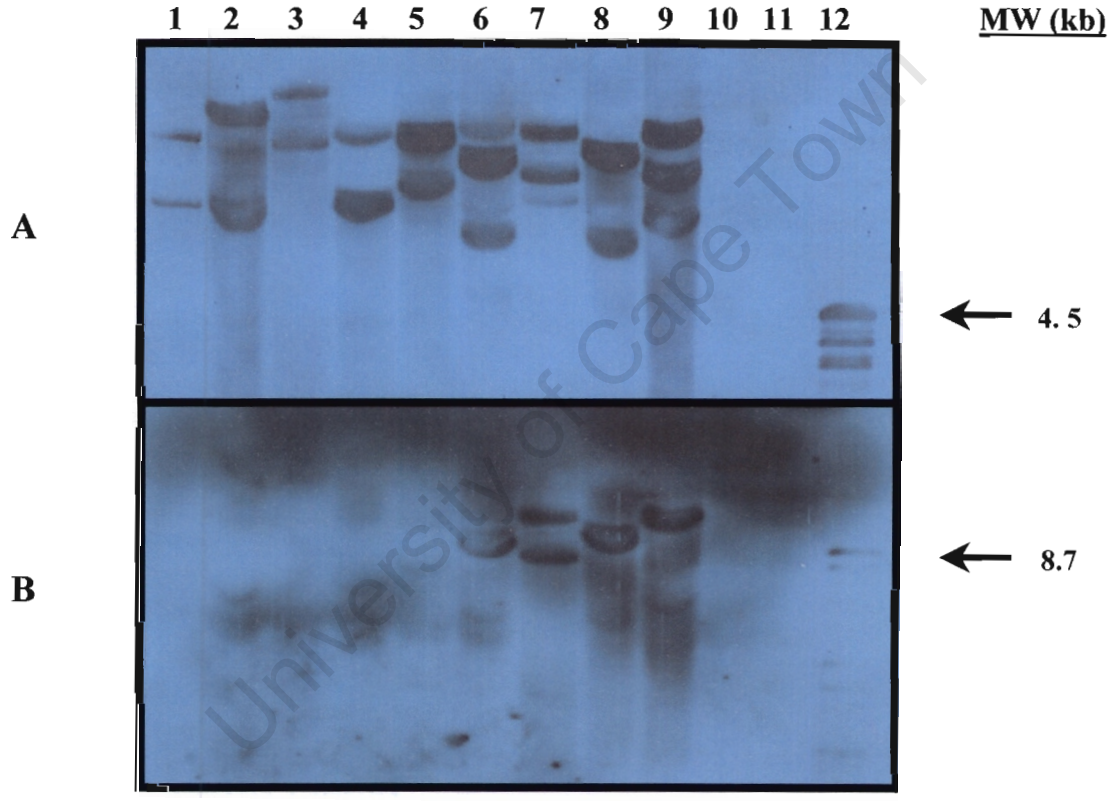

Figure 2.3 Southern blot analysis of *B. fragilis* genomic DNA containing Tn*4400'* or cointegrates using the (**A**) *tetQ* probe, and (**B**) *ermF* probe. Molecular mass markers are shown on the right (kb). Lanes 1-5, *B. fragilis* Tn*4400'* mutants 1a to 1e genomic DNA digested with *Pvu*II, respectively; Lanes 6-10, *B. fragilis* cointegrate mutants 2a to 2e genomic DNA digested with *Pvu*II, respectively; Lane 11, *B. fragilis* 638R genomic DNA digested with *Pvu*II; Lane 12, pYT646B digested with *Bgl*II.

The 0.9 kb *Sac*I-*Eco*RV internal *tetQ* probe (Fig 2.2) hybridized to the genomic DNA of the five Tn*4400'* mutants resulting in two bands, as expected for a single insertion (Fig 2.3, Lanes 1-5A). No major signals were visible for these Tn*4400'* mutants when probing with the 0.75 kb *Hin*dIII-*Hin*dIII internal *ermF* probe (Fig 2.3, Lanes 1-5B). The *tetQ* probe and *ermF* probe hybridized to the genomic DNA of four of the five cointegrate mutants, 2a to 2d (Fig 2.3, Lanes 6-9A and Lanes 6-9B, respectively). Mutant 2e did not result in a signal with any of the probes (Fig 2.3, Lane 10A and B) and was probably a false positive. The *tetQ* DNA probe hybridized to the positive control, pYT646B digested with *Bgl*II (Fig 2.2), resulting in multiple bands due to incomplete digestion, with the main band of an expected size of 4.5 kb (Fig 2.3, Lane 12A). The *ermF* probe also hybridised to the positive control as shown by the expected 8.7 kb band (Fig 2.3, Lane 12B). Genomic DNA from WT *B. fragilis* 638R was used as a negative control and did not hybridise to either of the two probes (Fig 2.3, Lane 11A and B).

Since the Tn*4400*' mutants (Mutants 1a to 1e) all had two signals when probed with the *tetQ* probe, this implied they had only one copy of the *tetQ* gene, showing a single integration of the inverse transposon. The sizes of the two bands were different for each transposon mutant, suggesting that integration of the transposon occurred randomly into different chromosomal loci. The absence of the *ermF* gene in these mutants confirmed their nature as true inverse transposon mutants. The first four cointegrate mutants (Mutants 2a to 2d) showed more than 2 signals, however some bands were very faint, suggesting partial digestion of the genomic DNA. This suggested that a single copy of the *tetQ* gene was present. These mutants were shown to be cointegrate mutants by the presence of both the *tetQ* and *ermF* genes in the same chromosomal *Pvu*II fragment, as shown by the fact that the same sized bands are detected with the two probes. This shows that these mutants contain a cointegrate integration, where the entire pYT646 plasmid is integrated at one site in the chromosome, rather than a double integration of a normal Tn*4400* transposon, containing *ermF*, and an inverse Tn*4400'* transposon, containing *tetQ*, into different loci of the chromosome. The growth of cointegrate mutants 2e on tetracycline and erythromycin cannot be explained, since it lacks both genes.

2.4.2 Isolation of metronidazole resistant and sensitive *B. fragilis* and *B. thetaiotaomicron* Tn*4400'* mutants

2.4.2.1 Screening of the *Bacteroides* mutant libraries for metronidazole mutants

In order to isolate *Bacteroides* genes involved in resistance to metronidazole, all 5 pools from the *B. thetaiotaomicron* and *B. fragilis* 638R Tn*4400'* mutant library were screened for metronidazole and mitomycin C sensitive or resistant mutants. No drug sensitive or resistant *B. fragilis* 638R Tn*4400'* mutants were identified. However, four *B. thetaiotaomicron* mutants were isolated. These were a metronidazole (Met) and mitomycin C (MTC) sensitive mutant, Mutant A (Table 2.3), a metronidazole sensitive *B. thetaiotaomicron* Mutant B, and two metronidazole resistant *B. thetaiotaomicron* mutants, Mutants C and D (isolated by Ms Lynthia Paul), both with a metronidazole MIC on plates of 8 μg/ml (Table 2.3). These four *B. thetaiotaomicron* mutants were further analysed with a view to their being used to identify *B. fragilis* homologous genes.

Table 2.3. Susceptibility to metronidazole and mitomycin C of *B. thetaiotaomicron* transposon mutants. A representative set of data is shown from 10 experiments.

B. thetaiotaomicron **Tn*4400'* mutants**	**Metronidazole MIC[a] (μg/ml)**	**Mitomycin C MIC (μg/ml)**	**Met/ MTC Phenotype**
B. thetaiotaomicron	0.5	0.3	WT
Mutant A	0.2	0.05	Met^s/MTC^s
Mutant B	0.3	0.3	Met^s
Mutant C	8	ND	Met^r
Mutant D	8	ND	Met^r

a Minimal inhibitory concentration (MIC) determined on BHIS agar plates anaerobically.

ND Not determined.

Metronidazole (Met); Mitomycin C (MTC); Sensitive (s); Resistant (r)

2.4.2.2 Metronidazole Broth resistant assays

The survival of the four *B. thetaiotaomicron* mutants in different concentrations of metronidazole was compared to that of the wild type *B. thetaiotaomicron.* Mutant A was most sensitive to metronidazole, reaching 10% survival (as a percentage of absorbance at 0 µg/ml metronidazole) at approximately 0.75 µg/ml (Fig 2.4). The metronidazole sensitivity of Mutant B was very similar to that of Mutant A. WT *B. thetaiotaomicron* had a 10% survival at 1.25 µg/ml. These results were very reproducible.

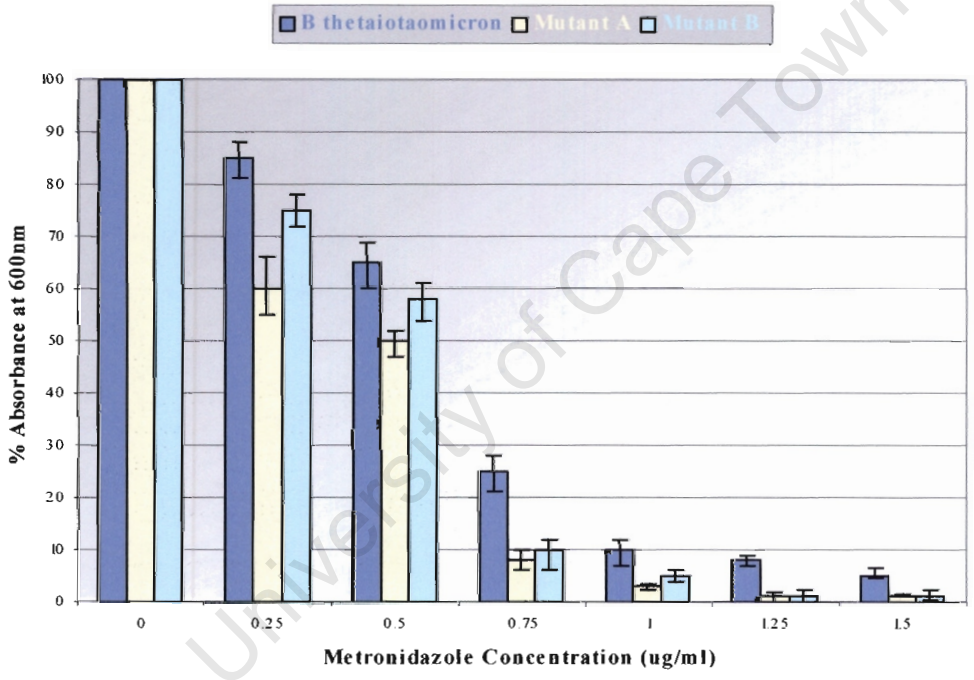

Figure 2.4 Susceptibility in broth of *B. thetaiotaomicron* Mutants A and B at different concentrations of metronidazole. *B. thetaiotaomicron* was used as a measure of WT metronidazole resistance. Error bars represent the ±S.D. of the mean.

Mutants C and D were both highly resistant to metronidazole as compared to the wild type *B. thetaiotaomicron*, with 10% survival closer to 1.75 µg/ml (Fig 2.5).

These results confirmed Mutant A and B as metronidazole sensitive mutants and Mutant C and D as metronidazole resistant, relative to WT *B. thetaiotaomicron.*

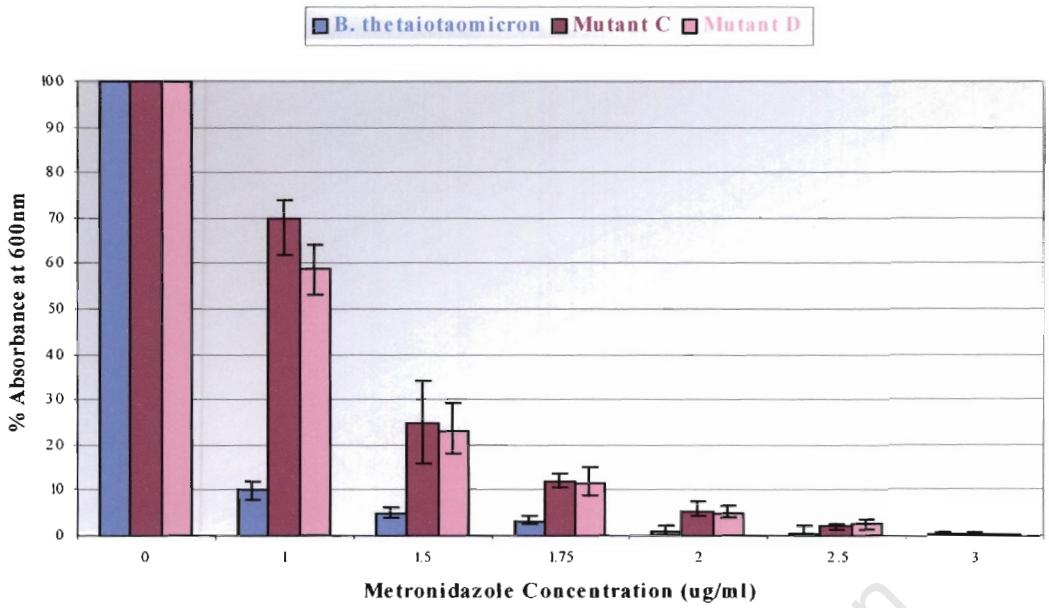

Figure 2.5 Susceptibility in broth of *B. thetaiotaomicron* Mutants C and D at different concentrations of metronidazole. *B. thetaiotaomicron* was used as a measure of WT metronidazole sensitivity. Error bars represent the ±S.D. of the mean.

2.4.2.3 Analysis of integration number of transposon mutants A, B, C and D

It was necessary to demonstrate that the phenotype of these mutants was due to disruption of a single gene. Southern hybridization analysis was used to identify the number of transposon integration sites in each *B. thetaiotaomicron* mutant A, B, C and D. Hybridisation of the 900 bp *Sac*I-*Eco*RV *tetQ* probe (Fig 2.2) to *Pvu*II-digested genomic DNA from a Tn*4400*' mutant containing a single insertion within its chromosome would give 2 bands. The results are shown in Figure 2.6. The WT *B. thetaiotaomicron* genomic DNA did not hybridise to the probe (Lane 2), showing the absence of the transposon in the chromosome. Mutants D (Lane 3), C (Lane 4) and B (Lane 5), all contained a single insertion of the inverse transposon Tn*4400*' at different sites within their chromosome, as shown by the two, different sized bands. Hybridisation of the probe to Mutant D genomic DNA resulted in a doublet made up of 2 bands of very similar sizes (Lane 3). Mutant A, however, (Lane 6) contained 3 bands, two of which seemed to be the same size as Mutant B. This suggested the possibility of two different integration events had occurred within the chromosome, one of which could be the same as in Mutant B. Mutant A was plated on erythromycin and tetracycline and found to be resistant to both antibiotics (results not shown). It is very likely that Mutant A is a double mutant,

containing a normal Tn*4400'* insertion, the same as in Mutant B, as well as a partial cointegrate or a normal Tn*4400* transposon, containing only part of the *tetQ* gene which would explain the third band. Plasmid rescue and sequencing of the chromosomal fragments were done to identify the genes disrupted by the transposon in each of the mutants.

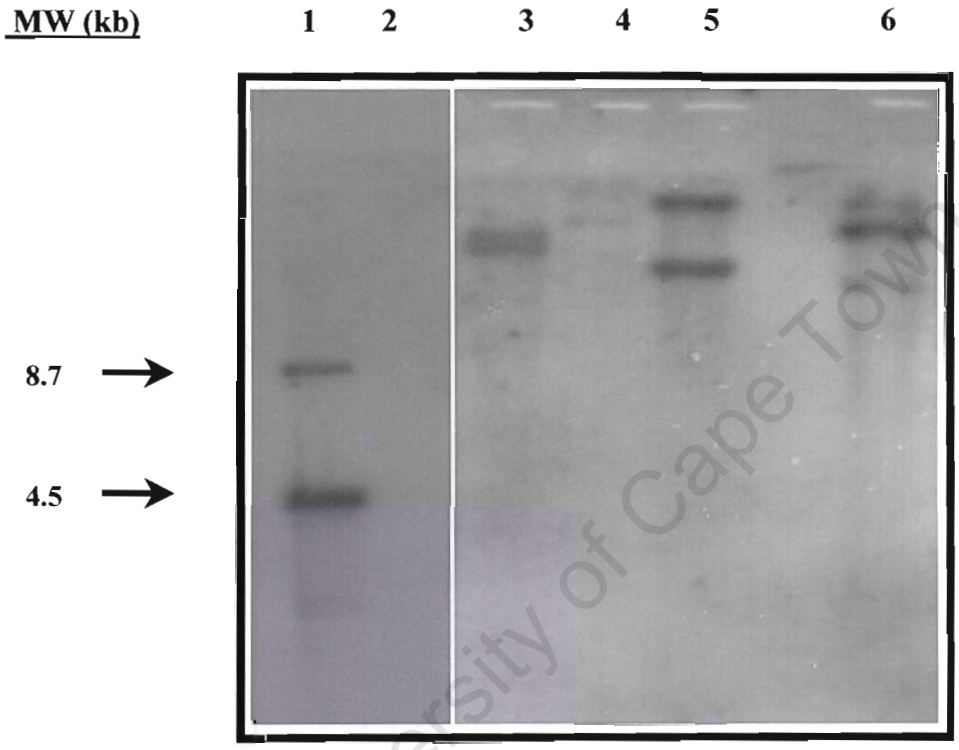

Figure 2.6 Southern blot analysis of *B. thetaiotaomicron* Tn*4400'* mutants A, B, C and D genomic DNA using the *tetQ* probe. Molecular mass markers are shown on the left (kb). Lane 1, pYT646B digested with *Bgl*II; Lane 2, *B.thetaiotaomicron* genomic DNA digested with *Pvu*II; Lane 3-6, Mutants D, C, B, and A genomic DNA digested with *Pvu*II, respectively.

2.4.3 Sequence analysis of disrupted genes from Mutants A, B, C, D, and relationship to metronidazole phenotype

2.4.3.1 Sequence analysis of the disrupted gene in Mutant B

Plasmid rescue of the chromosomal region flanking the Tn*4400'* insertion site in Mutant B was done. Digestion of the chromosome of the mutant using *Hin*dIII and religation of

that fragment yielded a plasmid containing part of the inverse transposon and a chromosomal fragment (Fig 2.1, Section 2.3.8). A single plasmid of approximately 9 kb in size was isolated. Sequence analysis of the Mutant B chromosomal DNA adjacent to the inverse transposon revealed that Tn*4400'* had integrated 650 bp within the *B. thetaiotaomicron* gene BT1339. The BT1339 gene product, annotated as an undecaprenyl-phosphate-alpha-*N*-acetylglucosaminyltransferase in the *B. thetaiotaomicron* VPI-5482 genome (Accession Number NC_004663.1), had sequence identity to a glycosyltransferase group 4 family protein PG0106 from *Porphyromonas gingivalis* (37% identity and 59% similarity in 362 amino acid residues), TagO, a putative undecaprenyl-phosphate-*N*-acetylglucosaminyltransferase from *Bacillus subtilis* (31% identity and 47% similarity in 328 residues), and to BT2888, a putative glycosyltransferase from *B. thetaiotaomicron* VPI-5482 (45% identity and 65% similarity in 340 residues).

The BT1339 gene product of 368 amino acids contained the Rfe family conserved domain. This family is made up of members such as the *E. coli* WecA (formerly known as Rfe), *B. subtilis* TagO as well as the *B. fragilis* WecA (also known as WcfL) among others (Amer and Valvano, 2001; Saldo *et al.*, 2002). Members of this family are crucial in multi-enzyme complexes involved in synthesis of surface polysaccharides in both prokaryotes and eukaryotes (Amer and Valvano, 2001). The capsular polysaccharide has been implicated in promoting drug exclusion and Fernebro *et al.* (2004) showed that capsular polysaccharides contributed to antibiotic tolerance in pneumococci. It is, therefore, possible that capsular modification is playing a role in the metronidazole sensitivity of Mutant B. It is, then, of interest to review the literature on the part played by this family of enzymes in capsular polysaccharide biosynthesis and to see whether *B. thetaiotaomicron* and *B. fragilis* might have similar systems.

The bacterial members of the Rfe family catalyse the first step in the synthesis of surface polysaccharides, the linking of a membrane-associated undecaprenyl-phosphate to *N*-acetylhexosamine-1-phosphate. The product formed is used as an acceptor for the addition of sugars by other glycosyltransferases, to form the polysaccharide repeating subunits. The best-characterized Rfe family protein is the *E. coli* WecA. It contains 11 transmembrane regions with 6 cytosolic regions, motifs I to V and the C-terminal region

(Amer and Valvano, 2001). These cytosolic regions are also found in the other members of the family, including BT1339 gene product (Fig 2.7). Within motif II and III, there are two highly conserved aspartic acid residues ($D_{106 \text{ and } 107}$, and D_{170} and D_{173} in BT1339, respectively, Fig 2.7). These are thought to be involved in interactions with the undecaprenyl-phosphate-N-acetylhexosamine products as well as with Mg^{2+} and Mn^{2+} ions needed for enzymatic activity, and for catalytic activity, respectively (Amer and Valvano, 2002). Within motif V, there are highly conserved residues, such as an arginine (R_{284} in BT1339, Fig 2.7) and HIHH sequence involved in binding the nucleotide substrate, UDP-*N*-acetylhexosamine ($HIHH_{298\text{-}301}$ in BT1339, Fig 2.7) (Anderson *et al.*, 2000).

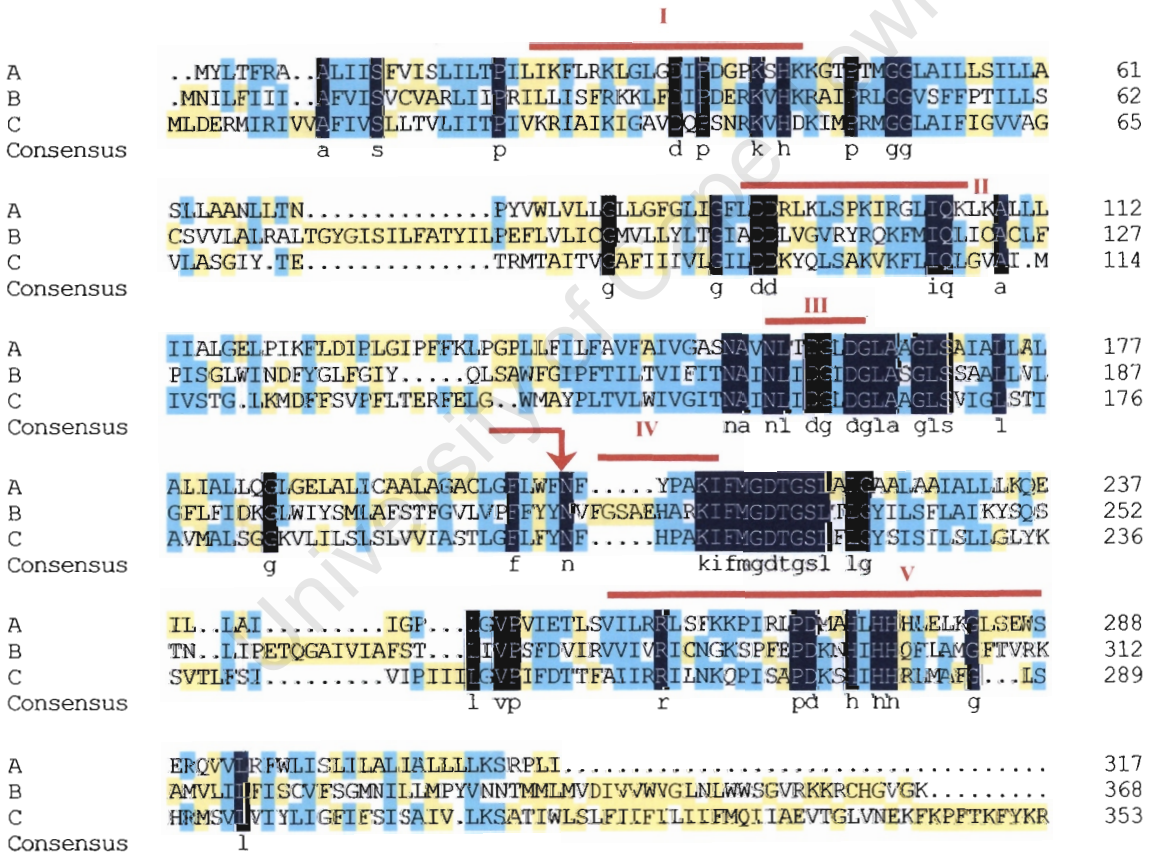

Figure 2.7 Multiple sequence alignment of the *B. thetaiotaomicron* BT1339 gene product with the Rfe family proteins. **A:** Rfe proteins consensus (Accession number COG0472); **B:** *B. thetaiotaomicron* BT1339 gene product (NP_810252); **C:** *B. subtilis* TagO (NP_391433). The inverse transposon is inserted into BT1339 gene product following amino acid residue 216, as shown by the red arrow. Cytosolic motifs I to V are underlined in red. Shading identity shown as 100% (black), 50% (blue), 33% (yellow), with the consensus depicted by lower case below the amino acid alignment.

BT1339 forms part of locus 4 (Fig 2.8), one of the seven polysaccharide biosynthesis loci present in *B. thetaiotaomicron* (Xu *et al.*, 2003). Since disruption of this gene in *B. thetaiotaomicron* results in the metronidazole sensitivity phenotype, it is interesting to examine the *B. fragilis* genome for the presence of a homologous gene which might serve a similar function.

Capsular polysaccharide biosynthesis loci have been well characterized in *B. fragilis* (reviewed in Section 1.1.4.1). Both the *B. fragilis* and *B. thetaiotaomicron* capsular polysaccharide biosynthesis loci contain homologous genes coding for carbohydrate biosynthetic enzymes, such as flippases and glycosyltransferases. However, the *B. thetaiotaomicron* genetic arrangement within the loci is different to the *B. fragilis* arrangement, and the *B. thetaiotaomicron* genes homologous to the *B. fragilis* ones are not clustered in the same loci as in *B. fragilis* but are scattered within the different *B. thetaiotaomicron* loci. This is clearly seen by the fact that *B. thetaiotaomicron* contains 7 such loci while *B. fragilis* contains 9 loci. The BT1339 gene codes for an undecaprenyl-phosphate-alpha-*N*-acetylglucosaminyltransferase. As mentioned previously, this is the initiating enzyme within the pathway. Almost every capsule biosynthesis locus in *B. thetaiotaomicron* and *B. fragilis* contains a gene coding for that type of enzyme. The closest *B. fragilis* homologue to BT1339 is BF3464 of *B. fragilis* 9343 (72% identity, 81% similarity in 365 amino acid residues), coding for a putative LPS biosynthesis related glycosyltransferase within the PS_H/PS_3 locus (gene c, Fig 2.8), and BF3665 of *B. fragilis*YCH46 (72% identity and 82% similarity in 363 amino acid residues), coding for an undecaprenyl-phosphate-alpha-*N*-acetylglucosaminyltransferase within the PS-8 locus (Kuwahara *et al.*, 2004).

In *B. fragilis*, capsule polysaccharide biosynthesis genes are transcribed as an operon from a promoter upstream of *upxY* (Krinos *et al.*, 2001). *B. thetaiotaomicron* seems to have a similar mechanism of regulation of these loci (Xu *et al.*, 2003; Comstock and Coyne, 2003). The lack of the undecaprenyl-phosphate-alpha-*N*-acetylglucosaminyltransferase in Mutant B would prevent formation of the capsular polysaccharide formed by locus 4, since this enzyme is the initiating enzyme for the formation of the polysaccharide. *E. coli* mutants with a disruption in their *wecA* (*rfe*) gene are defective in synthesis of their surface polysaccharides (Meier-Dieter *et al.*, 1990;

Barua *et al.*, 2002). One of these mutants was found to be sensitive to acetic acid (Barua *et al.*, 2002). It was suggested that surface polysaccharides were indispensable for resistance to acetic acid and other short chain fatty acids. There are no reports in the literature linking sensitivity to metronidazole to the loss of capsular polysaccharides. Britz and Wilkinson (1979) isolated *B. fragilis* mutants highly and moderately resistant to metronidazole. Both types of mutants were unable to activate the drug, but the highly resistant mutants were also found to have larger capsules. There is a possibility that the thick capsule decreases the entry of metronidazole into the cell by acting as a physical barrier. Visual microscopy indicated the presence of a thick capsule in Mutant B. Loss of a polysaccharide involved in capsule formation in Mutant B might affect the capsule structure and composition, allowing better entry of the metronidazole and therefore causing it to be more sensitive than the WT parent.

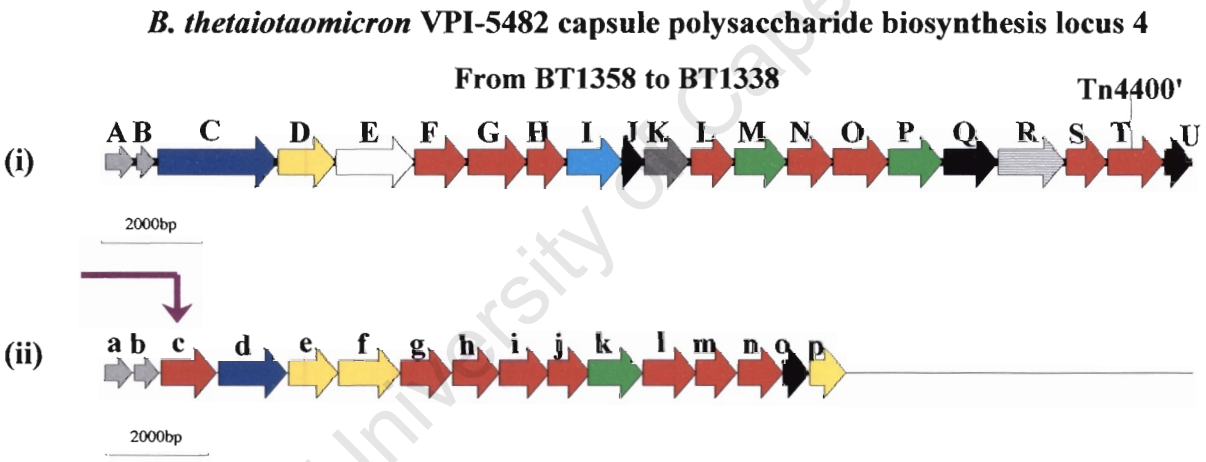

Figure 2.8 Genetic arrangement of the (i) *B. thetaiotaomicron* VPI-5482 capsule polysaccharide biosynthesis locus 4 containing BT1339 and (ii) *B. fragilis* 9343 PS_H/PS_3 locus containing the BT1339 homologue. Insertion of the inverse transposon Tn4400' within BT1339 (T) in Mutant B is shown by a thin black line. The *B. fragilis* 9343 homologue to BT1339, BF3464 (c) is indicated by a purple junction arrow. *B. thetaiotaomicron* genes BT1358 to BT1338 are represented by elements A-U. *B. fragilis* 9343 genes BF3466 to 3451 are represented by elements a-p. Genes a and b represent uphY and uphZ respectively, while A and B represent the *B. thetaiotaomicron* upxY and upxZ homologues. Glycosyltransferases are shown in red, transport proteins in blue, epimerases in black, dehydratases in turquoise, dehydrogenases in horizontal stripes, flippases in white, synthases in white cross-hatching, capsule proteins in green and hypothetical proteins in yellow.

2.4.3.2 Sequence analysis of the disrupted genes in Mutant A

In section 2.4.2.3, it was suggested that the double mutant A might share an integration site with Mutant B, in addition to a further integration event. In order to investigate the validity of this assumption, identification of the genes mutated in Mutant A was attempted by the *Hin*dIII plasmid rescue technique (Fig 2.1, Section 2.3.8). Plasmid rescue yielded two different species of plasmids, one of approximately 9 kb in size and the other 5.8 kb. This confirmed the previous result which suggested that Mutant A was a double insertion mutant. Plasmid rescue was attempted using *Pvu*II, a restriction enzyme which does not cut within pYT646, however this was unsuccessful.

Sequencing of the linearised *Hin*dIII-digested 5.8 kb plasmid revealed a sequence with identity to vector pBSL168 followed by the β-lactamase protein. This was expected and corresponded to the transposon end of the *Hin*dIII-digested fragment, containing the *bla* gene and the IS*4400*R (Fig 2.2). Sequencing of the other end of the plasmid resulted in sequence with identity to the end of the *ermF* gene from Tn4551, followed by the end of the *tetX* gene in the opposite orientation. This corresponded exactly to the sequence of pYT646B following the *Hin*dIII site at the end of the *ermF* gene (Fig 2.2). This plasmid therefore contained the *Hin*dIII internal fragment of pYT646B, containing the ampicillin resistance gene (Fig 2.9). Since pYT646B is unable to replicate in *Bacteroides*, this could only be a result of a cointegrate being present within the chromosome of Mutant A. The region pf pYT646 where the cross-over occurred for the integration of the cointegrate in the chromosome could not be identified. Southern hybridisation and PCR was used to attempt to find it (Results not shown). The presence of the second integration prevented the identification of this region, and therefore the chromosomal fragment disrupted by this cointegrate could not be identified.

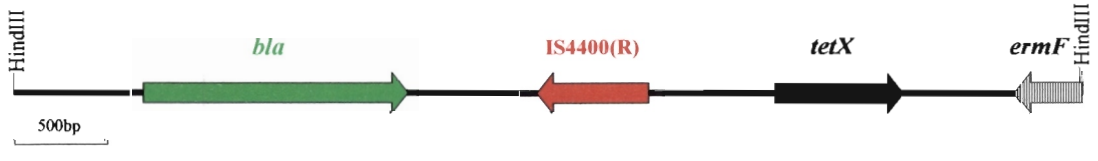

Figure 2.9 Map of the 5.8 kb rescue plasmid from Mutant A, representing an internal fragment of pYT646B. The *ermF* gene is truncated and only the terminal part of the gene is present in this plasmid.

Sequence analysis of the chromosomal region adjacent to the IS4400(R) of the 9 kb plasmid revealed that Tn*4400'* had integrated 650 bp downstream of the ATG start codon of *B. thetaiotaomicron* gene BT1339. This meant that Tn*4400'* had integrated at the exact same position, within the same gene in both Mutant A and B, an event which is unlikely to have happened, unless it is a hotspot for integration. A more likely explanation for this result would be that Tn*4400'* integrated into the *B. thetaiotaomicron* chromosome, 650 bp from the start of BT1339 forming Mutant B. Following a few rounds of cell division, a Mutant B cell had a cointegrate plasmid inserted at a different position in its chromosome, forming Mutant A. Mutant A is also sensitive to Mitomycin C relative to Wildtype *B. thetaiotaomicron* while Mutant B is not (Table 2.3). This suggests that the sensitivity to Mitomycin C might be due to disruption of the unknown chromosomal region by insertion of the cointegrate.

2.4.3.3 Sequence analysis of the disrupted gene in Mutant C

Mutant C was shown to have a single insertion of Tn*4400'* (Fig 2.6, Lane 4), and this was confirmed by the rescue of a single species of plasmid, of approximately 4.4 kb in size. Sequencing of the chromosomal DNA adjacent to the inverse transposon identified the insertion site as 1500 bp downstream of the ATG of *B. thetaiotaomicron* gene BT4755. Gene BT4755 had an open reading frame of 2612 bp, encoding a putative protein of 870 amino acid residues. This putative protein was annotated in the published sequence as a 2-component system sensor histidine kinase (Accession No NP_813666) but had low sequence identity to other histidine kinases and DNA mismatch repair proteins (Table 2.4). The BT4755 gene product contained the HATPase_c conserved domain in the last 100 amino acid residues of the protein. This domain is the histidine kinase domain of the sensor protein involved in phosphorylation (Xu *et al.*, 2004). The N-terminus of the predicted protein had the HtpG conserved domain of molecular chaperones. However, the N-terminus of the protein also seemed to have similarity to DNA mismatch repair proteins (Table 2.4).

BT4755 was followed by an open reading frame, BT4756 whose 231-residue product had low sequence identity to a response regulator from *Cytophaga hutchinsonii* (36% identity in 68 amino acid residues) (Fig 2.10). This suggested that BT4755 and BT4756 formed

part of a classical two-component system as described by Xu *et al.* (2004). The gene immediately preceding BT4755 was described as a putative cytosine-specific methyltransferase and contained both DNA_methylase and Cyt_C5_DNA_meth conserved domains. These genes were preceded by a cluster of proteins similar to DNA methylases and methyltransferases (Fig 2.10).

Table 2.4. Sequence identity and similarity of BT4755 gene product to proteins from various organisms.

Gene Product	**% Identity**	**% Similarity**	**BT4755 residues with identity**
Wolinella succinogenes hypothetical protein, signal transduction histidine kinase	26% in 414 residues	47% in 414 residues	Residue 15 to 412
Erwinia carotovora putative signal transduction protein	27% in 236 residues	44% in 236 residues	Residue 15 to 194
Erwinia crotovora hypothetical protein weakly similar to *S. pombe* DNA mismatch repair protein	31% in 149 residues	47% in 149 residues	Residue 26 to 149
Streptococcus mutans UA159 possible DNA mismatch repair protein	25% in 425 residues	44% in 425 residues	Residue 5 to 429
Streptococcus mutans UA159 putative two-component sensor protein	23% in 160 residues	43% in 160 residues	Residue 700 to 857

The fact that BT4755 is located immediately downstream of a putative cytosine-specific methyltransferase, and its N-terminus has sequence similarity to DNA mismatch proteins suggests that BT4755 is part of a two-component system possibly involved in methylation of DNA and mismatch repair. This suggests a possible connection between mismatch repair and metronidazole DNA damage. No genes with significant identity or similarity to BT4754, BT4755 nor BT4756 were found in *B. fragilis* NCTC 9343.

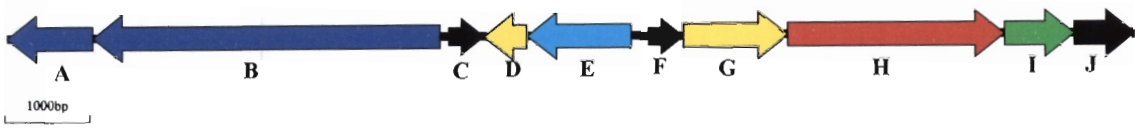

Figure 2.10 Genetic arrangement of the *B. thetaiotaomicron* VPI-5482 region containing BT4755. BT4748 to BT4757 are represented by elements A-J. A: similarity to putative DNA methylase; B: putative DNA methylase; C: hypothetical protein; D: similarity to methyltransferases; E: conserved protein with similarity to proteins adjacent to DNA topoisomerases and methylases; F: hypothetical protein; G: putative cytosine-specific methyltransferase (BT4754); H: Histidine kinase (BT4755); I: similarity to response regulator (BT4756); J: hypothetical protein.

2.4.3.4 Sequence analysis of the disrupted gene in Mutant D

As shown in Figure 2.6 (Lane 3), Mutant D also contained a single insertion of the inverse transposon Tn*4400'*. Plasmid rescue of the flanking chromosomal DNA fragment resulted in a 4.5 kb plasmid. Analysis of the DNA sequence at the junction between the transposon and the Mutant D chromosomal DNA revealed that Tn*4400'* had integrated in an intergenic region, 11 bp downstream of the putative stop codon of *B. thetaiotaomicron* gene BT3767 and 131 bp upstream of the putative start codon of BT3768. The 1154 bp BT3767 gene codes for a putative protein of 384 amino acids, described in the annotated *B. thetaiotaomicron* VPI-5482 genome as a lactaldehyde reductase, also known as FucO. This is followed by the 899 bp BT3768 gene coding for a protein with 299 amino acid residues with sequence identity to an AraC/XylS Family transcriptional regulator. These two genes are part of the putative *B. thetaiotaomicron* rhamnose gene cluster (Fig 2.12, E). *B. thetaiotaomicron* is able to grow on L-fucose and L-rhamnose (Salyers and Pajeau, 1989), however little is known on the exact assimilation pathways of these 2 sugars. In order to analyse a possible reason why a disruption in this pathway might result in metronidazole resistance, the well-studied *E. coli* L-fucose and L-rhamnose assimilation pathways are used as the paradigm for further comparison.

In *E. coli*, L-rhamnose and L-fucose are metabolized in parallel (Fig 2.11) (Boronat and Aguilar, 1981). Each pathway has a permease (FucP and RhaT for fucose and rhamnose respectively), an isomerase (FucI and RhaA), a kinase (FucK and RhaB) and an aldolase (FucA and RhaD). The genes for these enzymes are in two different gene clusters in the

chromosome (Fig 2.12, A and D). The last step, catalysed by the aldolase cleaves the 6-carbon sugars into dihydroxyacetone-phosphate and L-lactaldehyde (Fig 2.11). Dihydroxyacetone is channeled onto the general metabolism to generate energy. Under aerobic conditions, L-lactaldehyde is oxidized to L-lactate by an NAD-dependent lactaldehyde dehydrogenase, encoded by the *ald* gene, and then to pyruvate by a flavin-linked lactate dehydrogenase (Fig 2.11). Under anaerobic conditions, L-lactaldehyde has to be sacrificed as a hydrogen acceptor and it is reduced to L-1,2-propanediol (Fig 2.11). This is catalysed by an NAD-linked oxidoreductase, FucO. The gene coding for this enzyme, *fucO*, is part of the fucose regulon (Fig 2.12, A).

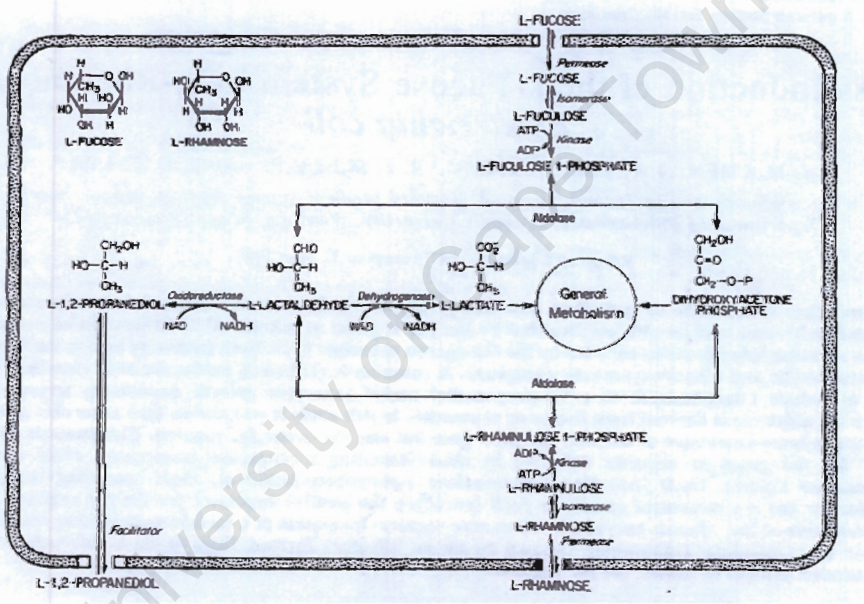

Figure 2.11 The catabolism pathways for L-fucose and L-rhamnose in *E. coli* (Chen and Lin, 1984).

The *E. coli fuc* regulon consists of two divergent operons, *fucPIK* and *fucAO* (Fig 2.12, A). These are under the positive control of FucR, which is activated by L-fuculose-1-phosphate, as well as CRP, the cyclic AMP receptor protein (Chen *et al.*, 1989; Podolny *et al.*, 1999). L-rhamnose induces the *rha* regulon (Power, 1967) and anaerobically it also induces expression of not only *fucO* but also the whole *fuc* regulon (Chen and Lin, 1984; Chen *et al.*, 1987). L-lactaldehyde is needed for cross-induction of the L-fucose enzymes by rhamnose (Chen *et al.*, 1987). It is thought to interact with FucR to induce the *fuc* regulon.

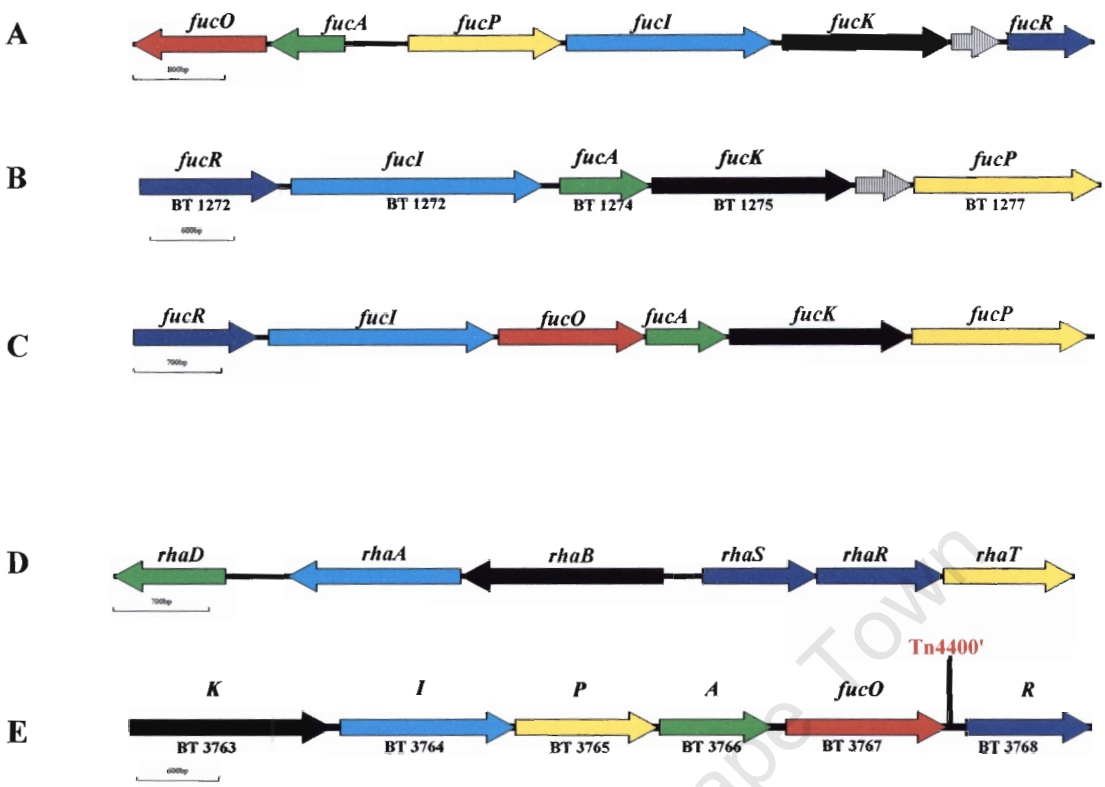

Figure 2.12. L-fucose and L-rhamnose dissimilation pathway genes in *E. coli*, *B. thetaiotaomicron* and *B. fragilis*. **A:** *E. coli fuc* regulon; **B:** *B. thetaiotaomicron* fucose regulon; **C:** *B. fragilis* putative fucose operon; **D:** *E. coli rha* regulon; **E:** *B. thetaiotaomicron* putative rhamnose gene cluster. Black, kinases; turquoise, isomerases; yellow, permeases; green, aldolases; red, reductases; blue, regulatory proteins; striated white arrows, hypothetical proteins.

Under aerobic conditions, FucO is present in the cell but is inactive (Chen and Lin, 1984). This is due to the fact that FucO is a group III Fe^{2+}-dependent alcohol dehydrogenase (Lu *et al.*, 1998). This group of enzymes contains a putative iron-binding motif with 3 conserved His residues (Fig 2.13, red arrows), 2 of them in a HXXXH motif (Obradors *et al.*, 1998). These residues are necessary for catalytic activity. A conserved Cys residue (Fig 2.13, blue arrow) is involved in protein stabilization. During aerobic growth, H_2O_2 reacts with Fe^{2+} forming the highly reactive $OH^{\cdot}$ radical (Lu *et al.*, 1998). This radical is thought to damage certain amino acid residues near the catalytic centre, causing decreased affinity for NAD. This is thought to be an adaptive self-destruct mechanism to prevent depletion of L-lactaldehyde and NADH aerobically.

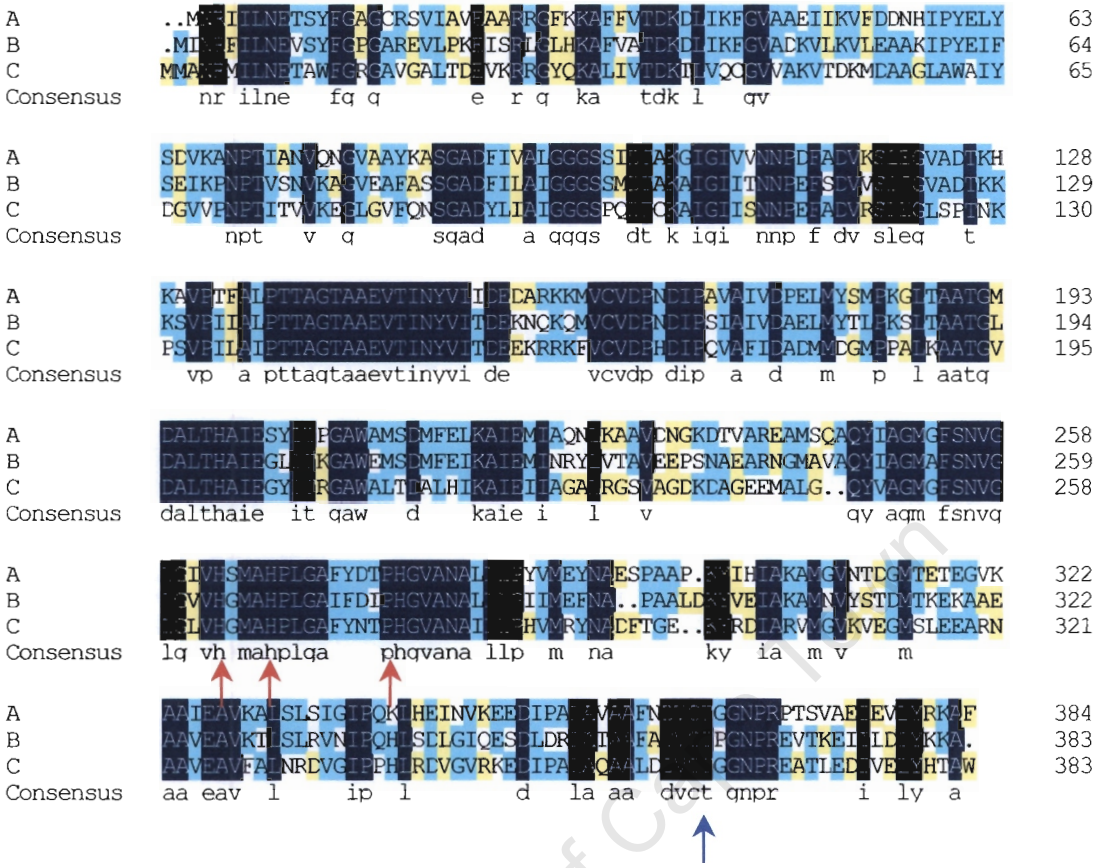

Figure 2.13. Multiple sequence alignment of the *B. thetaiotaomicron* BT3767 gene product with other FucO proteins. **A:** *B. thetaiotaomicron* BT3767 gene product (Accession number NP_812678); **B:** *B. fragilis* NCTC 9343 putative FucO; **C:** *E. coli* FucO (AAB40449). The red arrows indicate conserved His residues and the blue arrow, a conserved Cys residue. Shading identity is represented as 100% in black with the consensus as lower case below the alignment, 50% in blue and 33% in yellow.

The *B. thetaiotaomicron* VPI-5482 published genome data was used to analyse the putative L-rhamnose and L-fucose pathways. As is the case with *E. coli*, *B. thetaiotaomicron* contains two different pathways for the dissimilation of these two sugars but they both share the FucO protein. Unlike *E. coli*, the *fucO* gene is part of the putative rhamnose operon (Fig 2.12, E). The fucose regulon consists of 2 operons, *fucRIAK* and *fucP* (Fig 2.12, B) (Hooper *et al.*, 1999). In the absence of L-fucose, FucR binds to the *fucRIAK* promoter causing repression of those genes. L-fucose prevents FucR from binding to its own promoter allowing expression of *fucRIAK*. The putative rhamnose operon contains putative genes for the dissimilation of L-rhamnose (Fig 2.12, E), including *fucO*, whose gene product is necessary for both L-fucose and L-rhamnose

utilization as is the case in *E. coli*. The putative rhamnose operon contains a regulator, designated *R*. It is very likely a positive regulator since it is part of the AraC/XylS Family of transcriptional regulators which tend to be positive regulators (Gallegos *et al*., 1997). Within the intergenic region between *fucO* and the regulator, there are various putative consensus sequences for *Bacteroides* promoters, suggesting the possibility that the regulator has its own promoter (data not shown). Insertion of the inverse transposon downstream of the *fucO* gene could have disrupted expression of the regulator assuming it had its own promoter. Assuming it was a positive regulator of the rhamnose operon, there would not be expression of any of the genes, including *fucO*. The inverse transposon might have affected *fucO*, preventing formation of an active FucO protein.

In *Prevotella ruminicola*, L-rhamnose is fermented to acetate, formate, succinate and 1,2-propanediol (Turner and Robertson, 1979). L-lactaldehyde is one of the intermediates of this fermentation, suggesting a similar fermentation mechanism as that in *E. coli*. *B. fragilis* is known to have a very similar metabolism to *P. ruminicola*, as shown by their carbohydrate utilisation and end products of such utilisation (Macy *et al*., 1978). Assuming *B. thetaiotaomicron* also follows a similar pathway, disruption of FucO would force the pathway into lactate production. Metronidazole resistant *Bacteroides fragilis* mutants unable to activate the drug were found to have increased levels of lactate (Britz and Wilkinson, 1979; Narikawa *et al*., 1991). This was also the case in metronidazole resistant *Clostridium perfringes* (Sindar *et al*., 1982). All of these mutants were also shown to have slower growth, a characteristic also present in Mutant D (data not shown). The disruption of the lactaldehyde reductase, FucO, could have disrupted the electron transfer of the cell, preventing activation of metronidazole. *B. fragilis* does not have a rhamnose pathway, however it does contain a fucose dissimilation pathway (Fig 2.12, C) containing a *fucO* gene. It would be interesting to see whether disruption of that gene would also increase the resistance of the bacterium to metronidazole.

2.5 Conclusions

Due to its genetic intractability, construction of a large, representative *B. fragilis* transposon mutant library was not successful and no metronidazole mutants were isolated. The fact that introduction of foreign DNA into *B. fragilis* is difficult (Salyers *et al.*, 1999) makes the use of transposon mutagenesis problematic. It is necessary to introduce the transposon at very high frequency, to get a high number of insertions into the chromosome in order to have a large enough pool which might include the genes of interest. This is difficult to achieve in *B. fragilis*.

For this reason, *B. thetaiotaomicron* was chosen for construction of the transposon bank. *B. fragilis* and *B. thetaiotaomicron* are part of the same genus and at the time of this research project, they were thought to be closely related. The assumption was made that any genes of interest identified using the *B. thetaiotaomicron* mutant bank may have a *B. fragilis* homologue of similar function. This would then allow further characterisation of the *B. fragilis* homologue by direct mutagenesis. The published genome of *B. thetaiotaomicron* (Xu *et al.*, 2003), and both the incomplete genome sequence of *B. fragilis* NCTC 9343 (www.sanger.ac.uk/Projects/B_fragilis) and the recently published sequence of *B. fragilis* YCH46 (Kuwahara *et al.*, 2004), however reveal that these two organisms are not as genetically similar as previously thought. The genome size of *B. fragilis* is around 5.2 Mb while that of *B. thetaiotaomicron* is 6.26 Mb. Browsing through the genomes also reveals a large number of genes which are unique to each species. This diversity was evident in the identification of the disrupted genes in the *B. thetaiotaomicron* metronidazole mutants. In order to identify *B. fragilis* genes involved in metronidazole resistance, a more direct approach would be more useful in the future, such as the construction of specific mutants by insertion or deletion mutations.

The isolation of four *B. thetaiotaomicron* mutants with a different metronidazole MIC as the wildtype gives an insight into some possible mechanisms of metronidazole resistance. Two mutants with increased sensitivity to metronidazole were isolated, Mutant A and Mutant B. Both these mutants had an insertion of the transposon within the same gene, suggesting that these were siblings. Mutant A however, had an additional cointegrate insertion in a different chromosomal locus. Due to the lack of suitable restriction enzymes, the cointegrate locus could not be identified. Salyers *et al.* (2000) reported that

transposon Tn*4351* was able to insert by itself or carrying the vector forming a cointegrate. The two different types of insertions occurred at a frequency of approximately 50%. Tang and Malamy (2000) also reported cointegrate formation with the inverse transposon Tn*4400'* used in this study, however the frequency of cointegrate formation was much lower than that found with Tn*4351* or that found with Tn*4400'* in this study.

The Tn*4400'* transposon insertion present in Mutant A and Mutant B was within one of the seven capsule polysaccharide loci of *B. thetaiotaomicron*. *B. fragilis* contains nine capsule polysaccharide and these are different to those from *B. thetaiotaomicron*. It was, therefore, not possible to compare the disrupted polysaccharide locus of *B. thetaiotaomicron* with the *B. fragilis* loci. Although the identification of a *B. thetaiotaomicron* gene involved in metronidazole resistance cannot be extrapolated directly to *B. fragilis*, it can give insight into possible mechanisms of metronidazole resistance. Disruption of a capsular polysaccharide locus caused increased metronidazole sensitivity in *B. thetaiotaomicron* Mutants A and B. This suggests that the capsule might be involved, possibly preventing entry of metronidazole into the cell. Further work is needed to test this hypothesis, such as doing a comparison of drug uptake in the wildtype *B. thetaiotaomicron* and Mutants A and B.

Metronidazole resistant mutants of *B. thetaiotaomicron* were also identified. Mutant C contained an insertion within a gene coding for a putative histidine kinase. This two-component sensor gene was located within a cluster of cytosine-specific methyltransferases and possible mismatch genes. Homologues of the putative histidine kinase gene or the downstream and upstream genes, coding for the receptor component and the methyltransferase respectively, however were not present in *B. fragilis*.

B. thetaiotaomicron Mutant D was also more resistant to metronidazole than the wild type. The transposon insertion was not within a coding region but within the intergenic region between the lactaldehyde reductase and transcriptional regulator of the *B. thetaiotaomicron* putative rhamnose gene cluster. The transposon insertion was only 11 nucleotides downstream of the stop codon of the lactaldehyde reductase gene, also known as *fucO*, and 131 bp upstream of ATG of the transcriptional regulator. Insertion of the transposon could have had various effects leading to lack of expression of the FucO

protein. Assuming the rhamnose locus is an operon and the transcriptional regulator does not have its own promoter, insertion of the transposon downstream of *fucO* might cause a polar effect by interrupting transcription of the regulator. The regulator is likely to be a positive regulator since it forms part of the AraC Family of positive regulator (Gallegos *et al.*, 1997). This would then decrease expression of the rhamnose operon and therefore decreased expression of *fucO*. Various putative promoter sequences have been identified upstream of the transcriptional regulator suggesting it has its own promoter. Insertion of the transposon might have disrupted promoter sequences needed for efficient expression of the regulator, once again causing decreased expression of the operon. Assuming *B. thetaiotaomicron* follows a similar fucose/rhamnose pathway to *E. coli* (Chen and Li, 1984), decreased levels of lactaldehyde reductase in the cell would cause disruption of the electron flow, as well as increased levels of lactate. This phenomenon has been found in *Clostridium* and *B. fragilis* cells resistant to metronidazole (Sindar *et al.*, 1982; Britz and Wilkinson, 1979; Narikawa *et al.*, 1991), suggesting a similar mechanism in Mutant D. *B. fragilis* does not have genes for rhamnose metabolism, however it has a cluster of genes that may be involved in fucose metabolism containing the *fucO* homologue. Disruption of the *B. fragilis fucO* by insertion or deletion mutations would confirm the hypothesis that the phenotype seen in Mutant D is due to lack of expression of FucO, directly or indirectly. Analysis of both *B. thetaiotaomicron* and *B. fragilis fucO* mutants might give some insight the pathway(s) used by the cell to activate metronidazole.

Genetic studies are helpful to prove hypotheses supported by biochemical analysis as well as to discover novel pathways which are not identified using a biochemical approach. Transposon mutagenesis is a good method to achieve this. However, transposon mutagenesis is difficult in *B. fragilis*, and the use of close relatives such as was done in this study is sometimes not successful. Since no DNA repair genes involved in metronidazole resistance were identified, other genetic approaches were explored in Chapters 3 and 4, such as complementation of *E. coli* DNA repair mutants, to identify *B. fragilis* DNA repair genes involved in metronidazole resistance.

CHAPTER 3

Cloning of a *B. fragilis* gene involved in metronidazole resistance in *E. coli*

3.1 Abstract

Metronidazole, a DNA damaging agent, is the antibiotic of choice for treating *B. fragilis* infections. Isolation of *B. fragilis* bfl DNA repair genes involved in metronidazole resistance was attempted by multicopy suppression of *E. coli* DNA repair mutants with a *B. fragilis* gene bank. A construct, pAN2, conferring mitomycin C and metronidazole resistance on *E. coli uvrA*$^-$, in the presence and absence of oxygen, was isolated. This construct was also able to increase the resistance of *E. coli uvrB*$^-$ to metronidazole, both aerobically and anaerobically, but had no effect on *E. coli uvrC*$^-$ or *recA*$^-$. However, pAN2 did not affect the resistance of any of the *E. coli* mutants to ultraviolet light. Sequencing of pAN2 revealed the presence of three open reading frames, ORF1, ORF2 and ORF3. ORF1 coded for a truncated protein with high amino acid sequence similarity to the RecQ DNA helicases, involved in DNA repair, replication and suppression of illegitimate recombination caused by certain DNA damaging agents. Expressed in a divergent manner, ORF2 coded for a putative AraC Family transcriptional regulator. ORF3 was located downstream of ORF2 and coded for a protein with 23% sequence identity to dipeptidyl peptidase III metalloproteases. Deletion studies showed that ORF2 alone was sufficient to confer metronidazole and mitomycin C resistance on *E. coli uvrA*$^-$ and *uvrB*$^-$. This suggests the possibility that ORF2 might be interacting with *E. coli* genes in a heterologous manner.

3.2 Introduction

Metronidazole is one of the major drugs used to combat *Bacteroides fragilis* infections. Metronidazole resistance mechanisms have been studied with the focus mainly on the genes involved in reduction and activation of the drug (reviewed in Section 1.2 of Chapter 1). Other possible resistance mechanisms, such as the repair by DNA repair enzymes of the DNA damage caused by metronidazole, have not been addressed. To date, no *B. fragilis* DNA repair genes involved in metronidazole resistance have been isolated although a *recA* mutant of *B. thetaiotaomicron* has been shown to be metronidazole sensitive (Cooper *et al.*, 1997). In addition, *E. coli* nucleotide excision repair mutants have been shown to be more sensitive to metronidazole than the wild type isogenic strain (Yeung *et al.*, 1984; Jackson *et al.*, 1984).

The DNA damaging agent, mitomycin C, causes interstrand DNA crosslinks (Iyer and Szybalski, 1963). This type of damage is repaired by the nucleotide excision repair system (Friedberg *et al.*, 1995; Van Houten *et al.*, 1986). Since *E. coli* nucleotide excision repair mutants are more susceptible to metronidazole (Yeung *et al.*, 1984; Jackson *et al.*, 1984), there is a possibility that both metronidazole and mitomycin C damage is repaired using the same enzymes. Increased resistance to mitomycin C could, therefore, be used as an indirect selection procedure for metronidazole repair genes. Since metronidazole is only activated anaerobically (Ingham *et al.*, 1980), mitomycin C also allows the screening to be done under aerobic conditions. The gene(s) isolated could then be further analysed anaerobically for resistance to metronidazole, as done by Dachs *et al.* (1995).

This approach was used in the present study. *E. coli* nucleotide excision repair mutants were used to screen a *B. fragilis* DNA library on mitomycin C, with the intention of finding *B. fragilis* DNA repair genes involved in metronidazole resistance. Although transcription of *B. fragilis* genes in *E. coli* can be inefficient, presumably due to differences in transcriptional signals (Smith *et al.*, 1992). Despite these differences, a large number of *B. fragilis* genes have been isolated by complementation into *E. coli* mutants, such as the *B. fragilis glnA* (Southern *et al.*, 1986), *gdhB* (Abrahams and Abratt, 1998), *KatB* (Rocha and Smith, 1995) and the DNA repair *recA* (Goodman *et al.*, 1987).

3.3 Materials and Methods

3.3.1 Bacterial strains and plasmids

B. fragilis bf1 was described by Mossie *et al.* (1979). The *E. coli* isogenic strains used in the screening for DNA repair genes are listed in Table 3.1. *E. coli* JM109 (Yanisch-Perron *et al.*, 1985) was used for transformation of recombinant plasmids. The *B. fragilis* gene library was constructed using the suicide vector pEcoR251 (Appendix 2) (Zabeau and Stanley, 1982) as described by Southern *et al.* (1986). The recombinant plasmid pAN2, and its deletion derivatives, contained *B. fragilis* DNA cloned into pEcoR251. Plasmid pMT104, which contains an insertion within the *Eco*RI gene of pEcoR251 inactivating it (Wehnert *et al.*, 1992), was used as a negative control.

Table 3.1. *E. coli* K12 strains used for screening *B. fragilis* gene library.

***E. coli* strain**	**Genotype**	**Relevant genotype**	**Reference or source**
AB1157	*rac*$^-$, *ara*, *argE*, del(*gpt-proA*)62, *galK*, *hisG*, *kdgK*, *lacY*1, *leuB*6, *mgl*51, *mtl*1, *qsr*, *rfbD*1, *rpsL*, *supE*44, *thi*1, *thr*1, *tsx*33, *xyl*5	*uvr*$^+$ *recA*$^+$	ATCC 29055 DeWitt and Adelberg (1962)
AB1884	*uvrC*34 (AB1157 derivative)	uvrC$^-$	Howard-Flanders *et al.*, 1966
AB1885	*uvrB*5 (AB1157 derivative)	*uvrB*$^-$	Howard-Flanders *et al.*, 1966
AB1886	*uvrA*6 (AB1157 derivative)	*uvrA*$^-$	Howard-Flanders *et al.*, 1966
AB2463	*recA*13 (AB1157 derivative)	*recA*$^-$	Howard-Flanders *et al.*, 1966

3.3.2 Media and growth conditions

B. fragilis was grown as described in Section 2.3.1. *E. coli* strains were grown aerobically as described in Section 2.3.1. Screening of the gene library was conducted on LB agar (Sambrook *et al.*, 1989) supplemented with ampicillin (Ap) (100 μg/ml), and mitomycin C (MTC) at a concentration range of 0 to 1.0 μg/ml as described in the text.

3.3.3 Construction of the *B. fragilis* recombinant DNA library and screening for DNA repair genes

The construction of the *B. fragilis* DNA library has been described by Southern *et al.* (1986). Recombinant plasmids from pools of clones (about 10 000 per pool) were transformed into competent *E. coli* AB1884 (*uvrC*$^-$), AB1885 (*uvrB*$^-$), AB1886 (*uvrA*$^-$) and AB2463 (*recA*$^-$) mutants. Mitomycin C resistant transformants were selected on LB agar containing 100 µg/ml Ap and MTC at concentrations above the minimum inhibitory concentration (MIC) for each mutant, 0.6 µg/ml for AB1886; 0.8 µg/ml for AB1885; 0.8 µg/ml for AB1884; and 0.25 µg/ml for AB2463. *E. coli* AB1157 (pMT104) was used as the wild type repair control.

3.3.4 General recombinant DNA procedures

Procedures for plasmid and genomic DNA extraction, and DNA manipulations and modifications were described in Section 2.3.2.

3.3.5 Measurement of cell survival during mitomycin C treatment

Stationary phase cultures of *E. coli* AB1886 (pAN2), as well as *E. coli* AB1157 and AB1886 containing pMT104, were diluted 10^{-2} into one-quarter strength Ringer's solution (22.5% NaCl, 1.05% KCl, 1.2% CaCl, 0.5% $NaHCO_3$) containing mitomycin C at 10 µg/ml. Samples were removed at various time intervals and suitable dilutions were plated on LB agar.

3.3.6 Mitomycin C and metronidazole resistance assays

Stationary phase cultures of the *E. coli* strains described in Table 3.1, containing pAN2 or pMT104, were diluted appropriately and plated onto LB agar containing Ap (100 µg/ml) and either mitomycin C (0-2.4 µg/ml) or metronidazole (0-1000 µg/ml) to a colony density of 100 cfu/plate. The MIC was determined after 24 hours incubation at 37°C aerobically. For anaerobic MIC determination, the cultures were plated onto prereduced

yeast-tryptone (YT) agar supplemented with 0.5% glucose and 0.2% sodium nitrate, containing Ap (100 μg/ml) and metronidazole (0-260 μg/ml) and incubated for 48 h at 37°C in an anaerobic chamber.

3.3.7 Ultraviolet (UV) resistance assays

Stationary phase *E. coli* cells were diluted appropriately and plated onto LB agar containing Ap (100 μg/ml). The inoculated plates were irradiated with a Cole-Parmer, 9815-Series UV lamp (Cole-Parmer Instruments Co, Chicago) at a UV (254nm) dose rate of 1.0 Joules.m^{-2}.sec^{-1}, as measured with a Blak-Ray® UV Meter (UltraViolet Products Inc., California). The MIC was determined after 24 h incubation at 37°C (Hanada *et al.*, 2000).

3.3.8 Southern hybridization

Southern hybridization of *B. fragilis* genomic DNA was performed as in Section 2.3.5.

3.3.9 Nucleotide sequencing

Subclones of pAN2 were constructed using the plasmid vector pBluescript II SK(+) (Stratagene, La Jolla). Sequencing reactions were performed as described in Section 2.3.9, using the universal M13 sequencing forward and reverse primers. The *B. fragilis* 9343 and 638R preliminary genome sequences produced by the Sanger Centre Sequencing group (www.sanger.ac.uk/projects/b_fragilis) were also used for analysis.

3.3.10 PCR of *B. fragilis* bfl ORF1 and ORF2

To clone the complete ORF1, the *B. fragilis* bfl *recQ* homologue (Section 3.4.5), two oligonucleotide primers were designed based on the preliminary genome sequence of *B. fragilis* 9343 (www.sanger.ac.uk/projects/b_fragilis): These were the *recQ*F forward primer, 5'-CCT TAG CTG AAT AGT CCG-3' at positions –409 to –391 upstream of the ORF1 ATG start codon, and the *recQ*R reverse primer, 5'-CCT ATT GCA ATT GGC

AGC-3' at positions +1956 to +1939 downstream of the ORF1 ATG start codon. PCR amplification was done in 50 µl reaction volumes containing 100 ng of *B. fragilis* chromosomal DNA, 0.8 µM of each primer, 2.5 mM Mg^{2+}, 250 µM deoxynucleotide triphosphates and 2.5U *Taq* I polymerase (Roche Diagnostics). The samples were amplified for 30 cycles as follows: 94°C for 1.5 min; 50°C for 1 min and 72°C for 1.5 min with a final elongation at 72°C for 5 min. PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics). The amplified product was blunted and ligated into pEcoR251 (Zabeau and Stanley, 1982) to construct pRecQ.

To clone ORF2, the transcriptional regulator homologue (Section 3.4.5), two primers were designed based on the preliminary genome sequence of *B. fragilis* 9343 (www.sanger.ac.uk/projects/b_fragilis): These were the *RegBgl*II forward primer, 5'-GGT TGT GGA AGA TCT CTT CC-3' at positions –306 to –287 upstream of ORF2 ATG start codon and the *AraCEnd* reverse primer, 5'-CAA GAT TAC GGT TGC AGC-3' at positions +599 to +582 downstream of ORF2 ATG start codon. PCR amplification, DNA purification and cloning were done as for ORF1, resulting in the recombinant plasmid pE2Reg.

3.3.11 Construction of deletion subclones of pAN2 for resistance assays

Plasmids pANBB, pANC and pANH were constructed by subcloning the 2.5 kb *Hin*dIII-*Bgl*II fragment, the 1.4 kb *Hin*dIII-*Pvu*II fragment and the 1.9 kb *Hin*dIII-*Bam*HI fragment from pAN2 respectively into pEcoR251. Plasmids pRecQ and pE2Reg were described in Section 3.3.10.

3.4 Results and Disussion

3.4.1 Isolation of *B. fragilis* DNA repair genes by complementation in *E. coli* DNA repair mutants

Mitomycin C (MTC) causes DNA damage which is repaired by the nucleotide excision repair system in *E. coli* (Van Houten *et al.*, 1986). In order to isolate *B. fragilis* DNA repair genes, a *B. fragilis* gene library (Southern *et al.*, 1986) was screened for increased mitomycin C resistance in *E. coli* DNA repair mutants AB1886 (*uvrA*$^-$), AB1885 (*uvrB*$^-$), AB1884 (*uvrC*$^-$) and AB2463 (*recA*$^-$). Ten *E. coli* AB1886 *uvrA*$^-$ transformants were isolated on plates containing 0.6 µg/ml MTC. Plasmid DNA was isolated from each transformant and restriction analysis of the plasmids showed that they were all the same species (data not shown). The recombinant plasmid was designated pAN2. Retransformation of pAN2 into *E. coli* AB1886 *uvrA*$^-$ confirmed that the plasmid conferred both Ap and MTC resistance. The restriction map of pAN2 (Fig 3.1) revealed that a 4.5 kb DNA fragment of *B. fragilis* genomic DNA was inserted into pEcoR251. The presence of the recombinant plasmid did not alter the growth rate of the *E. coli* host.

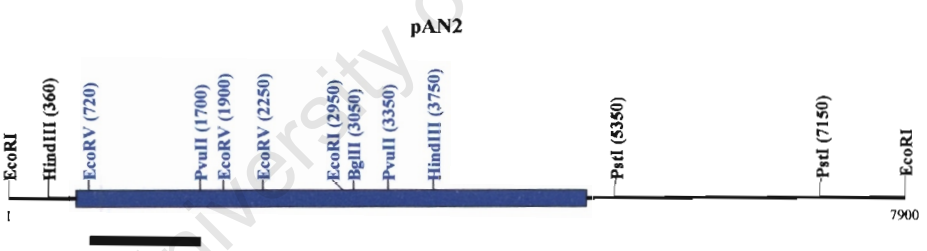

Figure 3.1. Restriction enzyme map of pAN2. Blue line, *B. fragilis* genomic DNA insert; thin black line, pEcoR251 vector; solid bar, *Eco*RV-*Pvu*II fragment used as a probe for Southern hybridization analysis.

3.4.2 Measurement of cell survival after mitomycin C treatment

The survival of *E. coli* AB1886 *uvrA*$^-$ containing pAN2, exposed to 10 µg/ml MTC was compared to that of *E. coli* AB1886 *uvrA*$^-$ transformed with the control plasmid pMT104 (Fig 3.2). *E. coli* AB1157 (pMT104) was the wildtype control. *E. coli* AB1886 (pAN2) showed increased resistance to MTC compared to *E. coli* AB1886 (pMT104), but did not reach the levels of the wildtype AB1157 (pMT104). This could be due to reduced

expression or incomplete complementation of the *B. fragilis* gene in *E. coli*. However, it is also possible that the gene involved in conferring resistance did not complement the *uvrA*⁻ mutation specifically. In order to ascertain this, it was necessary to assess the effect of pAN2 in the other *E. coli* DNA repair mutants.

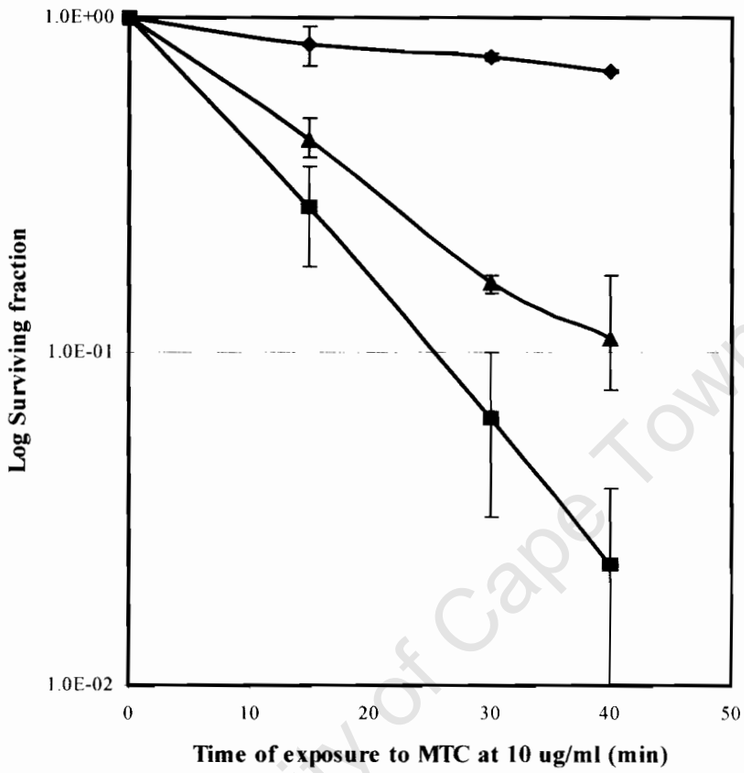

Figure 3.2. Mitomycin C survival curve of *E. coli* AB1886 transformed with pAN2 (▲) or the control plasmid pMT104 (■). *E. coli* AB1157 transformed with pMT104 was used as a measure of WT resistance to MTC (♦).

3.4.3 Mitomycin C, metronidazole and UV resistance assays

The possibility of pAN2 conferring resistance to various DNA damaging agents on other *E. coli* DNA repair mutants was investigated. Transformation with pAN2 conferred MTC resistance on *E. coli* AB1885 *uvrB*⁻ as well as *E. coli* AB1557, while no effect was seen

in *E. coli* AB1884 *uvrC*⁻ and *E. coli* AB2463 *recA*⁻ (Table 3.2). Transformation with pMT104, the control plasmid, did no influence the MIC's of the *E. coli* DNA repair mutants. This data showed no variation at all in the 10 replicate experiments.

Table 3.2. Susceptibility of *E. coli* strains transformed with pAN2 to various DNA damaging agents. A representative set of data is shown from 10 experiments.

***E. coli* strain**	**Mitomycin C MIC[a] (μg/ml)**	**Metronidazole MIC (μg/ml)**		**UV MIC (Joules.m^{-2})**
		O_2[b]	**AnO_2[b]**	
AB1157 (pAN2)	2.4	800	ND	40
AB1157 (pMT104)	1.8	800	ND	40
AB1886 (pAN2)	0.7	400	50	1.5
AB1886 (pMT104)	0.5	300	35	1.5
AB1885 (pAN2)	1.1	950	250	2.5
AB1885 (pMT104)	0.7	600	220	2.5
AB1884 (pAN2)	0.7	300	ND	2.5
AB1884 (pMT104)	0.7	300	ND	2.5
AB2463 (pAN2)	0.25	45	ND	ND
AB2463 (pMT104)	0.25	45	ND	ND

a The minimal inhibitory concentration (MIC) to the various DNA damaging agents was determined on LB agar plates under aerobic conditions, unless specified.

b The MIC's were determined under aerobic conditions (O_2) or anaerobic conditions in prereduced media (AnO_2).

ND Not determined.

Metronidazole resistance was increased in *E. coli* AB1886 *uvrA*⁻ and AB1885 *uvrB*⁻ containing pAN2, but not in *E. coli* AB1884 *uvrC*⁻, AB2463 *recA*⁻ nor AB1157 (Table 3.2). The metronidazole MIC's *E. coli* AB1886 (pAN2) and AB1885 (pAN2) were determined under anaerobic conditions. This was done to ensure that the metronidazole resistance was due to the damage caused by the reduced active metronidazole and not to damage by Reactive Oxygen Species formed during oxidation of reduced metronidazole into inactive metronidazole in the presence of O_2. The presence of pAN2 increased the

resistance to metronidazole in both *E. coli uvrA*⁻ and *uvrB*⁻ by a small but reproducible amount as determined by 10 replicate experiments. The ability of pAN2 to confer resistance to far UV irradiation was also investigated in the *uvr E. coli* mutants. None of the *uvr E. coli* mutants were more resistant to UV irradiation when transformed with pAN2 (Table 3.2). The MIC's of the *E. coli* strains transformed with the control plasmid pMT104 were the same as those with no plasmid.

The finding that the presence of pAN2 conferred higher MTC resistance to both *E. coli uvrA*⁻ and *uvrB*⁻ suggested that the gene(s) in the pAN2 insert was not complementing the *E. coli* mutation specifically. However, no increased resistance was noticed for *E. coli uvrC*⁻ or *recA*⁻ mutants. In the Nucleotide Excision Repair system of *E. coli*, the $UvrA_2$-UvrB complex is thought to bind to DNA, where the UvrA dimer recognises the DNA lesion and targets the UvrB subunit to the site of damage (Lin and Sancar, 1992). UvrC is involved in causing incisions of the DNA lesion, once the UvrB-DNA complex is formed (reviewed in Section 1.3.2, Chapter 1). The *B. fragilis* genes on pAN2 involved in conferring MTC resistance might be involved in recognition of DNA damage caused by MTC and, therefore, complementing the *uvrA*⁻ and *uvrB*⁻ mutants but not the *uvrC*⁻ *E. coli*. Apart from their role in Nucleotide Excision Repair (NER), UvrA and UvrB seem to have other functions in the cell. They are thought to be involved in suppression of illegitimate recombination in a pathway that is independent from the NER (Hanada *et al.*, 2000). DNA damaging agents which cause breaks in the DNA strand(s) directly, such as metronidazole, or indirectly by repair of the crosslinks, such as MTC, induce illegitimate recombination. UvrA and UvrB have also been implicated in a DNA replication backup system that replaces Polymerase I (PolI) and where UvrC is not involved (Moolenaar *et al.*, 2000). The possibility of the *B. fragilis* gene(s) on pAN2 complementing these functions of UvrA and UvrB cannot be discarded.

The pAN2 construct increased the resistance of the *uvrA*⁻ and *uvrB*⁻ *E. coli* mutants to metronidazole under aerobic and anaerobic conditions. This suggests that the resistance is to metronidazole damage itself and not to Reactive Oxygen Species generated under aerobic conditions by futile cycling.

3.4.4 Southern hybridization analysis

Southern hybridization analysis was used to confirm that the pAN2 insert DNA originated from *B. fragilis*. A 980 bp *EcoR*V-*Pvu*II internal DNA fragment from pAN2 was used as a probe (Fig 3.1) and hybridized to the *B. fragilis* genomic DNA digested with *EcoR*V-*Pvu*II, resulting in a single band of an expected size of ~1 kb (Fig. 3.3, Lane 2). The equivalent sized band was detected for pAN2 DNA digested with the same restriction endonucleases (Fig 3.3, Lane3). *B. fragilis* genomic DNA digested with *EcoR*V alone resulted in a single band of ~1.2 kb, as expected from Figure 3.1 (Fig 3.3, Lane1). The pAN2 probe did not hybridise to pMT104 DNA (Fig 3.3, Lane 4). This confirmed the origin of the pAN2 insert as *B. fragilis* DNA.

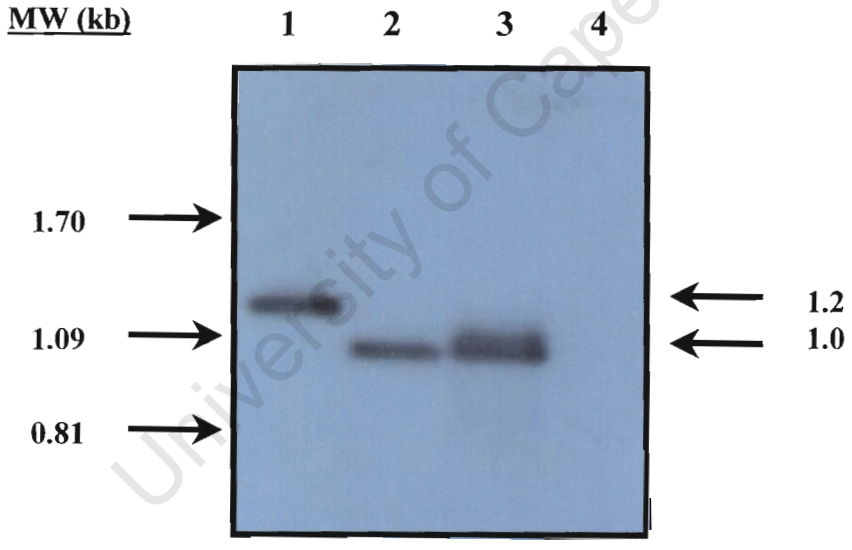

Figure 3.3 Southern blot analysis of *B. fragilis* genomic DNA and plasmid DNA probed with the internal *EcoR*V-*Pvu*II fragment of pAN2. Molecular mass markers in kilobases (kb) are shown on the left. Lane 1, *B. fragilis* bf1 genomic DNA digested with *EcoR*V; Lane 2, *B. fragilis* bf1 genomic DNA digested with *EcoR*V-*Pvu*II; Lane 3, pAN2 *EcoR*V-*Pvu*II; Lane 4, pMT104 *EcoR*V-*Pvu*II.

3.4.5 Deletion studies of pAN2 and nucleotide sequencing

In order to investigate the DNA regions responsible for the increased MTC and metronidazole resistance conferred by pAN2, deletion plasmids were constructed and

MTC and metronidazole sensitivity phenotypes of the various plasmids were determined in *E. coli* AB1886 (*uvrA*⁻) and AB1885 (*uvrB*⁻) (Fig 3.4). Only *E. coli* transformed with plasmid pANBB, which contained the first 2500 bp of the insert, conferred the same level of resistance as those containing pAN2. Cells transformed with pANC, which contained the first 1400 bp of the pAN2 insert and pANH, containing the last 1900 bp of the insert, showed levels of resistance similar to those with the control plasmid pMT104.

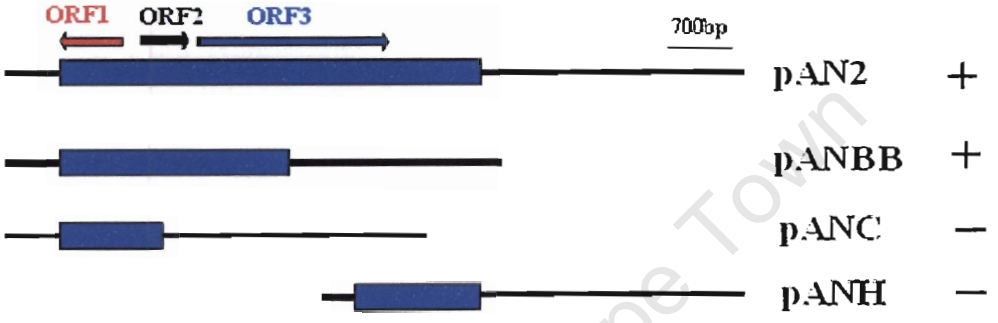

Figure 3.4 Deletion plasmids of pAN2 showing MTC and metronidazole resistance phenotypes in *E. coli* AB1886 (*uvrA*⁻) and AB1885 (*uvrB*⁻): +, increased resistance as compared to *E. coli* transformed with control plasmid pMT104; -, same level of resistance as with pMT104. Plasmids pANBB, pANC and pANH were derived by subcloning insert DNA fragments into pEcoR251 in the same orientation as pAN2. The pEcoR251 vector (thin black line) and the insert DNA (bold blue line) are shown. The arrows show the position and orientation of the 3 ORFs.

The active insert DNA region in pANBB was fully sequenced on both strands by construction of subclones in pSK(+) (not shown). This region contained an incomplete open reading frame (ORF1), truncated at the C-terminus; a complete ORF (ORF2) and a third ORF (ORF3) that was complete in pAN2, but only the N-terminus was present in pANBB (Fig 3.4). This suggested that ORF3 was not involved in conferring resistance since disruption of that gene did not affect the resistance phenotype (Fig 3.4, pANBB). Disruption of ORF2 caused loss of activity (Fig 3.4, pANC), implying the involvement of that gene in resistance, while the role of the truncated ORF1 in resistance was not clear and required further analysis.

3.4.5.1 Genetic analysis of ORF2

The percentage G+C of ORF2 was 39%, which was on the limit of the *B. fragilis* genomic DNA range of 39-48%, as determined by Holdeman *et* al. (1984). Translation of ORF2 started from a potential ATG start codon (nucleotide position 181) to a TAA stop codon (nucleotide position 645) (Fig 3.5).

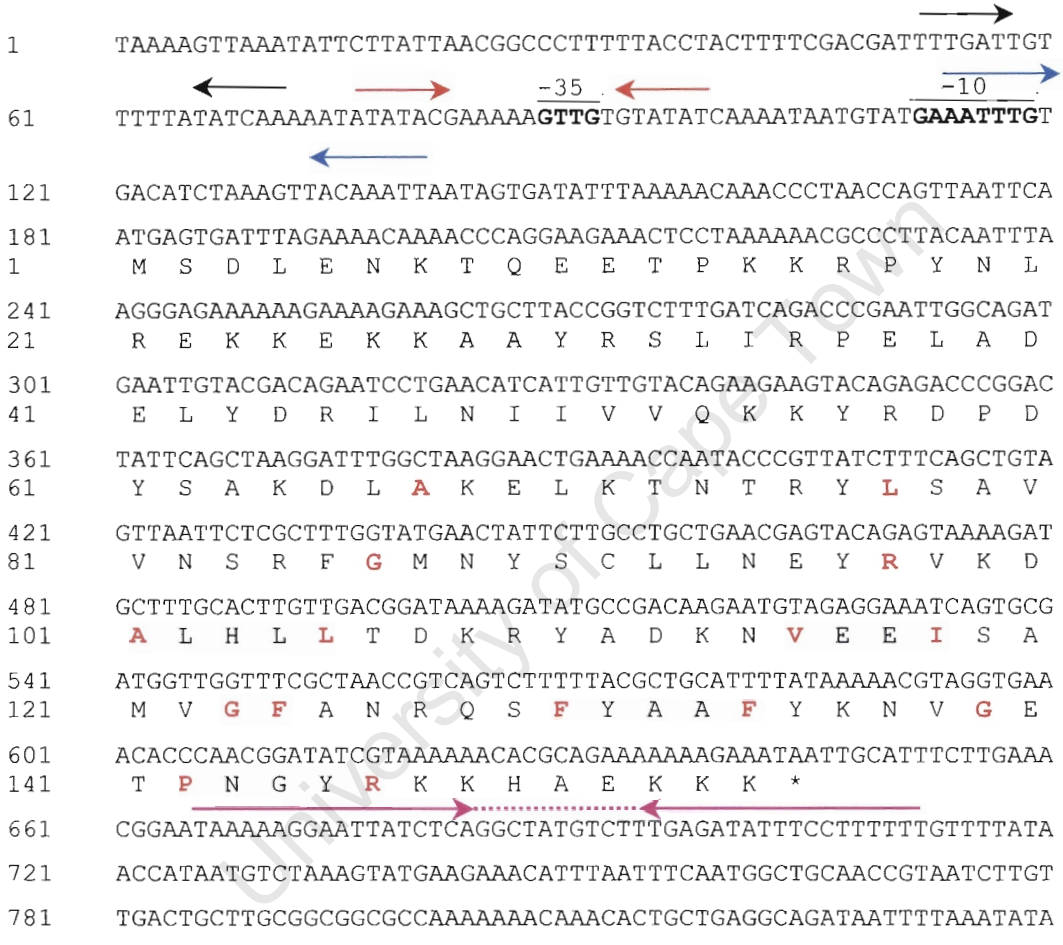

Figure 3.5 Nucleotide sequence of ORF2. The deduced amino acid sequence is shown underneath the corresponding codon sequences. The conserved amino acids to the AraC/XylS family of transcriptional regulators are shown in red. The upstream promoter sequence is shown, indicating the putative –10 and –35 promoter sequences. Arrows (black, blue and red) indicate inverted repeats in the promoter region. The downstream nucleotide sequence shows the putative factor independent terminator (pink arrows).

ORF2 was 465 bp in size and was able to encode a putative protein of 154 amino acids with a deduced molecular weight of 18.1 kiloDaltons (kDa) and sequence identity to the

AraC/XylS family of transcriptional regulators. This is significant since a number of these transcriptional regulators have been involved in multidrug resistance systems and response to alkylating agents (Gallegos *et al.*, 1997).

No typical *E. coli* ribosomal binding site was found upstream of the start codon of ORF2 (Shine and Dalgarno, 1974). Promoter sequences from various organisms differ slightly from the *E. coli* consensus promoter regions, but they have an overall similarity and conserved bases. However, due to their early evolutionary divergence from the main eubacterial branch (Woese, 1987), *B. fragilis* promoters contain unconventional motifs (Weisburg *et al.*, 1985). Weisburg *et al.* (1985) identified *Bacteroides* ribosomal binding sites from the 16S rRNA sequences and found them to be identical to the consensus sequence of *E. coli* (5'-GGAGGAA). However, other *B. fragilis* genes lack the typical ribosomal binding sites, such as *recA* (Goodman and Woods, 1990), *glnA* (Hill *et al.*, 1989) and *gdhB* (Abrahams and Abratt, 1998). The promoter region of ORF2 revealed the presence of sequences similar to the *B. fragilis* promoter consensus sequences, a –10 (TGAAATTTG) and a –35 (GTTG) (Bayley *et al.*, 2000) (Fig 3.5). Three sets of inverted repeats were identified in the promoter region and these might be involved in transcriptional regulation of ORF2. Termination of transcription in *E. coli* is either factor-dependent or factor-independent. A region containing a nearly perfect inverted repeat of 17 bp separated by 11 bp was identified downstream of the stop codon, able to form a stem-loop structure. This was followed by a T-rich region, suggesting the presence of a factor independent terminator (Rosenberg and Court, 1979; Brendel and Trifonov, 1984).

3.4.5.2 Genetic analysis of ORF1

ORF1 was situated upstream of ORF2 (Fig 3.4) and transcribed in the opposite direction, with a 213 bp intergenic region. The incomplete ORF1 started with a potential ATG start codon (nucleotide position 181, Fig 3.6) and was 669 bp in size. The truncated ORF1 encoded a polypeptide of 223 amino acids, with sequence identity to the RecQ DNA helicases from prokaryotes and eukaryotes. The presence of a RecQ helicase may be significant due to its involvement in DNA repair (Hanada *et al.*, 1997; Chakraverty *et al.*, 1999).

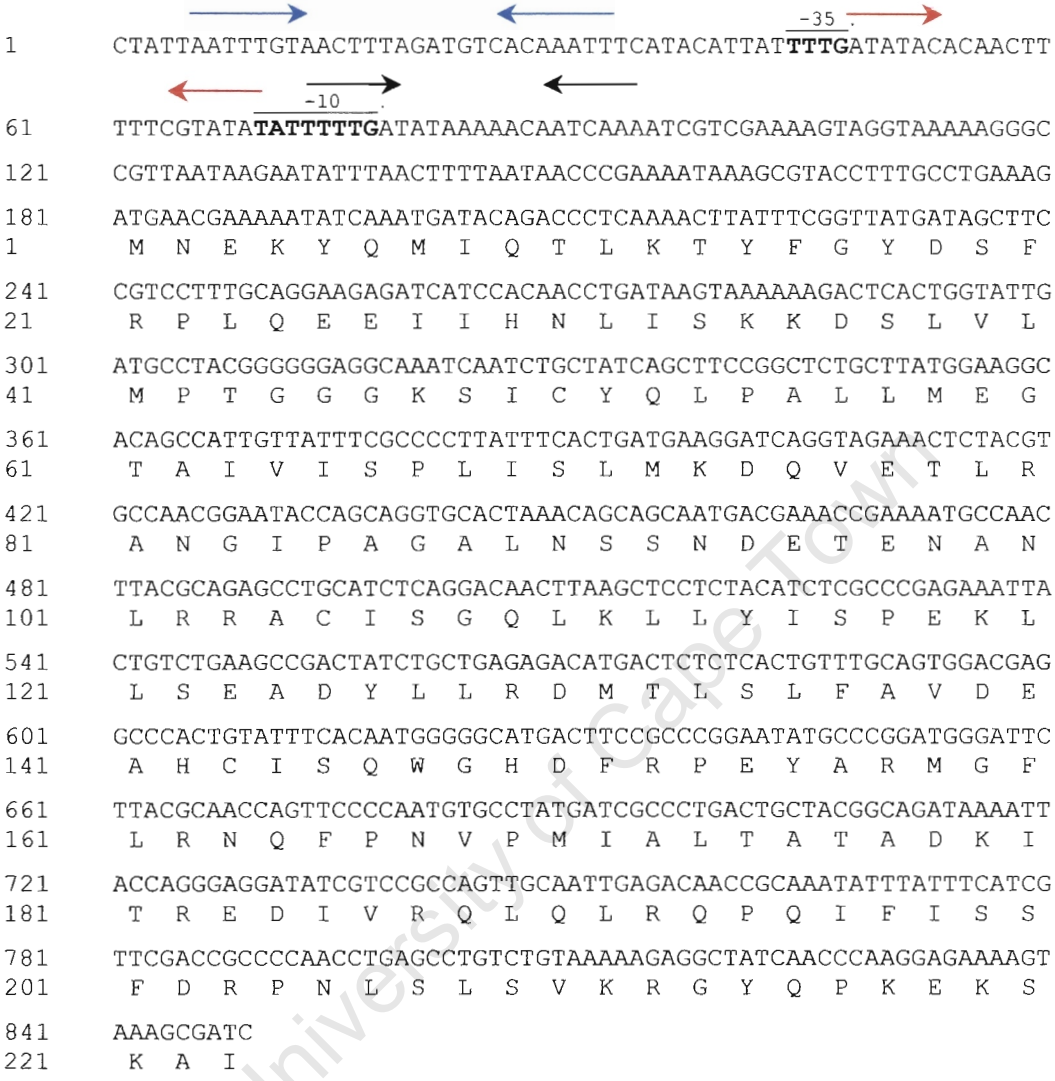

Figure 3.6 Nucleotide sequence of the incomplete ORF1as found in pAN2. The deduced amino acid sequence is shown underneath the corresponding codons. The upstream promoter sequence is shown, indicating the –10 and –35 promoter sequences. Arrows indicate inverted repeats in the promoter.

No conventional ribosomal binding site was found upstream of the start codon of ORF1, but the –10 (TATTTTTG) and –35 (TTTG) promoter sequences similar to the *B. fragilis* consensus promoter sequences were identified (Fig 3.6). Since ORF1 and ORF2 are separated by 213 bp and are transcribed divergently, the same three sets of inverted repeats found in the promoter of ORF2 were also present in the 5' upstream region of

ORF1 (Fig 3.6). As in ORF2, one set was found upstream of the –35 promoter sequence, while the other were located near the –35 and –10 promoter regions, which may once again involve a regulatory role.

In order to obtain the complete nucleotide sequence of ORF1, a BLAST search of the preliminary genome sequences of *B. fragilis* 9343 and 638R (www.sanger.ac.uk/projects/b_fragilis) was done. The *B. fragilis* bf1 669 bp sequence of the truncated ORF1 in pAN2 was 100% identical to the corresponding sequence of *B. fragilis* 9343 and 638R. Using these sequences, primers were designed and the complete *B. fragilis* bf1 ORF1 was amplified. In all 3 *B. fragilis* strains, ORF1 was found to be 1824 bp in size and capable of encoding a protein of 607 amino acids with a predicted molecular mass of 68.7 kDa. The G+C content of ORF1 was 49%.

3.4.5.3 Genetic analysis of ORF3

The ATG putative start codon of ORF3 started 92 bp downstream of ORF2 and was transcribed in the same orientation (Fig 3.4). Disruption of this gene in pANBB (Fig 3.4), did not alter the resistance phenotype of *E. coli uvrA*$^-$ or *uvrB*$^-$ harboring this plasmid, suggesting that ORF3 was not involved in metronidazole or MTC resistance. The nucleotide sequence from pANBB identified an ORF of 1318 bp with no stop codon, suggesting that the sequence of ORF3 was incomplete. The preliminary genome sequence of *B. fragilis* 9343 was used to obtain the complete nucleotide sequence of ORF3. The complete ORF3 was found to be 2052 bp in size, with a TAA stop codon, and a G+C content of 46%. ORF3 was able to code for a protein of 683 amino acids with a molecular mass of 76.9 kDa. The predicted amino acid sequence of ORF3 was used to search for homology in the NCBI database using the BLAST function (www.ncbi.nlm.nih.gov). This revealed homology to dipeptidyl peptidase III of eukaryotes with the highest identities to dipeptidyl peptidase III from *Leishmania major* (23%), *Drosophila melanogaster* (23%), *Rattus norvegius* (24%) and *Mus musculus* (24%). Dipeptidyl peptidases are zinc metallopeptidases with a conserved zinc-binding domain, HELLGH (Fukasawa *et al.*, 1999), which hydrolyse Arg-Arg-β-naphthylamides (Fukasawa *et al.*, 1998). The deduced amino acid sequence of ORF3 contained the zinc-binding domain

(amino acid position 456), HECLGH (data not shown), confirming its possible function as a zinc metallopeptidase.

3.4.5.4 Cloning and analysis of complete ORF1 and ORF2

The truncated ORF1 alone was not able to confer resistance to the *E. coli* mutants (Fig 3.4, pANC). In order to determine whether the complete ORF1 or ORF2 were involved in resistance, the 2 genes were subcloned separately into pEcoR251 and transformed into the *E. coli uvrA* and *uvrB* mutants. ORF2 alone was sufficient to confer both metronidazole and MTC resistance to the *E. coli uvrA*$^-$ or *uvrB*$^-$ mutants (Fig 3.7). The fact that ORF2 had high sequence identity to the AraC family of transcriptional regulators suggested the possibility that it was regulating *E. coli* genes which were, in turn, causing the resistance phenotype.

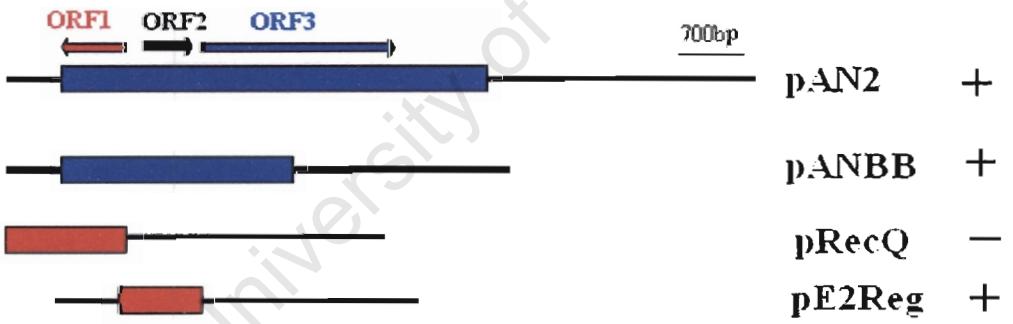

Figure 3.7 Deletion plasmids of pAN2 showing MTC and metronidazole resistance phenotypes in *E. coli* AB1886 (*uvrA*$^-$) and AB1885 (*uvrB*$^-$): +, increased resistance as compared to *E. coli* transformed with control plasmid pMT104; -, same level of resistance as with pMT104. The complete ORF1 and ORF2 were amplified (red bold line) and subcloned into pEcoR251 (thin black line) to form constructs pRecQ and pE2Reg, respectively. The arrows show the position and orientation of the 3 ORF's.

3.4.6 Analysis of the amino acid sequence of ORF2

As mentioned in section 3.4.5, the deduced amino acid sequence of ORF2 revealed homology to the AraC/XylS family of transcriptional regulators. This is one of the most

common families of positive regulators and it is characterized by a highly conserved region of 100 amino acid residues that constitutes the DNA binding domain (Gallegos *et al.*, 1997). The consensus for the family is shown in Table 3.3. The *B. fragilis* bfl AraC regulator (ORF2) contained most of the amino acids from the consensus sequence (Table 3.3 and Fig 3.8).

Table 3.3 Comparison of the amino acid consensus sequence for the AraC family of transcriptional regulators and ORF2.

AraC Family consensus[a]	A-----S---L---F----G----------R---A---L--------I/V—I/V----GF/Y-----F---FR/K---G—P---R
ORF2 conserved residues[b]	A---------L---------G----------R---A---L--------- V— I ---- GF -----F---F ---- G—P---R

a AraC family consensus sequence as described by Gallegos *et al.* (1997) where – represents any amino acid.

b ORF2 conserved amino acid residues from position 67 to 146 in Fig 3.8.

From the multiple sequence alignment of AraC transcriptional regulators, it was clear that most of the above residues were highly conserved in the members of the family (Fig 3.8). The DNA binding regulatory domain of the AraC transcriptional regulators MarA and Rob from *E. coli* have been well studied. These proteins are involved in regulating transcription of stress tolerance genes. The DNA binding domain consists of 7 α-helices that fold in such a way as to form two helix-turn-helix (HTH) motifs (Manzanera *et al.*, 2000; Rhee *et al*, 1998; Kwon *et al.*, 2000). In all 3 proteins, helix-2 and 3, and helix-5 and 6 form the HTH motifs, with helix-4 linking these two subdomains (Fig 3.8). The second HTH motif shows the highest homology between the members of the family (Manzanera *et al.*, 2000, Gallegos *et al.*, 1997). The crystal structure of MarA bound to its own promoter DNA showed the two HTH motifs binding to adjacent segments of the major groove causing a slight bend of the DNA (Rhee *et al.*, 1998). The structure of Rob bound to *micF* promoter DNA showed a similar situation, where the first HTH motif was in the major groove but the second HTH motif lay on the surface of the

DNA without bending the DNA (Kwon *et al.*, 2000). However, the two HTH motifs of the Rob DNA binding domain were superimposable on MarA (Martin and Rosner, 2001).

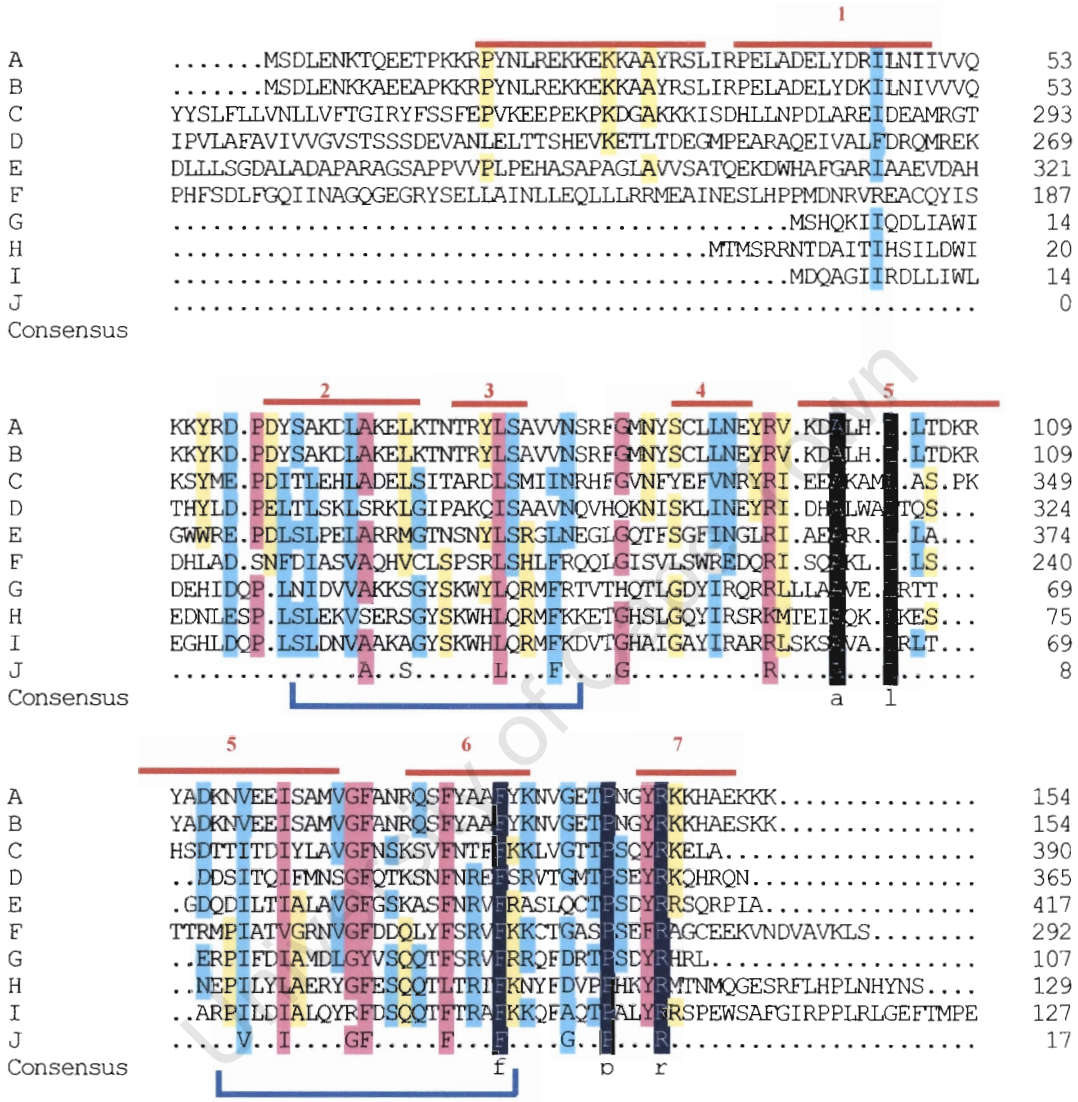

Fig 3.8 Multiple sequence alignment of the *B. fragilis* bfl putative AraC regulator (ORF2) with other AraC/XylS transcriptional regulators. **A:** *B. fragilis* bfl ORF2; **B:** *B. thetaiotaomicron* putative AraC regulator (accession number AAO76954); **C:** *Microbulbifer degradans* putative AraC regulator (ZP_00316144); **C:** *Vibrio cholerae* putative AraC regulator (F82398); **E:** *Xanthomonas campestris* AraC transcriptional regulator (AAM43294); **F:** *E. coli* AraC (CAA23507); **G:** *E. coli* SoxS (P22539); **H:** *E. coli* MarA (BAA15233); **I:** *E. coli* Rob (NP_418813); **J:** AraC Family conserved amino acid residues. The ORF2 residues forming α-helices are shown by a red line over them. The blue line shows the two helix-turn-helix motifs of the AraC transcriptional regulators. Shading identityy is represented as 100% in black, 75% pink, 50% in blue and 33% in yellow.

Gallegos *et al.* (1997) proposed that the high degree of conservation of 17 residues extending over 75 amino acids suggests that the DNA binding domain of these regulators have identical tertiary structure. Analysis of the possible secondary structure of the putative *B. fragilis* bf1 AraC regulator predicted the presence of 8 α-helices (Fig 3.8, red line above the amino acid residues). The first α-helix lay outside the region of homology, at the N-terminal of the protein. The other 7 α-helices (numbered 1 to 7 in Fig 3.8) were located within the conserved region. Helix-2 and 3 spanned over the region containing the HTH motif in the AraC regulators, suggesting the possibility of these helices forming a HTH motif. This suggestion was supported by the fact that this region followed most of the characteristics for a putative HTH motif, laid out by Brennan and Matthews (1989). In the same way, the end of helix-5 and helix-6 were situated in the same region as the second, highly conserved HTH motif and contained all the requirements for formation of a HTH motif, including a glycine residue at position 9 of the HTH motif. Since these transcription regulators are only highly conserved in their DNA-binding regulatory domain, comparison of the overall sequence of the bf1 AraC regulator and other AraC transcriptional regulators revealed very low identities. The highest identities were to the putative AraC transcriptional regulators from *B. thetaiotaomicron* (95% overall amino acid identity), *Microbulbifer degradans* (32% identity over 140 amino acids of the conserved region), *V. cholerae* (32% identity over 110 residues of the conserved region) and *X. campestris* (32% identity over 94 amino acids of the conserved domain).

3.4.7 Analysis of the amino acid sequence of ORF1

As mentioned in section 3.4.5, the deduced amino acid sequence of ORF1 revealed similarity to the RecQ family of DNA helicases, found in both prokaryotes and eukaryotes. In this study, ORF1 was not shown to be involved in the metronidazole resistance phenotype but since it may be involved in DNA repair, replication and suppression of illegitimate recombination in *B. fragilis*, it was analysed further.

The RecQ proteins belong to the superfamily II of helicases which contain 7 highly conserved motifs, I, Ia, II, III, IV, V and VI (Fig 3.8) (Gorbalenya *et al.*, 1989). Motif I is found exclusively in ATP/GTP binding proteins (Hodgman, 1988). It represents the

consensus sequence A for the NTP- binding motif as described by Walker *et al.* (1982). The consensus sequence consists of the following amino acid residues: GxxxxGK(T)xxxxxx(I/V), where x is any amino acid, and is preceded by a basic residue, mostly K or R (Walker *et al.*, 1982). In the helicases from superfamily II, the first conserved G from sequence A is replaced by a bulky residue, such as A or M, as is the case in the RecQ DNA helicases (Fig 3.9) (Gorbalenya *et al.*, 1989). Motif I is thought to form a loop which binds one of the phosphates from the triphosphate (Hodgman, 1988). Motif II contains the DEAD/H box (Gorbalenya *et al.*, 1989). The DEAD box is found in a large family of RNA helicases (Linder *et al.*, 1989). However a variant of this motif, DExH is found in other helicases of superfamily II (Gorbalenya *et al.*, 1989). The conserved D residue of motif II is thought to interact with Mg^{2+}, which in turn binds the other phosphate from the triphosphate. This is required for hydrolysis of ATP (Subramanya *et al.*, 1996). Motifs Ia, III, IV and V are less strictly conserved (Gorbalenya *et al.*, 1989). Motif Ia was identified by Lane (1988) and possibly interacts with the DNA (Subramanya *et al.*, 1996). Motif III may be involved in coupling the process of ATP hydrolysis with helicase activity. Motif VI contains an abundance of positively charged residues that might be involved in nucleic acid binding (Gorbalenya *et al.*, 1989). The DExH family of helicases contains the sequence QxxGRxxR in motif VI, while the sequence for the DEAD family is HxxGRxxR. The DNA binding helicases contain a conserved tyrosine residue in motif VI (Hodgman, 1988). All the proteins in superfamily II contain a common activity of nucleic acid-dependent NTPase and helicase.

The RecQ family members have three conserved regions: the Helicase domain, which spans from the N-terminus to motif VI; the RecQ-conserved domain (RecQ-CT), which is C-terminal to the Helicase domain; and the Helicase-and-RNase-D-C-terminal domain (HRDC), which is proposed to be involved in stable DNA binding (Morozov *et al.*, 1997; Bernstein and Keck, 2003). The Helicase and RecQ-Ct regions form a composite domain involved in the DNA-dependent ATPase and helicase activities (Bernstein and Keck, 2003). Within that domain a novel RecQ Family N-terminal motif has been proposed, motif 0, which is also important for catalytic activity. Motif 0 starts at residue 19 of the *E. coli* RecQ protein (residue 10 of the *B. fragilis* bf1 deduced amino acid sequence of ORF1, Figure 3.9), LxxxFGxxxFRxxQ.

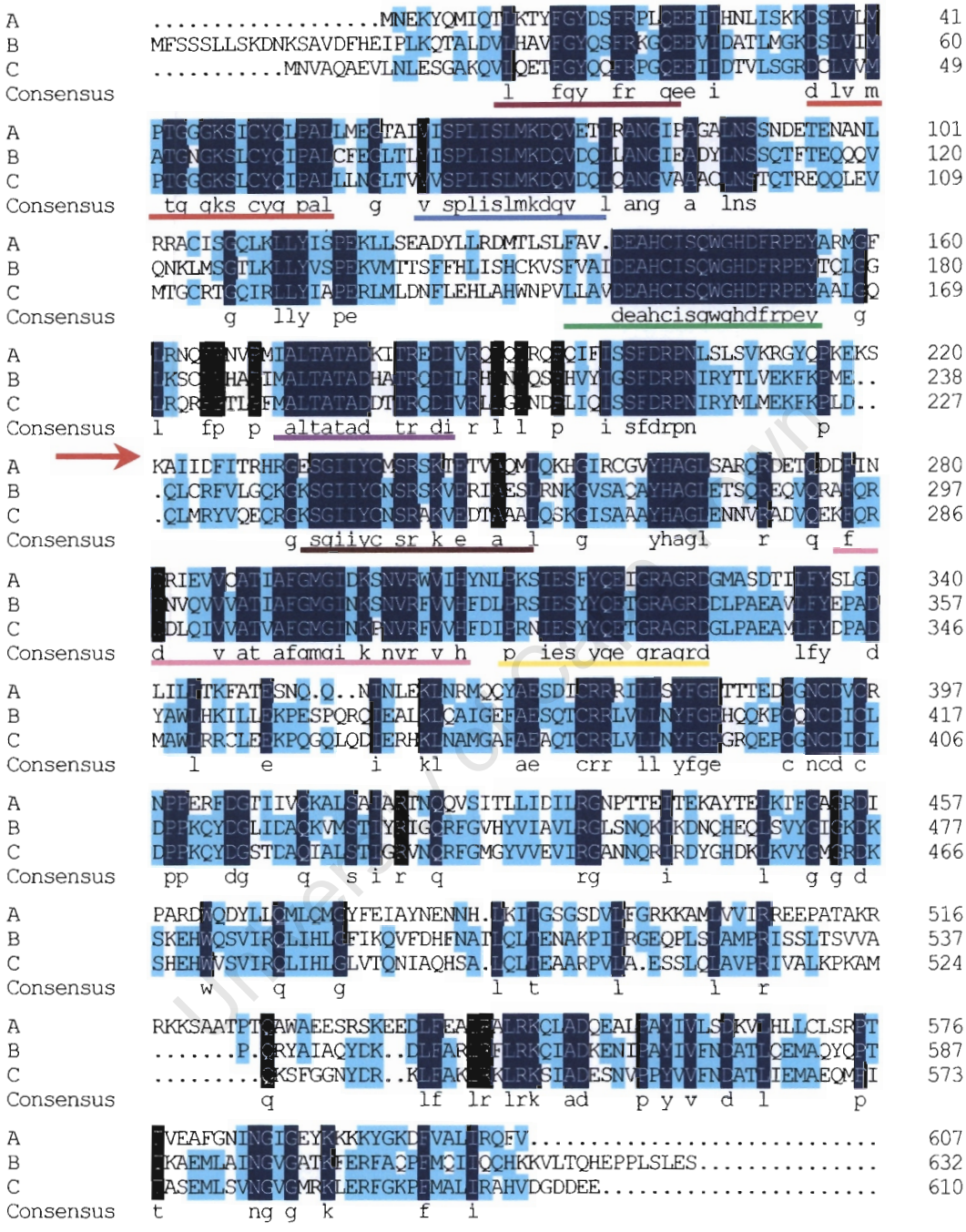

Figure 3.9 Multiple sequence alignment of the complete *B. fragilis* bf1 RecQ homologue (ORF1) with other RecQ DNA helicases. **A:** *B. fragilis* bf1 ORF1; **B:** *Pasteurella multocida* RecQ (accession number AAK03511); **C:** *Escherichia coli* RecQ (AAA67618); *B. fragilis* bf1 ORF1 is truncated at amino acid residue 223 in pAN2, as indicated by the red arrow. Motifs 0 (brown), I (red), Ia (blue), II (green), III (purple), IV (black), V (pink), and VI (yellow) are underlined.

As shown in Figure 3.9, the amino acid sequence of the complete ORF1 from *B. fragilis* bf1 contained all 8 conserved motifs. Its sequence revealed high identities to both prokaryote and eukaryote DNA helicases. The highest identities are shown in Table 3.4. The truncated putative ORF1 protein derived from pAN2 would only contain the first 220 amino acid residues and the first 5 motifs, 0, I, Ia, II and III (Fig 3.9, indicated by a red arrow). It is not likely that this truncated protein would be active since the complete Helicase and RecQ-Ct domain is necessary for ATPase and helicase activity (Bernstein and Keck, 2003) and the first 220 residues only represent two thirds of the Helicase domain.

Table 3.4 Sequence identity and similarity of complete ORF1 to RecQ homologues from other organisms.

RecQ homologue	% Identity[a]	% Similarity[b]	Length of protein (Amino acids)
Bacteroides thetaiotaomicron VPI-5482 (Xu *et al.*, 2003) Accession N° AAO76955	77% in 601 residues	86% in 601 residues	620
Pasteurella multocida (May *et al.*, 2001) Accession N° AAK03511	44% in 579 residues	63% in 579 residues	632
E. coli 0157:H7 (Welch *et al.*, 2002) Accession N° BAB38175	42% in 604 residues	59% in 604 residues	611
H. influenzae Rd KW20 (Fleischmann *et al.*, 1995) Accession N° AAC22387	43% in 580 residues	62% in 580 residues	619
Yersinia pestis KIM (Parkhill *et al.*, 2001) Accession N° CAC93301	42% in 612 residues	59% in 612 residues	610
E. coli K12 (Irino *et al.*, 1986) Accession N° AAA67618	42% in 604 residues	59% in 604 residues	610

Analysis using the preliminary genome sequences of *B. fragilis* 9343 and 638R (www.sanger.ac.uk/projects/b_fragilis) and the recently published genome of *B. fragilis* YCH46 (Kuwahara *et al.*, 2004) revealed the presence of two additional open reading frames that also had high sequence similarity to the RecQ DNA helicases (Table 3.5). All

three *B. fragilis* RecQ homologues in both strains contained the 7 conserved motifs of the superfamily II of DNA helicases and were highly similar within the N-terminus up to motif VI. *B. fragilis* RecQ homologue #3 contained a stretch of approximately 180 amino acid residues following motif VI, with no similarity to the other two *B. fragilis* RecQ homologues or to the *E. coli* RecQ. *E. coli* as well as lower eukaryotes such as yeast contain a single RecQ helicase; however higher eukaryotes have multiple RecQ helicases, humans having 5 different ones (Bachrati and Hickson, 2003). The presence of three RecQ homologues in the *B. fragilis* genome is interesting and raises various questions of what their various roles might be.

Table 3.5 Comparison of *B. fragilis* NCTC 9343 and *B. fragilis* YCH46 (Kuwahara *et al.*, 2004) RecQ homologues.

Name of ORF	***B. fragilis* ORF1 homologue**	***B. fragilis* RecQ homologue #2**	***B. fragilis* RecQ homologue #3**
***B. fragilis* 9343 gene draft annotation**	BF 3249	BF 3892	BF3706
***B. fragilis* YCH46 gene annotation**	BF 3427	BF 4076	BF3933
Length of predicted protein (amino acids)	607	726	634
% Identity to *E. coli* RecQ	43.7%	39.6%	39.8%
Preceding ORF	Putative AraC transcriptional regulator (ORF2 homologue)	ORF with similarity to *B. subtilis* inosine-5'-monophosphate dehydrogenase, GnaB	ORF with similarity to *P. gingivalis* single strand DNA specific exonuclease RecJ

3.5 Conclusions

E. coli nucleotide excision repair mutants have increased sensitivity to metronidazole (Yeung *et al.*, 1984; Jackson *et al.*, 1984), suggesting the involvement of the nucleotide excision repair system in repairing metronidazole damage. Since mitomycin C is active in the presence of oxygen and the DNA damage caused is repaired by the nucleotide excision repair system (Van Houten *et al.*, 1986), it was used to screen a *B. fragilis* genomic library in *E. coli*, aerobically. This was perhaps not the ideal selection method for *B. fragilis* genes involved in metronidazole resistance and future screening of those genes should be done in *E. coli* nucleotide excision repair mutants, anaerobically in metronidazole containing media. The availability of the *B. fragilis* 9343 and 638R incomplete genomes, as well as the recently published genome of *B. fragilis* YCH46 (Kuwahara *et al.*, 2004) should also allow the direct cloning of targeted DNA repair genes and analysis of their possible involvement in metronidazole resistance.

In the search for *B. fragilis* DNA repair genes involved in resistance to metronidazole, a construct from a *B. fragilis* genomic library was isolated. The construct, pAN2 contained 3 open reading frames, ORF1, ORF2 and ORF3. ORF2 alone was able to confer both metronidazole and MTC resistance to *E. coli uvrA*$^-$ or *uvrB*$^-$. The deduced amino acid sequence of ORF2 had homology to the AraC Family of transcriptional regulators. This family comprises largely of positive regulators involved in a diversity of functions, from carbon metabolism, to pathogenesis, to stress tolerance including multidrug resistance and DNA repair (Gallegos *et al.*, 1997). They are divided into 2 types, those in which the signal receptor is in the same polypeptide as the regulatory function and those in which the regulator and the signal receptor controlling expression of the regulator are found on separate genes. Examples of the former are AraC from *E. coli* involved in arabinose utilization and XylS from *P. putida* involved in benzoate catabolism. *E. coli* MarA, involved in multidrug resistance and SoxS, superoxide resistance, are examples of the latter type of regulators. The regulators involved in carbon metabolism stimulate transcription in response to effector molecules and are usually around 300 amino acids in length. They act as dimers and have a C-terminal conserved DNA binding domain and a N-terminal dimerisation and sugar-binding domain (Martin and Rosner, 2001). Those involved in pathogenesis do not usually bind effector molecules but respond to the

environment (Gallegos *et al.*, 1997). These are involved in adhesion, cell capsule, invasions, siderophores and other virulence factors. Stress tolerance regulators are involved in alkylation, oxidative stress, tolerance to antibiotics or organic solvents as well as transition from exponential to stationery phase. These need to be overproduced in order have a regulatory effect. They act as monomers and contain the DNA binding domain alone or have an additional domain (Martin and Rosner, 2001). There is a possibility that *B. fragilis* ORF2 is involved in stress tolerance and since it is only 154 amino acid residues in length, the protein would probably be only a transcriptional regulator without a signal receptor domain.

The fact that *B. fragilis* ORF2 on its own was able to induce resistance raises the possibility that *B. fragilis* ORF2 is regulating *E. coli* genes in a heterologous manner. *E. coli* MarA, SoxS and Rob are AraC transcriptional regulators involved in stress tolerance. MarA, involved in multidrug resistance, is a 129 amino acids protein (Cohen *et al.*, 1993), and SoxS, responsible for oxidative stress tolerance, is a 106 residues polypeptide (Li and Demple, 1994). Rob, which binds to the right border of the *oriC* of *E. coli* as well as being involved in superoxide and drug tolerance, is 289 amino acid residues in length and unlike the other two, contains another domain apart from the conserved DNA binding domain (Skarstad *et al.*, 1993). These three regulators are homologous but not identical and have slightly different functions. They are independently regulated but they all regulate a common set of over 20 genes in the *E. coli* chromosome (Martin *et al.*, 1999) which result in multiple antibiotic resistance, superoxide resistance and organic solvent tolerance. Examples of the target genes are *nfo*, coding for the DNA repair endonuclease IV; *micF*, which causes reduction of OmpF, one of the major porins; *acrAB*, involved in antibiotic efflux, *fpr*, a NADPH:ferredoxin oxidoreductase and *nfsA* and *nfnB*, two of the *E. coli* oxygen-insensitive nitroreductases, among others (Martin and Rosner, 2002). The fact that 3 different AraC transcriptional regulators activate similar genes suggests the possibility that *B. fragilis* ORF2 is also regulating *E. coli* genes which are usually regulated by an *E. coli* AraC transcriptional regulator. In order to test this hypothesis in future work, microarray technology could be used to identify *E. coli* genes which are induced in the presence of overexpressed *B. fragilis* ORF2 and to identify those genes

which might be, directly or indirectly involved in metronidazole and mitomycin C resistance.

The fact that pAN2 conferred metronidazole and mitomycin C resistance only to *E. coli* *uvrA*⁻ and *uvrB*⁻ and not *uvrC*⁻ nor *recA*⁻ suggested that ORF2 was regulating a gene or genes which suppressed the *uvrA* and *uvrB* mutations. In *E. coli*, RecQ as well as UvrAB are involved in suppression of illegitimate recombination and it is thought that they share a pathway (Hanada *et al.*, 2000). In *E. coli*, RecQ is also involved in the alternative pathway for repair of double strand breaks, the type of DNA damage caused by metronidazole (reviewed in Section 1.3.1, Chapter 1). ORF1 was not involved in conferring metronidazole resistance on the *E. coli* DNA repair mutants, however, the presence of this *recQ* homologue, upstream of ORF2 and expressed divergently suggested the possibility that ORF1 and ORF2 might interact at a regulatory level in *B. fragilis* itself. The regulation of both ORF1 and ORF2 in *B. fragilis* and their possible role in metronidazole survival was, therefore, analysed in *B. fragilis* bf1.

CHAPTER 4

Transcriptional Regulation of a *B. fragilis* gene involved in metronidazole resistance in *E. coli*

4.1 Abstract

The transcriptional regulation of the *B. fragilis* bfl ORF2 gene involved in mitomycin C and metronidazole resistance in *E. coli uvrA*⁻ and *uvrB*⁻ mutants was analysed, as well as the regulation of the *B. fragilis* bfl ORF1 gene, found upstream of ORF2 and expressed divergently. Primer extension analysis identified the ORF2 transcriptional start site as an adenine, 59 bp upstream of the ORF2 start codon. Sequences resembling the *B. fragilis* promoter consensus motifs were identified upstream of the transcriptional start site and possible regulatory inverted repeats were recognised overlapping the –10 and –35 promoter sequences. Expression of ORF2 in *B. fragilis* revealed a single 600 nucleotides transcript. Exposure of *B. fragilis* cells to metronidazole caused single stranded breaks, but there was no difference in the transcription of ORF2 before or after metronidazole treatment. Transcription of ORF1 in *B. fragilis* was, however, dramatically decreased following exposure to the drug.

4.2 Introduction

A *B. fragilis* bf1 gene involved in metronidazole resistance in *E. coli uvrA*⁻ and *uvrB*⁻ cells was isolated as described in Chapter 3. This gene encoded an AraC/XylS transcriptional regulator, a family of regulators known to be involved in many cellular processes, including multidrug resistance and DNA damage repair (Gallegos *et al.*, 1997). The DNA sequence immediately upstream of this gene contained putative *B. fragilis* promoter sequences as well as possible regulatory inverted repeats. A *recQ* homologue was identified further upstream of this gene, and transcribed in a divergent manner. However, this homologue did not seem to be involved in conferring metronidazole resistance on the *E. coli* cells.

There is a possibility that *B. fragilis* ORF2 was regulating *E. coli* genes which in turn confered increased metronidazole resistance on the *E. coli*. However, working in a heterologous host does not give insight on the function and regulation of ORF2, and whether ORF2 might also be involved in metronidazole resistance in *B. fragilis*. DNA is thought to be the main target of the reduced cytotoxic metronidazole intermediate (Edwards, 1977), reviewed in Section 1.2 of Chapter 1. It would, therefore, be interesting to study the *in vivo* effect of metronidazole on the DNA damage of *B. fragilis* DNA, as well as the response of the transcription of 2 putative target genes involved in metronidazole survival.

This chapter reports the analysis of a putative transcriptional start and a putative promoter of the ORF2 gene, as well as identification of a transcript by Northern blot. The effect of metronidazole on *B. fragilis* cells was studied *in vivo* and the damage and possible repair on the DNA of metronidazole treated *B. fragilis* cells was analysed. Expression in *B. fragilis* of the ORF2 gene, involved in metronidazole resistance in *E. coli* DNA repair mutants, as well as the ORF1 *recQ* homologue, was studied at those same conditions.

4.3 Materials and Methods

4.3.1 Bacterial strains, plasmids growth conditions

B. fragilis was grown as described in Section 2.3.1. Metronidazole was added to the media at a final concentration of 10 μg/ml. *E. coli* JM109 strain (Yanisch-Perron *et al.*, 1985) and LK112λ strain containing the relevant plasmid were grown as described in Section 2.3.1. Ampicillin was added at a final concentration of 100 μg/ml. Plasmids pAN2 and pMT104 were described in Chapter 3 (Section 3.3.1).

4.3.2 General recombinant DNA procedures

These procedures were described in section 2.3.2.

4.3.3 RNA extraction

Total RNA from *B. fragilis* bfl and *E. coli* was isolated from 30 ml mid-exponential phase cultures according to the method of Aiba *et al.* (1981). The quality of the RNA was confirmed by electrophoresis in 1.5% denaturing formaldehyde agarose gel (Fourney *et al.*, 1988) and quantified using spectrophotometric methods.

4.3.4 Primer extension analysis

The transcriptional start site of ORF2 was identified by nonradioactive automated primer extension using a Cy5-labeled primer PEReg (5'-GTC GTA CAA TTC ATC TGC-3'), corresponding to positions +132 to +114 downstream of the putative ATG start codon of ORF2. *B. fragilis* bfl RNA (70 μg) was precipitated and dissolved in 100 μl of HP buffer (40 mM PIPES, pH 6.4; 1 mM EDTA, pH 8.0; 400 mM NaCl, 80% deionised formamide). PEReg primer (5 pmol) was added to the RNA and the nucleic acids were denatured at 95°C for 10 min and hybridized overnight at 42°C. The annealed nucleic acids were precipitated with ice-cold ethanol and resuspended in 20 μl of RTB buffer [4 μl AMV Reverse Transcriptase buffer (Promega), 0.5 mM dNTP's, 40 U RNazin (Roche Diagnostics), 2 μg Actinomycin D] and the reverse transcription reaction was done using

20 U of AMV Reverse Transcriptase (Promega) at 42°C for 2 hours. Addition of 1 µl 0.5 M EDTA (pH 8.0) terminated the reaction and the RNA was degraded with 10 µg RNase A at 37°C for 30 min. The cDNA was precipitated and resuspended in 5 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6). Amersham STOP buffer (5 µl) was added. The primer extension products were sequenced using the ALFexpress Automated DNA Sequencer (Pharmacia Biotech) and analysed together with the sequence products using the same primer PEReg.

4.3.5 Construction of DNA probes for Northern hybridisation

B. fragilis bf1 16S rRNA gene probe. The 16S rRNA gene from bf1 was amplified using the universal bacterial primers for 16S rRNA, F1 forward primer, 5'-AGA GTT TGA TCI TGG CTC AG-3' (positions 8-27) and R5 reverse primer, 5'-ACG GIT ACC TTG TTA CGA CTT-3' (positions 1512-1492). PCR amplification was done in 50 µl reaction volumes containing 100 ng of *B. fragilis* chromosomal DNA, 0.5 µM of each primer, 1.5 mM Mg^{2+}, 150 µM deoxynucleotide triphosphates and 2.5U *Taq* I polymerase (Roche Diagnostics). The samples were amplified for 30 cycles as follows: 95°C for 0.5 min; 54.5°C for 0.5 min and 71°C for 1.5 min with a final elongation at 71°C for 3 min. PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics). The amplified product was cloned into the pGEM®-T Easy Vector (Promega). The *B. fragilis* 16s rRNA gene probe was released from the vector by digestion with restriction endonuclease *Not*I and DIG-labeled as described in section 3.3.8.

B. fragilis bf1 ORF1 probe. The ORF1 gene was amplified as described in section 3.3.10. An internal 1.29 kb *Pvu*II-*Eco*RV DNA fragment was isolated and DIG-labeled as described in Section 2.3.5.

B. fragilis bf1 ORF2 probe. ORF2 was amplified as described in section 3.3.10. A 200 bp *Pvu*II-*Eco*RV DNA fragment was purified and DIG-labeled as described in Section 2.3.5.

4.3.6 Northern hybridisation analysis

B. fragilis RNA (70 µg) and *E. coli* RNA (15 µg) were separated by electrophoresis in a 1.5% denaturing formaldehyde agarose gel and visualized by ethidium bromide staining. The RNA was transferred onto a nylon membrane (Roche Diagnostics) using an adapted version of the transfer method by Koetsier *et al.* (1993), where 20X SSC (3 M NaCl; 300 mM sodium citrate, pH 7.0) was used as the transfer solution. The transferred RNA was UV cross-linked using an Ultraviolet Crosslinker (RPN 2500/2501, Amersham Life Sciences). Hybridization with the DIG-labeled ORF2 DNA probe was performed for overnight at 50°C in EasyHyb hybridization buffer (Roche Diagnostics). Chemiluminescent signals were detected using CDP-Star™ (Roche Diagnostics), according to the manufacturer's instructions.

4.3.7 Measurement of cell survival during metronidazole treatment

Mid-logarithmic *B. fragilis* bf1 cells were exposed to metronidazole at different concentrations (5-50 µg/ml) and incubated at 25°C. Samples were removed at various time intervals and suitable dilutions were plated on supplemented BHI agar.

4.3.8 Measurement of cell survival during and following metronidazole treatment

B. fragilis bf1 cells were grown anaerobically to mid-logarithmic phase. Metronidazole (10 µg/ml) was added to the culture at time 0 (T_0) and incubated at 25°C for 15 min (T_{15}). The culture was centrifuged at 5000 rpm for 5 min and the cell pellet was resuspended in fresh medium without metronidazole, and incubated at 37°C. Samples were removed after 45 min (T_{60}), 30 min (T_{90}), and 60 min intervals (T_{150}, T_{210} and T_{270}). Viable cell numbers were determined by making suitable dilutions of the samples and plating them on supplemented BHI agar. Cells were also processed for DNA and RNA extraction for detection of single strand breaks and RNA Slot Blot hybridization, respectively (see below).

4.3.9 Detection of single strand DNA breaks

B. fragilis bf1 chromosomal DNA (1 µg) extracted at times T_0, T_{15}, T_{60} and T_{90} (Section 4.3.7) was loaded on a 0.5% alkaline denaturing agarose gel. The gel was prepared in neutral buffer (50 mM NaCl, 1 mM EDTA) and once solidified equilibrated for 1 hour in alkaline buffer (30 mM NaOH, 2 mM EDTA). Alkaline tracking dye (0.12% Bromocresol green, 25% glycerol, 0.5 M NaOH added just before use) was used to load the samples. The samples were electrophoresed at 20 volts overnight and the gel was neutralized in 0.1 M Tris buffer, pH 8.0 for 30 min followed by ethidium bromide staining.

4.3.10 RNA Slot Blot hybridisation

Equal amounts of *B. fragilis* RNA (20 µg) at times T_0, T_{15}, T_{60} and T_{90} (Section 4.3.7) and *E. coli* RNA (10 µg) extracted from mid-logarithmic *E. coli* cells not treated with metronidazole, were loaded in duplicate into a slot blot apparatus (SlotBlot, Hoefer Scientific Instruments, San Francisco) and transferred onto a nylon membrane (Roche Diagnostics). The transferred RNA was UV cross-linked. Hybridization with the DIG-labeled DNA probes was performed overnight at 50°C in EasyHyb hybridization buffer (Roche Diagnostics). Signals were detected using chemiluminescent detection with CSPD® (Roche Diagnostics), according to the manufacturer's instructions.

4.4 Results and Discussion

4.4.1 Primer Extension analysis

In Chapter 3, putative *B. fragilis* promoter consensus sequences were identified upstream of the ATG start codon of ORF2 (Section 3.4.5). Primer extension analysis was performed in order to identify the *in vivo* transcriptional start site of ORF2 and possibly confirm the typical *Bacteroides* promoter sequences. A single primer extension product (Fig 4.1a) identified the transcriptional start site to be an Adenine 59 bp upstream of the ATG start codon of ORF2 (Fig 4.1c, noted as +1). The *B. fragilis* promoter consensus –10 motif was identified (GAAATTTG), separated from the –35 motif (GTTG) by 20 nucleotides (Fig 4.1c). Three sets of inverted repeats were identified in the promoter region, 5'-TTTTGAT-3' (nucleotide positions 51 and 67 in Fig 4.1c, black), 5'-ATATAC-3' (nucleotide positions 76 and 93, Fig 4.1c, red) and 5'-AATTTGT-3' (nucleotide positions 114 and 134, Fig 4.1c, blue). The first set of inverted repeats was located upstream of the –35 promoter sequence, while the last two sets spanned the –35 and –10 promoter regions, respectively.

In a study done by Bayley *et al.* (2000) on *B. fragilis* promoters, the preferred transcriptional initiation site was a purine, in most cases an Adenine. This supports the above result for the transcriptional start site of ORF2. The –10 promoter sequence of ORF2 had significant similarity to the –7 *B. fragilis* consensus motif (TAnnTTTG) described by Bayley *et al.* (2000). This motif was followed by a T, which once again is in accordance with the findings of Bayley *et al.* (2000) who found this motif followed by a T or C in most cases. The beginning of the –35 promoter sequence of ORF2 was found 20 bp upstream from the en d of the –10 sequence, within the consensus spacing (Bayley *et al.*, 2000). This promoter sequence once again was highly similar to the *B. fragilis* –33 consensus motif (TTTG).

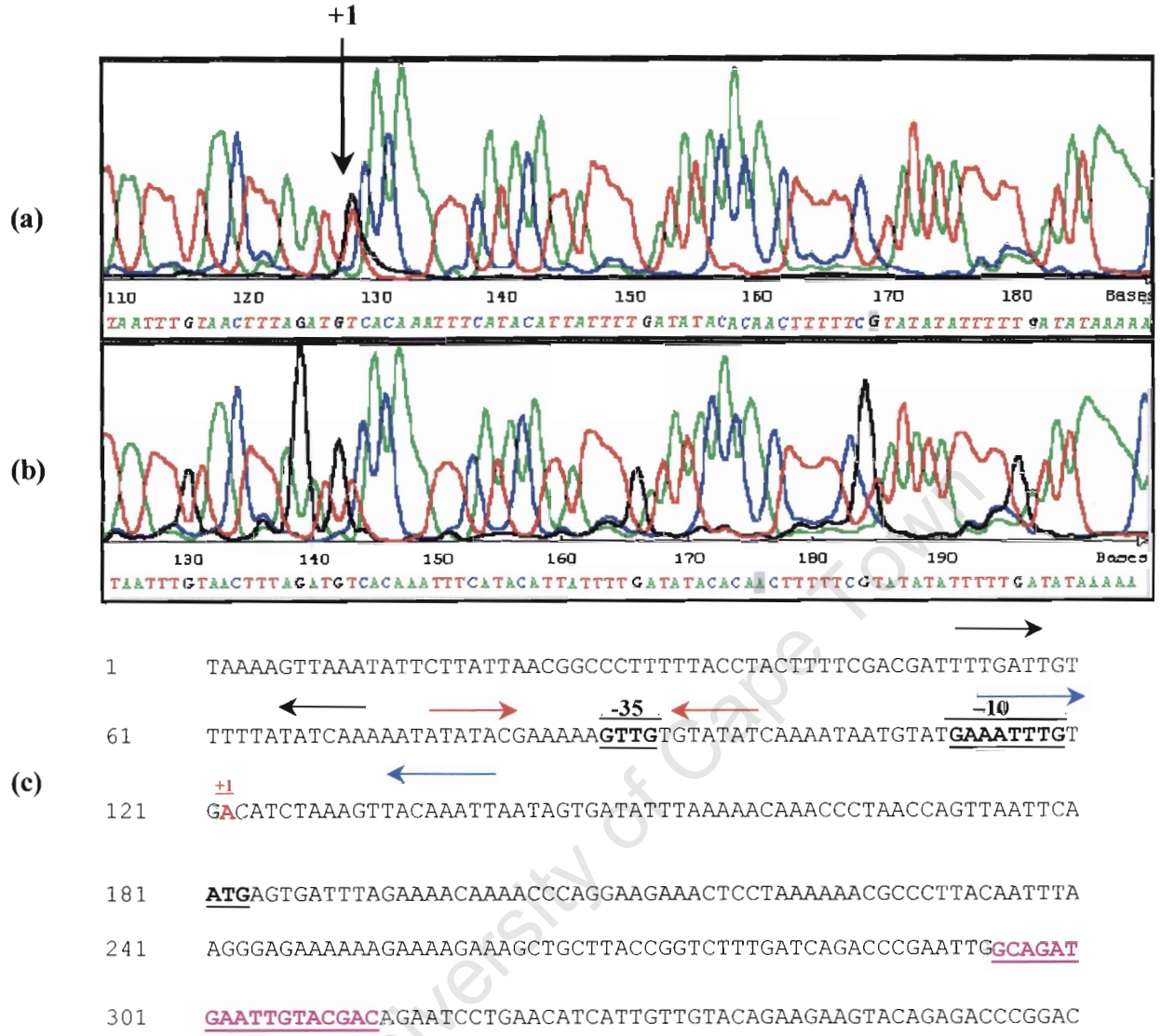

Figure 4.1 Identification of the *B. fragilis* ORF2 transcriptional start site by primer extension analysis. **(a)** Primer extension reaction product (black line) and A, T and C sequencing reaction products (green, red and blue lines, respectively). The transcriptional start (+1) site is indicated by an arrow. **(b)** Chromatogram of the corresponding DNA sequencing reaction using the same primer PEReg (underlined in pink). **(c)** Nucleotide sequence of the putative promoter region of ORF2: the ATG start codon is underlined at position 181, the transcription start site is in red (+1), the putative-10 and –35 motifs are underlined. The 3 sets of inverted repeats are shown by arrows.

4.4.2 Northern hybridisation analysis

Northern blot analysis of *B.* fragilis RNA was carried out, to investigate whether the mRNA transcribed from the transcriptional start site identified above contained the ORF2 as a single transcript. *B. fragilis* total RNA hybridized to the *Pvu*II-*Eco*RV 200 bp ORF2 probe, resulting in a single band of approximately 600 nucleotides (nt) (Fig. 4.2A, Lane 3). The ORF2 specific probe hybridized to RNA from *E. coli* JM109 containing pAN2, resulting in two bands of 600 nt and 1900 nt in size (Fig 4.2A, Lane1). *E. coli* JM109 transformed with pMT104 did not result in any hybridization signals (Fig 4.2A, Lane2).

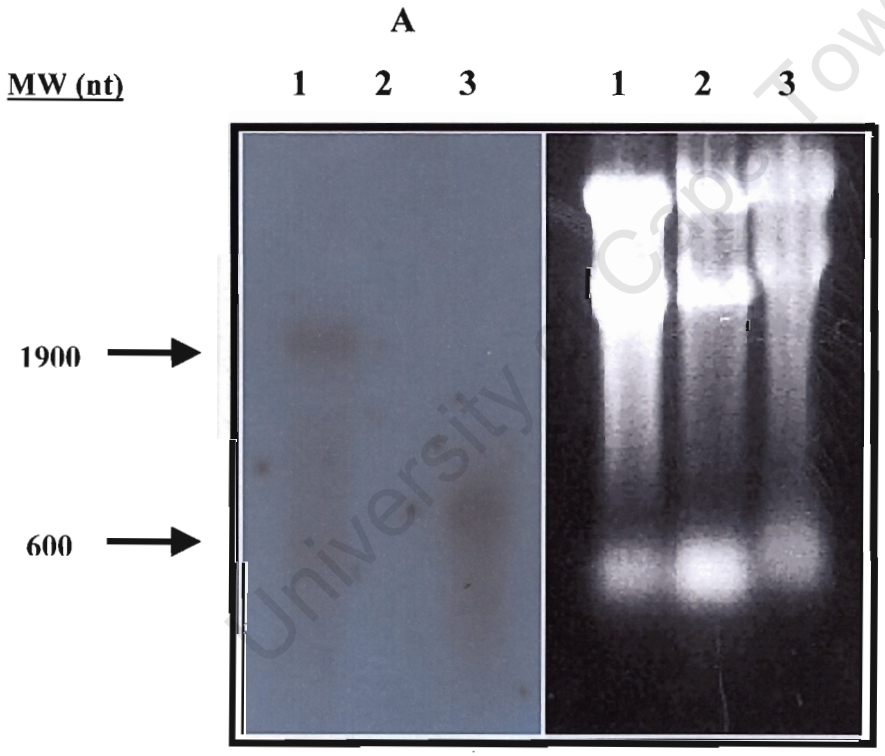

Figure 4.2 Northern blot analysis of expression of ORF2 mRNA in *B. fragilis* bf1 and *E. coli*. Molecular mass markers are on the left (nt). The radiograph of total RNA probed with ORF2 probe (**A**), and the electrophoresed total RNA in a denaturing agarose gel (**B**). Lane 1, *E. coli* JM109 (pAN2); Lane 2, *E. coli* JM109 (pMT104); Lane 3, *B. fragilis* bf1.

The ORF2 *B. fragilis* mRNA transcript was approximately 600 nucleotides in size. This agreed with the expected size of a monocistronic ORF2 transcript from the transcriptional

start site to the putative rho-independent terminator discussed in Chapter 3, Section 3.4.5.1. The same sized transcript was made in *E. coli* cells containing the ORF2 gene, implying that the *E. coli* transcriptional machinery recognized the ORF2 promoter. This is not always the case for *B. fragilis* genes in *E. coli* (Smith *et al.*, 1992). Another transcript of 1900 nucleotides in size was expressed by the *E. coli* cells. This transcript was likely to be expressed from the λ promoter found upstream of the insert in pAN2, containing approximately 500 nucleotides of pEcoR251 vector sequence from the λ transcriptional initiation site to the beginning of the insert, 700 nucleotides of the truncated ORF1 sequence and ORF2 sequence up to the putative terminator.

To confirm that ORF2 was expressed in *E. coli* from its own promoter and not the λ vector promoter, expression of the gene in *E. coli* LK112λ containing the repressor of the vector promoter was investigated. Expression of ORF1 in *E. coli* was also studied.

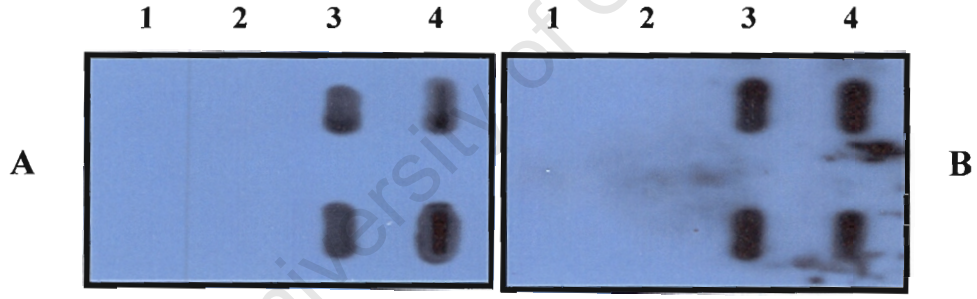

Figure 4.3 RNA slot blot analysis of the ORF2 (A) and ORF1 (B) genes in *E. coli* JM109 and LK112λ. **A:** RNA was probed with a 200 bp ORF2-specific probe. **B:** RNA was probed with a 1.29 kb ORF1-specific probe. Lane 1, *E. coli* JM109 (pMT104); Lane 2, *E. coli* LK112λ (pMT104); Lane 3, *E. coli* JM109 (pAN2); Lane 4, *E. coli* LK112λ (pAN2).

The ORF2 and ORF1 genes were expressed in mid-logarithmic *E. coli* JM109 and LK112λ cells containing pAN2 (Fig 4.3, Lanes 3-4A and 3-4B) and there was no hybridisation signal with *E. coli* JM109 and LK112λ carrying the pMT104 control plasmid (Fig 4.3, Lanes 1-2A and 1-2B). *E. coli* LK112λ contains the Cro repressor preventing transcription from the λ promoter present in the vector region of pAN2. This

confirmed that the ORF2 transcript was made from its own promoter in *E. coli*. Due to its orientation in pAN2, ORF1 could only be transcribed from its own promoter. This suggested that *E. coli* recognised both the ORF1 and ORF2 promoters. The ORF2 and ORF1 probes were specific for *B. fragilis* ORF2 and ORF1 respectively, as shown by the negative signal in *E. coli* cells lacking pAN2.

The presence of ORF2 in *E. coli uvrA*⁻ and *uvrB*⁻ cells grown in medium containing metronidazole causes increased metronidazole resistance (Chapter 3), most likely due to expression of ORF2. The above results show that ORF2 is expressed in *E. coli* and *B. fragilis* cells in the absence of metronidazole. It is, therefore, of interest to investigate the relationship between ORF2 expression and metronidazole treatment in *B. fragilis* cells.

4.4.3 Measurement of cell survival and identification of DNA strand breaks after metronidazole treatment

Exposure to metronidazole must be done on actively growing cells since metronidazole needs to get activated to cause DNA damage. *B. fragilis* cells were exposed to different concentrations of metronidazole to identify a sub-lethal concentration of metronidazole that would allow the study of the expression of ORF2 and ORF1 without killing of the population.

Exposure of *B. fragilis* cells to 50 µg/ml metronidazole caused complete death of the population within 30 min (Fig 4.4, (X) symbols). Within 10 min of exposure to metronidazole at 20 µg/ml, there was 1-log decrease in the population, with a 2-log decrease following 30 min exposure (Fig 4.4, (▲) symbols). Following 25 min metronidazole exposure at 10 µg/ml, there was approximately a 1-log decrease in the surviving fraction (Fig 4.4, (■) symbols), while exposing cells to 5 µg/ml metronidazole for 40 min did not even cause a 1-log decrease in survival (Fig 4.4, (♦) symbols). In order to induce DNA repair genes, damage of the DNA is necessary without complete killing of the population. For this reason, a concentration of 10 µg/ml seemed high enough to cause limited damage, with the potential for recovery of the population.

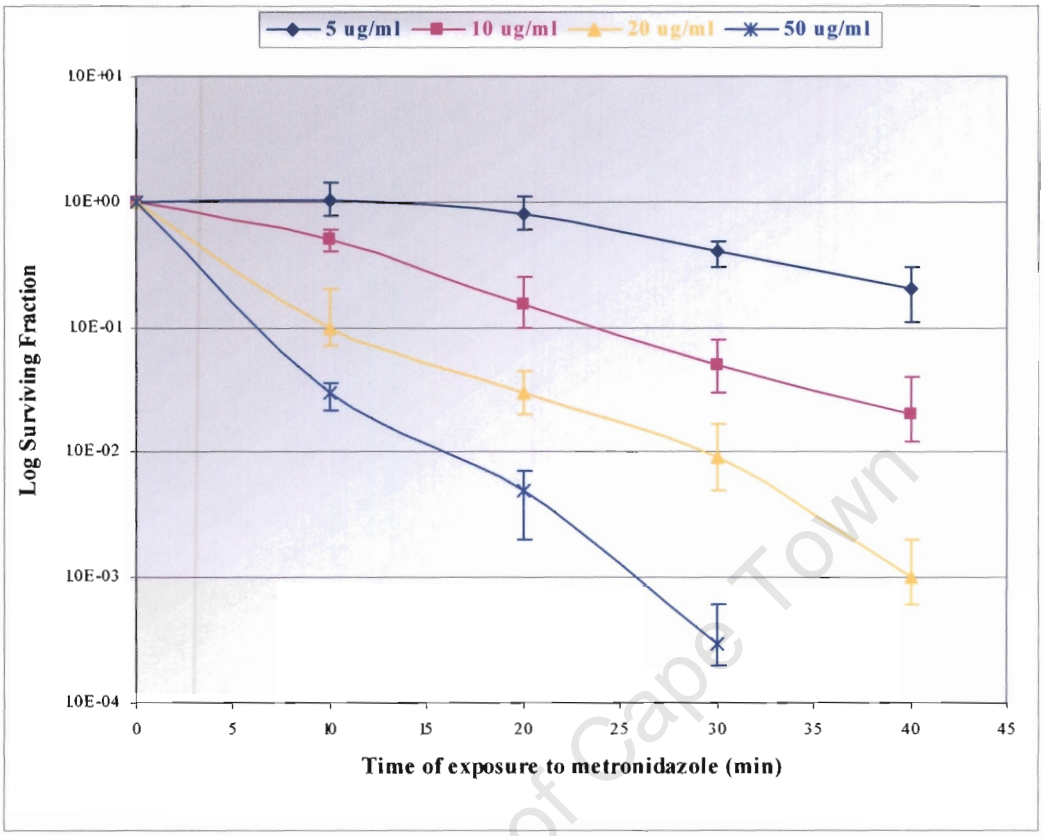

Figure 4.4 Survival of *B. fragilis* bf1 cells exposed to metronidazole at 5 µg/ml (♦); 10 µg/ml (■); 20 µg/ml (▲) and 50 µg/ml (X). The experiment was performed 3 times in duplicate.

In order to investigate the expression of ORF2 in *B. fragilis* in response to the presence or absence of metronidazole, the effect of 10 µg/ml metronidazole treatment on the survival and DNA damage of *B. fragilis* cells, as well as the recovery of the population on metronidazole-free medium was investigated. Exposure for 15 min caused a 5-fold decrease in cell numbers (Fig 4.5, T_{15} (■) symbols). After 135 min growth in fresh BHI medium, the cell numbers had increased 3-fold from T_{15} (Fig 4.5, T_{150} (■) symbols). After 255 min following metronidazole exposure, cell numbers had increased 10-fold from T_{15} (Fig 4.5, T_{270} (■) symbols).

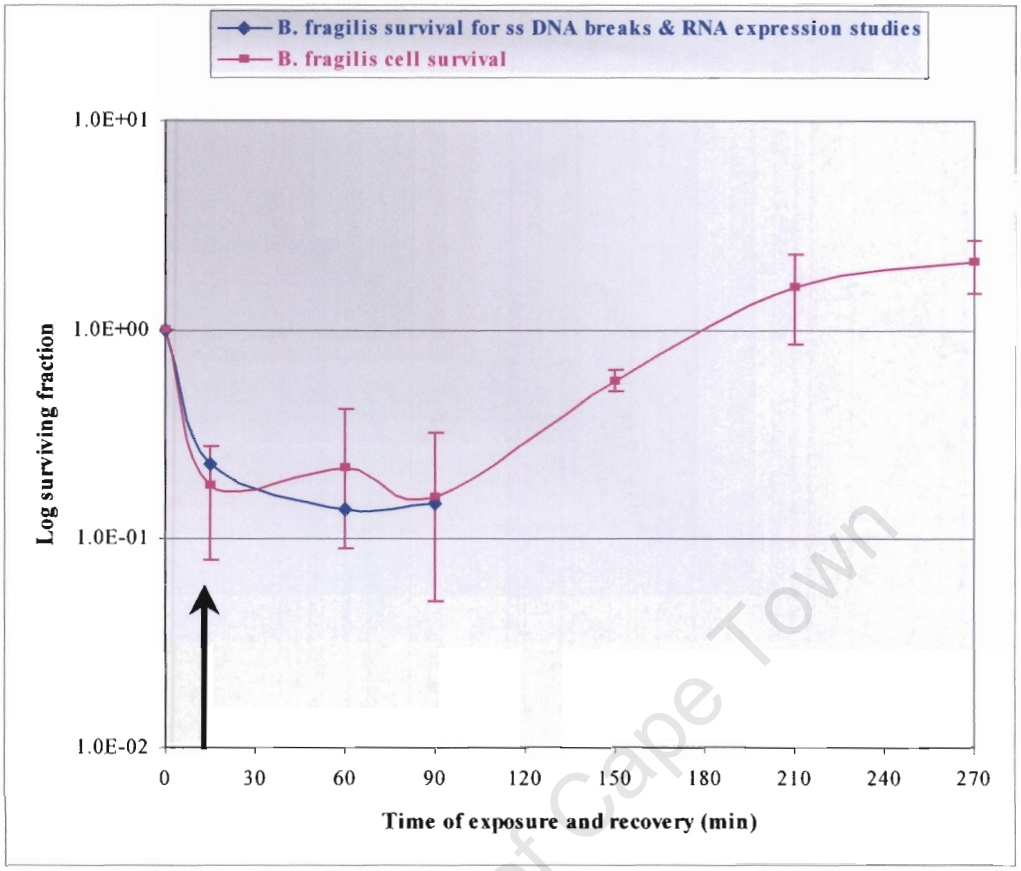

Figure 4.5 Survival of B. *fragilis* bf1 cells after exposure to metronidazole at 10 µg/ml. *B. fragilis* cells were exposed to metronidazole for 15 min (black arrow), after which cells were resuspended in fresh medium and incubated anaerobically at 37°C. The experiment was performed 3 times in duplicate (■). Samples at T_0, T_{15}, T_{60} and T_{90} were used for detection of single stranded DNA breaks and ORF1 and ORF2 expression studies (♦), and these were performed in duplicate.

Survival of the population, during and after metronidazole treatment, is dependent on the rate of DNA damage versus the rate of DNA repair by those cells. Replication of undamaged actively growing cells also affects survival. As expected, exposure of *B. fragilis* cells to metronidazole decreased the number of cells approximately half a log. However, once in metronidazole-free medium, the population recovered. This was largely due to replication of undamaged cells, as well as repair of the DNA and recovery of damaged cells to a lesser extent.

Since metronidazole causes single strand DNA breaks (Edwards, 1977; Knight *et al.*, 1978; Rowley *et al.*, 1979), the extent of damage was measured by visualization of the single strand breaks on a denaturing agarose gel. Single strand DNA breaks are held together by the double stranded DNA helix and are not detected on a normal agarose gel. However, alkaline denaturing gels separate the two strands of the helix and single strand breaks would result in low molecular weight DNA.

Total DNA of *B. fragilis* bf1 cells, grown in metronidazole followed by recovery in medium without metronidazole (Fig 4.5, (♦) symbols), was extracted at times T_0, T_{15}, T_{60} and T_{90}, and loaded on a denaturing agarose gel. Exposure to metronidazole of *B. fragilis* bf1 culture resulted in a reduction in high molecular weight chromosomal DNA on the denaturing alkaline gel (Fig 4.6, Lane 2A). This damage was due to single strand DNA breaks since there was no DNA degradation of the same sample on the non-denaturing gel (Fig 4.6, Lane 2B). At T_{60}, following 45 min of repair, there was an increase in single strand breaks (Fig 4.6, Lane3A). After 75 min of recovery, at T_{90}, the smear caused by the low molecular weight single strand breaks started to decrease compared to T_{60} (Fig 4.6, Lane 4A).

Prior to metronidazole exposure, chromosomal DNA from *B. fragilis* cells was mostly high molecular weight in both denaturing and non-denaturing conditions, indicating minimal single strand and double strand breaks. Following 15 minutes of metronidazole exposure at 10 μg/ml, lower molecular weight DNA fragments were present in the denaturing gel, indicating an increase in single strand breaks; however in the non-denaturing gel, DNA was still intact, suggesting that metronidazole did not cause double strand breaks. Edwards (1977) found that metronidazole caused both single and double stranded DNA breaks *in vitro*, however Rowley *et al.* (1979) only found single strand breaks being produced *in vitro*. The *B. fragilis* cells were allowed to recover in metronidazole-free medium for 45 minutes; however there seemed to be an increase in single strand breaks. This could be attributed to the action of repair enzymes that either nick the DNA in order to remove and repair the damage, or degrade the damaged DNA such as in the nucleotide excision repair (reviewed in Section 1.3, Chapter 1). An increase in high molecular weight DNA was observed in the denaturing gel after 75 minutes in fresh medium, suggesting a decrease in single strand breaks. This indicated

that either the damaged DNA had been repaired or/and the synthesis of new DNA by replication of undamaged cells occurred.

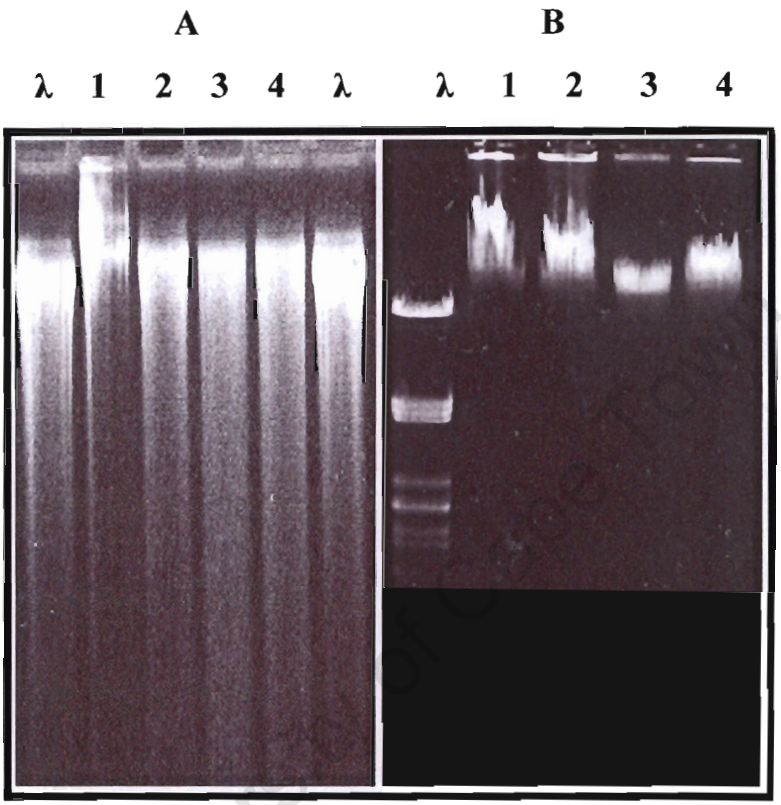

Figure 4.6 Detection of single strand DNA breaks in genomic DNA from *B. fragilis* bf1 cells. Denaturing alkaline gel (**A**) and non-denaturing agarose gel (**B**). Lane 1, DNA extracted from untreated cells at T_0; Lane 2, DNA from cells exposed to metronidazole for 15 min at T_{15}; Lane 3, DNA from cells at T_{60}; Lane 4, DNA from cells at T_{90}. (**A**) Linear 52 kb λ DNA was used as a marker of intact double stranded DNA. (**B**) *Pst*I digested λ DNA was used as a size marker.

4.4.4 RNA Slot Blot analysis of ORF2 and ORF1

ORF2 is expressed in *B. fragilis* under mid-logarithmic growth conditions in nutrient-rich medium (Fig 4.2, Lane 3). RNA slot blot analysis was carried out in order to investigate whether the level of ORF2 expression was upregulated following metronidazole treatment and whether ORF1 was possibly involved in the DNA repair. *B. fragilis* cells

were exposed to sub-lethal levels of metronidazole (10 μg/ml) for 15 minutes and allowed to recover in BHI broth for 75 minutes after metronidazole exposure (Fig 4.5, (♦) symbols). Total RNA was extracted from the same *B. fragilis* culture from which DNA was extracted for single strand break analysis and at the same time points T_0, T_{15}, T_{60} and T_{90}.

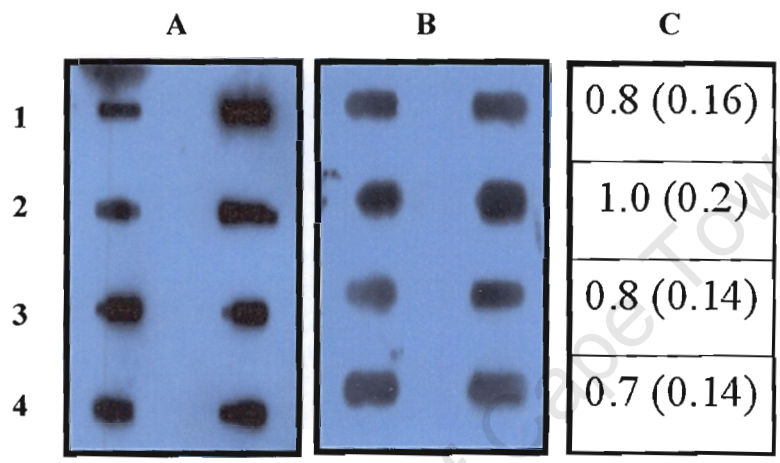

Figure 4.7 RNA slot blot analysis of the ORF2 mRNA in *B. fragilis* bf1. **A.** RNA was probed with a 1.5 kb *B. fragilis* bf1 16S rRNA probe. **B.** RNA was probed with a 200 bp ORF2-specific probe. **C.** mRNA levels expressed as ratios of the ORF2-specific hybridisation signal to the 16S rRNA hybridisation signal. Experiments were performed in duplicate, and standard deviations are shown in parentheses. Lane 1: *B. fragilis* cells at T_0; Lane 2: *B. fragilis* cells at T_{15}; Lane 3: *B. fragilis* cells at T_{60}; Lane 4: *B. fragilis* cells at T_{90}.

The ORF2 gene was expressed in *B. fragilis* cells, at the same level irrespective of metronidazole treatment (Fig 4.7, Lanes 1-4B and C). A *B. fragilis* bf1 DNA probe of the 16S rRNA gene was used as an RNA concentration control (Fig 4.7, Lanes 1-4A). There seemed to be a slight increase in expression of the ORF2 gene in *B. fragilis* following metronidazole treatment (Fig 4.7, Lane 2B and C), however the increase was not significant as shown by the standard deviation. The difference in ORF2 expression at the different conditions was negligible and there was no evidence of regulation of the ORF2 gene under the conditions tested.

The ORF1 gene was expressed at low levels in mid-logarithmic *B. fragilis* cells (Fig 4.8, Lane 1B and C) and these levels decreased following metronidazole exposure and during the 75-min recovery period (Fig 4.8, Lanes 2-4B and C).

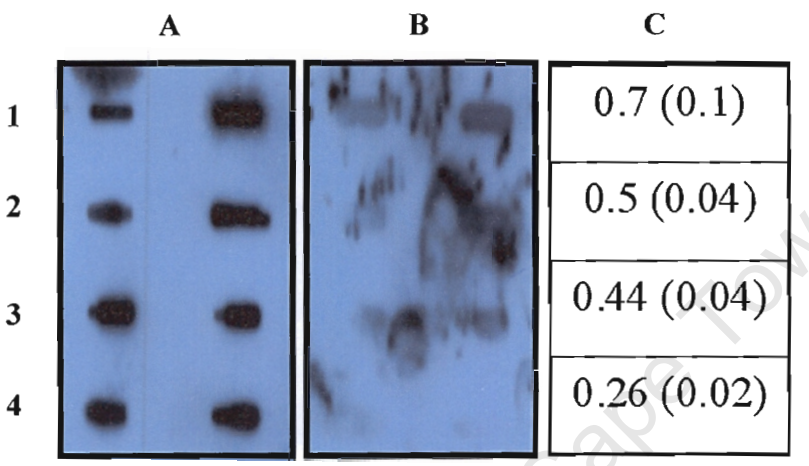

Figure 4.8 RNA slot blot analysis of the ORF1 mRNA in *B. fragilis* bf1. **A.** RNA was probed with a 1.5 kb *B. fragilis* bf1 16S rRNA probe. **B.** RNA was probed with a 1.29 kb ORF1-specific probe. **C.** mRNA levels expressed as ratios of the ORF1-specific hybridisation signal to the 16S rRNA hybridisation signal. Experiments were performed in duplicate, and standard deviations are shown in parentheses. Lane 1: *B. fragilis* cells at T_0; Lane 2: *B. fragilis* cells at T_{15}; Lane 3: *B. fragilis* cells at T_{60}; Lane 4: *B. fragilis* cells at T_{90}.

This suggests downregulation of ORF1 in response to metronidazole exposure, possibly due to the DNA damage. This contradicts the hypothesis that ORF1 might be involved in DNA repair and suggests that its expression following DNA damage to the cell might be detrimental and needs to be decreased.

4.5 Conclusions

To gain more understanding on the *B. fragilis* putative AraC/XylS transcriptional regulator ORF2, regulation of the gene at the transcription level was analysed. The expression of this gene, as well as ORF1, the *recQ* homologue, was determined in *B. fragilis*.

ORF2 was expressed in *E. coli* from a promoter upstream of ORF2 as well as from the λ pEcoR251 vector promoter. This indicated that *E. coli* transcriptional machinery recognised the promoter sequences of ORF2. Transcriptional signals in *B. fragilis* are different from those in *E. coli*, and *B. fragilis* genes are sometimes transcribed inefficiently in *E. coli* if at all (Smith *et al.*, 1992). However, this does not seem to be the case for ORF2.

Mid-logarithmic *B. fragilis* cells expressed ORF2 at constitutive levels and there was no significant increased transcription of this gene following metronidazole treatment. There seemed to be a slight increase in expression of ORF2 immediately following metronidazole exposure, however, due to the errors encountered in the technique used, the difference was not significant. A more accurate technique for promoter expression analysis, such as the use of promoterless reporter gene systems, would demonstrate more specifically whether ORF2 expression was changed in response to metronidazole. The AraC transcriptional regulators involved in stress tolerance need to be overproduced in order to exert their regulatory effect (Gallegos *et al.*, 1997). Certain stress signals cause overexpression of these regulators. In *E. coli*, ORF2 was present in a multicopy plasmid and its expression was, therefore, increased without an inducing signal. It is very likely that the conditions used for the RNA slot blot analysis in *B. fragilis* are not the stress signal needed to overexpress ORF2. Conditions for inducibility of metronidazole resistance in *B. fragilis* have yet to be established. It is possible that in *B. fragilis*, metronidazole is not the inducing signal for overexpression of ORF2. Identification of genes controlled by ORF2 might shed a light on the possible function of the ORF2 regulator and might help to identify the stress signal which triggers ORF2 overproduction. Overexpression as well as construction of an ORF2 inactivated mutant in *B. fragilis* would give insight on the function of this regulator in *B. fragilis*.

The expression of the *recQ* homologue ORF1 was of interest due to the fact that ORF1 and ORF2 share a divergent promoter and the possibility of the *recQ* homologue being involved in DNA repair. ORF1 was also expressed in *E. coli* cells from its own promoter. In *B. fragilis*, expression of ORF1 decreased following exposure of the cells to metronidazole and remained low during the recovery period. Following exposure to DNA damaging agents, DNA synthesis is temporarily inhibited in *E. coli* (Setlow *et al.*, 1963; Livneh *et al.*, 1993). Some of the inhibition is due to the replication fork not being able to go beyond the DNA lesion. However, it is also due to inhibition of new rounds of replication from the origin of replication *oriC* (Verma *et al.*, 1989). This is not due to DNA lesions on *oriC* but occurs to allow the damage to be repaired and prevent errors when synthesizing new DNA. This inhibition of replication could be done by repression of genes involved in replication. It is possible that DNA replication in *B. fragilis* is also inhibited following DNA damage. This would explain a decrease in the transcription of the ORF1 gene, if it had to play a role in replication of DNA. Humans have 5 different RecQ helicases and some of them are thought to act at different steps in DNA replication and interact with components of the DNA replication machinery (Bennet and Keck, 2004). The conserved non-catalytic domain of the human RecQ helicase WRN has a role in DNA replication intermediate processing (Sharma *et al.*, 2004). The fact that *B. fragilis* has three different RecQ homologues allows for the possibility that one of them, ORF1, might be involved in DNA replication. A more detailed analysis is required in order to establish whether ORF1 and ORF2 do, in fact, play a role in the response of *B. fragilis* cells to metronidazole treatment.

CHAPTER 5

General Conclusions

Metronidazole is the most widely used antibiotic in the treatment of anaerobic infections, including those caused by *Bacteroides fragilis*. The drug is activated into a cytotoxic intermediate under anaerobic conditions, and is thought to cause DNA damage leading to cell death. Research on the possible development of resistance has focused on the activation of the drug into its active intermediate, while very little work has been done on the mechanisms involved in repair of the metronidazole DNA damage. The aim of the study reported in this thesis was to identify *Bacteroides* genes involved in repair of the DNA damage caused by metronidazole.

The initial stages of this study preceded the publication of the genomes of *B. thetaiotaomicron* VPI-5482 (Xu *et al.*, 2003) and *B. fragilis* YCH46 (Kuwahara *et al.*, 2004), as well as the access to the completed but yet unpublished *B. fragilis* 9343 and 638R genome (www.sanger.ac.uk/projects/b_fragilis). Therefore, indirect identification approaches were used. These involved identification of genes implicated in metronidazole resistance or sensitivity in *Bacteroides* itself by construction of *Bacteroides* transposon mutants, and by identification of the *Bacteroides* genes in *E. coli* by multicopy suppression of *E. coli* DNA repair mutants. As genomic sequence information became available, this was integrated into the analysis of the experimental findings.

Attempts to isolate *B. fragilis* metronidazole mutants were not successful. The approach was then adopted of identifying the phenotypes in *B. thetaiotaomicon* mutants and then extrapolating the findings to *B. fragilis*. Isolation of *B. thetaiotaomicron* metronidazole mutants did result in the identification of genes involved in sensitivity or resistance to metronidazole. Some of the disrupted genes did not have close homologues in *B. fragilis*, and it was, therefore, not always possible to draw comparisons with *B. thetaiotaomciron*. Some of the *B. thetaiotaomicron* mutants isolated were, however, interesting and further analysis will be done on these. *B. thetaiotaomicron* metronidazole sensitive mutant B, with a disruption in a capsular polysaccharide biosynthetic gene, suggested that the

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Bacteroides capsule might be involved in preventing entry of metronidazole into the cell. Disruption of the gene, coding for a protein with identity to enzymes involved in catalysing the first step in the synthesis of surface polysaccharide in various prokaryotes (Amer and Valvano, 2001) would, very likely, affect the synthesis of the *B. thetaiotaomicron* capsular polysaccharide synthesised by the products of the genes in that specific polysaccharide locus 4. The capsular polysaccharide of *Bacteroides* might prevent entry of metronidazole into the cell by acting as a physical barrier. Genes homologous to the *B. thetaiotaomicron* gene disrupted in mutant B, were found in various capsular polysaccharide biosynthesis loci in *B. fragilis*. Disruption of individual capsular polysaccharides in *B. thetaiotaomicron* and *B. fragilis* by site-directed mutagenesis, and metronidazole uptake studies of the resulting mutants might explain the role of the *Bacteroides* capsule in the uptake of metronidazole.

One mechanism for metronidazole resistance in *Bacteroides* has been shown to be due to inefficient activation of metronidazole inside the cell. The enzyme thought to be involved in this is pyruvate:ferredoxin oxidoreductase. It is, however, possible that any metabolic enzyme involved in electron transfer might be able to donate electrons to metronidazole, activating it. Reduced activity of any of those enzymes might, therefore, increase the metronidazole resistance of the cell. This may be the case in *B. thetaiotaomicron* metronidazole resistant mutant D. Disruption of the intergenic region between *fucO*, coding for the lactaldehyde reductase enzyme, and the putative transcriptional regulator of the *B. thetaiotaomicron* rhamnose utilization gene cluster, might cause decreased lactaldehyde reductase activity. This might occur by decreased expression of the putative positive transcriptional regulator due to disruption of promoter sequences, which would then prevent expression of the *fucO* genes, as well as the other rhamnose utilisation genes. The rhamnose utilisation pathway of *B. thetaiotaomicron* has not been characterised, however, the genes identified suggest a similar pathway to *E. coli*. *B. fragilis* does not possess a rhamnose utilisation pathway, however it does have a fucose utilisation pathway containing the *fucO* gene. In *E. coli*, lack of activity of FucO would force the product of both L-fucose and L-rhamnose utilisation, L-lactaldehyde into L-lactate production, disrupting the normal electron flow of the cell. Metronidazole resistant *B. fragilis* mutants have been shown to produce increased levels of lactate (Britz

and Wilkinson, 1979, Narikawa *et al.*, 1991). It is, therefore, possible that FucO might be involved in activation of metronidazole in *Bacteroides*. Alternatively, disruption of the electron flow in Mutant D might cause decreased activity of pyruvate:ferredoxin oxidoreductase (POR) and therefore, decreased activation of metronidazole. Site-directed mutagenesis of the *fucO* gene in both *B. thetaiotaomicron* and *B. fragilis* would confirm whether the metronidazole resistant phenotype of *B. thetaiotaomicron* Mutant D is due to decreased expression of FucO. Lactate production assays, as well as measurement of the activity of FucO and POR are necessary to elucidate the mechanism of activation of metronidazole in Mutant D and the FucO-inactivated mutants.

Since no DNA repair genes were isolated using the transposon mutagenesis approach, a different, indirect approach was proposed, making use of functional complementation of *E. coli* DNA repair mutants using a *B. fragilis* gene library. *E. coli* is very useful for the identification of *B. fragilis* DNA repair genes for various reasons. *E. coli* is a facultative anaerobe allowing experiments to be done both aerobically and anaerobically, which mimics the *B. fragilis* environment. The DNA repair systems of *E. coli* have been well characterized to date, and there are a large number of *E. coli* repair mutants available for complementation studies. In this study, *E. coli* nucleotide excision repair mutants were, therefore, used to screen for *B. fragilis* genes involved in the repair of DNA damage induced by metronidazole.

Genes from *B. fragilis* bf1 which conferred increased metronidazole resistance on *E. coli* *uvrA* and *uvrB* mutants were cloned on a recombinant plasmid pAN2. The gene ORF2 was responsible for the phenotype, and encoded a putative protein of 154 amino acid residues and sequence identity to the AraC/XylS family of transcriptional regulators. These regulators are mostly positive regulators involved in diverse cellular functions such as metabolism, pathogenesis and stress tolerance (Gallegos *et al.*, 1997). In *E. coli*, there are AraC/XylS trancriptional regulators involved in DNA repair, such as Ada protein involved in repair of alkylated DNA, and antibiotic resistance, such as MarA, SoxS and Rob.

Although *E. coli* MarA, SoxS and Rob are non-identical AraC transcriptional regulators, with slightly different functions and regulation, they all regulate a common set of genes in *E. coli*. This demonstrates the flexibility of target genes in being regulated by a range of homologous AraC transcriptional regulators. AraC has highest identity to the *E. coli* SoxS, involved in superoxide resistance. It is possible that ORF2, expressed at high levels from a multicopy plasmid, is regulating *E. coli* genes, which are otherwise regulated by a homologous AraC transcriptional regulator, in a heterologous manner. Metronidazole causes single and double stranded breaks in the DNA (Edwards, 1977; Knight *et al.*, 1978) that are repaired in *E. coli* by the RecBCD pathway (reviewed in Section 1.3.1.3) or the RecE or RecF alternative pathways (reviewed in Section 1.3.1.4). The RecF pathway is known to work together with the nucleotide excision repair in repairing stalled replication forks (Courcelle *et al.*, 1999). The RecQ protein is a member of the RecF pathway and is involved in repair of double strand breaks (Courcelle and Hanawalt, 1999), as well as being involved in suppression of illegitimate recombination together with UvrA and UvrB (Hanada *et al.*, 2000). Interestingly, upstream of ORF2 and transcribed divergently in the *B. fragilis* chromosome, lies a RecQ homologue, ORF1. In this investigation, the *B. fragilis* ORF1 was not found to be involved in conferring metronidazole on the *E. coli uvrA* and *uvrB* mutants, however the presence of this RecQ homologue upstream of ORF2 is interesting. It is possible that ORF2 is regulating an *E. coli* repair gene, such as the *E. coli* RecQ, which would take over the function of either the UvrA or UvrB in repairing the DNA lesions caused by metronidazole, since they both share a pathway in suppression of illegitimate recombination (Hanada *et al.*, 2000). It would be interesting to identify the *E. coli* genes affected by ORF2 and the function of *B. fragilis* ORF2 in the *E. coli* mutants. Microarray analysis of the *E. coli uvrA* and *uvrB* mutants containing ORF2 might allow identification of such genes in *E. coli*, as well as microarray analysis in *B. fragilis* overexpressing the AraC regulator.

The function of ORF2 in *B. fragilis* and its possible role following metronidazole exposure are not clear. The AraC regulator ORF2 showed a minor increase in transcriptional expression following metronidazole exposure, however this increase was not significant. The method used, RNA slot blot analysis, was not accurate enough to detect small changes in transcription and the errors were too great. A different approach,

such as using the ORF2 promoter fused to a reporter gene would allow such differences to be measured more accurately.

It is interesting to note that the transcriptional expression of the RecQ homologue (ORF1) was decreased in *B. fragilis* following metronidazole exposure. During DNA damage, DNA replication is halted in *E. coli* (Setlow *et al.*, 1963) in order to give time for the damage to be efficiently repaired and prevent replication errors. There is a possibility that the same phenomenon occurs in *B. fragilis* and that genes involved in DNA synthesis might be downregulated. It is possible that ORF1, a RecQ homologue, would be involved in DNA replication and during DNA damage, its expression would be decreased. The published genome sequence of *B. fragilis* YCH46 (Kuwahara *et al.*, 2004) and the incomplete sequence of *B. fragilis* 9343 (www.sanger.ac.uk/Projects/B_fragilis) revealed the presence of three different RecQ homologues. It is possible that the different *B. fragilis* RecQ homologues may have different roles in the protection of the genetic material of the cell, one of them being DNA replication.

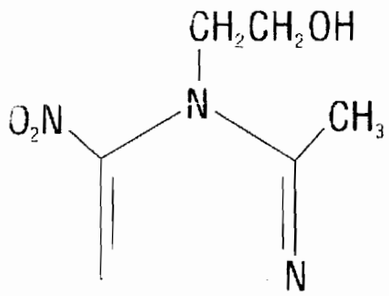
The functional complementation approach for identification of *B. fragilis* genes of interest will become more targeted in future due to the recent publication of the *B. fragilis* genome. This enables specific *B. fragilis* putative DNA repair genes to be cloned, using PCR techniques and their possible contribution to metronidazole resistance to be elucidated in *E. coli* repair mutants under anaerobic conditions. The DNA repair systems of *Bacteroides* have not been well-studied, especially in relation to metronidazole damage, but access to the *B. thetaiotaomicron* and *B. fragilis* genomes have allowed bioinformatic analysis to be done. Several candidate genes can now be identified and await functional investigation. Metronidazole causes single and double strand breaks in the DNA (Edwards, 1977), and it is known that *E. coli* nucleotide excision repair mutants have increased sensitivity to metronidazole (Yeung *et al.*, 1984; Jackson *et al.*, 1984). As mentioned in Chapter 1, section 1.3.3, *B. fragilis* has been shown to have an excision repair system (Abratt *et al.*, 1986), however, there is evidence that there might be another excision repair system involved in different types of DNA damage (Abratt *et al.*, 1985). Both *B. thetaiotaomicron* and *B. fragilis* contain two *uvrA* homologues, as well as homologues of *uvrB* and *uvrC*, (*B. thetaiotaomicron* genome Accession Number

NC_004663; *B.fragilis* incomplete genome sequence www.sanger.ac.uk/Projects/B_fragilis; *B. fragilis* YCH46 genome Accession Number NC_006347), suggesting the presence of a Nucleotide Excision Repair system.

A pathway similar to the RecF pathway of recombination might be present as shown by the fact that *recF*, *recO* and *recR* are present in both organisms. In *E. coli*, this pathway is involved in repair of daughter strand gaps as described in Section 1.3.1.2. Interestingly, the repair of double strand breaks, which in *E. coli* is repaired by the RecBCD pathway (Section 1.3.1.3) is likely to be repaired by a different system in *Bacteroides*. Even though both *B. thetaiotaomicron* and *B. fragilis* have a *recD* homologue, one of the subunits of Exonuclease V involved in the RecBCD pathway, there are no homologues of *recB* or *recC*. *Bacteroides* might use an alternative pathway for the repair of this damage, as *E. coli* does in the absence of functional RecBCD, such as the altered RecF pathway (Section 1.3.1.4). This is supported by the fact that both *Bacteroides* species contain homologues of the RecN, RecJ as well as the three different RecQ homologues. There is a possibility that one of the three RecQ homologues might be involved in repair of double stranded breaks.

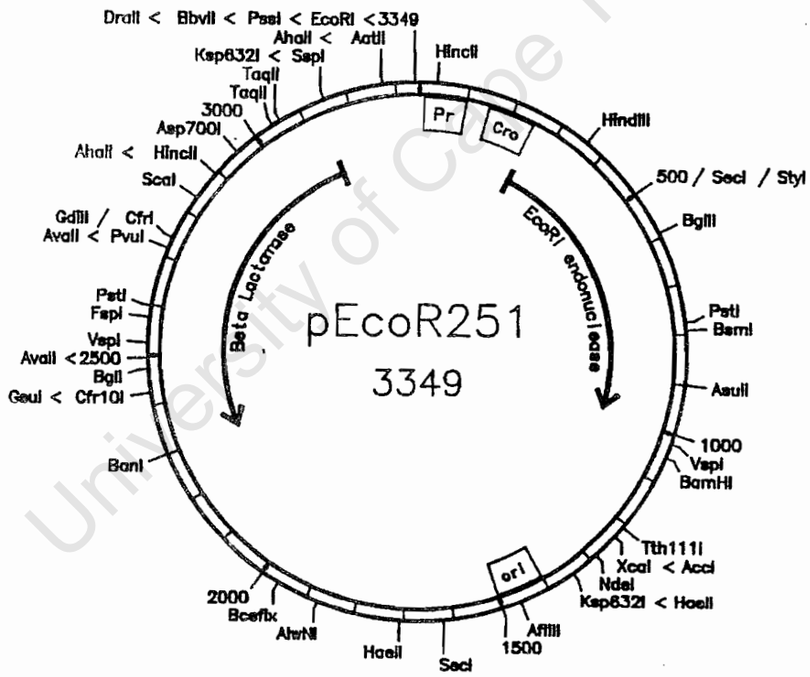
It is not yet clear what the interaction is between the *E. coli uvrA* and *uvrB* mutations and the *B. fragilis* ORF2 AraC transcriptional regulator, and whether there is a link between ORF2 and ORF1 in *B. fragilis*. A better understanding of *B. fragilis* DNA repair systems, and the functions of these *B. fragilis* DNA repair components is necessary to gain insight into the mechanisms involved in repair of metronidazole DNA damage and the possibility of resistance. Access to the *B. fragilis* genome will allow site-directed inactivation of the above-mentioned putative DNA repair genes to be performed. Studies can then be done on the effect of metronidazole on these mutants for elucidation of the possible role played by each in the repair of metronidazole-induced DNA damage.

APPENDIX 1



Chemical Structure of 1-2'-hydroxyethyl-2-methyl-5-nitroimidazole (Courtesy of 1998-2005 Galderma Laboratories, L.P. United States).

APPENDIX 2



Partial restriction map of suicide vector pEcoR251 (Zabeau and Stanley, 1982).

LITERATURE CITED

Abrahams GL and Abratt VR. (1998) The NADH-dependent Glutamate Dehydrogenase Enzyme of *Bacteroides fragilis* bf1 Is Induced by Peptides in the growth Medium. *Microbiology.* **144:** 1659-1657.

Abratt VR, Jones DT and Woods DR. (1985) Isolation and Physiological Characterization of Mitomycin C-Sensitive/UV-Sensitive Mutants in *Bacteroides fragilis*. *Journal of General Microbiology.* **131:** 2479-2483.

Abratt VR, Lindsey GL and Woods DR. (1986) Pyrimidine Dimer Excision Repair of DNA in *Bacteroides fragilis* Wild-Type and Mitomycin C-Sensitive/UV-Sensitive Mutants. *Journal of General Microbiology.* **132:** 2577-2581.

Abratt VR, Peak MJ, Peak JG, Santangelo JD and Woods DR. (1990) Induction and Repair of DNA Strand Breaks in *Bacteroides fragilis*. *Canadian Journal of Microbiology.* **36:** 490-494.

Aiba H, Adhya S and de Crombrugghe B. (1981) Evidence for Two Functional *gal* Promoters in Intact *Escherichia coli* cells. *Journal of Biological Chemistry.* **256:** 11905-11910.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acid Research.* **25:** 3389-3402.

Amer AO and Valvano MA. (2001) Conserved Amino Acid Residues Found in a Predicted Cytosolic Domain of the Lipopolysaccharide Biosynthesis Protein WecA Are Implicated in the Recognition of UDP-N-Acetylglucosamine. *Microbiology.* **147:** 3015-3025.

Amer AO and Valvano MA. (2002) Conserved Aspartic Acids Are Essential for the Enzymatic Activity of the WecA Protein Initiating the Biosynthesis of O-Specific Lipopolysaccharide and Enterobacterial Common Antigen in *Escherichia coli*. *Microbiology.* **148:** 571-582.

Anderson KL and Salyers AA. (1989) Biochemical Evidence that Starch Breakdown by *Bacteroides thetaiotaomicron* Involves Outer Membrane Starch-Binding Sites and Periplasmic Starch-Degrading Enzymes. *Journal of Bacteriology.* **171:** 3192-3198.

Anderson MS, Eveland SS and Price NPJ. (2000) Conserved Cytoplasmic Motifs that Distinguish Sub-Groups of the Polyprenol Phosphate:*N*-Acetylhexosamine-1-Phosphate Transferase Family. *FEMS Microbiology Letters.* **191:** 169-175.

Arthur M, Brisson-Noel A and Courvalin P. (1987) Origin and Evolution of Genes Specifying Resistance to Macrolide, Lincosamide and Streptogramin Antibiotics: Data and Hypotheses. *Journal of Antimicrobial Chemotherapy.* **20:** 783-802.

Bachrati CZ and Hickson ID. (2003) RecQ Helicases: Suppressors of Tumorigenesis and Premature Aging. *Biochemical Journal.* **374:** 577-606.

Baggio L and Morrison M. (1996) The NAD(P)H-Utilizing Glutamate Dehydrogenase of *Bacteroides thetaiotaomicron* Belongs to Enzyme Family I, and its Activity Is Affected by Trans-Acting Gene(s) positioned Downstream of GdhA. *Journal of Bacteriology.* **178:** 7212-7220.

Barua S, Yamashino T, Hasegawa T, Yokoyama K, Torii k and Ohta M. (2002) Involvement of Surface Polysaccharide in the Organic Acid Resistance of Shiga Toxin-Producing *Escherichia coli* O157:H7. *Molecular Microbiology.* **43:** 629-640.

Baughn and Malamy MJ. (2003) The Essential Role of Fumarate Reductase in Haem-Dependent Growth Stimulation of *Bacteroides fragilis*. *Microbiology.* **149:** 1551-1558.

Bayley DP, Rocha ER and Smith CJ. (2000) Analysis of *cepA* and other *Bacteroides fragilis* genes reveals a unique promoter structure. *FEMS Microbiology Letters.* **193:** 149-154.

Bennett RJ and Keck JL. (2004) Structure and Function of RecQ DNA Helicases. *Crit Rev. Biochem. Mol Biol.* **39:** 79-97.

Bernstein DA and Keck JL. (2003) Domain Mapping of *Escherichia coli* RecQ Defines the Roles of Conserved N- and C-Terminal Regions in the RecQ Family. *Nucleic Acids Research.* **31:** 2778-2785.

Boronat A and Aguilar J. (1981) Metabolism of L-Fucose and L-Rhamnose in *Escherichia coli*: Differences in Induction of Propanediol Oxidoreductase. *Journal of Bacteriology.* **147:** 181-185.

Brendel V and Trifonov EN. (1984) A Computer Algorithm for Testing Potential Prokaryotic Terminators. *Nucleic Acids Research.* **12:** 4411-4427.

Brennan RG and Matthews BW. (1989) The Helix-Turn-Helix DNA Binding Motif. *The Journal of Biological Chemistry.* **264:** 1903-1906.

Britz ML and Wilkinson RG. (1979) Isolation and Properties of Metronidazole-Resistant Mutants of *Bacteroides fragilis. Antimicrobial Agents and Chemotherapy.* **16:** 19-27.

Burt S, Meldrum S, Woods DR and Jones DT. (1978) Colonial Variation, Capsule Formation, and Bacteriophage Resistance in *Bacteroides thetaiotaomicron. Applied and Environmental Microbiology.* **35:** 439-443.

Campbell A. (1994) Comparative Molecular Biology of Lamboid Phages. *Annual Review of Microbiology.* **48:** 193-222.

Carlier JP, Sellier N, Rager MN and Reysset G. (1997) Metabolism of a 5-Nitroimidazole in Susceptible and Resistant Isogenic Strains of *Bacteroides fragilis. Antimicrobial Agents and Chemotherapy.* **41:** 1495-1499.

Cerkasovova A, Cerkasov J and Kulda J. (1984) Metabolic Differences Between Metronidazole Resistant and Susceptible Strains of *Tritrichomonas foetus. Molecular and Biochemical Parasitology.* **11:** 105-118.

Cerkasovova A, Cerkasov J and Kulda J. (1988) Resistance of Trichomonads to Metronidazole. *Acta Universitatis Carolinae-Biologica.* **30:** 485-503.

Chakraverty RK and Hickson ID. (1999) Defending Genome Integrity during DNA Replication: A Proposed Role for RecQ Family Helicases. *Bioessays.* **21:** 286-294.

Chang KC, Ho SW, Yang JC and Wang JT. (1997) Isolation of a Genetic Locus Associated with Metronidazole Resistance in *Helicobacter pylori. Biochemical and Biophysical Research Communications.* **236:** 785-788.

Chardon H, Ratignier A, Luneau r and Garriques B. (1977) Apparition de la Résistance au Metronidazole Chez *Bacteroides fragilis. Nouvelle Presse Médicale.* **6:** 2165.

Chen HW, Ruan B, Yu M, Wang JD and Julin DA. (1997) The RecD subunit of the RecBCD Enzyme from *Escherichia coli* Is a Single-Stranded DNA-Dependent ATPase. *Journal of Biological Chemistry.* **272:** 10072-10079.

Chen T, Dong H, Tang YP, Dallas MM, Malamy MH, Duncan MJ. (2000) Identification and Cloning of Genes from *Porphyromonas gingivalis* after Mutagenesis with a Modified Tn*4400'* Transposon from *Bacteroides fragilis. Infection and Immunity.* **68:** 420-423.

Chen Y, Kinouchi T, Kataoka K, Akimoto S, Ohnishi Y. (1995) Purification and Characterization of a Fibrinogen-Degrading Protease in *Bacteroides fragilis* Strain YCH46. *Microbiology and Immunology.* **39:** 967-977.

Chen YM and Lin ECC. (1984) Dual Control of a Common L-1,2-Propanediol Oxidoreductase by L-fucose and L-Rhamnose in *Escherichia coli. Journal of Bacteriology.* **157:** 828-832.
Chen YM, Lu Z and Lin ECC. (1987) NAD-Linked Aldehyde Dehydrogenase for Aerobic Utilization of L-Fucose and L-Rhamnose by *Escherichia coli. Journal of Bacteriology.* **169:** 3289-3294.

Chen YM, Lu Z and Lin ECC. (1989) Constitutive Activation of the *fucAO* Operon and Silencing of the Divergently Transcribed *fucPIK* Operon by an IS*5* Element in *Escherichia coli* Mutants Selected for Growth on L-1,2-Propanediol. *Journal of Bacteriology.* **171:** 6097-6105.

Cheng O, Yu MC, Reeves AR and Salyers AA. (1995) Identification and Characterization of a *Bacteroides* Gene, *csuF*, which Encodes an Outer Membrane Protein that Is Essential for Growth on chondroitin Sulphate. *Journal of Bacteriology.* **177:** 3721-3727.

Chow KH and Courcelle J. (2004) RecO Acts with RecF and RecR to Protect and Maintain Replication forks Blocked by UV-Induced DNA Damage in *Escherichia coli. Journal of Biological Chemistry.* **279:** 3492-3496.

Church DL, Bryant RD, Rabin HR and Laishley EJ. (1991) Physiological Effects of Metronidazole on *Clostridium pasteurianum. Journal of Antimicrobial Chemotherapy.* **28:** 221-228.

Church DL, Rabin HR and Laishley EJ. (1988) Role of Hydrogenase 1 of *Clostridium pasteurianum* in the Reduction of Metronidazole. *Biochemical Pharmacology.* **37:** 1525-1534.

Cohen SP, Hachler H and Levy SB. (1993) Genetic and Functional Analysis of the Multiple Antibiotic Resistance (*mar*) Locus in *Escherichia coli. Journal of Bacteriology.* **175:** 1484-1492.

Comstock LE and Coyne MJ. (2003) *Bacteroides thetaiotaomicron*: a Dynamic, Niche-Adapted Human Symbiont. *BioEssays.* **25:** 926-929.

Comstock LE, Coyne MJ, Tzianabos AO and Kasper DL. (1999a) Interstrain Variation of the polysaccharide B Biosynthesis Locus of *Bacteroides fragilis*: Characterization of the Region from Strain 638R. *Journal of Bacteriology.* **181:** 6192-6196.

Comstock LE, Coyne MJ, Tzianabos AO, Pantosti A, Onderdonk AB and Kasper DL. (1999b) Analysis of a Capsular polysaccharide Biosynthesis Locus of *Bacteroides fragilis. Infection and Immunity.* **67:** 3525-3532.

Cooper AJ, Kalinowski AP, Shoemaker NB and Salyers AA. (1997) Construction and Characterization of a *Bacteroides thetaiotaomicron recA* Mutant: Transfer of *Bacteroides* Integrated Conjugative Elements Is RecA Independent. *Journal of Bacteriology.* **179:** 6221-6227.

Courcelle J, Crowley D and Hanawalt PC. (1999) Recovery of DNA Replication in UV-Irradiated *Escherichia coli* Requires Both Excision Repair and RecF Protein Function. *Journal of Bacteriology.* **181:** 916-922.

Courcelle J, Donaldson JR, Chow KH and Courcelle CT. (2003) DNA Damage-Induced Replication Fork Regression and Processing in *Escherichia coli. Science.* **299:** 1064-1067.

Courcelle J and Hanawalt PC. (1999) RecQ and RecJ Process Blocked Replication Forks Prior to the Resumption of Replication in UV-Irradiated *Escherichia coli. Molecular and General Genetics.* **262:** 543-551.

Courcelle J and Hanawalt PC. (2001) Participation of Recombination Proteins in Rescue of Arrested Replication Forks in UV-Irradiated *Escherichia coli* Need Not Involve Recombination. *Proceedings of the National Academy of Sciences of the United States of America.* **98:** 8196-8202.

Cox MM. (1995) Alignment of 3 (but not 4) DNA Strands Within a RecA Protein Filament. *Journal of Biological Chemistry.* **270:** 26021-26024.

Coyne MJ, Kalka-Moll W, Tzianabos AO, Kasper DL and Comstock LE. (2000) *Bacteroides fragilis* NCTC9343 Produces at Least Three Distinct Capsular Polysaccharides: Cloning, Characterization, and Reassignment of Polysaccharide B and C Biosynthesis Loci. *Infection and Immunity.* **68:** 6176-6181.

Coyne MJ, Tzianabos AO, Mallory BC, Carey VJ, Kasper DL and Comstock LE. (2001) Polysaccharide Biosynthesis Locus Required for Virulence of *Bacteroides fragilis*. *Infection and Immunity.* **69:** 4342-4350.

Coyne MJ, Weinacht KG, Krinos CM and Comstock LE. (2003) Mpi Recombinase Globally Modulates the Surface Architecture of a Human Commensal Bacterium. *Proceedings of the National Academy of Sciences of the United States of America.* **100:** 10446-10451.

Dachs GU, Abratt VR and Woods DR. (1995) Mode of Action of Metronidazole and a *Bacteroides fragilis metA* Resistance Gene in *Escherichia coli*. *Journal of Antimicrobial Chemotherapy.* **35:** 483-496.

Degnan BA, Macfarlane S, Quigley ME and Macfarlane GT. (1997) Starch utilization by *Bacteroides ovatus* isolated from the Human Large Intestine. *Current Microbiology.* **34:** 290-296.

De Meo M, Vanelle P. Bernadini E, Laget M, Maldonado J, Jentzer O, Crozet MP nad Dumenil G. (1992) Evaluation of the Mutagenic and Genotoxic Activities of 48 Nitroimidazoles and Related Imidazole Derivatives by the Ames Test and the SOS Chromotest. *Environmental and Molecular Mutagenesis.* **19:** 167-181.

Despois R, Pinnet-Sindico S, Ninet L and Preud'homme J. (1956) Three Antibiotics of Different Groups Produced by the Same Strain of *Streptomyces*. *Journal of Microbiology.* **21:** 76.

DeWitt SK and Adelberg EA. (1962) The Ocurrence of a Genetic Transposition in a Strain of *Escherichia coli*. *Genetics.* **47:** 577-585.

Diniz CG, Farias LM, Carvalho MA, Rocha ER, Smith CJ. (2004) Differential Gene Expression in a *Bacteroides fragilis* Metronidazole-Resistant Mutant. *Journal of Antimicrobial Chemotherapy.* **54:** 100-108.

Diniz CG, Santos SG, Pestana ACNR, Farias LM and Carvalho MAR. (2000) Chromosomal Breakage in the *Bacteroides fragilis* Group Induced by Metronidazole Treatment. *Anaerobe.* **6:** 149-153.

Dykers JR. (1975) Hazards of Metronidazole. *New England Journal of Medicine.* **293:** 454.

Edwards DI. (1977) The Action of Metronidazole on DNA. *Journal of Antimicrobial Chemotherapy.* **3:** 43-48.

Edwards DI. (1980) *Antimicrobial Drug Action*, p235-243. MacMillan Press Ltd., London.

Edwards DI, Dye M and Carne H. (1973) The Selective Toxicity of Antimicrobial Nitroheterocyclic Drugs. *Journal of General Microbiology.* **76:** 135-145.

Edwards DI and Mathison GE. (1970) The Mode of Action of Metronidazole Against *Trichomonas vaginalis*. *Journal of General Microbiology.* 63: 297-302.

Fernandez de Henestrosa A, Ogi T, Aoyagi S, Chafin D, Hayes JJ, Ohmori H and Woodgate R. (2000) Identification of Additional Genes Belonging to the LexA Regulon in *Escherichia coli*. *Molecular Microbiology.* **35:** 1560-1572.

Fernebro J, Anderson I, Sublett J, Morfeldt E, Novak R, Tuomanen E, Normark S and Normark BH. (2004) Capsular Expression in *Streptococcus pneumoniae* Negatively Affects Spontaneous and Antibiotic-Induced Lysis and Contributes to Antibiotic Tolerance. *Journal of Infectious Diseases.* **189:** 328-338.

Fleischmann RD, Adams MD, White O, clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb J, Dougherty BA, Merrick JM, McKenney K, Sutton GG, FitzHugh W, Fields CA, Gocayne JD, Scott JD, Shirley R, Liu LI, Glodek A, Kelley JM, Weidman JF, Phillips CA, Spriggs T, Hedblom E, Cotton MD, Utterback T, Hanna MC, Nguyen DT, Saudek DM, Brandon RC, Fine LD, Fritchman JL, Fuhrmann JL, Geoghagen NS, Gnehm CL, McDonald LA, Small KV, Fraser CM, Smith HO and Venter JC. (1995) Whole-Genome Random Sequencing and Assembly of *Haemophilus influenzae* Rd. *Science.* **269:** 496-512.

Fourney RM, Miyskoshi J, Day RS III, and Paterson MC. (1988) Northern blotting: efficient RNA staining and transfer. *Focus.* **10:** 5-7.

Franco AA. (2004) The *Bacteroides fragilis* Pathogenicity Island Is Contained in a Putative Novel Conjugative Transposon. *Journal of Bacteriology.* **186:** 6077-6092.

Frank BP and Dennis LK. (1977) *Bacteroides fragilis* Subspecies in Clinical Isolates. *Annuals of the International Journal of Medicine.* **86:** 569-571.

Friedberg EC, Walker GC and Siede W. (1995) DNA Repair and Mutagenesis. *American Society for Microbology Press, Washington DC,* 191-232, 440-456.

Fukasawa K, Fukasawa KM, Iwamoto H, Hirose J and Harada M. (1999) The HELLGH Motif of Rat Liver Dipeptidyl Peptidase III Is Involved in Zinc Coordination and the Catalytic Activity of the Enzyme. *Biochemistry.* **38:** 8299-8303.

Fukasawa K, Fukasawa KM, Kanai M, Fujii S, Hirose J and Harada M. (1998) Dipeptidyl Peptidase III is a Zinc Metallo-Exopeptidase. Molecular Cloning and Expression. *Biochemical Journal.* **329:** 275-282.

Gallegos MT, Schleif R, Bairoch A, Hoffman K and Ramos JL. (1997) AraC/XylS Family of Transcriptional Regulators. *Microbiology and Molecular Biology Reviews.* **61:** 393-410.

Gibson FC 3rd, Onderdonk AB, Kasper Dl and Tzianabos AO. (1998) Cellular Mechanism of Intraabdominal Abscess Formation by *Bacteroides fragilis*. *Journal of Immunology.* **160:** 5000-5006.

Gibson SA and Macfarlane GT. (1988a) Studies on the Proteolytic Activity of *Bacteroides fragilis*. *Journal of General Microbiology.* **134:** 19-27.

Gibson SA and Macfarlane GT. (1988b) Characterization of Proteases formed by *Bacteroides fragilis*. *Journal of General Microbiology.* **134:** 2231-2240.

Godoy VG, Dallas MM, Russo TA and Malamy MH. (1993) A Role for *Bacteroides fragilis* Neuraminidase in Bacterial Growth in Two Model Systems. *Infection and Immunity.* **61:** 4415-4426.

Goodman HJK, Parker JR, Southern JA and Woods DR. (1987) Cloning and Expression in *Escherichia coli* of a *recA*-Like Gene from *Bacteroides fragilis*. *Gene.* **58:** 265-271.

Goodman HJK and Woods DR. (1990) Molecular Analysis of the *Bacteroides fragilis recA* Gene. *Gene.* **94:** 77-82.

Goodwin A, Kersulyte D, Sisson G, Veldhuyzen van Zanten SJO, Berg DE and Hoffman PS. (1998) Metronidazole Resistance in *Helicobacter pylori* Is Due to Null Mutations in a Gene (*rdxA*) that Encodes an Oxygen-Insensitive NADPH Nitroreductase. *Molecular Microbiology.* **28:** 383-393.

Gorbalenya AE, Koonin EV, Donchenko AP and Blinov VM. (1989) Two Related Superfamiliesn of Putative Helicases Involved in Replication, Recombination, Repair and Expression of DNA and RNA Genomes. *Nucleic Acids Research.* **17:** 4713-4729.

Green MH, Greenberg J and Donch J. (1969) Effect of a *recA* Gene on Cell Division and Capsular Polysaccharide Production in a *lon* Strain of *Escherichia coli*. *Genetical Research.* **14:** 159-162.

Griffin IV, TJ and Kolodner RD. (1990) Purification and Preliminary Characterization of the *Escherichia coli* K-12 RecF Protein. *Journal of Bacteriology.* **172:** 6291-6299.

Guiney DG, Hasegawa P and Davis CE. (1984) Plasmid Transfer from *Escherichia coli* to *Bacteroides fragilis*: Differential Expression of Antibiotic Resistance Phenotypes. *Proceedings of the National Academy of Sciences of the United States of America.* **81:** 7203-7206.

Gupta RS. (2004) The Phylogeny and Signature Sequences Characteristics of *Fibrobacteres*, *Chlorobi*, and *Bacteroidetes*. *Critical Reviews in Microbiology.* **30:** 123-143.

Gutacker M, Valsangiacomo C, Bernasconi MV and Piffaretti JC. (2002) RecA and *glnA* Sequences Separate the *Bacteroides fragilis* Population Into Two Genetic Divisions Associated with the Antibiotic Resistance Genotypes *cepA* and *cfiA*. *Journal of Medical Microbiology.* **51:** 123-130.

Haggoud A, Reysset G, Azeddoug H and Sebald M. (1994) Nucleotide Sequence Analysis of Two 5-Nitroimidazole Resistance Determinants from *Bacteroides* Strains and of a New Insertion Sequence Upstream of the Two Genes. *Antimicrobial Agents and Chemotherapy.* **38:** 1047-1051.

Handa N, Bianco PR, Baskin RJ and Kowalczykowski SC. (2005) Direct Visualisation of RecBCD Movement Reveals Cotranslocation of the RecD Motor After Chi Recognition. *Molecular Cell.* **17:** 745-750.

Hanada K, Iwasaki M, Ihashi S and Ikeda H. (2000) UvrA and UvrB Suppress Illegitimate Recombination: Synergistic Action with RecQ Helicase. *Proceedings of the National Academy of Sciences of the United States of America.* **97:** 5989-5994.

Hanada K, Ukita T, Kohno Y, Saito K, Kato JI and Ikeda H. (1997) RecQ DNA Helicase Is a Suppressor of Illegitimate Recombination in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America.* **94:** 3860-3865.

Hayashi H, Sakamoto M and Benno Y. (2002) Phylogenetic Analysis of the Human Gut Microbiota Using 16S rDNA Clone Libraries and Strictly Anaerobic Culture-Based Methods. *Microbiology and Immunology.* **46:** 535-548.

Herren CD, Rocha ER and Smith CJ. (2003) Genetic Analysis of an Important oxidative Stress Locus in the Anaerobe *Bacteroides fragilis*. *Gene.* **316:** 167-175.

Hill RT, Parker JR, Goodman HJK, Jones DT and Woods DR. (1989) Molecular Analysis of a Novel Glutamine Synthetase of the Anaerobe *Bacteroides fragilis*. *Journal of General Microbiology.* **135:** 3271-3279 .

Hodgman TC. (1988) A New Superfamily of Replicative Proteins. *Nature.* **333:** 22-23.

Hof H and Sticht-Groh V. (1984) Antibacterial Effects of Niridazole: its Effect on Microaerophilic *Campylobacter*. *Infection.* **12:** 36-39.

Holdeman LV, Kelly RW and Moore WEC. (1984) Genus I. *Bacteroides* Castellani and Chalmers 1919,1959. In *Bergeys Manual of Systematic Bacteriology*, pp 604-631. Edited by Krieg, N. R. and Holt, J. G. Williams and Wilkins. Baltimore.

Holdeman LV and Moore WEC. (1972) Anaerobe Laboratory Manual. 4th Ed. Virginia Polytechnic Institute and State University Anaerobe Laboratory, Blacksburg, VA.

Hooper LV, Midtvedt T and Gordon JI. (2002) How Host-Microbial Interactions Shape the Nutrient Environment of the Mammalian Intestine. *Annual Review of Nutrition.* **22:** 283-307.

Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG and Gordon JI. (2001) Molecular Analysis of Commensal Host-Microbial Relationships in the Intestine. *Science.* **291:** 881-884.

Hooper LV, Xu J, Falk PG, Midtvedt T and Gordon JI. (1999) A Molecular Sensor that Allows a Gut Commensal to Control its Nutrient Foundation in a Competitive Ecosystem. *Proceedings of the National Academy of Sciences of the United States of America.* **96:** 9833-9838.

Howard-Flanders P, Boyce RP and Theriot L. (1966) Three Loci in *Escherichia coli* K-12 that Control the Excision of Pyrimidine Dimers and Certain Other Mutagen Products from DNA. *Genetics.* **53:** 1119-1136.

Ingham HR, Eaton S, Venables CW and Adams PC. (1978) *Bacteroides fragilis* Resistant to Metronidazole After Long-Term Therapy. *Lancet. i*: 214.

Ingham HR, Hall CJ, Sisson PR, Tharagonnet D and Selkon JB. (1980) The Activity of Metronidazole Against Facultatively Anaerobic Bacteria. *Journal of Antimicrobial Chemotherapy.* **6:** 343-347.

Ings RMJ, McFadzean JA and Ormerod WE. (1974) The Mode of Action of Metronidazole in *Trichomonas vaginalis* and Other Microorganisms. *Biochemical Pharmacology.* **23:** 1421-1429.

Irino N, Nakayama K and Nakayama H. (1986) The *recQ* Gene of *Escherichia coli* K12: Primary Structure and Evidence for SOS Regulation. *Molecular and General Genetics.* **205:** 298-304.

Ish-Horowicz D and Burke JF. (1981) Rapid and Efficient Cosmid Cloning. *Nucleic Acids Research.* **9:** 2989-2998.

Ivancic-Bace I, Salaj-Smic E and Brcic-Kostic K. (2005) Effects of recJ, recQ and recFOR mutations on recombination in nuclease-deficient recB recD double mutants of *Escherichia coli*. *Journal of Bacteriology.* **187:** 1350-1356.

Iyer VN and Szybalski W. (1963) A Molecular Mechanism of Mitomycin C Action: Linking of Complementary DNA Strands. *Proceedings of the National Academy of Sciences of the United States of America.* **50;** 335-362.

Jackson D, Salem a and Coombs GH. (1984) The In-Vitro Activity of Metronidazole Against Strains of *Escherichia coli* with Impaired DNA Repair Systems. *Journal of Antimicrobial Chemotherapy.* **13:** 227-236.

Jorgensen MA, Manos J, Mendz GL and Hazell SL. (1998) The Mode of Action of Metronidazole in *Helicobacter pylori*: Futile Cycling or Reduction? *Journal of Antimicrobial Chemotherapy.* **41:** 67-75.

Joseph JW and Kolodner R. (1983) Exonuclease VIII of *Escherichia coli*. II. Mechanism of Action. *Journal of Biological Chemistry.* **258:** 10418-10424.

Kasper DL, Lindberg AA, Weintraub A, Onderdonk AB and Lonngren J. (1984) Capsular Polysaccharides and Lipopolysaccharides from Two Strains of *Bacteroides fragilis*. *Reviews of Infectious Diseases.* **6:** 25-29.

Kim BT, Kim WS, Kim YS, Linhardt DT and Kim DH. (2000) Purification and Characterization of a Novel Heparinase from *Bacteroides stercoris* HJ-15. *Journal of Biochemistry.* **128:** 323-328.

Kim JM, Kim YJ and Cho YJ. (2000) Synergy of *Bacteroides fragilis* and *Escherichia coli* in the Induction of KC Gene Expression in Mouse Peritoneal Tissues. *Scandinavian Journal of Infectious Diseases.* **32:** 643-649.

Knight RC, Rowley DA, Skolimowski IM and Edwards DI. (1979) Mechanism of Action of Nitroimidazole Antimicrobial and Antitumour Radiosensitizing Drugs. *International Journal of radiation Biology.* **36:** 367-377.

Knight RC, Skolimowski IM and Edwards DI. (1978) The Interaction of Reduced Metronidazole with DNA. *Biochemical Pharmacology.* **27:** 2089-2093.

Knox RJ, Edwards DI and Knight RC. (1984) The Mechanism of Nitroimidazole Damage to DNA: Colourimetric Evidence. *International Journal of Radiation Oncology, Biology, Physics.* **10:** 1315-1318.

Koch RL, Chrystal EJT, Beaulieu BB Jr and Goldman P. (1979) Acetamide: A Metabolite of Metronidazole Formed by the Intestinal Flora. *Biochemical Pharmacology.* **28:** 3611-3615.

Koch RL and Goldman P. (1979) The Anaerobic Metabolism of Metronidazole Forms N-(2-Hydroxyethyl)-Oxamic Acid. *Journal of Pharmacology and Experimental Therapeutics.* **208:** 406-410.

Koetsier PA, Schorr J and Doerfler W. (1993) A Rapid Optimized Protocol for Downward Alkaline Southern Blotting of DNA. *Biotechniques.* **15:** 260-262.

Krinos CM, Coyne MJ, Weinacht KG, Tzianabos AO, Kasper DI and Comstock LE. (2001) Extensive Surface Diversity of a Commensal Microorganism by Multiple DNA Inversions. *Nature.* **414:** 555-558.

Kulda J, Tachezy J and Cerkasovova A. (1993) *In Vitro* Induced Anaerobic Resistance to Metronidazole in *Trichomonas vaginalis*. *Journal of eukaryotic Microbiology.* **40:** 262-269.

Kuwahara T, Yamashita A, Hirakawa H, Nakayama H, Toh H, Okada N, Kuhara S, Hattori M, Hayashi T and Ohnishi Y. (2004) Genomic Analysis of *Bacteroides fragilis* Reveals Extensive DNA Inversions Regulating Cell Surface Adaptation. *Proceedings of the National Academy of Sciences of the United States of America.* **101:** 14919-14924.

Kuzminov A. (1999) Recombinational Repair of DNA Damage in *Escherichia coli* and Bacteriophage λ. *Microbiology and Molecular Biology Reviews.* **63:** 751-813.

Kwon DH, El-Zataari FAK, Kato M, Osato MS, Reddy R, Yamaoka Y and Graham DY.a (2000) Analysis of *rdxA* and Involvement of Additional genes Encoding NAD(P)H Flavin Oxidoreductase (FrxA) and Ferredoxin-Like Protein (FdxB) in Metronidazole Resistance of *Helicobacter pylori*. *Antimicrobial Agents and Chemotherapy.* **44:** 2133-2142.

Kwon DH, Osato MS, Graham DY and El-Zataari FA. B (2000) Quantitative RT-PCR Analysis of Multiple Genes Encoding Putative Metronidazole Nitroreductases from *Helicobacter pylori*. *International Journal of Antimicrobial Agents.* **15:** 31-36.

Kwon HJ, Bennik MHJ, Demple B and Ellenberger T. (2000) Crystal Structure of the *Escherichia coli* Rob Transcription Factor in Complex with DNA. *Nature Structural Biology.* **7:** 424-430.

Lacey SL, Moss SF and Taylor GW. (1993) Metronidazole Uptake by Sensitive and Resistant Strains of *Helicobacter pylori*. *Journal of Antimicrobial Chemotherapy.* **32:** 393-400.

Lane D. (1988) Enlarged Family of Putative Helicases. *Nature.* **334:** 478.

Lee BI, Kim KH, Park SJ, Eom SH, Song HK and Suh HW. (2004) Ring-Shaped Architecture of RecR: Implications for Its Role in Homologous Recombinational DNA Repair. *EMBO Journal.* **23:** 2029-2038.

Lehman IR and Nussbaum R. (1964) The Deoxyribonuclease of *Escherichia coli*. V. On the Specificity of Exonuclease I (Phosphodiesterase). *Journal of Biological Chemistry*. **239:** 2628-2636.

Li Z and Demple B. (1994) SoxS, An Activator of Superoxide Stress Genes in *Escherichia coli*: Purification and Interaction with DNA. *Journal of Biological Chemistry*. **269:** 18371-18377.

Lin JJ and Sancar A. (1992) (A)BC Excinuclease: the *Escherichia coli* Nucleotide Excision Repair Enzyme. *Molecular Microbiology*. **6:** 2219-2224.

Linder P, Lasko PF, Ashburner M, Leroy P, Nielsen PJ, Nishi K, Schnier J and Slonimski PP. (1989) Birth of the D-E-A-D Box. *Nature*. **337:** 121-122.

Linn SP, Chan T and Salyers AA. (1983) Isolation and Characterization of Two Chondroitin Lyases from *Bacteroides thetaiotaomicron*. *Journal of Bacteriology*. **156:** 859-866.

Lindmark DG and Müller M. (1976) Antitrichomonad Action, Mutagenicity, and Reduction of Metronidazole and Other Nitroimidazoles. *Antimicrobial Agents and Chemotherapy*. **10:** 476-482.

Little JW, Edmiston SH, Pacelli LZ and Mount DW. (1980) Cleavage of the *Escherichia coli* Lexa Protein by the RecA Protease. *Proceedings of the National Academy of Sciences of the United States of America*. **77:** 3225-3229.

Little JW and Mount DW. (1982) The SOS Regulatory System of *Escherichia coli*. *Cell*. **29:** 11-22.

Liu Y and Fletcher HM. (2001) Environmental Regulation of *recA* Gene Expression in *Porphyromonas gingivalis*. *Oral Microbiology and Immunology*. **16:** 136-143.

Livneh Z, Cohen-Fix O, Skaliter R and Elizur T. (1993) Replication of Damaged DNA and the Molecular Mechanism of Ultraviolet Light Mutagenesis. *Crit. Rev. Biochem. Mol. Biol.* **28:** 465-513.

Lloyd RG and Buckman C. (1985) Identification and Genetic Analysis of *sbcC* Mutations in Commonly Used *recBC sbcB* Strains of *Escherichia coli* K-12. *Journal of Bacteriology*. **164:** 836-844.

Lockerby DL, Rabin HR and Laishley EJ. (1985) Role of the Phosphoroclastic Reaction of *Clostridium pasteurianum* in the Reduction of Metronidazole. *Antimicrobial Agents and Chemotherapy*. **27:** 863-867.

Lu Z, Cabiscol E, Obradors N, Tamarit J, Ros J, Aguilar j and Lin ECC. (1998) Evolution of an *Escherichia coli* Protein with increased Resistance to Oxidative Stress. *Journal of Biological Chemistry*. **273:** 8308-8316.

Macfarlane GT, Allison C, Gibson SA and Cummings JH. (1988) Contribution of the Microflora to Proteolysis in the Human Large Intestine. *Journal of Applied Bacteriology*. **64:** 37-46.

Macfarlane GT, Macfarlane S and Gibson GR. (1992) Synthesis and Release of proteases by *Bacteroides fragilis*. *Current Microbiology*. **24:** 55-59.

Macy JM, ljungdahl LG and Gottschalk G. (1978) pathway of Succinate and propionate formation in *Bacteroides fragilis*. *Journal of Bacteriology*. **134:** 84-91.

Madiraju MVVS and Clark AJ. (1992) Evidence for ATP Binding and Double-Stranded DNA Binding by *Escherichia coli* RecF Protein. *Journal of Bacteriology*. **174:** 7705-7710.

Manzanera M, Marques S and Ramos JL. (2000) Mutational Analysis of the Highly Conserved C-Terminal Residues of the XylS Protein, a Member of the AraC Family of Transcriptional Regulators. *FEBS Letters*. **476:** 312-317.

Marteau P, Pochart P, Doré J, Béra-Maillet C, Bernalier A and Corthier G. (2001) Comparative Study of Bacterial Groups Within the Human Cecal and Fecal Microbiota. *Applied and Environmental Microbiology.* **67:** 4939-4942.

Martin RG, Gillete WK, Rhee S and Rosner JL. (1999) Structural Requirements for Marbox Function in Transcriptional Activation of *mar/sox/rob* Regulon Promoters in *Escherichia coli*: Sequence, Orientation and Spatial relationship to the Core Promoter. *Molecular Microbiology.* **34:** 431-441.

Martin RG and Rosner JL. (2001) The AraC Transcriptional Activators. *Current Opinion in Microbiology.* **4:** 132-137.

Martin RG and Rosner JL. (2002) Genomics of the *marA/soxS/rob* Regulon of *Escherichia coli*: Identification of Directly Activated Promoters by Application of molecular Genetics and Informatics to Microarray Data. *Molecular Microbiology.* **44:** 1611-1624.

Masterson C, Boehmer PE, McDonald F, Chaudhury S, Hickson ID and Emmerson PT. (1992) Reconstitution of the Activities of the RecBCD Holoenzyme of *Escherichia coli* from the Purified Subunits. *Journal of Biological Chemistry.* **267:** 13564-13572.

May BJ, Zhang Q, Li LL, Paustian ML, Whittam TS and Kapur V. (2001) Complete Genomic Sequence of *Pasteurella multocida*, Pm70. *Proceedings of the National Academy of Sciences of the United States of America.* **98:** 3460-33465.

Meier-Dieter U, Starman r, Barr K, Mayer H and Rick PD. (1990) Biosynthesis of Enterobacterial Common Antigen in *Escherichia coli*. Biochemical Characterization of Tn10 Insertion Mutants Defective in Enterobacterial Common Antigen Synthesis. *Journal of Biological Chemistry.* **265:** 13490-13497.

Michael NP, Brehm JK, Anlezark GM and Minton NP. (1994) Physical Characterization of the *Escherichia coli* B Gene Encoding Nitroreductase and its Over-Expression in *Escherichia coli* k12. *FEMS Microbiology Letters.* **124:** 195-202.

Moolenaar GF, Moorman C and Goosen N. (2000) Role of the *Escherichia coli* Nucleotide Excision Repair Proteins in DNA Replication. *Journal of Bacteriology.* **182:** 5706-5714.

Moolenaar GF, van Rossum-Fikkert S, van Kesteren M and Goosen N. (2002) Cho, a Second Endonuclease Involved in *Escherichia coli* Nucleotide Excision Repair. *Proceedings of the National Academy of Sciences of the United States of America.* **99:** 1467-1472.

Moore RA, Beckthold B and Bryan LE. (1995) Metronidazole Uptake in *Helicobacter pylori*. *Can. Journal of microbiology.* **41:** 746-749.

Moore WE and Holdeman LV. (1974) Human Fecal Flora: the Normal Flora of 20 Japanese-Hawaiians. *Applied Microbiology.* **27:** 961-979.

Moore WEC, Cato EP and Holdeman LV. (1978) Some Current Concepts of Intestinal Bacteriology. *American Journal of Clinical Nutrition.* **31:** S33-S42.

Morimatsu K and Kowalczykowski SC. (2003) RecFOR Proteins Load RecA onto Gapped DNA to Accelerate DNA Strand Exchange: A Universal Step of Recombinational Repair. *Molecular Cell.* **11:** 1337-1347.

Morozov V, Mushegian AR, Koonin EV and Bork P. (1997) A Putative Nucleic Acid-Binding Domain in Bloom's and Werner's Syndrome Helicases. *Trends in Biochemical. Sciences.* **22:** 417-418.

Mortenson LE, Valentine RC and Carnahan JE. (1963) Ferredoxin in the Phosphoroclastic Reaction of Pyruvic Acid and its relation to Nitrogen Fixation in *Clostridium pasteurianum*. *Journal of Biological Chemistry.* **238:** 794-800.

Mossie KG, Jones DT, Robb FT and Woods DR. (1979) Characterization and mode of action of a bacteriocin produced by a *Bacteroides fragilis* strain. *Antimicrobial Agents Chemotherapy.* **16:** 724-30.

Müller M. (1983) Mode of Action of Metronidazole on anaerobic Bacteria and Protozoa. *Surgery.* **93:** 165-171.

Müller M and Lindmark DG. (1976) Uptake of Metronidazole and its Effect on Viability in trichomonads and *Entamoeba invadens* under Anaerobic and Aerobic Conditions. *Antimicrobial Agents and Chemotherapy.* **9:** 696-700.

Nakayama K, Irino n and Nakayama H. (1985) The *recQ* Gene of *Escherichia coli* K12: Molecular Cloning and Isolation of Insertion Mutants. *Mol. Gen. Genet.* **200:** 266-271.

Narikawa S. (1986) Distribution of Metronidazole Susceptibility Factors in Obligate Anaerobes. *Journal of Antimicrobial Chemotherapy.* **18:** 565-574.

Narikawa S, Suzuki T, Yamamoto M and Nakamura M. (1991) Lactate Dehydrogenase Activity as a Cause of Metronidazole Resistance in *Bacteroides fragilis* NCTC 11295. *Journal of Antimicrobial Chemotherapy.* **28:** 47-53.

Noble JI and Tally FP. (1984) Metronidazole. *Antimicrobial Therapy*, p255-263. Raven Press, New York.

Obradors N, Cabiscol E, Aguilar J and Ros J. (1998) Site-Directed Mutagenesis Studies of the Metal-Binding Center of the Iron-Dependent Propanediol oxidoreductase from *Escherichia coli*. *European Journal of Biochemistry.* **258:** 207-213.

Odelson D, Rasmussen J, Smith CJ and Macrina FL. (1987) Extrachromosomal Systems and Gene Transmission in Anaerobic Bacteria. *Plasmid.* **17:**87-109.

Onderdonk AB, Cisneros RL, Finberg R, Crabb JH and Kasper DL. (1990) Animal Model System for Studying Virulence of and Host Response to *Bacteroides fragilis*. *Reviews of infectious Diseases.* **12:** 169-177.

Onderdonk AB, Kasper DL, Cisneros RL and Bartlett JG. (1977) The Capsular Polysaccharide of *Bacteroides fragilis* as a Virulence Factor: Comparison of the Pathogenic Potential of Encapsulated and Unencapsulated Strains. *Journal of Infectious Diseases.* **136:** 82-89.

Pan N and Imlay JA. (2001) How Does Oxygen Inhibit Central Metabolism in the Obligate anaerobe *Bacteroides thetaiotaomicron*. *Molecular Microbiology.* **39:** 1562-1571.

Pantosti A, Tzianabos AO, Onderdonk AB and Kasper DL. (1991) Immunochemical Characterization of Two Surface Polysaccharides of *Bacteroides fragilis*. *Infection and Immunity.* **59:** 2075-2082.

Pantosti A, Tzianabos AO, Reinap BG, Onderdonk AB and Kasper DL. (1993) *Bacteroides fragilis* Strains Express Multiple Capsular Polysaccharides. *Journal of Clinical Microbiology.* **31:** 1850-1855.

Parkhill J, Wren BW, Thomson NR, Titball RW, Holden MTG, Prentice MB, Sebaihia M, James KD, Churcher C, Mungall KL, Baker S, Basham D, Bentley SD, Brooks K, Cerdeno-Tarraga AM, Chillingworth T, Cronin A, Davies RM, Davis P, Dougan G, Feltwell T, Hamlin N, Holroyd S, Jagels K, leather S, Karlyshev AV, Moule S, Oyston PCF, Quail M, Rutherford K, Simmonds M, Skleton J, Stevens K, Whitehead S and Barrell BG. (2001) Genome Sequence of *Yersinia pestis*, the Causative Agent of Plague. *Nature.* **413:** 523-527.

Paster BJ, Dewhirst FE, Olsen I and Fraser GJ. (1994) Phylogeny of *Bacteroides*, *Prevotella*, and *Porphyromonas* Spp. And Related Bacteria. *Journal of Bacteriology.* **176:** 725-732.

Park BH and Levy SB. (1988) The Cryptic Tetracycline Resistance Determinant on Tn*4400* Mediates Tetracycline Degradation as well as Efflux. *Antimicrobial Agents and Chemotherapy.* **32:** 1797-1800.

Patrick S, Lutton DA and Crockard AD. (1995) Immune Reactions to *Bacteroides fragilis* Populations with Three different Types of Capsule in a Model of Infection. *Microbiology.* **141:** 1969-1976.

Patrick S, Parkhill J, McCoy L, Lennard N, Larkin MJ, Collins M and Blakely G. (2003) Multiple Inverted DNA Repeats of *Bacteroides fragilis* that Control Polysaccharide Antigenic Variation Are Similar to the Hin Region Inverted Repeats of *Salmonella typhimurium*. *Microbiology.* **149:** 915-924.

Patrick S, Reid JH and Larkin MJ. (1984) The Growth and Survival of Capsulate and Non-Capsulate *Bacteroides fragilis in Vivo* and *In Vitro*. *Journal of Medical Microbiology.* **17:** 237-246.

Pavicic MJ, van Winkelhoff AJ, Pavicic-Temming YA and de Graaff J. (1995) Metronidazole Susceptibility Factors in *Actinobacillus actinomycetemcomitans*. *Journal of Antimicrobial Chemotherapy.* **35:** 263-269.

Perez-Reyes E, Kalyanaraman B and Mason RP. (1980) The Reductive Metabolism of Metronidazole and Ronidazole by Aerobic Liver Microsomes. *Molecular Pharmacology.* **17:** 239-244.

Peterson FJ, Mason RP, Hovsepian J and Holtzman JL. (1979) Oxygen-Sensitive and –Insensitive Nitroreduction by *Escherichia coli* and Rat Hepatic Microsomes. *Journal of Biological Chemistry.* **254:** 4009-4014.

Plant CW and Edwards DI. (1976) The Effect of Tinidazole, Metronidazole and Nitrofurazone on Nucleic Acid Synthesis in *Clostridium bifermentans*. *Journal of Antimicrobial Chemotherapy.* **2:** 203-209.

Podolny V, Lin ECC and Hochschild A. (1999) A Cyclic AMP Receptor Protein Mutant That Constitutively Activates an *Escherichia coli* Promoter Disrupted by an IS*5* Insertion. *Journal of Bacteriology.* **181:** 7457-7463.

Power J. (1967) The L-Rhamnose Genetic System in *Escherichia coli* K-12. *Genetics.* **55:** 557-568.

Privitera G, Dublanchet A and Sebald M. (1979) Transfer of Multiple Antibiotic Resistance Between Subspecies of *Bacteroides fragilis*. *Journal of Infectious Diseases.* **139:** 97-101.

Radman M. (1974) Phenomenology of an Inducible Mutagenic DNA Repair Pathway in *Escherichia coli*: SOS Repair Hypothesis. *Molecular and Environmental Aspects of Mutagenesis.* Charles C. Thomas Publishers, Springfield. pp128-142.

Rasmussen BA, Gluzman Y and Tally FP. (1990) Cloning and Sequencing of the Class-B β-Lactamase Gene (*ccrA*) from *Bacteroides fragilis* TAL3636. *Antimicrobial Agents and Chemotherapy.* **34:** 1590-1592.

Rasmussen BA, Gluzman Y and Tally FP. (1991) *Escherichia coli* Chromosomal Mutations That Permit Direct Cloning of the *Bacteroides fragilis* Metallo-β-Lactamase Gene, *ccrA*. *Molecular Microbiology.* **5:** 1211-1219.

Reeves A, Wang G and Salyers AA. (1997) Characterization of Four Outer Membrane Proteins that Play a Role in utilization of Starch by *Bacteroides thetaiotaomicron*. *Journal of Bacteriology.* **179:** 643-649.

Rhee S, Martin RG, Rosner JL and Davies DR. (1998) A Novel DNA-Binding Motif in MarA: The First Structure for an AraC Family Transcriptional Activator. *Proceedings of the National Academy of Sciences of the United States of America.* **95:** 10413-10418.

Robert MC, Moncla B and Kenny GE. (1987) Chromosomal DNA Probes for Identification of *Bacteroides* Species. *Journal of General Microbiology.* **133:** 1423-1430.

Robillard NJ, Tally FP and Malamy MH. (1985) Tn*4400*, a Compound Transposon Isolated from *Bacteroides fragilis* Functions in *Escherichia coli*. *Journal of Bacteriology.* **164:** 1248-1255.

Rocha ER, Owens G Jr and Smith CJ. (2000) The Redox-Sensitive Transcriptional Activator OxyR Regulates the Peroxide Response Regulon in the Obligate Anaerobe *Bacteroides fragilis*. *Journal of Bacteriology*. **182:** 5059-5069.

Rocha ER, Smith A, Smith CJ and Brock JH. (2001) Growth Inhibition of *Bacteroides fragilis* by Hemopoxin: Proteolytic Degradation of Hemopoxin to Overcome Heme Limitation. *FEMS Microbiology Letters*. **199:** 73-78.

Rocha ER and Smith CJ. (1995) Biochemical and Genetic Analyses of a Catalase from the Anaerobic Bacterium *Bacteroides fragilis*. *Journal of Bacteriology*. **177:** 3111-3119.

Rocha ER and Smith CJ. (2004) Trancriptional Regulation of the *Bacteroides fragilis* Ferritin Gene (*ftnA)* by Redox Stress. *Microbiology*. **150:** 2125-2134.

Rosenberg M. and Court D. (1979) Regulatory Sequences involved in the Promotion and Termination of RNA Transcription. *Annual Review of Genetics*. **13:** 319-353.

Rosenkranz HS and Speck WT. (1975) Mutagenicity of Metronidazole: Activation by Mammalian Liver Microsomes. *Biochemical and Biophysical Research Communication*. **66:** 520-525.

Rowley DA, Knight RC, Skolimowski IM and Edwards DI. (1979) The Effect of Nitroheterocyclic Drugs on DNA: An *In Vitro* Model of Cytotoxicity. *Biochemical Pharmacology*. **28:** 3009-3013.

Rowley DA, Knight RC, Skolimowski IM and Edwards DI. (1980) The Relationship Between Misonidazole Cytotoxicity and Base Composition of DNA. *Biochemical Parasitology*. **29:** 2095-2098.

Russo TA, Thompson JS, Goddoy VG and Malamy MH. (1990) Cloning and Expression of the *Bacteroides fragilis* TAL2480 neuraminidase Gene, *nanH*, in *Escherichia coli*. *Journal of Bacteriology*. **172:** 2594-2600.

Rustia M and Shubik P. (1972) Induction of Lung Tumours and Malignant Lymphomas in Mice by Metronidazole. *Journal of the National Cancer Institute*. **48:** 721-729.

Salyers AA. (1984) *Bacteroides* of the Human Lower Intestinal Tract. *Annual Review of Microbiology*. **38:** 293-313.

Salyers AA, Bonheyo G and Shoemaker NB. (2000) Starting a New Genetic System: Lessons from *Bacteroides*. *Methods*. **20:** 35-46.

Salyers AA and Kotarski SF. (1980) Induction of Chondrotin Sulphate Lyase Activity in *Bacteroides thetaiotaomicron*. *Journal of Bacteriology*. **143:** 781-788.

Salyers AA and Pajeau M. (1989) Competitiveness of Different Polysaccharide Utilization Mutants of *Bacteroides thetaiotaomicron* in the Intestinal Tracts of Germfree Mice. *Applied and Environmental Microbiology*. **55:** 2572-2578.

Salyers AA and Shoemaker NB. (1995) Conjugative Transposons: The Force behind the Spread of Antibiotic Resistance Genes among *Bacteroides* Clinical Isolates. *Anaerobe*. **1:** 143-150.

Salvers AA, Shoemaker N, Cooper A, D'Elia J and Shipman J. (1999) Genetic Methods for *Bacteroides* species. *Genetic Methods for Diverse Prokaryotes. Methods in Microbiology 29*, (ed. Sockett, M.C.M.S.a.R.E.) (Academic Press, London) **29:** 229-249.

Salyers AA, Vercelloti J, West s and Wilkins T. (1977a) Fermentation of Mucin and Plant Polysaccharide by Strains of *Bacteroides* from the Human Colon. *Applied and Environmental Microbiology*. **33:** 319-322.

Salyers AA, West S, Vercelloti J and Wilkins T. (1977b) Fermentation of Mucins and Plant Polysaccharides by Anaerobic Bacteria from the Human Colon. *Applied and Environmental Microbiology.* **34:** 529-533.

Sambrook JT, Fritsch EF and Maniatis T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sandler SJ and Clark AJ. (1994) RecOR Suppression of *recF* Mutant Phenotypes in *Escherichia coli* K-12. *Journal of Bacteriology.* **176:** 3661-3672.

Sanger F, Nicklen S and Coulson AR (1977) DNA Sequencing with Chain-Terminating Inhibitors. *Proceedings of the National Academy of Sciences of the United States of America.* **74:** 5463-5467.

Savage DC. (1977) Microbial Ecology of the Gastrointestinal Tract. *Annual Review of Microbiology.* **31:** 107-133.

Schlegel HG. (1986) Special Fermentations. *General Microbiology*, 6th Edition, 265-305. Cambridge University Press, Cambridge.

Sears CL. (2001) The Toxins of *Bacteroides fragilis*. *Toxicon.* **39:** 1737-1746.

Setlow RB, Swenson PA and Carrier WL. (1963) Thymine Dimers and Inhibition of DNA Synthesis by Ultraviolet Irradiation of Cells. *Science.* **142:** 1464-1466.

Sghir A, Gramet G, Suau A, Rochet V, Pochart P and Doré J. (2000) Quantification of Bacterial Groups Within Human Fecal Flora by Oligonucleotide Probe Hybridization. *Applied and Environmental Microbiology.* **66:** 2263-2266.

Shah HN and Collins MD. (1983) Genus *Bacteroides*: A chemotaxonomical Perspective. *Journal of Applied Bacteriology.* **55:** 403-416.

Shah HN and Collins MD. (1989) Proposal to Restrict the Genus *Bacteroides* (Castellani and Chambers) to *Bacteroides fragilis* and Closely Related Species. *International Journal of Systematic Bacteriology.* **39:** 85-87.

Sharma S, Sommers JA and Brosh RM Jr. (2004) *In Vivo* Function of the Conserved Non-Catalytic Domain of Werner Syndrome Helicase in DNA Replication. *Hum. Mol. Genet.* **13:** 2247-2261.

Shine J and Dalgarno L. (1974) The 3' terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. *Proceedings of the National Academy of Sciences of the United States of America.* **71:** 1342-1346.

Shinn DLS. (1962) Metronidazole in Acute Ulcerative Gingivitis. *Lancet.* **1:** 1191.

Shipman J, Berleman J and Salyers AA. (2000) Characterization of Four Outer Membrane Proteins Involved in Binding Starch to the Cell Surface of *Bacteroides thetaiotaomicron*. *Journal of Bacteriology.* **182:** 5365-5372.

Shoemaker N, Getty C, Gardner JF and Salyers AA. (1986) Tn4351 Transposes in *Bacteroides* spp. and Mediates the Integration of Plasmid R751 into the *Bacteroides* Chromosome. *Journal of Bacteriology.* **165:** 929-936.

Sindar P. Britz ML and Wilkinson RG. (1982) Isolation and Properties of Metronidazole Resistant Mutants of *Clostridium perfringens*. *Journal of Medical Microbiology.* **15:** 503-509.

Singleton MR, Dillingham MS, Gaudier M, Kowalczykowski SC, Wigley DB. (2004) Crystal Structure of RecBCD enzyme reveals a machine for processing DNA breaks. *Nature.* **432:** 187-193.

Sisson G, Jeong JY, Goodwin A, Bryden L, Rossler N, Lim-Morrison S, Raudonikiene A, Berg DE and Hoffman PS. (2000) Metronidazole Activation Is Mutagenic and Causes DNA Fragmentation in *Helicobacter pylori* and in *Escherichia coli* Containing a Cloned *H. pylori rdxA*$^{+}$ (Nitroreductase) Gene. *Journal of Bacteriology*. **182:** 5091-5096.

Skarin A and Mårdh PA. (1981) Scanning Electron Microscopic Examination of *Bacteroides fragilis* and *Gardnerella vaginalis* After Exposure to Concentration Gradients of Metronidazole and Tinidazole. *Scandinavian Journal of Infectious Diseases*. **26:** 54-59.

Skarstad K, Thöny B, Hwang DS and Kornberg A. (1993) A Novel Binding Protein of the Origin of the *Escherichia coli* Chromosome. *Journal of Biological Chemistry*. **268:** 5365-5370.

Smith CJ. (1995) Genetic Transformation of *Bacteroides Spp.* Using Electroporation. *Methods in Molecular Biology*. **47:** 161-169.

Smith CJ, Parker A and Rogers MB. (1990) Plasmid Transformation of *Bacteroides Spp.* By Electroporation. *Plasmid*. **24:** 100-109.

Smith CJ, Rogers MB and McKee ML. (1992) Heterologous Gene Expression in *Bacteroides fragilis*. *Plasmid*. **27:** 141-154.

Smith CJ, Tribble GD and Bayley DP. (1998) Genetic Elements of *Bacteroides* Species: A Moving Story. *Plasmid*. **40:** 12-29.

Smith GRS, Kunes SM, Schultz DW, Taylor A and Triman KL. (1981) Structure of Chi Hotspots of Generalized Recombination. *Cell*. **24:** 429-436.

Smith KA and Salyers AA. (1989) Cell-Associated Pullulanase from *Bacteroides thetaiotaomicron*: Cloning, Characterization, and Insertional Mutagenesis to Determine Role in Pullulan Utilization. *Journal of Bacteriology*. **171:** 2116-2123.

Smith KA and Salyers AA. (1991) Characterization of a Neopullulanase and an Alpha-Glucosidase from *Bacteroides thetaiotaomicron* 95-1. *Journal of Bacteriology*. **173:** 2962-2968.

Smith KC. (2004) Recombinational DNA Repair: The Ignored Repair Systems. *Bioessays*. **26:** 1322-1326.

Smith MA and Edwards DI. (1995) Redox Potentials and Oxygen Concentration As Factors in the Susceptibility of *Helicobacter pylori* to Nitro heterocyclic Drugs. *Journal of Antimicrobial Chemotherapy*. **35:** 751-764.

Soldo B, Lazarevic V and Karamata D. (2002) *tagO* Is Involved in the Synthesis of all Anionic Cell-Wall Polymers in *Bacillus subtilis* 168. *Microbiology*. **148:** 2079-2087.

Southern JA, Parker JR and Woods DR. (1986) Expression and purification of glutamine synthetase from *Bacteroides fragilis*. *Journal of General Microbiology* **132:** 2827-2835.

Speer BS, Bedzyk L and Salyers AA. (1991) Evidence That a Novel Tetracycline Resistance Gene Found on Two *Bacteroides* Transposons Encodes an NADP-Requiring Oxidoreductase. *Journal of Bacteriology*. **173:** 176-183.

Speer BS and Salyers AA. (1990) A tetracycline Efflux Gene on *Bacteroides* Transposon Tn*4400* Does Not Contribute to Tetracycline Resistance. *Journal of Bacteriology*. **172:** 292-298.

Spies M, Bianco PR, Dillingham MS, Handa N, Baskin RJ and Kowalczykowski SC. (2003) A Molecular Throttle: the Recombination Hotspot Chi Controls DNA Translocation by the RecBCD Helicase. *Cell*. **114:** 647-654.

Suau A, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD and Doré J. (1999) Direct Analysis of Genes Encoding 16S rRNA from Complex Communities Reveals Many Novel Molecular Species Within the Human Gut. *Applied and Environmental Microbiology.* **65:** 4799-4807.

Subramanya HS, Bird LE, Brannigan JA and Wigley DB. (1996) Crystal Structure of a Dexx Box DNA Helicase. *Nature.* **384:** 379-383.

Tally FP and Malamy MH. (1982) Mechanism of Antimicrobial Resistance and Resistance Transfer in Anaerobic Bacteria. *Scandinavian Journal of Infectious Diseases.* **35:** 37-44.

Tang YP, Dallas MM and Malamy MH. (1999) Characterization of the *Batl* (*Bacteroides* Aerotolerance) Operon in *Bacteroides fragilis*: Isolation of a *B. fragilis* Mutant with Reduced Aerotolerance and impaired Growth in *in vivo* Model Systems. *Molecular Microbiology.* **32:** 139-149.

Tang YP and Malamy MH. (2000) Isolation of *Bacteroides fragilis* Mutants with In Vivo Growth Defects by Using Tn*4400'*, a Modified Tn*4400* Transposition System, and a New Screening Method. *Infection and Immunity.* **68:** 415-419.

Taylor AF and Smith GR. (1995a) Monomeric RecBCD Enzyme Binds and Unwinds DNA. *Journal of Biological Chemistry.* **270:** 24451-24458.

Taylor AF and Smith GR. (1995b) Strand Specificity of nicking of DNA at Chi Sites by RecBCD Enzyme. Modulation by ATP and Magnesium Levels. *Journal of Biological Chemistry.* **270:** 24459-24467.

Taylor AF and Smith GR. (2003) RecBCD Enzyme Is a DNA Helicase with Fast and Slow Motors of Opposite Polarity. *Nature.* **423:** 889-893.

Telander-Muskavitch KM and Linn S. (1982) A Unified Mechanism for the Nuclease and Unwinding Activities of the *recBC* Enzyme of *Escherichia coli*. *Journal of Biological Chemistry.* **257:** 2641-2648.

Theis K, Chen PJ, Skorvaga M, Van Houten B and Kisker C. (1999) Crystal Structure of UvrB, a DNA Helicase Adapted for Nucleotide Excision Repair. *EMBO Journal.* **18:** 6899-6907.

Theis K, Skorvaga M, Machius M, Nakagawa N, Van Houten B and Kisker C. (2000) The Nucleotide Excision Repair Protein UvrB, a Helicase-Like Enzyme with a Catch. *Mutation Research.* **460:** 277-300.

Thompson SA and Blaser MJ. (1995) Isolation of the *Helicobacter pylori recA* Gene and Involvement of the *recA* Region in Resistance to Low pH. *Infection and Immunity.* **63:** 2185-2193.

Trinh S, Haggoud A, Reysset G and Sebald M. (1995) plasmids pIP419 and pIP421 from *Bacteroides*: 5-Nitroimidazole Resistance Genes and Their Upstream Insertion Sequence Elements. *Microbiology.* **141:** 927-935.

Trinh S and Reysset G. (1996) Detection by PCR of the *nim* genes Encoding 5-Nitroimidazole Resistance in *Bacteroides* Spp. *Journal of Clinical Microbiology.* **34:** 2078-2084.

Turner KW and Robertson AM. (1979) Xylose, Arabinose, and Rhamnose Fermentation by *Bacteroides ruminicola*. *Applied and Environmental Microbiology.* **38:** 7-12.

Tzianabos AO, Finberg RW, Wang Y, Chan M, Onderdonk AB, Jennings HJ and Kasper DL. (2000) T Cells Activated by Zwitterionic Molecules Prevent Abscesses Induced by Pathogenic Bacteria. *Journal of Biological Chemistry.* **275:** 6733-6740.

Tzianabos AO, Onderdonk AB, Zaleznik DF, Smith RS and Kasper DL. (1994) Structural Characteristics of Polysaccharides that Induce Protection Against Intra-Abdominal Abscess Formation. *Infection and Immunity.* **62:** 4881-4886.

Tzianabos AO, Pantosti A, Baumann H, Brisson JR, Jennings HJ and Kasper DL. (1992) The Capsular Polysaccharide of *Bacteroides fragilis* Comprises Two Ionically Linked Polysaccharides. *Journal of Biological Chemistry.* **267:** 18230-18235.

Umezu K, Nakayama K and Nakayama H. (1990) *Escherichia coli* RecQ Protein Is a DNA Helicase. *Proceedings of the National Academy of Sciences of the United States of America.* **87:** 5363-5367.

Van den Eynde H, De Baere R, Shah HN, Gharbia SE, fox GE, Michalik J, Van de Peer Y and De Wachter R. (1989) 5S ribosomal Ribonucleic Acid Sequences in *Bacteroides* and *Fusobacterium*: Evolutionary Relationships Within these Genera and Among Eubacteria in General. *International Journal of Systematic Bacteriology.* **39:** 78-84.

Van Houten B, Gamper H, Holbrook SR, Hearst JE and Sancar A. (1986) Action Mechanism of ABC Excision Nuclease on a DNA Substrate Containing a Psoralen Crosslink at a Defined Position. *Proceedings of the National Academy of Sciences of the United States of America.* **83:** 8077-8081.

Van Tassel RL and Wilkins TD. (1978) Isolation of Auxotrophs of *B. fragilis*. *Canadian Journal of Microbiology.* **24:** 1619-1621.

van Zwet AA, Thijs JC and de Graaf B. (1995) Explanations for High Rates of Eradication with Triple Therapy Using Metronidazole in Patients Harboring Metronidazole-Resistant *Helicobacter pylori* Strains. *Antimicrobial Agents and Chemotherapy.* **39:** 250-252.

Varel VH and Bryant MP. (1974) Nutritional Features of *Bacteroides fragilis* subsp. *Fragilis*. *Applied Microbiology.* **28:** 251-257.

Veldhuyzen van Zanten SJO, Sherman PM and Hunt RH. (1997) *Helicobacter pylori*: New Developments and Treatments. *Canadian Medical Association Journal.* **156:** 1565-1574.

Verdijk RM, Wilke M, Beslier V, Kloosterman A, Brand A, Goulmy E and Mutis T. (2004) *Escherichia coli*-Nitroreductase Suicide Gene Control of Human Telomerase Reverse Transcriptase-Transduced Minor Histocompatibility Antigen-Specific Cytotoxic T Cells. *Bone Marrow Transplant.* **33:** 963-967.

Verma M, Moffat KG and Egan JB. (1989) UV Irradiation Inhibits Initiation of DNA Replication of *oriC* in *Escherichia coli*. *Mol. Gen. Genet.* **216:** 446-454.

Vines RR, Perdue SS, Moncrief JS, Sentz DR, Barroso LA, Wright RL and Wilkins TD. (2000) Fragilysin, the Enterotoxin from *Bacteroides fragilis*, Enhances the Serum Antibody Response to Antigen Co-administered by the Intranasal Route. *Vaccine.* **19:** 655-660.

Voogd CE, van der Stel JJ and Jacobs JJJAA. (1974) The Mutagenic Action of Nitroimidazoles. I Metronidazole, Nimorazole Dimetridazole and Ronidazole. *Mutation Research.* **26:** 483-490.

Wagner R and Meselson M. (1976) Repair Tracts in Mismatched DNA Heteroduplexes. *Proceedings of the National Academy of Sciences of the United States of America.* **73:** 4135-4139.

Walker JE, Saraste M, Runswick MJ and Gay NJ. (1982) Distantly Related Sequences in the α- and β-Subunits of ATP Synthase, Myosin, Kinases and Other ATP-Requiring Enzymes and a Common Nucleotide Binding Fold. *EMBO Journal.* **1:** 945-951.

Wang Y, Kalka-Moll WM, Roehrl MH and Kasper DL. (2000) Structural Basis of the Abscess-Modulating Polysaccharide A2 from *Bacteroides fragilis*. *Proceedings of the National Academy of Sciences of the United States of America.* **97:** 13478-13483.

Weaver J, Whitehead TR, Cotta MA, Valentine PC and Salyers AA. (1992) Genetic Analysis of a locus on the *Bacteroides ovatus* Chromosome which Contains Xylan Utilization Genes. *Applied and Environmental Microbiology.* **58:** 2764-2770.

Wehnert GU, Abratt VR and Woods DR. (1992) Molecular analysis of a gene from *Bacteroides fragilis* involved in metronidazole resistance in *Escherichia coli. Plasmid.* **27:** 242-5.

Weisburg WG, Oyaizu Y, Oyaizu H and Woese CR. (1985) Natural Relationship Between *Bacteroides* and *Flavobacteria. Journal of Bacteriology.* **164:** 230-236.

Welch RA, Burland V, plunkett GD, Redford P, Roesch P, Rasko DA, Buckles EL, Liou SR, Boutin A, Hackett J, Stroud D, mayhew GF, Rose DJ, Zhou S, Schwartz DC, Perna NT, Mobley HLT, Donnenberg MS and Blattner FR. (2002) Extensive Mosaic Structure Revealed by the Complete Genome Sequence of Uropathogenic *Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America.* **99:** 17020-17024.

Whiteway J, Koziarz P, Veall J, Sandhu N, Kumar P, Hoecher B and Lambert IB. (1998) Oxygen-Insensitive Nitroreductases: Analysis of the Roles of *nfsA* and *nfsB* in Development of Resistance to 5-Nitrofuran Derivatives in *Escherichia coli. Journal of Bacteriology.* 180: 5529-5539.

Willis AT, Fergusson IR, Jones PH, Phillips KD, Teasle PV, Berry RB, Fiddian RV, Graham DF, Harland DHC, Innes DB, Mee MW, Rothwell-Jackson RL, Sutch I, Kilbey C and Edwards D. (1976) Metronidazole in Prevention and Treatment of *Bacteroides* Infections After Appendectomy. *British Medical Journal* **1:** 318-321.

Willis AT, Fergusson IR, Jones PH, Phillips KD, Teasle PV, Fiddian RV, Graham DF, Harland DHC, Hughes DFR, Knight D, Mee WM, Pashby N, Rothwell-Jackson RL, Sachdeva AK, Sutch L, Kilbey C and Edwards D. (1977) Metronidazole in Prevention and Treatment of *Bacteroides* Infections in elective Colonic Surgery. *British Medical. Journal* **1:** 607-610.

Willis AT, Jones PH, Phillips KD and Gotobed G. (1978) Metronidazole Resistance Among *Bacteroides fragilis. Lancet. i:* 721.

Witkin E. (1969) Ultraviolet Induced Mutation and DNA Repair. *Annual Review of Microbiology.* **23:** 487-514.

Woese CR. (1987) Bacterial evolution. *Microbiological Reviews.* **51:** 221-271.

Wright M, ButtinG and Hurwitz J. (1971) The Isolation and Characterization from *Escherichia coli* of Adenosine Triphosphate-Dependent Deoxyribonuclease Directed by *recB, C* Genes. *Journal of Biological Chemistry.* **246:** 6543-6555.

Xu J, Bjursell MK, Himrod J, Deng S, Carmichael LK, Chiang HC, Hooper LV and Gordon JI. (2003) A Genomic View of the Human-*Bacteroides thetaiotaomicron* Symbiosis. *Science.* **299:** 2074-2076.

Xu J, Chiang HC, Bjursell MK and Gordon JI. (2004) Message from a Human Gut Symbiont: Sensitivity Is a Prerequisite for Sharing. *Trends in Microbiology.* **12:** 21-28.

Xu J and Gordon JI. (2003) Honor Thy Symbiont. *Proceedings of the National Academy of Sciences of the United States of America.* **100:** 10452-10459.

Yamamoto I, Abe A, Saito H and Ishimoto M. (1984) The Pathway of ammonia Assimilation in *Bacteroides fragilis. Journal of General and Applied Microbiology.* **30:** 499-508.

Yamamoto I, Saito H and Ishimoto M. (1987) Comparison of Properties of Glutamate Dehydrogenase in Members of the *Bacteroides fragilis* Group. *Journal of General and Applied Microbiology.* **33:** 429-436.

Yamamoto K, Inoue S and Kawanishi S. (1993) Site-Specific DNA Damage and 8-Hydroxydeoxyguanosine Formation by Hydroxylamine and 4-hydroxyaminoquinoline 1-Oxide in the Presence of Cu(II): Role of Active Oxygen Species. *Carcinogenesis.* **14:** 1397-1401.

Yanisch-Perron C, Viera J and Messing J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene.* **33:** 103-119.

Yeung TC, Beaulieu BB Jr, McLafferty MA and Goldman P. (1984) Interaction of Metronidazole with DNA Repair Mutants of *Escherichia coli*. *Antimicrobial Agents Chemotherapy.* **25:** 65-70.

Zabeau M and Stanley KK. (1982) Enhanced expression of cro-beta-galactosidase fusion proteins under the control of the PR promoter of bacteriophage lambda. *EMBO Journal.* **1:** 1217-24.

Zahoor A, Lafleur MVM, Knight RC, Loman H and Edwards DI. (1987) DNA Damage Induced by Reduced Nitroimidazole Drugs. *Biochemical Pharmacology.* **36:** 3299-3304.

Zenno S, Koike H, Kumar An, Jayaraman R, Tanokura M and Saigo K. (1996) Biochemical Characterization of NfsA, the *Escherichia coli* Major Nitroreductase Exhibiting a High Amino Acid Sequence Homology to Frp, a *Vibrio harveyi* Flavin Oxidoreductase. *Journal of Bacteriology.* **178:** 4508-4514.