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# **DNA REPAIR IN *BACTEROIDES* *FRAGILIS***

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## ABSTRACT

*Bacteroides fragilis* is a gut commensal in both humans and animals where it benefits the host through metabolizing indigestible compounds, stimulating the immune system and protecting against pathogen colonization. However, it is also an opportunistic pathogen, responsible for approximately half of anaerobic bacteraemias. Metronidazole is used to treat anaerobic infections. It diffuses into the cell as an inactive prodrug where it is reduced to form nitro anion and nitroso and hydroxylamine radicals. These chemically reactive compounds interact with DNA causing strand breaks and base mutations; the damage accumulates and leads to cell death. Mechanisms of metronidazole resistance in *B. fragilis* include decreased activity of oxidation/reduction enzymes, over-expression of multidrug efflux pumps and the conversion of metronidazole to non-toxic derivatives by nitroimidazole nitroreductases (encoded by *nim* genes). However, metronidazole resistance could also potentially be mediated by the over-expression or enhanced activity of DNA repair proteins. Thus, DNA repair in *B. fragilis* should be thoroughly investigated.

In many bacteria, RecA controls the expression of many DNA repair genes through the SOS response, and carries out homologous recombination repair, therefore, it is thought to be one of the principal DNA repair proteins in living cells and shows high conservation across species. A putative RecA protein has been identified in the genome of *B. fragilis*. Bioinformatic analysis of this protein revealed that it contained the predicted necessary motifs and amino acids for homologous recombination repair, and investigation of its genomic context suggested that *recA* formed part of an operon with its upstream genes. These genes coded for proteins which may be involved in assisting cells to survive oxidative stress.

A bioinformatic search for other DNA repair proteins in *B. fragilis* led to the identification of a range of proteins possibly involved in the repair of stalled replication forks and DNA strand breaks, processes which are dependent on RecA. Putative RecFOR proteins were discovered, along with three RecQ helicases and a RecJ endonuclease, all of which are involved in resolving stalled replication forks. For the repair of DNA strand breaks, the homologues for the *E. coli*

RecBCD proteins could not be identified, however, the *Lactococcus lactis* RexAB homologues were found to be present in the genome.

To investigate whether *B. fragilis* 638R *recA* was transcribed as an operon, RT-PCR was carried out, and the results revealed that *recA* was co-transcribed with its two upstream genes. Since the protein products of these genes could be important for oxidative stress responses, the inclusion of *recA* in this operon suggests a novel mechanism for coupling antioxidant and DNA repair processes.

To further investigate DNA repair in *B. fragilis* 638R, targeted insertional inactivation with the suicide vector pGERM was used to generate a *B. fragilis recA*<sup>-</sup> mutant. The mutation was confirmed with PCR, sequencing and Southern hybridisation. In spite of concerns that the plasmid could excise and recircularise, the insertion was found to be stable. This could be due to the fact that mutating *recA* abolishes homologous recombination in the cell as has been seen for the *Bacteroides thetaiotaomicron recA*<sup>-</sup> mutant.

A sub-population of the *B. fragilis recA*<sup>-</sup> mutant cells became abnormally elongated under normal growth conditions. This phenotype has been reported for *Bacillus subtilis* and *Streptococcus thermophilus recA*<sup>-</sup> mutants and was attributed to unresolved DNA damage preventing cell division. The *B. fragilis recA*<sup>-</sup> mutant was highly sensitive to ultraviolet radiation and metronidazole, pointing to *B. fragilis* RecA being involved in the repair of ultraviolet light and metronidazole-induced DNA damage. Since *B. fragilis* RecA repairs DNA strand breaks caused by metronidazole, it was hypothesised that over-expressing the gene on a multi-copy plasmid in *B. fragilis* wild type cells could lead to antibiotic resistance. The *B. fragilis recA* over-expressing strain was shown to have increased survival compared to the wild type. This is the first study that has shown that over-expression of a DNA repair protein in *B. fragilis* results in metronidazole resistance.

## ABBREVIATIONS

A	Adenine	l	Litre(s)
$\alpha$	Alpha	LB	Luria Bertani
ABC	ATP-binding cassette	Mg	Magnesium
AhpC	Alkyl hydroperoxide peroxidase	mg	Milligram(s)
Amp	Ampicillin	MIC	Minimum inhibitory concentration
ATP	Adenosine tri-phosphate	min	Minute(s)
$\beta$	Beta	ml	Millilitre(s)
BCP	Bacterioferritin co-migratory protein	Mob	Mobilisation
BfPAI	<i>B. fragilis</i> pathogenicity island	Mtz	Metronidazole
BHI	Brain heart infusion	MtzR	Mtz Resistant
BHISA	Supplemented BHI agar	MtzS	Mtz Sensitive
BHISB	Supplemented BHI broth	NAD(P)H	Nicotinamide adenine dinucleotide phosphate
bp	Base pair(s)	NER	Nucleotide Excision Repair
C	Cytosine	ng	Nano gram(s)
CDD	Conserved domains database	No.	Number
cDNA	Complementary DNA	OB	Oligomer binding
Cys	Cysteine	OD	Optical density
DIG	Digoxygenin	ORF	Open reading frame
DNA	Deoxyribonucleic acid	OSR	Oxidative stress response
Dnase	Deoxyribonuclease	PCR	Polymerase chain reaction
dsDNA	Double-stranded DNA	POR	Pyruvate oxidoreductase
Erm	Erythromycin	PSA	Polysaccharide
ETBF	Enterotoxigenic <i>B. fragilis</i>	RNA	Ribonucleic acid
FCB	<i>Flavobacteria-Chlorobium-Bacteroides</i>	rRNA	Ribosomal ribonucleic acid
fin	Fragilis invertible region	ROS	Reactive oxygen species
G	Gaunosine	sec	Second(s)
g	Gram(s)	ssDNA	Single-stranded DNA
gDNA	Gapped DNA	SF	Super family
Gent	Gentamicin	Strep	Streptomycin
h	Hour	T	Thymine
HhH	Helix-hairpin-helix	Tet	Tetracycline
IS	Insertion sequence	TSA	Thiol specific antioxidant
J	Joule(s)	$\mu$ g	Micro gram(s)
kDa	Kilo dalton(s)	UV	Ultraviolet radiation
L	Loop		

# CHAPTER 1

## LITERATURE REVIEW

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## 1.1. BACTEROIDES

### 1.1.1. INTRODUCTION TO THE GENUS BACTEROIDES

*Bacteroides* are gram negative gut symbionts that are present in humans and other mammals (Bakir *et al.*, 2006; Holdeman *et al.*, 1984; Miyamoto and Itoh, 2000; Wang *et al.*, 2003; Whitehead *et al.*, 2005). Morphologically, these obligate anaerobes are non-sporeforming, nonmotile rods with central or terminal swellings (Holdeman *et al.*, 1984). Their DNA mol % G+C ranges from 28-61, and carbon dioxide, haemin and vitamin K are required or preferred by many species during growth. *Bacteroides* produce the fermentation products succinate, acetate, lactate, formate and propionate, and biochemical tests are routinely used to classify *Bacteroides* species. However, 16S rRNA gene PCR and sequencing can also be used for identification as comparisons of these discriminate more efficiently between related bacteria (Song *et al.*, 2005).

The genus *Bacteroides* forms part of the *Flavobacteria-Chlorobium-Bacteroides* (FCB) group (Wang *et al.*, 2003). The FCB group forms three main bacterial phyla, which can be distinguished using 16S rRNA sequence comparisons (Gupta, 2004). No biochemical or other molecular characteristics can distinguish this group from other bacterial phyla. Because of their close evolutionary relationship, Gupta (2004) proposed that the FCB group should be placed in one phylum rather than three separate phyla.

*Bacteroides* species are amongst the earliest commensals to colonize the gut (Mazmanian *et al.*, 2005). Once they are established, they outnumber facultative anaerobic commensals by  $10^2$  to  $10^3$  orders of magnitude (Patrick *et al.*, 2003). Amongst these beneficial gut symbionts, *B. fragilis* stands out as a clinically important opportunistic pathogen (Sears, 2001). It is the most prevalent anaerobe isolated from clinical infections including abdominal abscesses and bloodstream infections even though it only accounts for 0.1-0.5% of colonic symbionts (Sears, 2001). *B. fragilis* causes approximately half of anaerobic bacteraemias, 19% of which are potentially fatal (Goldstein, 1996).

### 1.1.2. *BACTEROIDES FRAGILIS*: COMMENSAL AND OPPORTUNISTIC PATHOGEN

Although *B. fragilis* is an opportunistic pathogen, it plays an important role as a commensal in the gut. It benefits the host by metabolising indigestible compounds and providing essential nutrients (Mazmanian *et al.*, 2005). It also protects against pathogen colonization, and has been shown to inhibit the growth of the pathogen *Helicobacter pylori* *in vitro* (Krausse *et al.*, 2005; Mazmanian *et al.*, 2005). *B. fragilis* directs maturation of the host's immune system by secreting a specific polysaccharide (PSA), which was shown to correct imbalances between CD4<sup>+</sup> T helper 1 (T<sub>H1</sub>) and T helper 2 (T<sub>H2</sub>) cells in germ free mice (Mazmanian *et al.*, 2005). The CD4<sup>+</sup> T cells are important components of the mammalian immune system. T<sub>H1</sub> and T<sub>H2</sub> cells carry out opposing functions and a healthy immune system requires a balance between these two subtypes. PSA also stimulates development of spleen lymphocyte zones in germ free mice.

As an opportunistic pathogen, virulence factors assist *B. fragilis* in establishing itself at the site of infection. Cell surface polysaccharides are significant virulence factors implicated in abscess formation (Kuwahara *et al.*, 2004; Patrick *et al.*, 2003). Multiple inverted DNA repeat regions, known as fragilis invertible (fin) regions, activate and repress expression of different polysaccharide genes by inverting the promoters. This enables antigenic variation allowing *B. fragilis* to evade the immune system during an infection (Kuwahara *et al.*, 2004). Expression of transporters, signal transduction systems and carbon metabolism enzymes are also regulated through inversions resulting in an immediate response to environmental change. In addition to cell surface polysaccharides, *B. fragilis* sialidases are involved in abscess formation since these enzymes cleave  $\alpha$ -ketosidic linkages between glycosyl residues aiding invasion and tissue destruction (Briselden *et al.*, 1992). Various  $\alpha$ -enolases have also been shown to act as virulence factors, transforming bacteria into proteolytic and invasive pathogens, by converting plasminogen to plasmin (Sijbrandi *et al.*, 2005). The main functions of  $\alpha$ -enolases are to convert 2-phosphoglycerate to phosphoenolpyruvate making it an essential metabolic factor. This protein is also a component of RNA degradosome complexes, allowing it to influence transcription through processing mRNAs. The P46  $\alpha$ -enolase of *B. fragilis* was examined for plasminogen-binding activity, however, it did not function in this capacity. Since *B.*

*fragilis* can bind plasminogen, another protein must be responsible for this and a putative plasminogen-binding protein P60 was identified. Enterotoxigenic *B. fragilis* (ETBF) strains secrete fragilysin, a metalloprotease toxin similar to anthrax, tetanus and botulinum toxins (Wu *et al.*, 2006). Fragilysin is associated with diarrhoeal diseases in humans and animals and is thought to act by binding to receptors on polarized intestinal epithelial cells (Wu *et al.*, 2006), which results in morphological and physiological changes in host cells *in vitro* (Wu *et al.*, 2002). The toxin gene *bft* is located on a 6 kb pathogenicity island (BfPAI) and subsets of ETBF strains also contain a 12 kb region flanking the left side of BfPAI (Franco *et al.*, 1999). This flanking region has a putative mobilisation segment similar to that found on the 5-nitroimidazole resistance plasmid pIP417 and the clindamycin resistance plasmid pBFTM10. The region to the right of BfPAI codes for BfmC, a protein which shares significant similarity to the *H. pylori* TrsK and the *E. coli* TraD mobilisation proteins. Thus, BfmC could regulate the mobilisation of BfPAI into non-enterotoxigenic *B. fragilis* strains.

*B. fragilis* is highly aerotolerant compared to other anaerobes, which could be due its complex oxidative stress response (OSR) (Herren *et al.*, 2003; Rocha *et al.*, 1996). Aerotolerance may be important for virulence since the host immune system produces reactive oxygen species to defend against pathogens (Herren *et al.*, 2003). 2D gel electrophoresis revealed that an estimated 28 proteins are synthesised during the OSR (Rocha *et al.*, 1996). This protein profile was similar to that found for exposure to hydrogen peroxide. OxyR is a redox regulator which controls the expression a number of OSR genes, including the catalase gene, *katB*; the thioredoxin peroxidase gene, *tpx*; the heme containing peroxidase gene, *ccp*; and the RNA binding protein gene, *rbpA* (Herren *et al.*, 2003). Catalase prevents the toxic accumulation of hydrogen peroxide, while thioredoxin peroxidase reduces peroxide. Ccp could protect proteins and membrane lipids from peroxide damage and RbpA may regulate *tpx* through affecting mRNA stability. Tpx is a member of the thiol specific antioxidant (TSA) family; other members of this family in *B. fragilis* include alkyl hydroperoxide peroxidase C (AhpC) and bacterioferritin co-migratory protein (BCP). Another important OSR protein is superoxide dismutase which converts toxic O<sub>2</sub><sup>-</sup> to hydrogen peroxide and oxygen (Rocha *et al.*, 1996). Thus, the OSR allows *B. fragilis* to mount a rapid response to oxygen exposure.

Treatment of anaerobic infections is being hampered by the rise in antibiotic resistance (Salyers and Shoemaker, 1996). Wareham *et al.* (2005) reported a multidrug resistant *B. fragilis* strain which caused anaerobic sepsis and death. Pumbwe *et al.* (2007) have identified a sixteen tripartite RND-family of efflux pumps (BmeABC1-16) in *B. fragilis*. They demonstrated that deletion of *bmeR5* (the protein transcribed from this gene is a putative repressor of the BmeABC5 pump) in *B. fragilis* ADB77, resulted in a decreased susceptibility to ampicillin, cefoxitin, cefoperazone, ciprofloxacin, imipenem, cefoperazone, metronidazole, ethidium bromide and sodium dodecyl sulphate. Thus, overexpression of efflux pumps in *B. fragilis* could lead to multiple antibiotic resistance. Genes transferred on conjugative transposons are also responsible for many of the antibiotic resistance genes found in *Bacteroides* species (Salyers and Shoemaker, 1996), and these genes are stably maintained even in the absence of antibiotic selection (Shoemaker *et al.*, 2001). Added to this, *B. fragilis* has been reported to transfer antibiotic resistance genes to other *Bacteroides* species and *E. coli* (Rotimi *et al.*, 1981). Since *B. fragilis* carries many antibiotic resistance genes, it could aid in generating super antibiotic resistant pathogens. Eighty percent of *Bacteroides* species carry the tetracycline resistance gene *tetQ* and an increase in erythromycin resistance has been seen, with 70% of these cases being due to the presence of the *ermF* and *ermG* genes (Shoemaker *et al.*, 2001). *B. fragilis* also inactivates  $\beta$ -lactams by producing  $\beta$ -lactamases and penicillin binding proteins (Píriz *et al.*, 2004). Metronidazole (Mtz) is the preferred drug for treating anaerobic infections (Haggoud *et al.*, 1994), but *Bacteroides* strains have begun to develop a number of resistance mechanisms to combat this drug (Diniz *et al.*, 2004).

## 1.2. METRONIDAZOLE RESISTANCE IN BACTEROIDES AND OTHER BACTERIA

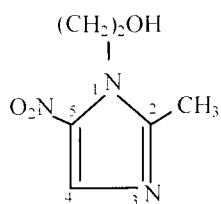
### 1.2.1. INTRODUCTION

Mtz is used to treat anaerobic infections at several body sites as well as gut conditions such as ulcerative colitis, inflammatory bowel disease and Crohn's disease. Characteristic of these conditions is an overgrowth of anaerobic bacteria like *B. fragilis* (Rafii *et al.*, 2003). However, the emergence of Mtz resistance (MtzR) mechanisms has increasingly compromised the effectiveness of treatment (Chang *et al.*, 1997). Researchers have begun investigating MtzR to understand the strategies

underlying resistance and thus develop new antibiotics that can circumvent these mechanisms (Land and Johnson, 1997).

### 1.2.2. STRUCTURE AND ACTIVATION OF METRONIDAZOLE

Mtz (Figure 1.1) has a nitroheterocyclic imidazole ring with nitrogen elements at position one and three (Land and Johnson, 1997; Trinh and Reysset, 1998). The first nitrogen has a hydroxyethyl side chain.



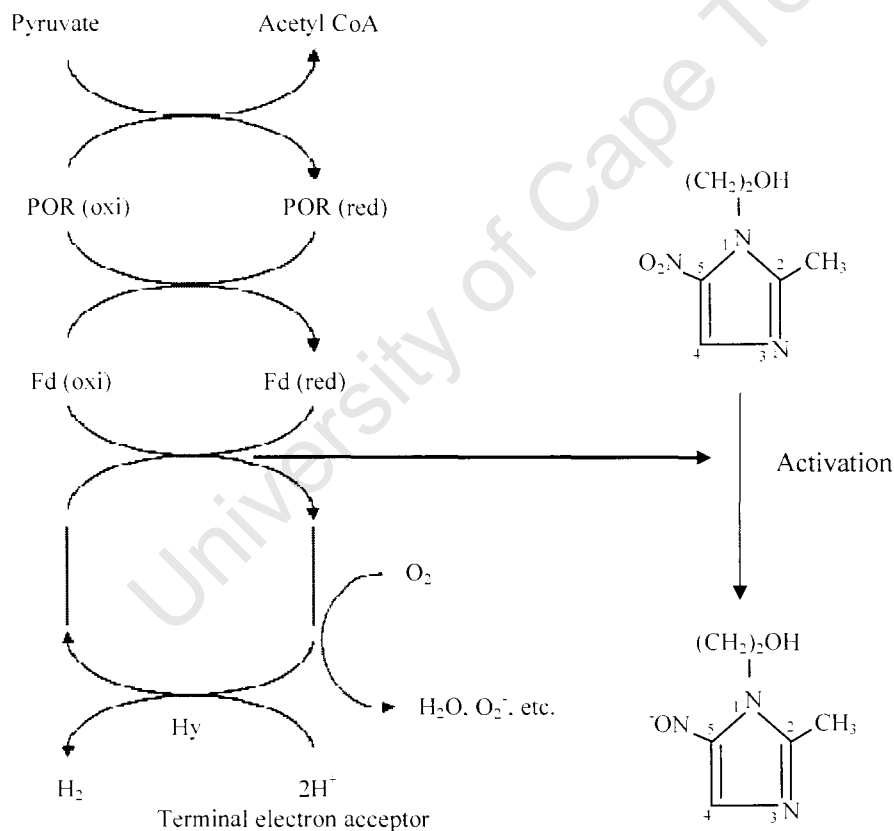
**Figure 1.1: Chemical structure of Metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole]**

(Land and Johnson, 1997; Trinh and Reysset, 1998)

The nitro group on the carbon group at position five, is reduced in anaerobic bacteria and protists (Jenks and Edwards, 2002; Trinh and Reysset, 1998) leading to production of the nitro anion and nitroso and hydroxylamine radicals (Jenks and Edwards, 2002). These intermediate substances are chemically reactive, short-lived compounds that interact with cellular components and macromolecules, resulting in cell death (Beaulieu *et al.*, 1981; Sisson *et al.*, 2000). These compounds can be further metabolized to non-toxic N-(2-hydroxyethyl) oxamic acid and acetamide (Chang *et al.*, 1997). As Mtz is reduced to active intermediates, a concentration gradient is established that favours the passive diffusion of further inactive Mtz pro-drug into the cell (Lindmark and Müller, 1976).

The electrons needed for the reductive activation of Mtz are generated during pyruvate catabolism (Jenks and Edwards, 2002; Land and Johnson, 1997). In anaerobic bacteria the decarboxylation of pyruvate is catalysed by the enzyme complex pyruvate oxidoreductase (POR) (Jenks and Edwards, 2002). An in-depth study of POR in *H. pylori* showed that it was a heterotrimeric complex (Hughes *et al.*, 1998). The four sub-units are PorA (48kDa), PorB (36kDa), PorC (24kDa) and PorD (14kDa). POR is an essential enzyme that is unstable in the presence of oxygen.

Aerobes use a pyruvate dehydrogenase complex to carry out pyruvate metabolism. In the process of metabolising pyruvate, POR is reduced, allowing it to oxidize ferredoxin or flavodoxin (Figure 1.2). These proteins then reduce a  $H^+$  which acts as a terminal electron acceptor. Since Mtz has a redox potential of  $-415\text{ mV}$ , it replaces the  $H^+$ , allowing the drug to be reduced and activated (Castelli *et al.*, 1997; Jenks and Edwards, 2002). The redox potentials of aerobic bacterial systems exceed  $-350\text{ mV}$ , making them insufficiently negative to reduce Mtz (Castelli *et al.*, 1997; Jenks and Edwards, 2002; Lindmark and Müller, 1976). This is the basis of the selective toxicity of Mtz against anaerobes since the redox potentials of anaerobic bacterial mechanisms range between  $-430$  and  $-460\text{ mV}$  (Castelli *et al.*, 1997).



**Figure 1.2: The pyruvate metabolism pathway allows for the activation of Mtz**

The proteins involved in the catabolism of pyruvate are on the left while arrows depict the path of electrons. Mtz activation is shown on the right. Fd, ferredoxin; Hy, hydrogenase; POR, pyruvate oxidoreductase; Mtz, Metronidazole; oxi, oxidized; red, reduced. (Jenks and Edwards, 2002; Land and Johnson, 1997)

Nitroreductases are another group of enzymes involved in reducing and activating Mtz (Jenks and Edwards, 2002; Sisson *et al.*, 2000). They use sequential two electron reduction reactions to reduce nitroheterocyclic compounds and produce nitroso derivatives and hydroxylamine (Jenks and Edwards, 2002; Land and Johnson, 1997). Oxygen-insensitive nicotinamide adenine dinucleotide phosphate (NAD(P)H) nitroreductase (RdxA), ferredoxin-like protein B (FdxB) and NAD(P)H flavin oxidoreductase (FrxA) are all examples of nitroreductases found in *H. pylori* and other anaerobes (Kwon *et al.*, 2000).

If oxygen is present in the cell, metronidazole undergoes a process known as 'futile cycling' (Jenks and Edwards, 2002). This process increases the cell's resistance to Mtz but can also result in cytotoxicity. Reduced Mtz forms active nitro anion radicals, which can be oxidized by oxygen causing them to revert back to the inactivated form of Mtz. Repeated cycles result in Mtz detoxification and superoxide radical anion formation. Superoxide dismutase converts superoxide radical anions to hydrogen peroxide, which can be changed by the catalase enzyme to water. However, if the superoxide ions and hydrogen peroxide interact in the presence of iron or copper, hydroxyl free radicals are generated which damage DNA causing cell death (Jenks and Edwards, 2002).

### 1.2.3. MODE OF ACTION OF METRONIDAZOLE IN BACTERIAL CELLS

*E. coli* and *B. fragilis* have been shown to undergo morphological changes leading to cell elongation when treated with Mtz (Jackson *et al.*, 1984; Skarin and Mardh, 1981). This is indicative of a stress response due to activated Mtz binding to DNA causing single-stranded (ss) and double-stranded (ds) DNA breaks (Sisson *et al.*, 2000; Trinh and Reysset, 1998). DNA fragmentation occurs in a dose-dependent manner which progresses over time and *in vitro* studies showed that strand breaks occur specifically at A/T residues (Trinh and Reysset, 1998). Mtz-induced DNA fragmentation has been illustrated in *B. fragilis* and *H. pylori* (Diniz *et al.*, 2004; Sisson *et al.*, 2000) and these DNA breaks inhibit DNA synthesis (Schapiro *et al.*, 2004). It has been hypothesized that DNA degradation could be taking place as a result of the ss and dsDNA breaks (Sisson *et al.*, 2000).

Mtz also causes transversion and transition base mutations (Sisson *et al.*, 2000). Since Mtz, itself, is mutagenic, its prolonged use could induce strains to develop antibiotic resistance or higher levels of virulence (Diniz *et al.*, 2003; Jenks and Edwards, 2002). Sisson studied Mtz mutagenesis in *H. pylori* rifampicin sensitive (RifS) and Mtz sensitive strains (Sisson *et al.*, 2000). The *H. pylori* Mtz sensitive strain had a minimum inhibitory concentration (MIC) of 1.5 µg/ml of Mtz. These cells were treated with Mtz and the frequency with which they became rifampicin resistant (RifR) was measured. Mutations to RifR occurred with a 6-fold frequency using 2 µg/ml of Mtz and a 12-fold frequency when 3 µg/ml of Mtz was used (Sisson *et al.*, 2000). MtzR strains with a Mtz MIC of 16 µg/ml were also tested. There was a 27-fold frequency of mutation at 25 µg/ml Mtz and a 166-fold frequency of mutation at 32 µg/ml of Mtz. These figures illustrated the powerful mutagenic action of Mtz. It is most likely that RifR cells contained mutations in *rpoB* which codes for the RNA polymerase β subunit (O' Neill *et al.*, 2006). Rifampicin targets this enzyme and causes cell death by inhibiting transcription.

Diniz *et al.* (2003) showed that *B. fragilis* cells pre-treated with 4 µg/ml of Mtz had increased virulence compared to controls when exposed to polymorphonuclear leukocytes. High levels of Mtz also caused increases in virulence as indicated by weight loss, increased inflammation and immunosuppression in infected mice (Diniz *et al.*, 2003). It is, therefore, evident that prolonged therapy with low or high Mtz concentrations could change the pathogenicity of the microbes being treated, and that acquired resistance to Mtz is often accompanied by an increase in virulence. Since Mtz is mutagenic, it is hypothesised, that the antibiotic itself is inducing these changes by random mutations (Diniz *et al.*, 2003; Trinh and Reysset, 1998).

#### 1.2.4. MECHANISMS OF METRONIDAZOLE RESISTANCE

A wide range of MtzR mechanisms has been described in bacteria. Decreased activity or total inactivation of electron transport chain components has been observed in *H. pylori* (Jenks and Edwards, 2002), and *B. fragilis* (Diniz *et al.*, 2004). Mutations in *rdxA*, as well as *frxA* and *fdxB* mutations, are often identified in MtzR *H. pylori* cells (Jenks and Edwards, 2002; Jeong *et al.*, 2000; Kwon *et al.*, 2000). Overexpression of multidrug efflux pumps in *B. fragilis* has also been implicated in MtzR (Pumbwe *et al.*, 2007).

### 1.2.5. METRONIDAZOLE RESISTANCE IN BACTEROIDES

Targeted mutagenesis in *B. fragilis* showed that the following genes were involved in certain MtzR cases: *porA* (encoding POR), *fld* (encoding flavodoxin) and *rdx* (encoding oxidoreductase) (Diniz *et al.*, 2004). The *porA/rdx* double mutation increased the Mtz MIC of the WT strain from 0.5 mg/l to 3.5 mg/l, and the *porA/fld* double mutation increased the Mtz MIC to 6 mg/l. The resistance mechanisms identified in this study involved decreasing the activity of enzymes involved in electron transfer reactions and oxidation or reduction. It was, therefore, hypothesized that a decrease in available electrons would result in decreased reduction and activation of Mtz (Diniz *et al.*, 2004).

In a separate study, clinically resistant *B. fragilis* strains were shown to exhibit decreased uptake and reduction of Mtz and decreased POR activity (Britz and Wilkinson, 1979). These strains exhibited altered metabolism, with increased lactate dehydrogenase activity. Thus, the bacterium compensated for decreased energy production by increasing its lactate dehydrogenase enzymatic activity (Narikwa *et al.*, 1991). Lactate dehydrogenase catalyses the fermentation of pyruvate to lactate (Diniz *et al.*, 2004).

The most widely described mechanism of resistance in *Bacteroides* is due to 5-nitroimidazole nitroreductases (encoded by *nim* genes) that confer Mtz resistance (MtzR) by converting Mtz to non-toxic amino derivatives (Diniz *et al.*, 2004, Fang *et al.*, 2002; Jeong *et al.*, 2000). The *nim* genes include *nimA*, *B*, *C*, *D*, *E* (Diniz *et al.*, 2004) and *F* (Löfmark *et al.*, 2005). They are found on plasmids but *nim* genes have also been found in the chromosomal DNA of 73% of MtzR *B. fragilis* bacteria (Diniz *et al.*, 2004). *NimB* has only been found on the chromosome but *nimA* was located on plasmid pIP417 (Haggoud *et al.*, 1994), as well as being found on the chromosome (Haggoud *et al.*, 2001). A specific determinant, an insertion sequence (IS) 1168, was found 14bp upstream of *nimA* and 12bp upstream of *nimB* (Haggoud *et al.*, 1994). Since IS 1168 is less than 20bp upstream of the genes, it points to IS-activated gene expression (Haggoud *et al.*, 1994). IS activation is also essential for *nim C* and *D* gene expression (Paula *et al.*, 2004), and *nim* genes are transferable by processes similar to conjugation (Haggoud *et al.*, 1994). A range of antibiotic resistance genes

often integrate into mobile elements (Paula *et al.*, 2004). so elements with *nim* genes can take up other antibiotic resistance genes as well. These elements will then confer super resistance on the bacteria that contain them and on the bacteria to which they are transferred to (Paula *et al.*, 2004).

De-repression of the tripartite RND-family efflux pump BmeABC5 lead to MtzR in *B. fragilis* ADB77 (Pumbwe *et al.*, 2007). BmeABC is composed of the membrane fusion protein (A), the pump (B) and the outer membrane channel (C). Deletion of the putative repressor gene *bmeR5* in ADB77, resulted in overexpression of the pump which lead to enhanced resistance to Mtz and other antibiotics. Thus, changes in efflux pump regulation in clinical strains could lead to MtzR (Pumbwe *et al.*, 2007).

#### 1.2.5.2. DNA REPAIR AS A MECHANISM OF METRONIDAZOLE RESISTANCE

Mtz exerts its bactericidal effect through generating ss and dsDNA breaks (Sisson *et al.*, 2000; Trinh and Reysset, 1998). Thus, the role of the DNA repair proteins UvrA, UvrB, RecA, RecB and RecC have been highlighted in the response to treatment with Mtz in bacteria such as *H. pylori* (Chang *et al.*, 1997), *B. thetaiotaomicron* (Cooper *et al.*, 1997) and *E. coli* (Dachs *et al.*, 1995; Yeung *et al.*, 1984). UvrA and UvrB are involved in excision repair which repairs strand breaks and other lesions (Van Houten *et al.*, 2002). The RecA protein is an important enzyme that mediates homologous recombination and, together with LexA, regulates the SOS response proteins in *E. coli* (Chang *et al.*, 1997; Friedberg *et al.*, 1995).

In *B. thetaiotaomicron*, a disruption in the *recA* gene generated a Mtz sensitive (MtzS) mutant (Cooper *et al.*, 1977). Since disrupting DNA repair proteins in *B. thetaiotamicron* resulted in MtzS cells, it can be hypothesised that enhanced DNA repair could lead to MtzR in anaerobic bacteria like *B. fragilis*. This has been reported for *H. pylori* where certain specific mutations in *recA* resulted in MtzR (Chang *et al.*, 1997). The mutated *H. pylori recA* locus had point mutations, which resulted in amino acid substitutions at two sites: tyrosine to histidine at position 103 and serine to aspartate at position 121. DNA repair in *B. fragilis* is only now being investigated as a possible MtzR mechanism, as a number of MtzR clinical isolates do

not contain *nim* genes or any of the previously described resistance mechanisms indicating the presence of alternative MtzR mechanisms.

### 1.3. DNA REPAIR

#### 1.3.1. INTRODUCTION

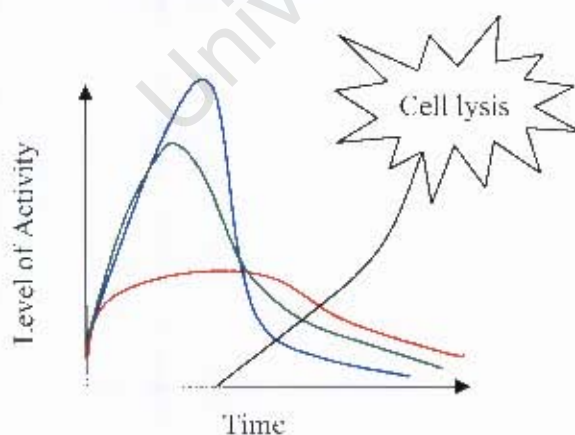
DNA, like other macromolecules in the cell, is constantly damaged during normal cell growth. However, for it to retain the information essential for transcription and replication, there must be cellular machinery present which will restore its integrity (Kuzminov, 1999; Truglio *et al.*, 2006). A wide array of DNA repair proteins have been identified in *E. coli*, for example, defence against the mutagenic base lesion 7,8-dihydro-8-oxo-guanine, is carried out by the mismatch repair proteins MutT, M, Y and S and endonuclease VIII (Nei) (Bai and Lu, 2007). DNA Photolyase repairs ultraviolet radiation (UV)-induced pyrimidine dimers by flipping them out of the DNA helix (Vande Berg and Sancar, 1998). Nucleotide excision repair also removes pyrimidine dimers and other bulky DNA lesions (Skorvaga *et al.*, 2002). RecA carries out homologous recombination and is the key protein in the RecFOR and RecBCD DNA repair pathways (Kuzminov, 1999). It is also an essential component of the SOS response to DNA damage. Excision repair and homologous recombination repair have been implicated in repairing Mtz-induced DNA damage and, together with the SOS response, will be discussed further in this review (Chang *et al.*, 1997; Cooper *et al.*, 1997). The focus will be on the *E. coli* DNA repair systems since these are currently the most extensively studied and serve as the paradigm for other bacterial systems.

#### 1.3.2. THE SOS RESPONSE

The SOS response has been extensively studied in *E. coli* where it is initiated by DNA damage and controls the expression of an estimated twenty genes involved in DNA repair (Keller *et al.*, 2001; Sander *et al.*, 2001). RecA plays a key role in induction of the SOS response, while LexA is the transcriptional repressor of the SOS response genes (Friedberg *et al.*, 1995; Kuzminov, 1999; Newmark *et al.*, 2005). LexA binds to a specific promoter sequence on these genes (known as the SOS box) to exert its repressor function (Janion, 2001). To induce the SOS response in *E. coli*, RecA must become activated by complexing with ssDNA molecules in the presence of nucleoside triphosphates (Smith, 1988). DNA damage halts replication which results in ssDNA

molecules becoming available for this induction process (Friedberg *et al.*, 1995). The newly activated RecA (termed RecA\*) promotes autoproteolytic cleavage of the repressor LexA. Cleaved LexA cannot repress transcription allowing for activation of the SOS response. As repair takes place, the levels of ssDNA molecules in the cell decreases, thus RecA\* reverts back to RecA. The LexA gene is autoregulated so LexA expression is also activated during the SOS response. As levels of RecA\* decrease, newly expressed LexA remains uncleaved resulting in renewed repression of the SOS response.

There are four tiers of gene expression during the SOS response as not all the SOS-regulated genes are induced at the same time to the same extent (Figure 3) (Kuzminov, 1999). The first level involves the excision repair genes, which deal with one-strand repair (Sommer *et al.*, 1998). If these proteins cannot repair the damage, then the second level genes, *recA* and *recN*, are overexpressed for enhanced recombinational repair (Kuzminov, 1999; Sommer *et al.*, 1998). The third tier genes are *umuD*, *umuC* and *sulA* (*sfiA*). UmuDC catalyzes translesion DNA synthesis and *sulA* (*sfiA*) inhibits cell division to allow extra time for repair (Mukherjee, *et al.*, 1998). If the cell still cannot cope, colicins are produced and cell lysis takes place (Kuzminov, 1999). This releases nutrients which benefit the surrounding cells allowing them increased chances of survival in harsh environments.



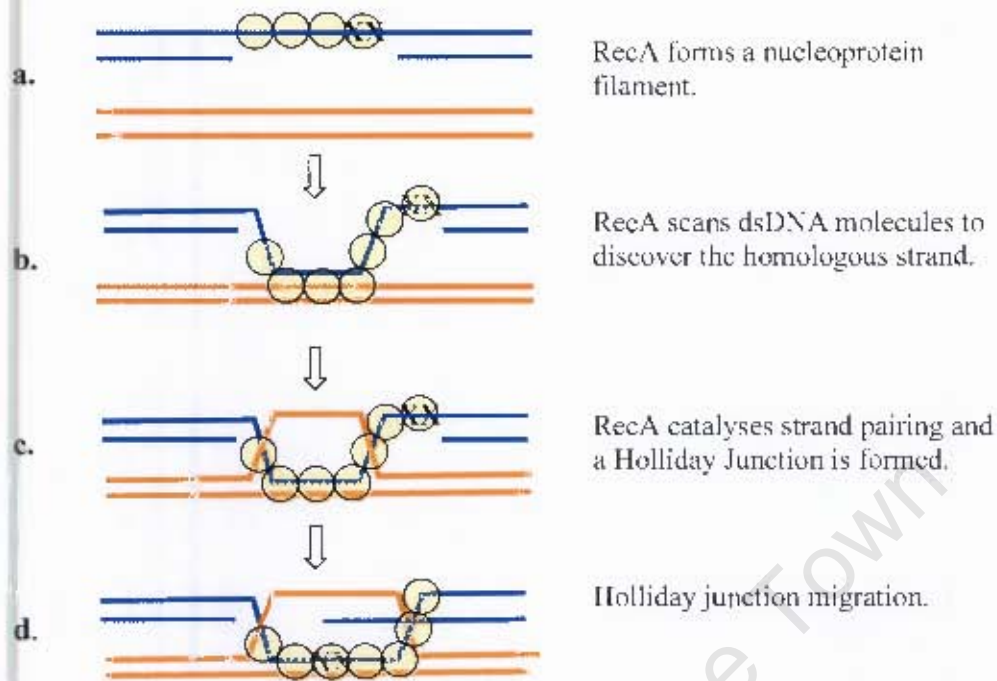
**Figure 1.3: Graph showing progression of induction of the four tiers of gene expression during the SOS response**

--- one-strand repair; — recombination repair; — translesion DNA synthesis and inhibition of cell division; — colicin production (Kuzminov, 1999).

Other bacteria have shown a DNA damage response similar to that of the SOS response in *E. coli* (Kuzminov, 1999). Examples includes *Bacillus subtilis* (Winterling *et al.*, 1998), *Pseudomonas fluorescens* (Jin *et al.*, 2007), *Salmonella enterica* serovar Typhimurium (Prieto *et al.*, 2007) and *Staphylococcus aureus* (Chen *et al.*, 2007).

### 1.3.3. THE RecA PROTEIN: A KEY ENZYME IN DNA REPAIR AND RECOMBINATION

RecA can be called the monarch of DNA repair as it is a homologous recombination repair enzyme which controls the expression of many other DNA repair proteins through the SOS response (Friedberg *et al.*, 1995; Kuzminov, 1999). During homologous recombination, RecA binds the damaged ssDNA in its primary DNA binding site to forms the nucleoprotein filament (Figure 1.4a) (Mazin and Kowalczykowski, 1998). The ssDNA-RecA filament forms rapidly with 1 000 monomers assembling per minute (Kuzminov, 1999). Every RecA monomer in the filament also binds one ATP molecule (Knight and McEntee, 1985). RecA then scans the neighbouring dsDNA by cradling it in the secondary DNA binding site (Figure 1.4b) (Cox and Lehman, 1982; Fulconis *et al.*, 2006; Sommer *et al.*, 1998). Once the homologous region has been identified, RecA catalyses pairing between the ssDNA and its complementary strand, but the opposite dsDNA strand is displaced (Figure 1.4c) (Kuzminov, 1999). Exchange occurs between the homologous DNA strands, allowing for repair (Figure 1.4c) and Holliday junction migration occurs (Figure 1.4d). ATP hydrolysis enables filament disassembly once repair has taken place.



**Figure 1.4: Model of homologous recombination**

RecA, yellow circles; XX, lesion; blue lines, parent DNA; orange lines, daughter DNA. RecA complexes with the damaged ssDNA molecules and facilitates identification of the complementary DNA strand. RecA then catalyses strand exchange and repair (Kuzminov, 1999).

Mutations in the *recA* gene have been made in *E. coli* (Larminat *et al.*, 1992; Lavery *et al.*, 1992; Sweasy and Witkin, 1991; Watanube-Akanuma *et al.*, 1997), *Bacteroides thetaiotaomicron* (Cooper *et al.*, 1997), *P. gingivalis* (Fletcher *et al.*, 1997), and *Mycobacterium bovis* (Sander *et al.*, 2001). These mutants often display increased sensitivity to DNA damaging agents and elongated cell morphologies.

*Deinococcus radiodurans* is known for its extreme resistance to DNA damaging agents, which is thought to be a result of enhanced recombination repair (Rajan and Bell, 2004). However, this bacterium does not exhibit an SOS response like that of *E. coli* and the novel regulatory protein IrrE controls expression of *recA* and regulates DNA repair rather than LexA (Jolivet *et al.*, 2006). The basal levels of RecA in *D. radiodurans* and *E. coli* are  $\pm 1\ 000$ /cell while induced levels can reach 100 000/cell (Jolivet *et al.*, 2006; Kuzminov, 1999). It is interesting to note that both bacteria have the same levels of RecA even though *D. radiodurans* displays extreme

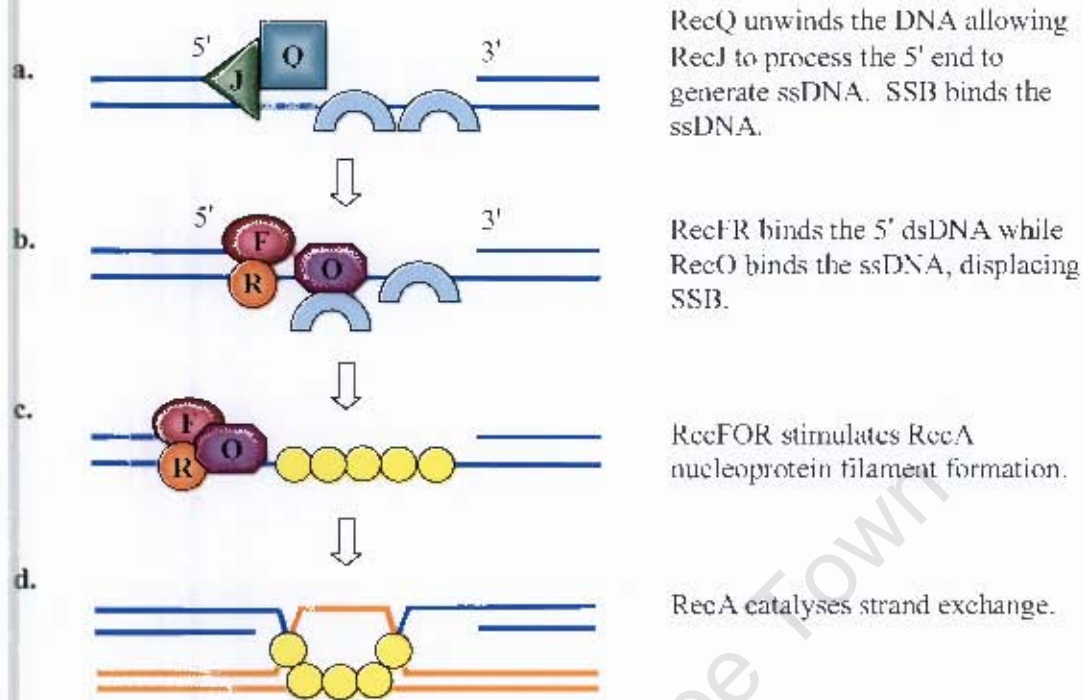
radioresistance. In *D. radiodurans*, the low level of RecA allows for repair but does not allow for cell survival in the face of catastrophic DNA damage in which case other repair mechanisms are used (Jolivet *et al.*, 2006).

#### 1.3.4. THE RecF PATHWAY: RESOLVING STALLED REPLICATION FORKS

The introduction of DNA lesions causes stalling of the replication process. The function of the RecF pathway is to repair stalled DNA replication forks (Leiros *et al.*, 2005). During replication, replisomes encounter lesions which block further progress along the template DNA (Kuzminov, 1999). The normal *E. coli* DNA polymerase (Pol III) cannot bypass these lesions directly. Instead, replication resumes further downstream, but gaps remain in the daughter strands. Recombination repair is used to fill in the gaps and the lesions are removed by excision repair.

The Rec proteins involved in this pathway are RecQ, RecJ, RecF, RecO, RecR and RecA (Kuzminov, 1999; Morimatsu and Kowalczykowski, 2003). RecJ is a 5'-3' single-stranded exonuclease while RecQ is a 3'-5' helicase (Courcelle, 2005; Leiros *et al.*, 2005). RecQ unwinds the DNA at the strand breaks to allow RecJ to exonucleolytically process the 5' end of the gapped DNA (gDNA), generating a 3' ssDNA tail which is quickly sequestered by single-stranded DNA-binding protein (SSB) (Figure 1.5a) (Morimatsu and Kowalczykowski, 2003). RecFOR activity is specific to gDNA containing a 5' terminus of a ssDNA-dsDNA junction. RecO binds the ssDNA, displacing SSB, although SSB remains indirectly bound to the ssDNA through its interaction with RecO (Figure 1.5b) (Inoue *et al.*, 2007). RecF and RecR form a complex on the 5' dsDNA end and then interact with RecO (Figure 1.5b) allowing the displacement of SSB and loading of RecA onto ssDNA (Figure 1.5c). This interaction stimulates DNA strand exchange (Figure 1.5d).

During synapsis, the RecA filaments match intact homologous duplexes to the single-stranded gap (Kuzminov, 1999). During the recombination process, DNA gyrase and topoisomerase I (Topo I) relieve supercoiling, DNA polymerase I repairs the gap and DNA ligases seals the nicks. Finally, RuvABC or RecG resolve the Holliday junction and remove the RecA filament.



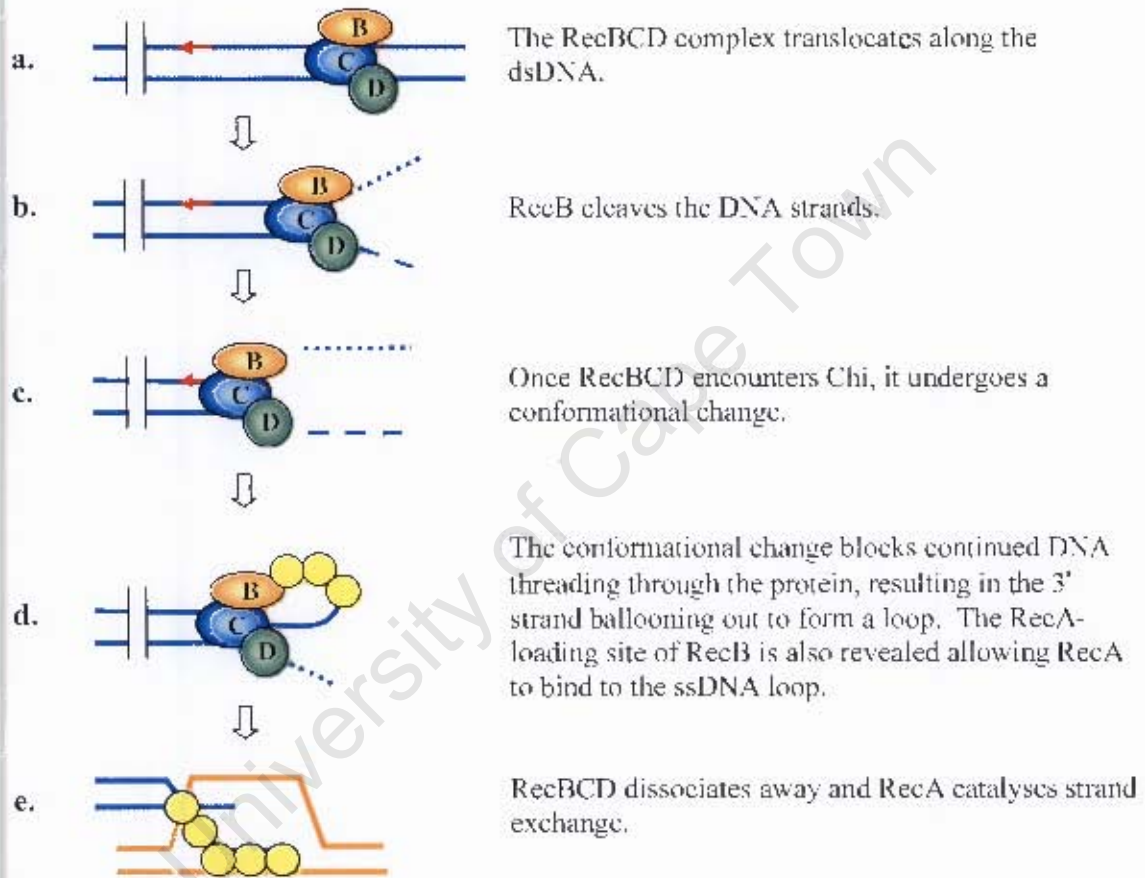
**Figure 1.5: Repair of ssDNA gaps**

RecQ, blue square; RecJ, green triangle; SSB, blue arcs; RecF, pink oval; RecO, purple hexagon; RecR, orange circle; RecA, yellow circles. The RecFOR complex recognizes the 5' dsDNA end of gapped DNA and binds to it, facilitating attachment of RecA to the gapped DNA (Inoue *et al.*, 2007; Morimatsu and Kowalczykowski, 2003).

### 1.3.5. THE RecBCD PATHWAY: REPAIR OF DOUBLE-STRANDED DNA BREAKS

The RecBCD pathway is involved in recombinational repair of double-strand DNA breaks and degradation of foreign DNA (Handa *et al.*, 2005; Singleton *et al.*, 2004). RecBCD is a holoenzyme comprised of three subunits (RecB, RecC and RecD) each with their own specific function (Singleton *et al.*, 2004). Both RecB and RecD are members of the Super family (SF) 1 helicases. RecB is a helicase with 3'-5' directionality, and it contains nuclease activity, while the RecD protein is a 5'-3' helicase. RecC is a 129 kDa protein involved in recognising the regulatory sequence Chi (Kuzminov, 1999; Singleton *et al.*, 2004). In *E. coli*, Chi has the sequence 5'-GCTGGTGG-3' (Kuzminov, 1999), whilst in *Bacillus subtilis* (Chédin *et al.*, 1998), *Lactococcus lactis* (Biswas *et al.*, 1995) and *Haemophilus influenza* (Sourice *et al.*, 1998) it differs but still remains short and asymmetric.

Figure 1.6 illustrates each step in the RecBCD holoenzymic recombinational repair pathway of *E. coli* (Handa *et al.*, 2005). RecBCD translocates along the DNA at 1 835 bp/sec (Figure 1.6a). As it does this, the RecB subunit cleaves the 3' strand frequently and the 5' strand infrequently (Figure 1.6b) (Singleton *et al.*, 2004).



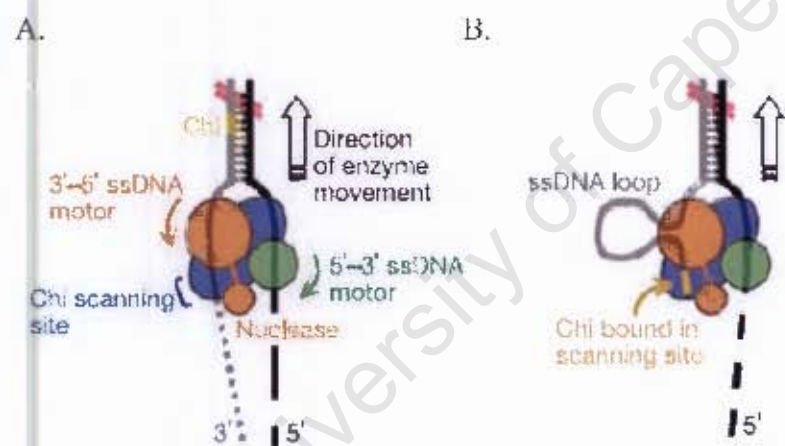
**Figure 1.6: Model for the RecBCD pathway**

RecB, orange; RecD, green; RecC, blue; RecA, yellow; Chi, red arrow; black lines, dsDNA break; blue lines, parent DNA; orange lines, daughter DNA; — — —, infrequently cleaved parent DNA; ······, frequently cleaved parent DNA. RecBCD undergoes a conformational change when it encounters Chi that allows it to promote RecA loading (Handa *et al.*, 2005).

When the holoenzyme encounters Chi, it pauses for five seconds, undergoes a conformational change (Figure 1.6c) and then moves on with altered nuclease activity and a two-fold reduced translocation speed (Figure 1.6d) (Handa *et al.*, 2005). The conformational change also exposes the RecA binding pocket allowing for RecA

loading and nucleofilament formation on the 3' strand (Figure 1.6d) (Churchill and Kowalczykowski, 2000; Handa *et al.*, 2005). RecA then carries out homologous recombination (Figure 1.6e) (Handa *et al.*, 2005).

Figure 1.7 shows a close-up view of the conformational changes caused by the Chi regulatory sequence (Singleton *et al.*, 2004). The 3' and 5' strands are fed into the RecBCD enzyme where they come into contact with the nuclease domain of RecB (Figure 1.7A). The holoenzyme encounters Chi which becomes bound to the scanning site (Figure 1.7B). This prevents further movement of the 3' strand into RecBCD, so it balloons out from RecB to form a ssDNA loop. RecA binds to this loop. The 5' strand does not need to compete with the 3' strand for access to the nuclease site, thus the 5' strand is cleaved more frequently.



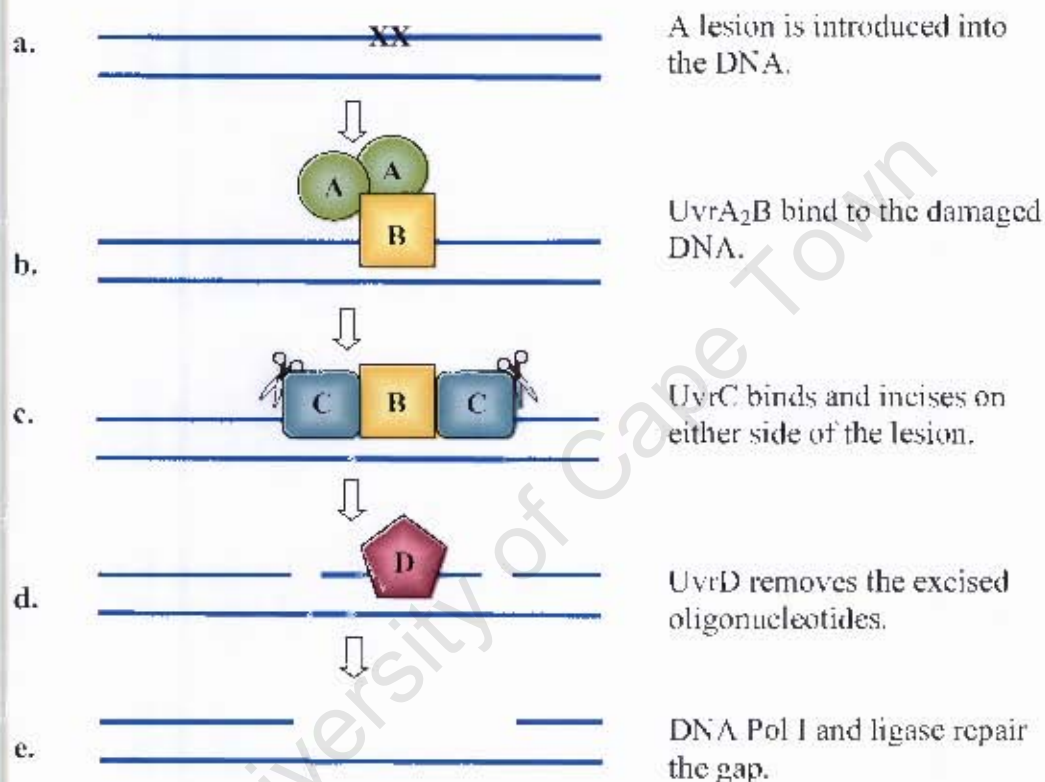
**Figure 1.7: Detailed diagram of Chi-induced changes to RecBCD**

RecB, orange; RecD, green; RecC, blue. RecB and RecD are the helicase motors while RecC houses the Chi scanning site (Singleton *et al.*, 2004).

### 1.3.6. THE UvrABC PATHWAY: NUCLEOTIDE EXCISION REPAIR

Nucleotide excision repair (NER) removes bulky DNA damage (such as thymine dimers) in both prokaryotes and eukaryotes (Eryilmaz *et al.*, 2006; Skorvaga *et al.*, 2002). NER has been thoroughly characterised in *E. coli* where the UvrABC nuclease carries out this form of repair (Skorvaga *et al.*, 2002). Besides exhibiting increased UV sensitivity, *E. coli* *uvrA*, *uvrB* and *uvrC* mutants, however, show increased sensitivity to metronidazole as well (Dachs *et al.*, 1995; Yeung *et al.*, 1984). Therefore, NER must also be able to repair DNA strand breaks although it is not

known how this is achieved. The SOS response controls *uvrA* and *uvrB* transcription but *uvrC* is regulated independently (Truglio *et al.*, 2006). To initiate NER, UvrA proteins dimerise. ATP binding stabilises UvrA dimerization while ATP hydrolysis causes dissociation (Figure 1.8) (Truglio *et al.*, 2006). UvrA<sub>2</sub> complexes with UvrB, which stimulates DNA damage recognition (Figure 1.8a-b) (Truglio *et al.*, 2006; Van Houten *et al.*, 2002).

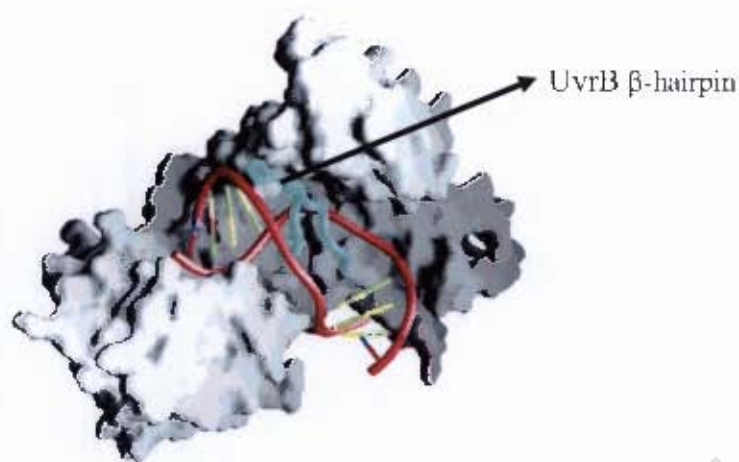


**Figure 1.8: Model of Nucleotide Excision Repair**

UvrA, green circles; UvrB, orange square; UvrC, blue squares; UvrD, purple pentagon; XX, DNA lesion (Truglio *et al.*, 2006; Van Houten *et al.*, 2002).

UvrB has a  $\beta$ -hairpin which inserts between the damaged DNA strands, securing the complex in place (Figure 1.9) (Skorvaga *et al.*, 2002). UvrA<sub>2</sub> then dissociates away, allowing UvrC to bind the UvrB-DNA complex (Figure 1.8c) (Van Houten *et al.*, 2002). The UvrC C-terminal domain incises eight nucleotides away from the DNA lesion on the 5' end and the N-terminal domain incises four or five nucleotides away from the DNA lesion on the 3' end (Truglio *et al.*, 2006; Van Houten *et al.*, 2002). The helicase, UvrD, removes the excised oligonucleotides (Figure 1.8d) (Van Houten

*et al.*, 2002). DNA repair is performed by DNA polymerase I enzyme and gaps in the DNA strand are sealed by DNA ligase (Figure 1.8e).



**Figure 1.9: UvrB surrounding the damaged DNA**

The UvrB  $\beta$ -hairpin (shown in blue) inserting between the DNA strands (Truglio *et al.*, 2006).

### 1.3.7. DNA REPAIR IN BACTEROIDES

In the past, studies on DNA repair in *Bacteroides* were hampered by the shortage of readily available genome information as well as a lack of appropriate genetic systems to generate gene specific mutations. Thus, chemically produced mutants were made and screened for deficiencies in repair. Abratt *et al.* (1985) used ethyl methane sulphonate mutagenesis to create random *B. fragilis* Bf-2 DNA repair mutants. Two mutants were generated, MTC25 and UVS9. The mitomycin C-sensitive mutant MTC25 was not sensitive to ultraviolet radiation (UV) while the UV-sensitive mutant UVS9 showed only low levels of mitomycin C sensitivity. These results suggested that there were two pathways for the repair of different forms of DNA damage in this bacterium. In further studies, the wild type and mutant strains were exposed to UV radiation in the presence or absence of chloramphenicol (Abratt *et al.*, 1986). In the presence of the antibiotic, repair still took place even though the rate of repair was halved. This pointed to the existence of a constitutive dimer excision repair system in *B. fragilis* Bf-2. UVS9 exhibited low levels of repair of UV-induced damage, which could be attributed to a second repair system present in the bacterium which was compensating for the mutation, or the mutation was a leaky and did not totally abolish the UV repair system. It was later found that regulated DNA strand breakage occurred in the *B. fragilis* Bf-2 wild type and DNA repair mutants in response to UV-

induced DNA damage (Abratt *et al.*, 1990). This alluded to the presence of a NER-type DNA repair pathway in *B. fragilis* where the DNA is nicked on either side of the dimer and the damaged piece removed. Homologous recombination repair was also identified in *B. fragilis* by the discovery of a *B. fragilis* Bf-1 *recA*-like gene which complemented the DNA repair deficient phenotype of an *E. coli recA* mutant (Goodman *et al.*, 1987). The complemented mutant was able to repair damage caused by UV, methyl methanesulfonate and 4-nitro-quinoline-1-oxide and carry out homologous recombination. Antibodies raised against *E. coli RecA* were used to probe for *B. fragilis RecA* in a western blot. The antibodies reacted with the *B. fragilis RecA* showing that both proteins were immunologically related. Further characterization of *B. fragilis* Bf-1 *recA* showed that the gene was 954 bp long and encoded a 35 545 kDa protein (Goodman and Woods, 1990). The promoter did not contain an SOS box. Expression of this gene, therefore, did not seem to be regulated by LexA, as seen in *E. coli*. The *recA* gene was also identified in *B. thetaiotamicron*, and mutation of this gene resulted in increased sensitivity of the cells to DNA damaging agents and oxygen (Cooper *et al.*, 1997).

The search for putative DNA repair genes in *Bacteroides* has been facilitated by the publication of the annotated genome sequences of *B. fragilis* YCH46 (Kuwahara *et al.*, 2004) NCTC 9343 and 638R (Cerdeño-Tárraga *et al.*, 2005) and *B. thetaiotamicron* VPI-5482 (Xu *et al.*, 2003). A few of the many examples of putative repair genes which have been identified in the published annotated genome of *B. fragilis* NCTC 9343 (www.ncbi.nih.gov) are: BF2634 (*uvrA1*), BF3159 (*uvrA2*), BF2642 (*uvrB*), BF0111 (*uvrC*), BF1180 (*recA*), BF2106 (*recR*), BF3706 (*recQ*), BF3892 (*recQ1*), BF3249 (*recQ2*) BF3705 (*recJ*) and BF1571 (*ssb*). This suggests that the DNA repair pathways of *B. fragilis* are similar to those found in *E. coli*, since a few of this bacterium's well-characterised excision repair and recombination repair proteins have been identified on the *B. fragilis* genome. Except for *recA*, there are no reports in the literature of functional characterisation of these genes or their possible roles in the repair of Mtz-induced DNA damage.

#### 1.4. AIMS AND OBJECTIVES

The antibiotic Mtz causes cell death by generating ss and dsDNA breaks. Thus, enhanced DNA repair mechanisms could potentially lead to MtzR in anaerobic

bacteria like *B. fragilis*. RecA is a major DNA repair protein and could play a role in dealing with Mtz damage and increasing MtzR. RecA activity is facilitated by the RecFOR and RecBCD pathways in *E. coli*. It was, therefore, of interest to identify possible homologues for these proteins in the *B. fragilis* genome through bioinformatic analysis. In *E. coli*, RecA function was first investigated through generating *recA*<sup>-</sup> mutants (Kuzminov, 1999) therefore, a preliminary analysis of RecA in *B. fragilis* for this study involved generating a *recA* mutant using the suicide vector pGERM. Once the mutation was confirmed with PCR, DNA sequencing and Southern hybridisation, the mutant was exposed to DNA damaging agents, especially Mtz, to assess its viability in contrast with the wild type strain. To study enhanced DNA repair which could lead to MtzR in *B. fragilis*, a *recA* over-expressing strain was constructed and its response to Mtz assessed in comparison to wild type cells. The aim of this project was to contribute to the knowledge of DNA repair and MtzR in *B. fragilis*.

**CHAPTER 2**

**BIOINFORMATIC ANALYSIS OF PUTATIVE PROTEINS  
INVOLVED IN RecA-MEDIATED DNA REPAIR**

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### *Abstract*

RecA is a major DNA repair protein involved in the SOS response and homologous recombination in *E. coli* and other bacteria. Bioinformatic analysis of the *B. fragilis* RecA, revealed that it contained the Walker A and Walker B motifs and other conserved regions and amino acids necessary for RecA functioning. Investigation of its surrounding genes suggested that *recA* formed part of an operon. The *B. fragilis* genome was further investigated for proteins which could possibly be involved in aiding RecA-mediated DNA repair. The RecFOR proteins, which repair stalled replication forks, which were annotated in the published genome, were examined along with three RecQ helicases and a RecJ endonuclease which are indispensable to this pathway. The RecBCD pathway, responsible for dsDNA break repair, could not be identified but it seems to have been replaced by the RexAB proteins which have been characterized in *L. lactis*.

University of Cape Town

## 2.1. INTRODUCTION

The sequenced *B. fragilis* NCTC 9343, 638R and YCH46 genomes confirmed the presence of a *recA* gene which had previously been identified and functionally characterised from a gene library of *B. fragilis* Bf-1 (Goodman *et al.*, 1987). The *B. fragilis* Bf-1 *recA* was able to complement an *E. coli recA* mutant by enhancing the mutant's cellular recombination and increasing its survival in the presence of various DNA damaging agents. *B. fragilis* RecA was also immunologically conserved with respect to the *E. coli* RecA, since antibodies raised against *E. coli* RecA cross-reacted with *B. fragilis* RecA. The *B. fragilis recA* gene sequence was determined and primer extension analysis identified the transcription start point of the gene (Goodman and Woods, 1990).

As reviewed in Chapter 1, RecA cannot function in isolation in conducting the repair of stalled replication forks and DNA breaks, since RecA nucleofilament formation is mediated by RecFOR (Morimatsu and Kowalczykowski, 2003) and RecBCD holoenzymes in *E. coli* (Singleton *et al.*, 2004). RecFOR is highly conserved amongst bacteria (Koroleva *et al.*, 2007) and this complex repairs stalled replication forks (Leiros *et al.*, 2005). RecBCD is involved with repairing dsDNA breaks (Singleton *et al.*, 2004), though this pathway has been replaced by AddAB (Zuñiga-Castillo *et al.*, 2004) or RexAB (Quiberoni *et al.*, 2001b) in other bacteria. To model RecA function in *B. fragilis* it would be of interest to analyse RecA function in the context of RecFOR and RecBCD (or AddAB/RexAB) homologues (as presented in the published genome) through further bioinformatic analysis. The work reported in this chapter, therefore, focuses on a comprehensive bioinformatic analysis of putative RecFOR, RecQ, RecJ, SSB, RecBCD, RexAB and LexA proteins which might function in conjunction with RecA in the repair of lesions caused by metronidazole (Mtz). The genetic context of *recA* is also described.

## 2.2. MATERIALS AND METHODS

### 2.2.1. BIOINFORMATIC ANALYSIS

The bacterial strains used for bioinformatic analysis are shown in Table 2.1. The protein and DNA sequences were obtained from the National Centre for Biotechnology Information ([www.ncbi.nih.gov](http://www.ncbi.nih.gov)), except for the sequences for *B. fragilis* 638R which were obtained from the Wellcome Trust Sanger Institute ([www.sanger.ac.uk](http://www.sanger.ac.uk)). BLAST 2.2.17 was used to calculate predicted percentage identity between protein sequences (Altschul *et al.*, 1997).

Conserved domains database (CDD) searches were used to identify conserved domains in the protein sequences (Marchler-Bauer and Bryant, 2004). Protein sequence alignments were carried out with DNAMAN version 4.13 (Lynnon BioSoft).

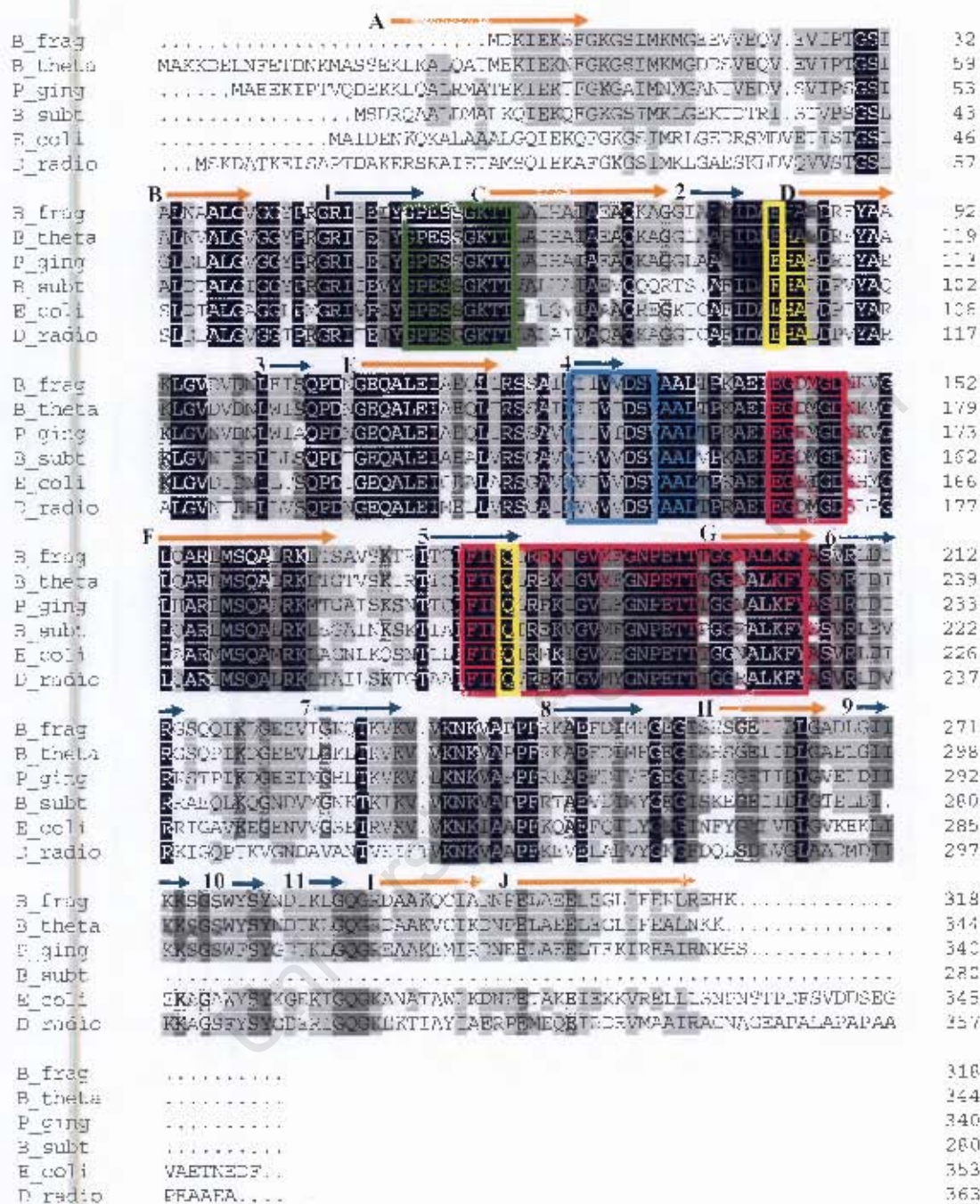
**Table 2.1: Bacterial strains and their genome accession numbers**

Bacterial Strain	Accession No.	Reference
<i>Bacillus subtilis</i> subsp. <i>Subtilis</i> str. 168	NC_000964	Kunst <i>et al.</i> , 1997
<i>Bacteroides fragilis</i> NCTC 9343	NC_003228	Cerdeño-Tárraga <i>et al.</i> , 2005
<i>Bacteroides fragilis</i> 638R	Not available	www.sanger.ac.uk
<i>Bacteroides thetaiotaomicron</i> VPI-5482	NC_004663	Xu <i>et al.</i> , 2003
<i>Bifidobacterium adolescentis</i> ATCC 15703	NC_008618	Suzuki <i>et al.</i> , 2006
<i>Bifidobacterium longum</i> NCC2705	NC_004307	Schell <i>et al.</i> , 2002
<i>Deinococcus radiodurans</i> R1	NC_001263	White <i>et al.</i> , 1999
<i>Enterococcus faecalis</i> V583	NC_004668	Paulsen <i>et al.</i> , 2003
<i>Escherichia coli</i> K-12	NC_000913	Blattner <i>et al.</i> , 1997
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> MG1363	NC_009004	El Karoui <i>et al.</i> , 1998
<i>Porphyromonas gingivalis</i> W83	NC_002950	Nelson <i>et al.</i> , 2003
<i>Salmonella typhimurium</i> LT2	NC_003197	McClelland <i>et al.</i> , 2001

## 2.3. RESULTS AND DISCUSSION

### 2.3.1. RecA

Crystallographic analysis of *E. coli* RecA has generated significant information on RecA structure and function. These studies have shown that RecA consists of three domains (Xing and Bell, 2004). The first domain forms the interface between RecA monomers in the nucleoprotein filament and consists of one  $\alpha$ -helix and one  $\beta$ -strand. A central  $\beta$ -sheet and six  $\alpha$ -helices make up the central domain, which binds ATP and ssDNA (Bell, 2005). The C-terminal domain folds into three  $\alpha$ -helices and three  $\beta$ -sheets for binding dsDNA. In RecA, the ATP binding region is found in the central domain (Bell, 2005). In this study, bioinformatic analysis was carried out on the *B. fragilis* NCTC 9343 RecA protein (the gene is annotated as BF1180). Figure 2.1 illustrates a multiple sequence alignment carried out between RecA proteins from *B. fragilis* NCTC 9343 and various other bacteria.

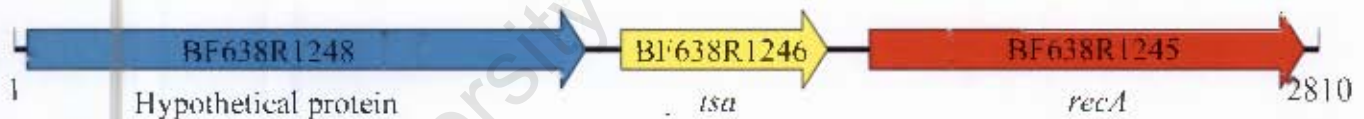


**Figure 2.1: Multiple RecA alignment**

B\_frag: *B. fragilis*; B\_theta: *B. theta*; P\_ging: *P. gingivalis*; B\_subt: *B. subtilis*; E\_coli: *E. coli*; D\_radio: *D. radiodurans*. Shading of amino acids: Black-identical, dark grey-highly similar, light grey-similar. Orange arrows, predicted alpha helices numbered A-J; blue arrows, predicted beta sheets numbered 1-11 (Rajan and Bell, 2004); green box, Walker A motif; blue box, Walker B motif; pink boxes, I.1 and I.2 (Chen *et al.*, 2007a); yellow boxes, glutamate (E) and glutamine (Q) involved in ATP binding (Bell, 2005).

*B. fragilis* RecA exhibited a predicted amino acid identity of 93% to *B. thetaiotaomicron*, 81% to *P. gingivalis*, 69% to *B. subtilis*, 62% to *E. coli*, and 61% to *D. radiodurans* RecA proteins as calculated by BLAST analysis (Altschul *et al.*, 1997). Like the *E. coli* RecA, *B. fragilis* RecA showed high conservation of Walker A (GPESGKT) and Walker B (Figure 2.1) which are signature motifs of ATP binding domains (Marchler-Bauer and Bryant, 2004). The Walker B motif can differ in specific sequence between proteins but it is characterised by four aliphatic residues followed by two negative residues (Marchler-Bauer and Bryant, 2004). Important residues for ATP binding are the glutamate (E) and glutamine (Q) boxed in yellow in Figure 2.1 (Bell, 2005). The L1 and L2 motifs (boxed in purple in the diagram) are involved in the binding of ssDNA (Chen *et al.*, 2007a). The sequence alignment shows a high degree of conservation of amino acid residues in L1 and L2 between the analysed bacteria.

Scrutiny of the arrangement of the genes around *B. fragilis* 638R *recA* revealed that it could be part of an operon (Figure 2.2, Table 2.2). In *B. fragilis*, *recA* (BF638R1245) is downstream of BF638R1248 and BF638R1246 (Figure 2.2). There is 82 bp between BF638R1248 and BF638R1246 and 97 bp between BF638R1246 and BF638R1245.



**Figure 2.2: Putative *recA* operon arrangement**

An investigation was carried out using a conserved domain database (CDD) search to determine whether the genes flanking *B. fragilis* 638R *recA* encoded proteins possibly related to RecA function. The hypothetical protein product of BF638R1248 was found to contain a homospermidine synthase domain (Marchler-Bauer and Bryant, 2004), which catalyses the synthesis of polyamine homospermidine from putrescine (Tholl *et al.*, 1996). In *E. coli*, polyamines and polyamine synthesis enzymes have been found to affect gene expression under oxidative stress (Jung and Kim, 2003). BF638R1246 has been annotated as encoding a putative thiol-specific antioxidant (TSA) enzyme. Three TSA peroxidases have been previously identified in *B. fragilis*: Alkyl hydroperoxide peroxidase (AhpC), BCP and Thioredoxin peroxidase (Tpx) (Chae *et al.*, 1994; Herren *et al.*, 2003). TSA peroxidases reduce peroxides to alcohols with the aid of a reduced thiol donor (Herren *et al.*, 2003). AhpC/TSA enzymes have been identified in four opportunistic pathogens, namely

*Enterococcus histolytica*, *H. pylori*, *Cryptosporidium parvum* and *B. fragilis* (Chae *et al.*, 1994). These enzymes may provide protection against the oxidative burst generated by macrophages and neutrophils during the host immune response to infection. Proteins involved in metal metabolism (e.g. ferritin and bacterioferritin) help prevent the formation of oxygen radicals and have been shown to be induced by oxidative stress (Sund *et al.*, 2008). Thus, in *B. fragilis*, the close proximity of these genes to *recA* could allow for an efficient co-ordinated response to oxidative stress: the protein encoded by BF638R1248 would regulate gene expression through polyamine synthesis. TSA could reduce peroxides to non-toxic intermediates while RecA repaired DNA damage caused by oxidative radicals. BLAST and CDD searches did not reveal any possible function for the hypothetical proteins encoded by BF638R1249 and BF638R1244. The annotation in *B. fragilis* 638R is different to the annotation for strains NCTC 9343 and YCH46 (Table 2.2). In YCH46 and NCTC 9343, BCP and TSA are classified as one gene with the BF638R1246 start site, therefore, for the purpose of clarity, only BF638R1246 is discussed in this thesis, not BF638R1247.

**Table 2.2: Genetic context of *recA* in *B. fragilis* 638R, NCTC 9343 and YCH46**

Strain	ORF number	Gene product	Location (bp)
638R	BF638R1249	Hypothetical protein	1497145-1498392
	BF638R1248	Hypothetical protein	1495857-1497047
	BF638R1247	BCP	1495328-1495771
	BF638R1246	TSA	1495328-1495789
	BF638R1245	RecA	1494274-1495314
	BF638R1244	Hypothetical protein	1493922-1494200
	NCTC 9343	BF1183	Hypothetical protein
BF1182		Hypothetical protein	1436557-1437750
BF1181		BCP/TSA	1436028-1436474
BF1180		RecA	1434974-1435930
BF1179		Hypothetical protein	1434627-1434908
YCH46	BF1216	Hypothetical protein	1448013-1449263
	BF1215	Hypothetical protein	1446725-1447918
	BF1214	BCP	1446196-1446642
	BF1213	RecA	1445142-1446185
	BF1212	Hypothetical protein	1444795-1445076

BCP: bacterioferritin co-migratory protein; TSA: thiol-specific antioxidant enzyme

The RecA protein does not function in isolation during recombination repair and requires either the RecFOR or RecBCD proteins to promote RecA loading onto ssDNA (Kuzminov, 1999). It has been reported that RecFOR, RecJ, RecQ, SSB, RecD and RexA homologues are present in the *B. fragilis* NCTC 9343 genome (Cerdeño-Tárraga *et al.*, 2005 supplementary material; www.sanger.ac.uk). In this study, bioinformatic analysis was used to confirm the putative annotation of the previously identified homologues (Cerdeño-Tárraga *et al.*, 2005 supplementary material; www.sanger.ac.uk). All the homologues for these genes are identical in *B. fragilis* 638R and NCTC 9343. Thus, even though the bioinformatic analysis was carried out on the *B. fragilis* NCTC 9343 protein sequences, it still has relevance to the functional work carried out in *B. fragilis* 638R for this project.

### 2.3.2. RecFOR PATHWAY INCLUDING RecQ, RecJ AND SSB

RecFOR proteins are involved in repairing stalled DNA replication forks (Leiros *et al.*, 2005). RecFOR homologues are identified at a higher rate in bacterial genomes than RecBCD homologues and amongst RecF, RecO and RecR, RecR shows the highest conservation between bacteria (Koroleva *et al.*, 2007).

#### 2.3.2.1. RecR

A multiple sequence alignment between the putative *B. fragilis* RecR (BF2106) and other RecR proteins showed conservation of three functionally important domains: the HhH motif, the Cys<sub>4</sub> zinc finger and the Walker B motif (Figure 2.3). The HhH motif (hxxhxGhGxxxAxxhh) and the Cys<sub>4</sub> zinc finger are involved in binding DNA while the Walker B motif is important for ATP hydrolysis (Lee *et al.*, 2004). The crystal structure of *D. radiodurans* RecR has been solved and, from this analysis, it seems that RecR forms a ring-shaped tetramer with a central hole through which the DNA slides. The putative *B. fragilis* RecR displays 92%, 44%, 38% and 49% predicted amino acid identity to *B. thetaiotaomicron*, *B. subtilis*, *E. coli* and *D. radiodurans* RecR respectively (Altschul *et al.*, 1997).



**Figure 2.3: Multiple RecR alignment**

B\_frag: *B. fragilis*; B\_theta: *B. theta*taotaomicron; B\_subt: *B. subtilis*; E\_coli: *E. coli*; D\_radio: *D. radiodurans*. Shading of amino acids: Black-identical, dark grey-highly similar, light grey-similar. Stars, Cys<sub>4</sub> zinc finger; pink box, HhI motif; blue box, Walker B motif (Lee *et al.*, 2004).

### 2.3.2.2. RecO

BLAST analysis confirmed *B. fragilis* BF0248 as a putative *E. coli* *recO* homologue, with the protein product sharing a rather low predicted amino acid identity of 28% with the *E. coli* RecO protein (Altschul *et al.*, 1997). A CDD search revealed that the protein contained a RecO domain which spanned its entire length (Figure 2.4) further confirming the BLAST results (Marchler-Bauer and Bryant, 2004).



**Figure 2.4: CDD Search result for *B. fragilis* BF638R0300**

*B. fragilis* BF0248, grey line; recO conserved domain, red oblong (Marchler-Bauer and Bryant, 2004).

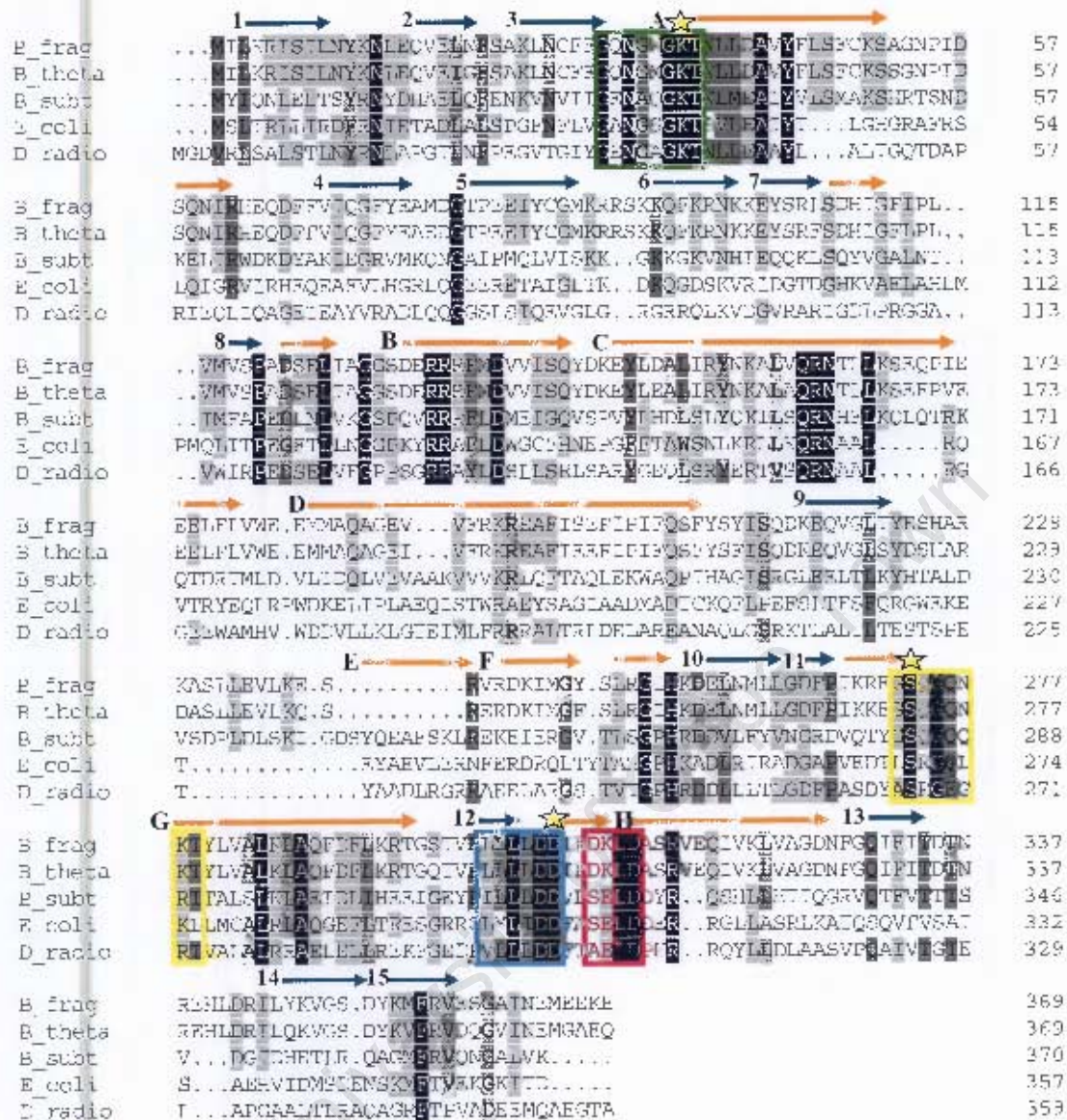
A multiple sequence alignment was carried out between the putative *B. fragilis* RecO and other RecO proteins (Figure 2.5). *D. radiodurans* RecO contains four important cysteine residues forming the DNA binding Cys<sub>4</sub> zinc finger motif, they are indicated by stars in



### 2.3.2.3. RecF

RecF is thought to direct the placement of RecO and RecR on the DNA, and together with RecR, RecF prevents RecA filaments from extending past ssDNA gaps (Koroleva *et al.*, 2007). The crystal structure of *D. radiodurans* RecF (Koroleva *et al.*, 2007), RecO (Leiros *et al.*, 2005) and RecR (Lee *et al.*, 2004) has been determined. Analysis of RecFOR structure and function points to RecF binding ATP and then forming a dimer on the DNA. This complex is then the base for two RecR dimers to form a tetramer clamp around the helix (Koroleva *et al.*, 2007).

A BLAST analysis using the *E. coli* RecF confirmed *B. fragilis* BF0877 as putative RecF-encoding homologue. *B. fragilis* BF0877 displayed a predicted amino acid identity of 92% to *B. thetaiotaomicron*, 31% to *B. subtilis*, 25% to *E. coli* and 26% to *D. radiodurans* RecF proteins (Altschul *et al.*, 1997). Figure 2.6 shows a multiple sequence alignment of these RecF proteins with functionally important residues boxed. The Walker A and B motifs and the D-loop and ABC transporter signature motifs are necessary for ATP binding and hydrolysis (Koroleva *et al.*, 2007; Marchler-Bauer *et al.*, 2007). Walker A forms a loop for binding the phosphates of nucleotides, the ABC transporter signature motif is thought to sense gamma phosphates whilst the Walker B and D-loop are involved in the ATP hydrolysis reaction (Marchler-Bauer *et al.*, 2007). The residues marked with stars have been shown to be essential for ATP binding or hydrolysis (Koroleva *et al.*, 2007). K36 has been mutated in *E. coli* and *D. radiodurans*, whilst D303 and S270 (*E. coli* numbering) have been mutated in *D. radiodurans* alone. The mutated proteins were not able to bind or hydrolyse ATP like their wild type counterparts. These residues are completely conserved across the alignment, pointing to their importance for RecF function.



**Figure 2.6: Multiple RecF alignment**

B\_frag: *B. fragilis*; B\_theta: *B. theta*; B\_subt: *B. subtilis*; E\_coli: *E. coli*; D\_radio: *D. radiodurans*. Shading of amino acids: Black-identical, dark grey-highly similar, light grey-similar. Orange arrows, alpha helices numbered A-II; Blue arrows; beta sheets numbered 1-15 (Koroleva *et al.*, 2007). Walker A motif, green box; Walker B motif, blue box; D-loop, pink box; ABC transporter signature motif, yellow box; stars, important residues (Koroleva *et al.*, 2007; Marchler-Bauer *et al.*, 2007).

### 2.3.2.4. RecQ AND RecJ

Both RecQ and RecJ are involved in the RecFOR pathway (Morimatsu *et al.*, 2003) (reviewed in Chapter 1). RecQ is a Super family 2 (SF2) 3'-5' helicase which unwinds the DNA at the strand breaks, allowing RecJ to exonucleolytically process the 5' end of the gDNA (Courcelle, 2005; Leiros *et al.*, 2005). Since, RecFOR activity is specific to gDNA containing a 5' terminus of a ssDNA-dsDNA junction, the RecFOR pathway cannot be initiated without RecQ and RecJ. In this study three putative *B. fragilis* RecQ-encoding genes were confirmed: BF3892 (*recQ1*), BF3249 (*recQ2*) and BF3706 (*recQ3*) (Altschul *et al.*, 1997). Figure 2.7 shows a section from a multiple sequence alignment between *E. coli* RecQ and the three putative RecQ proteins from *B. fragilis*. The seven signature motifs (numbered I-VI) of SF2 helicases have been labelled in the diagram (Bernstein *et al.*, 2003).



**Figure 2.7: Multiple RecQ alignment showing conserved helicase motifs**

*E. coli*: *E. coli*; *B. fragQ1*: *B. fragilis* RecQ1; *B. fragQ2*: *B. fragilis* RecQ2; *B. fragQ3*: *B. fragilis* RecQ3. Shading of amino acids: Black-identical, dark grey-highly similar, light grey-similar. Green boxes, helicase motifs numbered I-VI (Bernstein *et al.*, 2003).

The *B. fragilis* RecQ proteins show high conservation of these motifs. The putative *B. fragilis* RecQ proteins RecQ1, RecQ2 and RecQ3 have a deduced amino acid identity to the *E. coli* RecQ of 39%, 44% and 39% respectively (Altschul *et al.*, 1997). It is unusual that *B. fragilis* contains three putative RecQ homologues since other bacteria contain only one or two RecQ proteins or they are devoid of RecQ altogether (Nakayama, 2002).

*B. fragilis* BF3705 has been identified as a putative *recJ* gene ([www.sanger.ac.uk](http://www.sanger.ac.uk)). RecJ has been extensively studied in *E. coli* where it has been shown to be a Mg<sup>2+</sup>-dependent, 5'-3' ssDNA exonuclease indispensable to the RecFOR pathway and also affecting the efficiency of the RecBCD pathway (Sutera *et al.*, 1999). Seven conserved motifs have been recognized on the RecJ protein, which are thought to interact with Mg<sup>2+</sup> or the phosphates on the DNA backbone. These motifs are: 1. Dx Dx D(G); 2. D(NG); 3. DHH; 4. D; 5. Px<sub>2</sub>Nx<sub>3</sub>R; 6. (S)xR and 7. (GG)H. A protein alignment between *E. coli* RecJ and the putative *B. fragilis* RecJ shows the RecJ motifs are fully conserved between these two proteins (Figure 2.8). Furthermore, the putative *B. fragilis* genes encoding RecQ3 and RecJ overlap, suggesting that these two genes may be transcribed as an operon (Cerdeño-Tárraga *et al.*, 2005). This would allow for a co-ordinated response to DNA damage.

B_frag	MNHKANYREITDQEQAEISRALAQELGSEFWLGGFLVVRGGITKACDAKKEFRPOLPELHDP	60
E_coli	.....MKQCITQLRRREVDETADLPAEIPFLIRFLYASRGVRSACQLERSVKGMLEWQQLS	55
B_frag	FLMKEMDIAVERLDNMAMGKKERTILYGIYDWDGCTHAWATVYKFIQQFTSLLDYYIIPERY	119
E_coli	SVBK...AVEIILYNAFREGTRILAVGIFLADGMICITALEVLMRSLKCSMIDYLVENRE	111
B_frag	NEGYGSKKGVNYAETICVGLIIVLDGGTKAVEELAYAKEKGLDFILGDHHEVETLIFPA	179
E_coli	HDYGLSPEWVLCQAHACACGLITVVDNGLSSSHAGVEHARSLGEPVIVTDHHLGGLIFENA	171
B_frag	VATLAKHIDNTYBYTHISGGVGGKHKQAFASNGIEFHELIIE.....LLCIPPA	230
E_coli	PATLPMKIDCNFESKELAGVGVVYUMIDLETFLKDDQGWVDEENIAPNLAEILLVAPL	231
B_frag	SIASEVEMGENRITLAYGCKQLNMPVVELKALIDVCELSEKEITVSDIVFKGPRI	289
E_coli	GVADVELLANRITLWCGMSPTRACKCRPGKALLDANRDAQKLAASDLGFALGPRE	291
B_frag	NASGRICNGKEAVCLITLIDFSSALEKAGCINQYNETRKOLOKSMIBEANKIASEIE...	346
E_coli	NVAGELDDMSVGVALLLCONIGDPRVLANELDALNCTRKEIECGVQIEELTLNEKIDRSR	351
B_frag	.....GLADERSIVLYNEDNHEGVIGIASELIEIYYREAVVLRITLDMATGSARSVSG	400
E_coli	DILPGLAMYHPE.....WRCGVVGIASEIKERFHRVIAFAPAGDGLTKGSSRSVCG	405
B_frag	FDVYKATEKORDLEN.....FCGHTYAAGLSMKVENVQAFTERFFESFVVEPHL	449
E_coli	LHM.....LPLDLYPGMMLKFCGHAAACLSLREDKFKLEQQREFGRVLEWLD	458
B_frag	EQQSAVNDIDAEIDFKDITPKFENELKHFNEFCPENQVPEFCTHHVYDYGTSKVVGRDQ	509
E_coli	ESLLOGEVVSGLPGLSPAEMDMEVAQIRDACEWCCYFPELELDGHEKLLQCR.....	510
B_frag	ERIKLDVONKSNVMN.....GLAFQSSH.....VRYIKTKRSPDICYTIIE	553
E_coli	.....LVGERHILVWVEPVGGLLGLAENVDALWPDNGVREVQLAYKLDINEF...	561
B_frag	NTHKRCGEVQLQIEDIKPIE	572
E_coli	...RCNRQLQIICDNIWP	576

**Figure 2.8: RecJ alignment**

E\_coli: *E. coli*; B\_frag: *B. fragilis*. Shading of amino acids: Black-identical, dark grey-highly similar, light grey-similar. RecJ motifs are boxed in orange (Sutera *et al.*, 1999).

### 2.3.2.5. SINGLE-STRANDED DNA BINDING PROTEIN

Single-stranded DNA-binding protein (SSB) sequesters ssDNA from exonuclease digestion, protecting it until DNA repair can take place (Cox and Keck, 2004). However, gel shift experiments have shown that SSB-coated ssDNA enhances RecJ binding (Han *et al.*, 2006). Thus, it seems that SSB and RecJ form a co-complex with the DNA, RecJ then removes SSB and commences DNA degradation. In *B. fragilis*, BF1571 is annotated as *ssb* and the protein product shares a predicted amino acid identity of 50% with *E. coli* SSB (www.sanger.ac.uk). Figure 2.9 shows an alignment between these two proteins with the important residues marked by stars. SSB is topologically similar to other proteins that bind oligonucleotides and

oligosaccharides since the protein forms an oligomer-binding (OB) fold, the portion of the protein involved in the fold has been underlined in the diagram (Raghunathan *et al.*, 1997). SSB proteins interact to form dimers and then the dimers join to form tetramers, which bind ssDNA. The residues involved in dimer formation are marked with orange stars, while the residues for dimer-dimer interactions are marked with blue stars. *B. fragilis* SSB shows high conservation of these essential residues.

B_frag	...MSV <sup>***</sup> NK <sup>***</sup> VIL <sup>***</sup> IGN <sup>***</sup> VG <sup>***</sup> CDPEVRYLDITGEI <sup>*</sup> AVASFE <sup>*</sup> LAT <sup>**</sup> DRAYTL <sup>*</sup> SN <sup>*</sup> IQV <sup>*</sup> ERTEWH <sup>*</sup> NL <sup>*</sup> V	57
E_coli	MAS <sup>***</sup> GVNK <sup>***</sup> VIL <sup>***</sup> GNL <sup>***</sup> SDPEVRYMENGSAVANI <sup>*</sup> LAT <sup>**</sup> SHSWREKA <sup>*</sup> QEMK <sup>*</sup> .EQTEWH <sup>*</sup> EW	59
B_frag	<u>LRSLAE<sup>*</sup>AEKYVHK<sup>*</sup>GDKL<sup>*</sup>WECK<sup>*</sup>IRTRSY<sup>*</sup>DDCGAKR<sup>*</sup>WTECFW<sup>*</sup>...DNMEMLTPKGT<sup>*</sup>C</u>	114
E_coli	<u>LPGLAE<sup>*</sup>VASEYL<sup>*</sup>RKGSQV<sup>*</sup>LEGCI<sup>*</sup>RTRKW<sup>*</sup>.DCSCQLRY<sup>*</sup>IT<sup>*</sup>EW<sup>*</sup>VNVGGITMQLGGROG<sup>*</sup>C</u>	119
B_frag	SGSYAPAQQCAA <sup>*</sup> AFV.....R <sup>*</sup> PC <sup>*</sup> SD <sup>*</sup> EC <sup>*</sup> Q <sup>*</sup> EWSSQD <sup>*</sup> NSAD <sup>*</sup> DL <sup>*</sup> .F	161
E_coli	GAPACCNIGGG <sup>*</sup> CFGGNGQPQQ <sup>*</sup> PGGGN <sup>*</sup> QFSGGAQ <sup>*</sup> SR <sup>*</sup> PC <sup>*</sup> SS <sup>*</sup> FA <sup>*</sup> AF <sup>*</sup> SN <sup>*</sup> EP <sup>*</sup> MF <sup>*</sup> ED <sup>*</sup> DC <sup>*</sup> IF	177

**Figure 2.9: SSB alignment**

E\_coli: *E. coli*; B\_frag: *B. fragilis*. Shading of amino acids: Black-identical, dark grey-highly similar, light grey-similar. (\*)-ssDNA binding residues; (°)-dimer interface residues; (\*)-tetramer interface residues; blue line, OB fold (Marchler-Bauer and Bryant, 2004; Raghunathan *et al.*, 1997).

Thus, the bioinformatics analysis carried out in this study confirms that *B. fragilis* contains a RecFOR pathway equivalent to the pathway found in *E. coli*.

### 2.3.3. RecBCD PATHWAY

#### 2.3.3.1. RecB

RecBCD is involved in repairing dsDNA breaks (Singleton *et al.*, 2004). The role of RecB is to unwind and cleave the DNA thus, it is a member of SF1 helicases (Singleton *et al.*, 2004), and contains ATP-binding domains, helicase domains and nuclease domains (Zuñiga-Castillo *et al.*, 2004). Five of these domains are signature motifs of SF1 helicases and they are numbered I, Ia, II, V and VI (Seki *et al.*, 1994). Cerdeño-Tárraga *et al.* (2005 supplementary material) stated that a conventional RecB homologue had not been identified in the *B. fragilis* NCTC 9343 genome, however, the authors reported that BF2192 was a RexA homologue with a proposed similar helicase function to RecB (see section 2.3.3.3 below). In this study, BLAST analysis identified BF2192 as a putative RecB-encoding homologue (Altschul *et al.*, 1997). The putative *B. fragilis* RecB shows 21% predicted amino acid identity to the *E. coli* RecB protein which is due to the high conservation of the ATP-binding and helicase motifs

shown in Table 2.3. However, there was low sequence similarity between the C-terminal nuclease domains of *B. fragilis* RecB and *E. coli* RecB pointing to the possibility that the *B. fragilis* homologue can only function as a helicase (Table 2.3), and may not be a functional RecB protein.

Table 2.3: ATP-binding, helicase and nuclease domain sequence alignments of *E. coli* RecB and *B. fragilis* RecB

Domain	Motif	Species	Residues	Alignment
ATP-binding	I	<i>E. coli</i>	20-39	LTEASAGGKTFTEAALYLF
		<i>B. fragilis</i>	6-25	VYKASAGSGKTFTEAVEYIK
	II	<i>E. coli</i>	381-497	AMIDEFQDTDFQKPIF
		<i>B. fragilis</i>	379-395	VMIDEFQDTDFPQNDNF
Helicase	Ia	<i>E. coli</i>	57-75	EEILAVTFTAAATAELRGRIEFSNI
		<i>B. fragilis</i>	35-51	RQQLAVTFTKATAPKERILSQL
	-	<i>E. coli</i>	409-430	ELLGDPKQAIYAFRGADIFTY
		<i>B. fragilis</i>	419-440	SLIVGDVKEIYRMRNGDMGIL
	-	<i>E. coli</i>	550-562	RAKIDFVLYRSRQ
<i>B. fragilis</i>		551-563	KLMDIAFLYRKNK	
V	<i>E. coli</i>	734-752	HLVQIVTIIHKSGLFVPTV	
	<i>B. fragilis</i>	722-740	EGRIIEEIIHKSGLFPHIV	
VI	<i>E. coli</i>	793-808	ERLAEIDNLLYVALTR	
	<i>B. fragilis</i>	794-811	QLMVDNINLLYVAFTR	
Nuclease	-	<i>E. coli</i>	1061-1083	KGPELDLVFRHEEYLLDVKSN
		<i>B. fragilis</i>	971-992	VQEWYSGEWPLFNEKAIYRDK
	-	<i>E. coli</i>	1105-1118	EVDTQVQLVILAF
		<i>B. fragilis</i>	1022-1034	QVHKQVGGYMLLI

Shading of amino acids: Black-identical, dark grey-highly similar, light grey-similar (Zuñiga-Castillo *et al.*, 2004; Seki *et al.*, 1994).

Therefore, either the putative *B. fragilis* RecC or RecD proteins need to provide a nuclease domain, or the RecBCD pathway might be replaced in *B. fragilis* by the AddAB (Zuñiga-Castillo *et al.*, 2004) or RexAB pathways (Quiberoni *et al.*, 2001b), which have been found in other bacteria. These possibilities are discussed further as described below.

### 2.3.3.2. RecC AND RecD

In the RecBCD pathway, RecC splits the helix across its 'Pin' structure (to channel the strands through the holoenzyme) and recognises Chi, and RecD is a 5'-3' helicase (Handa *et al.*, 2005). In this investigation an *E. coli* RecC homologue could not be identified in the *B. fragilis* genome; nonetheless, BF2236 was identified as encoding a putative *E. coli* RecD homologue through BLAST analysis (Altschul *et al.*, 1997). However, a multiple sequence alignment between these two proteins showed that only four of the six described helicase motifs (Seki *et al.*, 1994) were conserved between the BF2236 protein product and *E. coli* RecD (Table 2.4). Thus, the putative *B. fragilis* RecD may not be involved in DNA repair and might have an unrelated helicase function.

**Table 2.4: Conserved helicase motifs between *E. coli* RecD and *B. fragilis* BF2236**

Species	Motif	Residues	Alignment
<i>E. coli</i>	I	167-185	LSVTSGGSGTGKTTIVRFL
<i>B. fragilis</i>		20-39	SLLEFSGSGTGKTTLRFLL
<i>E. coli</i>	Ia	199-212	IPTEAPTGKPAFARI
<i>B. fragilis</i>		46-59	MSVVAPTGKPAATNA
<i>E. coli</i>	II	162-182	HEQVILVDEKSMIDLMMSELI
<i>B. fragilis</i>		153-175	REGLVVIDEISMRADCLDAID
<i>E. coli</i>	V	534-551	WARTVHKSQGSSECHAAE
<i>B. fragilis</i>		366-385	WARTVHKSQGLTSENALI

Shading of amino acids: Black-identical, dark grey-highly similar, light grey-similar (Tancja *et al.*, 2002; Seki *et al.*, 1994).

Cerdeño-Lárraga *et al.* (2005) noted that another *B. fragilis* ORF (BF0679) encoded a putative RecD. They proposed that this RecD homologue might form a complex with a putative RexA protein to carry out dsDNA break repair. In this study, bioinformatic analysis between the putative *B. fragilis* RecD and the *E. coli* RecD showed that all the signature helicase motifs were conserved in the BF0679 protein product (Table 2.5). The proteins also shared a 28% predicted amino acid identity (Altschul *et al.*, 1997). Therefore, it is likely that BF0679 is a helicase in *B. fragilis* but experimental analysis is required to investigate whether it is involved in DNA repair.

Table 2.5: Conserved helicase motifs between *E. coli* RecD and *B. fragilis* BF0679

Species	Motif	Residues	Alignment
<i>E. coli</i>	I	167-185	TSVLSGGFGTGKTTVAAPL
<i>B. fragilis</i>		41-59	VEVLRGEAGTGKTSVWGLV
<i>E. coli</i>	Ia	199-212	ISLAAPTGAAARL
<i>B. fragilis</i>		70-83	SVLLAPTGRAAKVF
<i>E. coli</i>	II	162-182	HI DVIWVDEASMIELFMSPLI
<i>B. fragilis</i>		119-139	TRFDYLVDEASMISNEGLISGN
<i>E. coli</i>	V	534-551	WAMVWPKSGGSEFDHAAL
<i>B. fragilis</i>		415-432	YPMVWPKSGGQGWQVFL
<i>E. coli</i>	VI	559-573	EVVTFEAWYTAETRA
<i>B. fragilis</i>		443-457	TEVTEAWLYTAETRA

Shading of amino acids: Black-identical, dark grey-highly similar, light grey-similar (Taneja *et al.*, 2002; Seki *et al.*, 1994).

### 2.3.3.3. RexA

*B. fragilis* BF2192 has been annotated as a *rexA* homologue (Cerdeño-Tarraga *et al.*, 2005; B. Blakely, personal communication) as opposed to our identification of the ORF as a *recB* homologue. In *L. lactis*, the RecBCD pathway is replaced by RexAB. In this alternative pathway, RexA unwinds the DNA and both RexA and RexB display nuclease activity and degrade the DNA (Quiberoni *et al.*, 2001b). When RexAB encounters Chi, RexA nuclease activity is inactivated but RexB continues degrading the DNA, generating a ssDNA overhang for RecA loading. RexAB homologues have been identified in some gram-positive bacteria and in the gram-negative bacterium *P. gingivalis* (Quiberoni *et al.*, 2001a). *L. lactis* RexA and the BF2192 protein product share a predicted amino acid identity of 22% (Altschul *et al.*, 1997) and Table 2.6 shows the high sequence conservation between their ATP-binding and helicase motifs. The RexA nuclease consensus sequence is G(II)Dx<sub>12</sub>DYK(1D), and Figure 2.10 shows the possible location of this motif in the putative *B. fragilis* RexA (Quiberoni *et al.*, 2001a).

**Table 2.6: ATP-binding and helicases domain sequence alignments of *L. lactis* RexA and *B. fragilis* RexA**

Domain	Motif	Species	Residues	Alignment
ATP-binding	I	<i>L. lactis</i>	22-40	LVFASAGSGKIPFVLAGIVE
		<i>B. fragilis</i>	6-25	VVFASAGSGKIPFLAVEYII
	II	<i>L. lactis</i>	370-385	VRIDEVCDTSHTQERN
		<i>B. fragilis</i>	379-395	VRIDEFCDTSHTQERN
Helicase	Ia	<i>L. lactis</i>	50-72	RLFISLTFETRNAAVSGRMFLERNK
		<i>B. fragilis</i>	35-58	RQQLAVTFTVKATAMZERDLSLY
	-	<i>L. lactis</i>	395-415	LFVVGGRQSIYVFRDEGL
		<i>B. fragilis</i>	419-440	SLVVGGRQSIYVFRDEGL
	-	<i>L. lactis</i>	531-543	EPEDLAILVPSK
		<i>B. fragilis</i>	551-563	KRMDIATLVRER
V	<i>L. lactis</i>	743-761	NAGRWITFHRSRGLKFDYV	
	<i>B. fragilis</i>	722-740	ERLRFVSIHRSRGLKFDIV	
VI	<i>L. lactis</i>	824-841	TFALSEEHPVLYVAFTRA	
	<i>B. fragilis</i>	794-811	SLQLVQNLNLLYVAFTRA	

Shading of amino acids: Black-identical, dark grey-highly similar, light grey-similar (Zuñiga-Castillo *et al.*, 2004; Seki *et al.*, 1994).

B_frag	.....GLESKEAEQIIRSLVVKAF	965
L_lac	TLFDTEFGQFLSENYQETVKEAIFSHLRTOEFAKECYLVVGLDQGFVKLADKILP...	1115
B_frag	SLIEVQZMYSGEWRLFNECALINSKDKGVLQIRPFRVMEKNEQVWYDFKFGKANKPYS	1004
L_lac	.....PKDRFTNVKAIK.....IETPK	1104

**Figure 2.10: RexA nuclease domain alignment**

B\_frag: *B. fragilis*; L\_lac: *L. lactis*. Shading of amino acids: Black-identical, dark grey-highly similar, light grey-similar (Quiberoni *et al.*, 2001a).

The ATP-binding domains, helicase and nuclease motifs show slightly higher identity between the putative *B. fragilis* RexA and the *L. lactis* RexA than they do with the *E. coli* RecB. Thus, it seems possible that RecBCD may be replaced by the RexAB pathway in *B. fragilis*.

### 2.3.3.4. RexB

To locate the putative RexB homologue in *B. fragilis*, the gene upstream of BF2192 was investigated since *rexA* and *rexB* form an operon in *L. lactis* (G. Blakely, personal communication; Quiberoni *et al.*, 2001a). The BF2191 protein product was annotated as a hypothetical protein but a sequence alignment between RexB and this protein (Figure 2.11) revealed the presence of the RexB nuclease signature motif G(RI)DR(1)DX<sub>9-12</sub>(V)DYKS(S) (Quiberoni *et al.*, 2001a). *L. lactococcus* RexB and the putative *B. fragilis* RexB share 19% predicted amino acid identity (Altschul *et al.*, 1997).

B_frag	...VLPQLLPNDLCTAPPFMMHGRHQEVVEDIRLEGVYGLSLSLIEG...DRDMDKKEGTLRLV...	832
L_lact	...DLTESSCFELFK.....ELGMEFQWDE...G...DRDLDLSTD...YLG	907
B_frag	...DYKST.....GCSPKYPPANIKQLSLPAE.....GPNYIDITFLYR	867
L_lact	...ADYKESRANSEFALQEAAYDQLSL.....QFMVYLDWENLAPPNKEIWCALYLOEKNOPEM	961

**Figure 2.11: RexB nuclease domain alignment**

B\_frag: *B. fragilis*; L\_lact: *L. lactis*. Shading of amino acids: Black-identical, dark grey-highly similar, light grey-similar (Quiberoni *et al.*, 2001a).

Since the RecBCD pathway cannot be fully identified in the *B. fragilis* genome, it seems that RexAB may replace RecBCD for the repair of dsDNA breaks in *B. fragilis* (G. Blakely, personal communication).

### 2.3.4. LexA AND THE SOS RESPONSE

In certain gram-negative bacteria, such as *E. coli*, proteins involved in the SOS response contain an SOS box [5'-CTGT-(AT)<sub>4</sub>-ACAG-3'] in their promoter regions (Winterling *et al.*, 1998). This SOS box is the regulatory region where the LexA repressor protein binds (Kuzminov, 1999). Upon induction of the SOS response, RecA alleviates LexA repression by promoting LexA autocleavage and gene expression of LexA-regulated genes occurs (Kuzminov, 1999). In gram-positive bacteria, the SOS box [5'-GAAC-N<sub>4</sub>-GTTC-3'] contains no homology to the gram-negative regulatory sequence (Winterling *et al.*, 1998). This could be due to the LexA of gram-positive cocci and bacilli having a different DNA-terminal binding domain to the traditional gram-negative LexA. Winterling (1998) analysed the *B.*

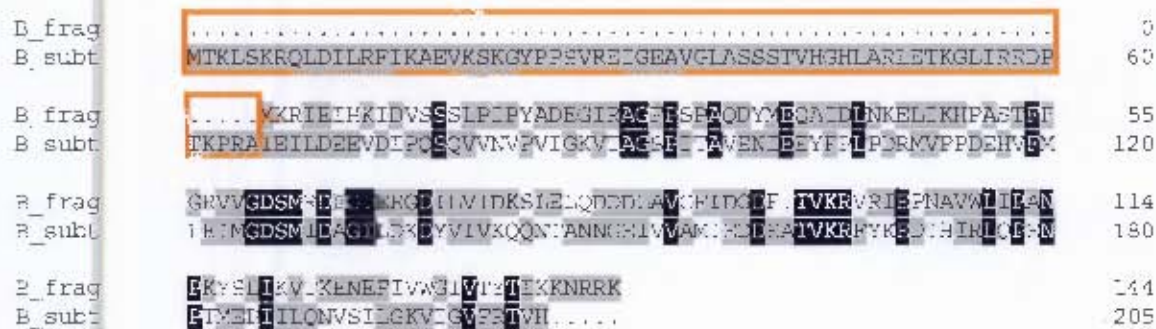
*fragilis recA* promoter region and identified two putative gram-positive-type SOS boxes, indicating potential gram-positive LexA-like SOS induction.

In the study reported here, BLAST searches of the *B. fragilis* genome using both the *E. coli* LexA and the *B. subtilis* LexA identified the protein product of BF1929 as a possible LexA candidate (Altschul *et al.*, 1997). The *E. coli* LexA shares 33% identity while the *B. subtilis* LexA shares 27% identity with this protein. However, the sequence alignments show that the LexA DNA binding domain is not present on the BF1929 protein product, hence this protein could, most likely, not function as a repressor of the SOS response (Figure 2.12 and Figure 2.13) (Fogh *et al.*, 1994). Thus, another as yet unidentified *B. fragilis* protein might function as LexA.



**Figure 2.12: *E. coli* LexA alignment**

B\_frag: *B. fragilis*; E\_coli: *E. coli*. Shading: Black-identical, dark grey-highly similar, light grey-similar. Orange box, DNA binding region (Marchler-Bauer *et al.*, 2007).



**Figure 2.13: *B. subtilis* LexA alignment**

B\_frag: *B. fragilis*; B\_subt: *B. subtilis*. Shading: Black-identical, dark grey-highly similar, light grey-similar. Orange box, DNA binding region (Marchler-Bauer *et al.*, 2007).

The protein product of BF1292 has been annotated as a putative UmuD/RumA DNA repair protein. Both UmuD (Hare *et al.*, 2006) and LexA (Kuzminov, 1999) undergo RecA-assisted autocleavage, thus they contain related peptidase domains. Figure 2.14 shows the high conservation between the BF1292 protein product and *E. coli* UmuD and these proteins share 40% identity (Altschul *et al.*, 1997). It, therefore, seems likely that BF1292 is a putative UmuD with similarity to LexA because of their related peptidase domains. Thus, there is no bioinformatic evidence for a *B. fragilis* LexA-like protein. Functional studies would be needed to confirm this conclusion.



**Figure 2.14: UmuD alignment**

B\_frag: *B. fragilis* UmuD; E\_coli: *E. coli* UmuD. Shading: Black-identical, dark grey-highly similar, light grey-similar.

## 2.4. CONCLUSION

Bioinformatic analysis was used to study RecA in the context of DNA repair homologues that might function together with RecA to repair strand breaks (Table 2.7). In *B. fragilis*, RecxAB seems to have replaced the *E. coli* RecBCD complex. RecA is indispensable to the RecFOR and RecxAB pathways. Bioinformatic analysis of *B. fragilis* RecA (BF1180) revealed that it shared a predicted amino acid identity of 93%, 62% and 81% identity with *B. thetaiotaomicron*, *E. coli* and *P. gingivalis* RecA sequences respectively. The BF1180 protein product also contained conserved Walker A and B motifs and other regions and amino acids necessary for RecA function. Three putative *B. fragilis* RecQ proteins were confirmed, which differs to other bacteria since they only have two RecQ proteins at most (Nakayama, 2002). *B. fragilis* RecQ3 could be the major RecQ helicase in the cell since it seems to form an operon with the putative RecJ. This could allow for concurrent transcription of *recQ3* and *recJ* in response to DNA damage. SSB has been annotated in the *B. fragilis* genome and it shows high conservation of the important functional amino acids. LexA could not be identified suggesting that *B. fragilis* either has a structurally different SOS regulator, or gene expression in response to DNA damage is regulated by a novel mechanism.

**Table 2.7: Candidate enzymes involved in DNA repair in *B. fragilis* NCTC 9343 (Accession no. NC\_003228)**

Name	ORF No.	Putative Function
RecA	BF1180	SOS derepressor, Homologous recombination
RecF	BF0877	Mediates RecA nucleofilament formation with RecO and RecR
RecO	BF0248	Mediates RecA nucleofilament formation with RecF and RecR
RecR	BF2106	Mediates RecA nucleofilament formation with RecF and RecO
RecQ1	BF3892	Helicase with 3'-5' directionality
RecQ2	BF3249	
RecQ3	BF3706	
RecJ	BF3705	5'-3' ssDNA exonuclease
SSB	BF1571	Binds ssDNA
RecB	BF2192	Nuclease with 3'-5' helicase activity
RecC	-	Recognizes Chi
RecD	BF0679	Helicase with 5'-3' unwinding activity
RexA	BF2192	Helicase and nuclease
RexB	BF2191	Nuclease
LexA	-	Regulation of the SOS response

(-), not identified. (Han *et al.*, 2006; Kuzminov, 1999; Leiros *et al.*, 2005; Mizrahi and Anderson, 1998; Quiberoni *et al.*, 2001a ; Singleton *et al.*, 2004).

Bioinformatic analysis only provides a preliminary indication of the possible function of putative proteins. Consequently, functional characterisation is needed to understand the true molecular properties of these proteins *in vivo*. Since RecA is the key DNA repair protein, mutation of the *recA* gene and analysis of the mutant phenotype would give fundamentally important insights into DNA repair in *B. fragilis*. This work is described in Chapters 3 and 4 of this thesis.

**CHAPTER 3**  
**MOLECULAR CHARACTERISATION OF *BACTEROIDES***  
***FRAGILIS recA***

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### *Abstract*

Transcriptional studies revealed that *B. fragilis recA* was transcribed as part of an operon. The other two genes that formed the operon with *recA* coded for proteins which could be involved with oxidative stress responses in the cell. Thus, the arrangement of these genes with *recA* could allow for merged antioxidant and DNA repair processes in *B. fragilis*. To assess the role of RecA with regards to DNA damage in *B. fragilis*, a *recA*<sup>-</sup> mutant was generated using targeted insertional inactivation using the suicide vector pGERM. The presence of the insertion in the chromosomal copy of the gene was confirmed with PCR, sequencing and Southern hybridisation and the mutation was found to be stable in the presence or absence of antibiotic selection.

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### 3.1. INTRODUCTION

RecA has been extensively characterised in *E. coli*, but in *B. fragilis*, further work needs to be carried out to understand fully how RecA functions in this bacterium. Prior to the publication of the sequenced *B. fragilis* genome, random mutagenesis and the analysis of gene banks were used to identify genes coding for potential DNA repair proteins. A *recA* gene was identified from a *B. fragilis* Bf-1 gene bank transformed into *E. coli* HB101 (*recA*<sup>-</sup>) and screened by testing for resistance to methyl methanesulfonate (Goodman *et al.*, 1987). One resistant *E. coli* transformant was identified and this clone was also seen to have increased resistance to ultraviolet (UV) irradiation and an increased frequency of recombination. Functional characterisation of *B. fragilis* RecA with respect to DNA damage in the host bacterium is also of interest. In order to do this it is necessary to create a stable *recA*<sup>-</sup> mutant.

Random mutagenesis involving chemical mutagens, UV irradiation or transposons have been used to generate *Bacteroides* mutants (Salyers *et al.*, 2000). However, these processes require extensive screening to identify interesting mutants and subsequently, the presence of multiple mutations or transposon insertions must be verified. New molecular techniques for targeted disruptions have simplified the generation of useful mutations in *Bacteroides* spp. In this study, insertional inactivation of the *B. fragilis* 638R *recA* gene was performed using the suicide vector pGERM (Salyers *et al.*, 2000) with a view to disrupting transcription of the *recA* gene resulting in a *recA*<sup>-</sup> mutant strain. *B. fragilis* 638R was used since it is particularly amenable to genetic manipulation.

The *recA* gene has been shown to form part of an operon in bacteria like *D. radiodurans* (Bonacossa de Almeida *et al.*, 2002) and *Mycobacterium smegmatis* (Vierling *et al.*, 2000). Bioinformatic analysis of the genes surrounding *B. fragilis* *recA* revealed that it could also form part of an operon (Chapter 2). The upstream genes coded for a hypothetical protein with a homospermidine synthase domain, and a thiol-specific antioxidant enzyme (TSA). Both these proteins could play a role in protecting *B. fragilis* cells from oxidative stress which damages cells through inactivating enzymes and generating DNA strand breaks (Miyoshi *et al.*, 2003). The combination of these genes in an operon with *recA* could be intended to fuse anti-oxidant and DNA repair functions for enhanced cell survival. It is of interest to determine if the gene cluster is, in fact, transcribed as an operon. Preliminary studies by C. J. Smith (personal communication) indicated that *B. fragilis* *recA* was possibly transcribed as part of an operon with these upstream genes.

## 3.2. MATERIALS AND METHODS

### 3.2.1. BACTERIAL STRAINS AND PLASMIDS

The strains and plasmids used are described in Table 3.1.

**Table 3.1: Description of bacterial strains and plasmids**

Strain/plasmid	Genotype/phenotype	Source/Reference
<b>Plasmids</b>		
pGERM	pUC19-based suicide vector	Shoemaker <i>et al.</i> , 2000
pGREC	pGERM containing <i>recA</i> internal fragment	This study
pLYL01	Mob <sup>-</sup> , Tet <sup>R</sup> Amp <sup>R</sup>	Li <i>et al.</i> , 1995
pLYLrecA	pLYL01 derivative containing <i>B. fragilis recA</i>	This study
<b>Strains</b>		
<i>Bacteroides fragilis</i>		
638R	Clinical strain, Rif <sup>R</sup> Gent <sup>R</sup>	Privitera <i>et al.</i> , 1979
638R <i>recA</i> <sup>-</sup>	638R derivative, <i>recA</i> <sup>-</sup> , Rif <sup>R</sup> Gent <sup>R</sup> Erm <sup>R</sup>	This study
<i>Escherichia coli</i>		
S17-1	RP4-2-Tc::Mu <i>aph</i> ::Tn7 <i>recA</i> <sup>-</sup> , Strep <sup>R</sup>	Simon <i>et al.</i> , 1983
S17-1 pGREC	RP4-2-Tc::Mu <i>aph</i> ::Tn7 <i>recA</i> <sup>-</sup> , Strep <sup>R</sup> Amp <sup>R</sup>	This study

Rif, rifampicin; Gent, gentamicin; Erm, erythromycin; Strep, streptomycin; Amp, ampicillin; Mob, mobilisation.

### 3.2.2. MEDIA AND GROWTH CONDITIONS

*E. coli* strains were grown in Luria-Bertani (LB) broth or on LB plates under aerobic conditions at 37°C (Maniatis *et al.*, 1982). *E. coli* cells harbouring plasmids were grown in LB supplemented with 100 µg/ml ampicillin (LB+amp). Brain heart infusion (BHI) agar medium was supplemented with 5 µg/ml haemin-menadione, 0.5 µg/ml cysteine and 1 µg/ml Na<sub>2</sub>CO<sub>3</sub> (BHISA) while BHI broth was supplemented with 5 µg/ml haemin-menadione and 4 µg/ml Na<sub>2</sub>CO<sub>3</sub> (BHISB). *B. fragilis* 638R strains were grown in BHISB or on BHISA at 37°C under anaerobic conditions (Holdeman and Moore, 1972). *B. fragilis* mutants were grown on BHISA including erythromycin at a concentration of 10 µg/ml (BHISA+erm).

### 3.2.3. TRANSCRIPTIONAL ANALYSIS OF *recA* AND UPSTREAM GENES

RNA was isolated from *B. fragilis* cells grown in BHISB to log phase. Log phase cells were also exposed to metronidazole (Mtz) (1 µg/ml) for one hour. RNA was extracted as previously described (Aiba *et al.*, 1981) except that phenol (pH 4.0) was used. Contaminating DNA was removed by DnaseI (Roche) treatment. Direct PCR of the unconverted RNA was carried out to confirm that contaminating DNA had been totally removed. The synthesis of the cDNA molecules was carried out using the First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Random primers were used for first strand cDNA amplification of RNA transcripts, followed by amplification of specific target genes using gene specific primers. GeneAmp PCR System 9700 was used for the cycling reactions (Perkin Elmer, Applied Biosystems). The PCR parameters were: initial denaturation of 95°C for 5 min, then 25 cycles of denaturation at 95°C for 30 sec, annealing at 53.8°C for 30 sec and elongation at 72°C for 3 min. A final elongation step was carried out at 72°C for 5 min.

Primers pairs HIF-HIR, BIF-BIR and RIF-RIR were used to generate internal PCR fragments of BF638R1248, BF638R1246 and BF638R1245 respectively (Table 3.2). To generate PCR fragments of intergenic regions, primers pairs FBRT-RBRT were used for BF638R1248 and BF638R1246 and FRA-RART were used for BF638R1246 and BF638R1245 (Table 3.2). PCR products were analysed with agarose electrophoresis using λ DNA (digested with *Pst*I) as a molecular size marker.

**Table 3.2: Description of PCR primers**

Name	Primer	Description
FRA	5'-GTA AAG <u>CTG CAG</u> ATG AAG TGA TCG-3' ( <i>Pst</i> I)	FRA and RRA amplify the full length <i>B. fragilis recA</i> BF638R1245 gene. Restriction enzyme sites (in brackets) are underlined.
RRA	5'-GGG <u>CAT GCC</u> TAT CGA GTT GG-3' ( <i>Sph</i> I)	
RIF	5'-CAG GTT CGA TAG CAC TGA ATG CTG C-3'	RIF and RIR amplify an internal fragment of <i>B. fragilis recA</i> BF638R1245.
RIR	5'-CGG ATT ACC GAA CAT TAC ACC G-3'	
HIF	5'-GTG AAA GCC ATC GGC AAT CCC-3'	HIF and HIR amplify an internal fragment of <i>B. fragilis</i> BF638R1248.
HIR	5'-CGA ATG TCA TCC AGA AAC GTG C-3'	
BIF	5'-GAG ATA AAG CCC CAG AAC TGC-3'	BIF and BIR amplify an internal fragment of <i>B. fragilis</i> BF638R1246.
BIR	5'-GAT GAT CCG TTC GAT AAC TCC C-3'	
FBRT	5'-CCG GCT ATG ATC GGT GCC-3'	FBRT and RBRT amplify the intergenic region between BF638R1248 and BF638R1246.
RBRT	5'-CGG CTT TAC GTA GCT CTG CG-3'	
RART	5'-CGT GGA TGG CCA GTG TCG-3'	FRA and RART amplify the intergenic region between BF638R1246 and BF638R1245.
M13F	5'-CGC CAG GGT TTT CCC AGT CAC GAC-3'	M13F and M13R in combination with gene specific primers allow verification of mutation in <i>B. fragilis</i> 638R <i>recA</i> (Yanisch-Perron <i>et al.</i> , 1985).
M13R	5'-GAG CGG ATA ACA ATT TCA CAC AGG-3'	

#### 3.2.4. DNA TECHNIQUES

*B. fragilis* genomic DNA was extracted using the high-salt buffer total DNA extraction method of Campbell and Yasbin (1984). All cloning reagents (enzymes, T4 polymerase and T4 ligase) were purchased from Fermentas. Plasmids were transformed into electrocompetent *E. coli* cells using electroporation (Tung and Chow, 1995). Electroporation was carried out with the Gene pulser apparatus (Bio-Rad laboratories, Richmond, Calif.) at 2.5kv, 200Ω and 25μF.

### 3.2.5. INSERTIONAL INACTIVATION OF BF638R1245

A *recA* internal fragment was obtained by PCR using primer pair RIF-RIR specific for BF638R1245 (Table 3.2). A GeneAmp PCR System 9700 was used for PCR reactions (Perkin Elmer, Applied Biosystems). The PCR parameters were as described previously except that the annealing temperature was 53°C. Blunt ends were created on the PCR fragment using T4 DNA Polymerase according to standard protocols (Maniatis *et al.*, 1982) and it was cloned into the pGERM *Sma*I site. The resulting plasmid, pGREC, was confirmed with restriction enzyme digests. *E. coli* S17-1 was transformed with plasmid pGREC. Mating of *E. coli* S17-1 and *B. fragilis* was performed as described by Shoemaker *et al.* (2000). Briefly, both strains were grown to an OD<sub>600</sub> of 0.2, the cultures were then mixed at a ratio of 3:1 (*B. fragilis*:*E. coli*) and centrifuged. The pellet was resuspended in 100 µl of LB and inoculated onto HAWP filters (Millipore), placed previously on BHISA plates without cysteine. The filters were incubated at 37°C aerobically overnight. Growth on filters was washed off with sterile, pre-reduced BHIS broth and incubated anaerobically for one hour. Aliquots of the rinses were plated onto BHISA agar containing erythromycin (10 µg/ml) and gentamicin (200 µg/ml). Plates were incubated anaerobically for two days at room temperature (25°C). Single colonies were analysed to confirm the mutation using PCR and primer pairs FRA-M13R and RRA-M13F with the same parameters described previously. The PCR products were sequenced.

### 3.2.6. DNA SEQUENCING

Sequencing was carried out using the fluorescent dideoxy-nucleotide chain termination method developed by Sanger *et al.* (1997) with the Big Dye terminator v3.1 Cycle Sequencing kit. These reactions were carried out according to the manufacturer's instructions and cycle sequenced on a GeneAmp PCR System 9700 (Perkin Elmer, Applied Biosystems). The sequencing reaction products were then analysed on an Applied Biosystems 3130 Genetic Analyser. The final sequences were analysed using BLAST 2.2.17 (Altschul *et al.*, 1997 and Schäffer *et al.*, 2001).

### 3.2.7. SOUTHERN HYBRIDISATION

A total of 5 µg of *B. fragilis* 638R *recA* mutant and wild type chromosomal DNA was digested to completion with *EcoRV*. The control plasmid DNA (5 ng) was digested with *Bam*HI. Electrophoresis of the target DNA and subsequent blotting on a nylon membrane (Amersham Biosciences) and probe hybridisation was carried out as described in the DIG Application Manual (Roche). The *recA* internal fragment (obtained by PCR using the primers RIF-RIR and labelled with DIG using random prime labelling) was used as probe. Membranes were exposed to X-ray Hyperfilm™ (Amersham Biosciences) and visualised by autoradiography.

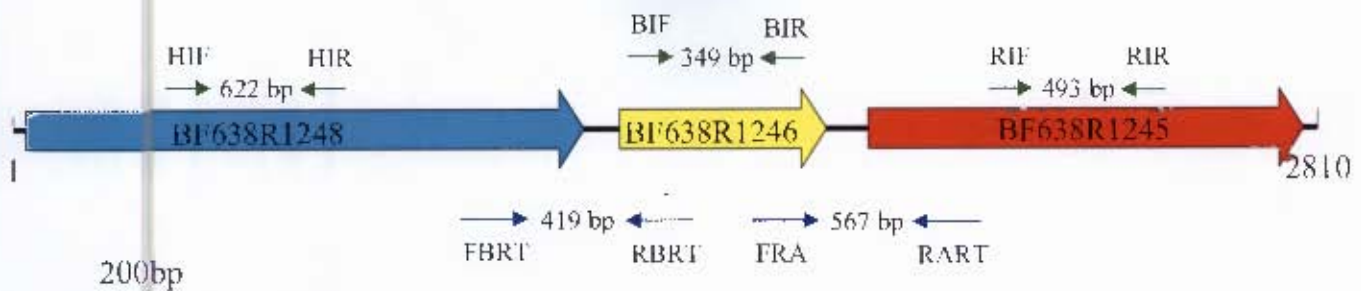
### 3.2.8. ASSESSMENT OF MUTANT STABILITY

*B. fragilis recA* cells were incubated anaerobically for 48 hr on BHISA+erm. Colonies from these plates were inoculated into BHISB with or without erythromycin and sub-cultured into fresh BHISB (under the same conditions) three times. Genomic DNA from cells from each sub-culture was subjected to PCR with primer pairs FRA-RRA, FRA-M13R and RRA-M13F and the parameters described above to verify the presence of pGREC in the chromosome.

## 3.3. RESULTS AND DISCUSSION

### 3.3.1. TRANSCRIPTIONAL ANALYSIS OF *recA* AND ITS UPSTREAM GENES

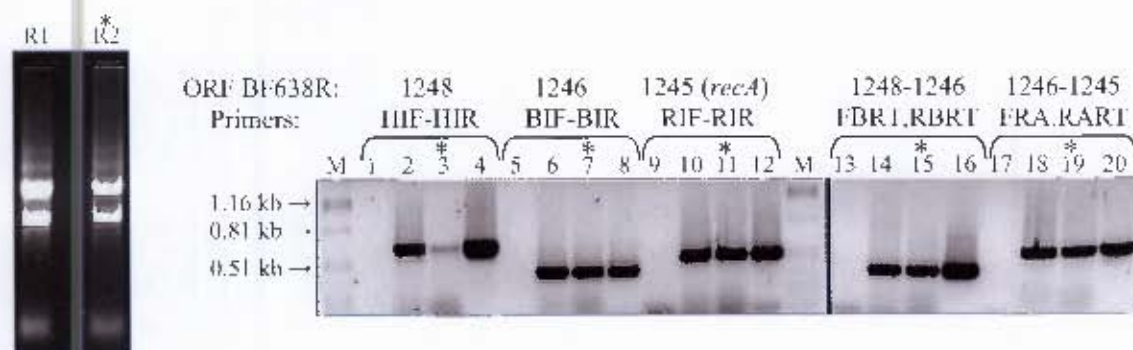
To determine whether BF638R1248, BF638R1246 and BF638R1245 were transcribed as an operon, a reverse-transcriptase PCR (RT-PCR) experiment was carried out. Five primer pairs were designed to amplify various regions of the gene cluster (Figure 3.1). Primer pairs HIF-HIR, BIF-BIR and RIF-RIR would amplify an internal fragment of BF638R1248, BF638R1246 (*tsa*) and BF638R1245 (*recA*) respectively (Figure 3.1). FBRT and RBRT could amplify the intergenic region between BF638R1248 and BF638R1246 while FRA and RART could amplify the intergenic region between *tsa* and *recA*. If FBRT-RBRT and FRA-RART gave a product from cDNA templates, this would indicate that the gene cluster was being transcribed as an operon.



**Figure 3.1: Genetic context of *B. fragilis* 638R *recA* and RT-PCR primer combinations**

The primers shown above are fully described in Table 3.2. Green arrows, primers amplifying internal gene fragments; blue arrows, primers amplifying intergenic regions.

A cDNA conversion was carried out on RNA extracted from log phase cultures. These cultures were exposed to inducing or non-inducing conditions involving exposure or lack of exposure to 1  $\mu$ g/ml Mtz for one hour. Direct PCR was carried out on unconverted RNA and no product was obtained, confirming that contaminating DNA had been removed during the RNA purification (results not shown). Lanes R1 and R2 show the RNA extracted from the cultures grown in the absence or presence of Mtz respectively (Figure 3.2). All the primer pairs described in Figure 3.1 gave a product with wild type genomic DNA, as can be seen in lanes 4, 8, 12, 16 and 20. PCR products of internal fragments were obtained from cDNA template using the primer pairs HIF-HIR, BIF-BIR and RIF-RIR (lanes 2, 3, 6, 7, 10 and 11), thus, all the genes were transcribed under both non-inducing and inducing conditions. Furthermore, FBRT-RBRT and FRA-RART produced a PCR product from the cDNA template (lanes 14, 15, 18 and 19); therefore, this gene cluster is transcribed as an operon. Semi-quantitative or quantitative RT-PCR needs to be carried out in order to clarify whether the operon is induced in the presence of Mtz.



**Figure 3.2: RT-PCR on RNA extracted from induced and non-induced cultures**

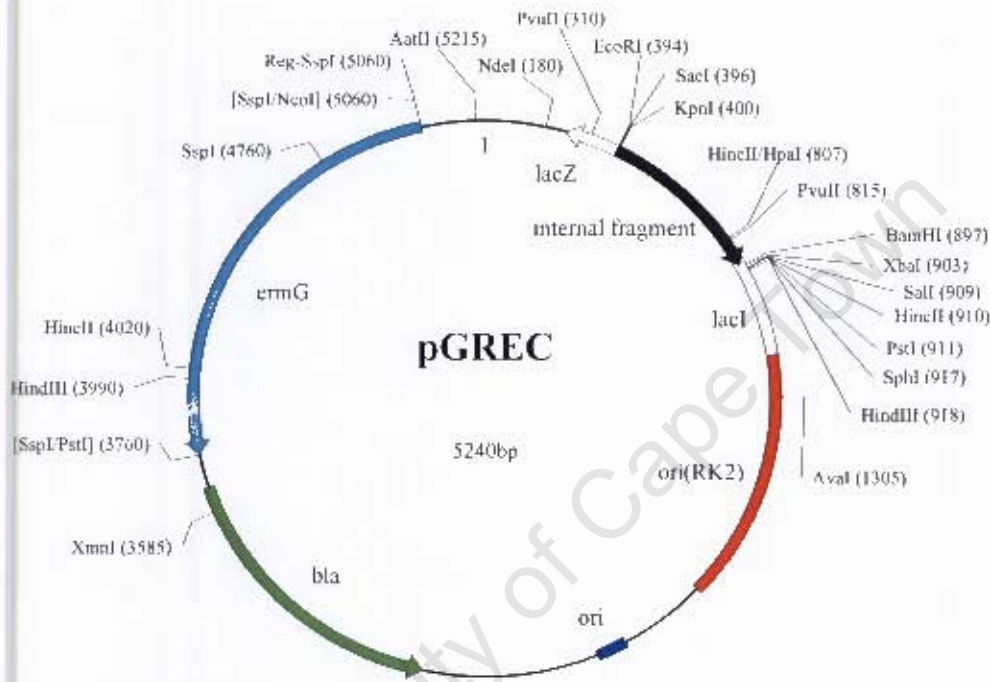
Lane R1, R2, RNA; M, Molecular size marker ( $\lambda$  DNA digested with *Pst*I); 1, 5, 9, 13, 17, no DNA control; 2, 3, 6, 7, 10, 11, 14, 15, 18, 19, cDNA; 4, 8, 12, 16, 20, genomic DNA; \*, metronidazole inducing conditions.

It is not clear why BF638R1248 and BF638R1246 seem to be involved with DNA repair, however, a possible link is the fact that they could be induced by oxidative stress. Both Mtz and oxidative stress cause DNA strand breaks, thus, these types of lesions might induce a DNA damage response from the *recA* operon.

The *recA* gene has been shown to form part of an operon in *D. radiodurans* (Bonacossa de Almeida *et al.*, 2002), *Streptococcus pneumoniae* (Mortier-Barrière *et al.*, 1998), *Streptomyces lividans* and *M. smegmatis* (Vierling *et al.*, 2000). In both *M. smegmatis* and *S. lividans*, *recA* and *recX* are co-transcribed as an operon (Vierling *et al.*, 2000). In *S. lividans* the operon is only transcribed in the presence of DNA damage while *recA* is constitutively expressed at basal levels under non-inducing conditions. This differs to *M. smegmatis* where both genes are expressed jointly at all times. RecX is thought to bind the nucleoprotein filament which leads to disassembly of RecA from the DNA, thus it functions as a negative regulator of RecA activity (Lusetti *et al.*, 2004). Consequently, RecX protects the cell from RecA over-expression toxicity (Vierling *et al.*, 2000). In *E. coli*, *recX* is downstream of *recA*, they are expressed under the same conditions but do not form an operon (Lusetti *et al.*, 2004). Since they are expressed simultaneously, this allows RecX to effectively control RecA-mediated homologous recombination. In *B. fragilis* NCTC 9343, the protein product of BF0454 is annotated as being a putative transcriptional regulator with limited similarity to *Pseudomonas aeruginosa* RecX, however, BF0454 is not clustered with *recA* on the genome. The *recA* gene in *D. radiodurans* forms an operon with *cinA* and *ligT* (Bonacossa de Almeida *et al.*, 2002). While in *S. pneumoniae*, *recA* forms an operon with *cinA*, *dinF* and *lytA* (Mortier-Barrière *et al.*, 1998). The *cinA* gene is a competence-induced and might code for a recombination accessory protein (Bonacossa de Almeida *et al.*, 2002). The arrangement of *cinA* and *recA* may be important for transformation and subsequent recombination of foreign DNA into the bacterial chromosome. The *ligT* gene codes for a 2'-5' DNA ligase (Bonacossa de Almeida *et al.*, 2002), *dinF* codes for a multidrug efflux pump in *Ralstonia solanacearum* (Brown *et al.*, 2007) and *lytA* codes for the pneumococcal autolysin (Mortier-Barrière *et al.*, 1998). The *recA* operon in *B. fragilis* presents a novel operon arrangement since the *B. fragilis recA* gene is clustered with putative oxidative stress response genes.

### 3.3.2. INSERTIONAL INACTIVATION OF BF638R1245 AND GENETIC CONFIRMATION AND ANALYSIS OF THE MUTANT

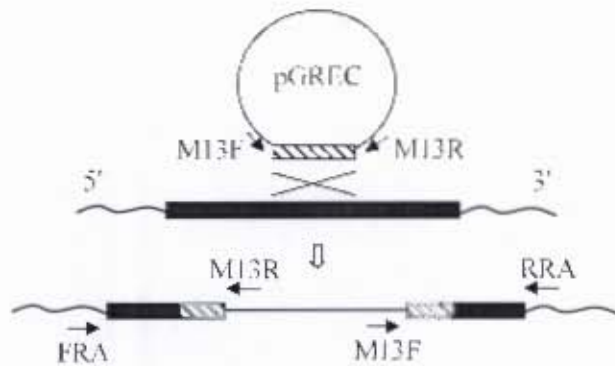
To test the function of *RecA*, a *B. fragilis recA* mutant was generated using targeted gene disruption. An internal fragment from *recA* was cloned into the suicide vector pGERM, to generate pGREC (Figure 3.3).



**Figure 3.3: Map of the pGREC suicide vector used for mutagenesis**

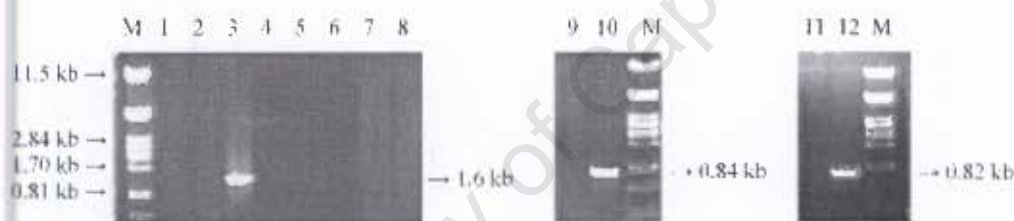
This map of pGREC shows the various genes and fragments essential for the mutagenesis process. The  $\beta$ -lactamase resistance gene (*bla*) and erythromycin resistance gene (*ermG*) allow for selection and maintenance of the plasmid in *E. coli* and *Bacteroides* respectively.

The *recA* internal fragment is homologous to a portion of the *B. fragilis* chromosomal *recA* gene, thus homologous recombination occurs in the recipient allowing the vector to integrate into the DNA, effectively disrupting it and generating a *recA* mutant (Figure 3.4). The assistance of Dr Lynthia Paul is acknowledged in generating the mutant.



**Figure 3.4: Schematic diagram illustrating targeted gene disruption**

The plasmid pGREC is shown with the *recA* internal fragment cross-hatched. Homologous recombination occurs between the chromosomal copy of *recA* (the black box) and the internal fragment (hatched box on pGREC). The internal fragment is duplicated on either side of the insertion. Binding site positions of the M13F and M13R primers (present on pGREC) and of FRA and RRA (on either side of the *recA* gene) are shown.



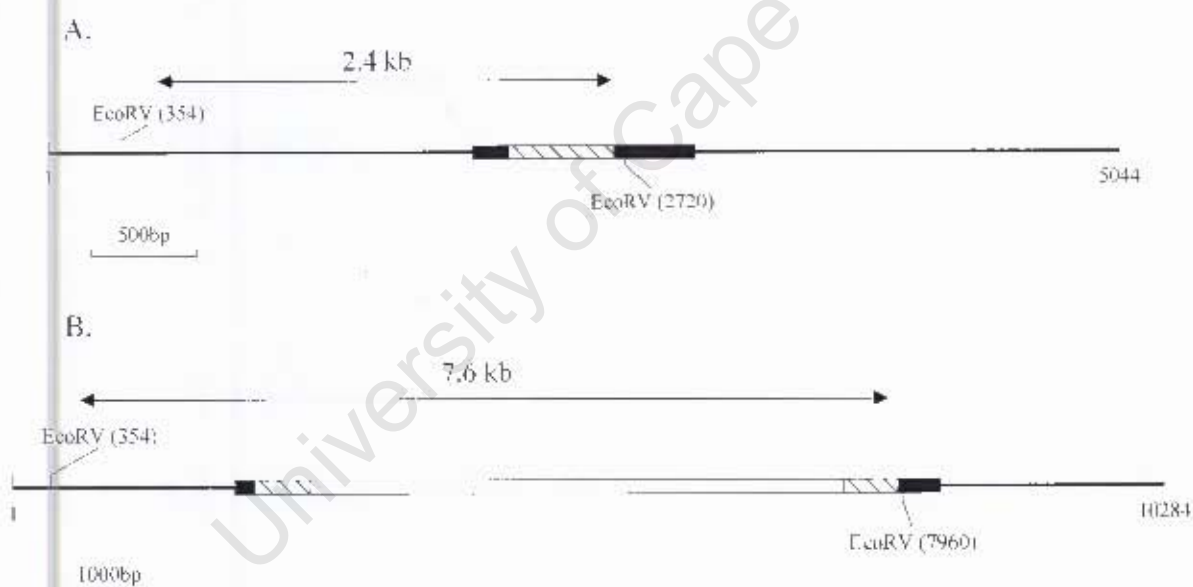
**Figure 3.5: PCR of genomic DNA from the wild-type *B. fragilis* 638R and the *B. fragilis* 638R *recA*<sup>-</sup> mutant**

The PCR products were obtained with the primers indicated. Lane M: Molecular size marker ( $\lambda$  DNA digested with *Pst*I); 1: FRA | no DNA; 2: RRA | no DNA; 3: FRA, RRA + wild type; 4: FRA, RRA + mutant; 5: M13F | no DNA; 6: M13R | no DNA; 7: M13F, M13R - wild type; 8: M13F, M13R + mutant; 9: FRA, M13R + wild type; 10: FRA, M13R + mutant; 11: RRA, M13F - wild type; 12: RRA, M13F + mutant.

PCR was carried out on the transconjugants to verify the mutation. Primers FRA-RRA would generate a 1.6 kb PCR product from wild type DNA and this can be seen in Figure 3.5, lane 3. However, if the disruption had been successful in the prospective mutant, PCR using the same primers on mutant DNA would not produce a 1.6 kb PCR product (size of the wild type *recA* gene) (lane 4). The results in Figure 3.5 show that an 838 bp was produced with primers FRA and M13R (lane 10) and RRA and M13F generated an 818 bp product (lane 12) for the mutant colonies. These primers did not give a product for the wild type genomic DNA as the plasmid DNA was not present in the chromosome (lanes 9 and 11). Thus, the PCR confirmed

the mutation. PCR products obtained from the mutant were also sequenced and the results further confirmed that the product sizes were correct and that the *recA* gene had been insertionally inactivated.

The mutation site was further verified using Southern hybridisation. *B. fragilis* wild type and *recA* mutant genomic DNA was digested to completion with *EcoRV*. The *B. fragilis* 638R genome sequence was used to predict the fragment sizes that would be obtained after digesting the wild type DNA (2.4 kb fragment containing the *recA* gene). From analysing the genome sequence and mapping the insertion of pGREC into the *recA* gene, it was calculated that the *recA* mutant DNA digestion would yield a 7.6 kb fragment containing the *recA* gene since the plasmid insertion added an additional 5.2 kb to the fragment (Figure 3.6).

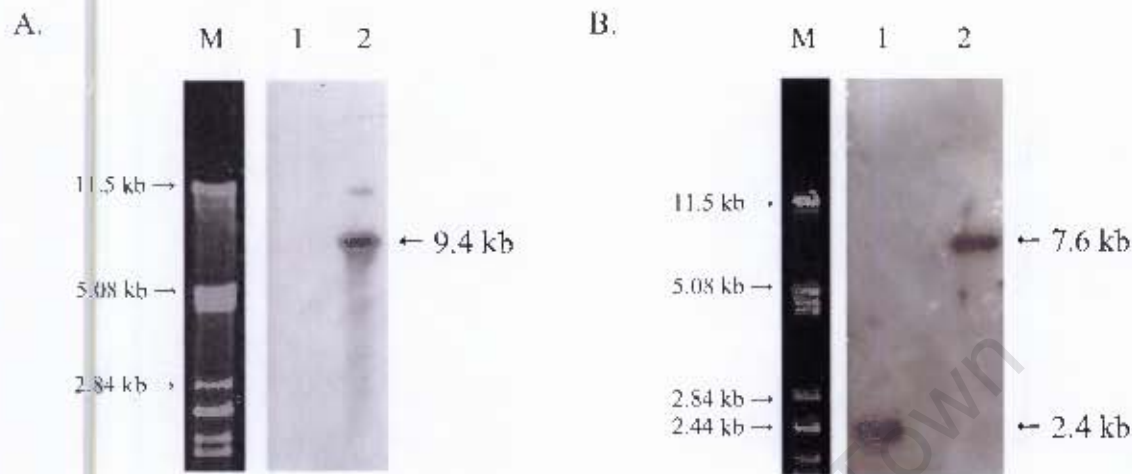


**Figure 3.6: Predicted restriction enzyme map of *B. fragilis* wild type and mutant *recA* genes**

Graphical representation of the *recA* wild type (A) and mutated genes (B). Black bar, *recA* gene; hatched bar, *recA* internal fragment; white bar, pGREC vector.

Southern hybridisation using an internal *recA* fragment confirmed the interruption of the gene with pGREC (Figure 3.7). The probe hybridised to a 2.4 kb fragment of genomic DNA isolated from the wild type (Figure 3.7B, lane 1). In contrast, the probe hybridised to a 7.6 kb *EcoRV* fragment from the mutant genomic DNA due to the integration of the suicide vector

into BF638R1245 (Figure 3.7B, lane 2). The probe was specific for the *recA* gene as can be seen in Figure 3.7A, lanes 1 and 2.



**Figure 3.7: Autoradiograph of genomic DNA from wild type *B. fragilis* and its derived *recA* mutant**

Autoradiographs obtained from Southern blots.

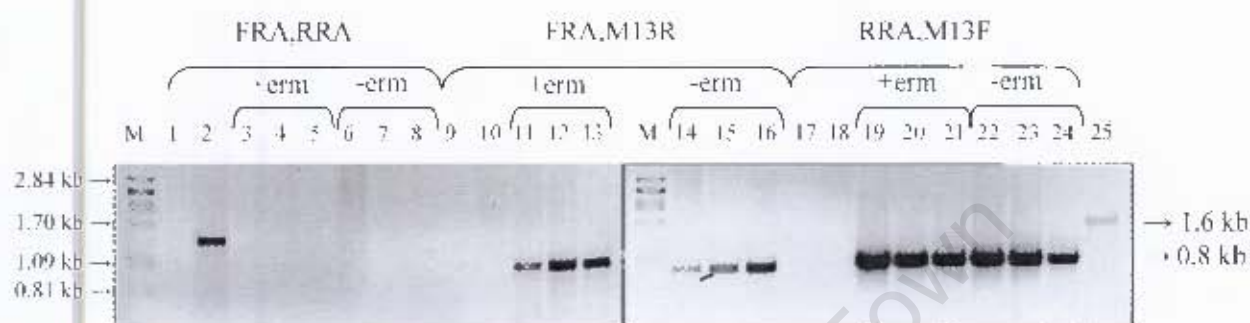
A. Controls. Lane M: Molecular size marker ( $\lambda$  DNA digested with *Pst*I); 1: negative control pLYL01 (digested with *Bam*HI); 2: positive control pLYL*recA* (digested with *Bam*HI).

B. Experimental. Lane M: Molecular size marker ( $\lambda$  DNA digested with *Pst*I); 1: wild type *recA* DNA (digested with *Eco*RV); 2: mutant *recA* DNA (digested with *Eco*RV).

### 3.3.3. ASSESSMENT OF THE STABILITY OF THE *recA* MUTATION

To test the stability of the mutant, this strain was sub-cultured three consecutive times in the presence or absence of erythromycin. The DNA of each culture was tested with PCR to determine if the gene-specific insertion was still present (Figure 3.8). Lanes 1-8 show if PCR reactions with FRA, RRA detected a wild type copy of the *recA* gene. Wild type genomic DNA was used as a positive control and it generated a 1.6 kb fragment with primers FRA and RRA (lane 2). However, this product was not detected with the mutant reactions in the presence (lanes 3-5) or absence of erythromycin (lanes 6-8). Thus, the wild type copy of the gene was not reconstituted during sub-culture of the *recA* mutant. The insertion was amplified using the primer pairs FRA and M13R (lanes 9-16) and RRA and M13F (lanes 17-24). Lanes 11-13 and 19-21 show the results for the experiment in the presence of selection while lanes 14-16 and 22-24 show the results for the experiment carried out without selection. In both the presence and absence of erythromycin, the mutation was stable. The inserted

plasmid could be detected in the *recA*<sup>-</sup> mutant but not in the wild type genomic DNA negative controls (lanes 10 and 18) where no insert DNA was present. Thus, the PCR confirmed that pGREC remained in the *B. fragilis* chromosome through each sub-culturing step in the presence or absence of the antibiotic.



**Figure 3.8: PCR of the wild-type *B. fragilis* 638R and of the *recA*<sup>-</sup> *B. fragilis* 638R**

Lane M: Molecular size marker ( $\lambda$  DNA digested with *Pst*I); 1: no DNA control; 2: wild type; 3-5: mutant after 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> transfer respectively; 6-8: mutant after 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> transfer respectively; 9: no DNA control; 10: wild type; 11-13: mutant after 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> transfer respectively; 14-16: mutant after 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> transfer respectively; 17: no DNA control; 18: wild type; 19-21: mutant 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> transfer respectively; 22-24: mutant after 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> transfer respectively; 25: M13F, M13R + pLYLrecA.

Insertional gene inactivation can generate unstable mutations since homologous recombination between the repeats can delete the insertion (Salyers *et al.*, 2000). However, when Cooper *et al.* (1997) constructed a *B. thetaotaomicron recA* mutant, it was defective in homologous recombination. Thus, the *B. fragilis recA* insertion mutant should be stable since it seems the absence of RecA leads to an absence of recombination.

### 3.4. CONCLUSION

RT-PCR showed that *recA* formed part of an operon with BF638R1248 and BF638R1246. This is novel and could allow for an efficient response to oxidative damage. Further analysis of the operon is required to understand the importance of the arrangement and co-expression of *recA* with these genes. It is possible that BF639R1248 and *usa* form a separate operon to *usa* and *recA* and this should be tested using RT-PCR and the primer pair HIF-RIR. Quantitative or semi-quantitative RT-PCR could be carried out to determine whether the operon is induced by Mtz. Additional experiments could also include mutating BF638R1248

and BF638R1246 and exposing these strains to DNA damaging agents and oxygen or hydrogen peroxide. The phenotype of these mutants would provide an indication of the importance of these protein products in DNA repair and oxidative stress. In this study, a *B. fragilis recA* mutant was generated by targeted gene disruption using pGERM. The mutation was confirmed by PCR, DNA sequencing and Southern hybridisation and the insertion was found to be stable in the presence and absence of antibiotic selection. Deletion mutants are often preferred to insertional mutants since the former are thought to be more stable. In this study, however, the insertion disrupted *B. fragilis recA*, abolishing homologous recombination and allowing for a stable mutation. In order to study the function of *B. fragilis* RecA in DNA repair, studies were undertaken where the mutant was exposed to DNA damaging agents and its survival compared to that of wild type cells. This work is described in Chapter 4.

University of Cape Town

**CHAPTER 4**  
**FUNCTIONAL CHARACTERISATION OF**  
***BACTEROIDES FRAGILIS recA***

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### Abstract

A *B. fragilis* 638R *recA*<sup>-</sup> mutant was generated using targeted insertional inactivation. The mutant displayed an elongated cell morphology compared to the wild type under normal growth conditions; this phenotype has been reported for *B. subtilis* and *Streptococcus thermophilus recA*<sup>-</sup> mutants. Immediately after first being isolated, the *recA*<sup>-</sup> mutant also exhibited temperature and hydrogen peroxide sensitivity. However, after repeated sub-culturing, the *recA*<sup>-</sup> mutant lost this phenotype and was able to adapt and cope with these factors. The *B. fragilis recA*<sup>-</sup> mutant showed decreased survival following exposure to ultraviolet radiation and metronidazole as compared to wild type cells. *B. fragilis* RecA, therefore, is involved in repairing thymine dimers caused by ultraviolet radiation as well as the ss and dsDNA breaks caused by metronidazole. Over-expression of *recA* on a multi-copy plasmid in wild type *B. fragilis* resulted in higher levels of metronidazole resistance relative to the untransformed cells. This indicates that over-expression of RecA may possibly lead to the metronidazole resistance seen in the clinical setting in the absence of other previously described resistance mechanisms.

#### 4.1. INTRODUCTION

During normal cell metabolism, oxygen radicals are released which damage DNA (Rocha *et al.*, 1996), and during DNA replication, errors may occur which affect the integrity of the DNA (Kuzminov, 1999). Thus, *E. coli* cells maintain basal levels of many DNA repair proteins, among them RecA, to cope with this intrinsic damage. However, *E. coli* cells are also sporadically exposed to external DNA damaging agents. This activates RecA to induce the SOS response, upregulating the expression of an estimated twenty genes, including RecA itself. During the SOS response, Sula inhibits cell division, providing time for the DNA repair proteins to deal with the damage. As a result, the cells filament since they continue to grow but do not divide until Sula is degraded (Mukherjee *et al.*, 1998).

Since RecA is a key DNA repair protein, it has been studied via mutation in many bacteria including *E. coli* (Kuzminov, 1999), *B. thetaiotaomicron* (Cooper *et al.*, 1997), *P. gingivalis* (Fletcher *et al.*, 1997) and *M. bovis* (Sander *et al.*, 2001). These mutants were exposed to DNA damaging agents such as ultraviolet radiation (UV), metronidazole (Mtz) and oxygen to study the role of RecA in repairing the lesions. The *B. thetaiotaomicron* *recA* mutant showed increased sensitivity to Mtz and oxygen compared to wild type cells (Cooper *et al.*, 1997). This was also seen in the *M. bovis* mutant, which showed an 80% decrease in viability compared to the wild type when exposed to Mtz as well as increased sensitivity to UV irradiation (Sander *et al.*, 2001). Similarly, the *P. gingivalis* *recA* mutant could not survive exposure to 2000  $\mu$ J of UV whilst the wild type had 40% survival (Fletcher *et al.*, 1997). These results point to a role for RecA in repairing UV, Mtz and oxygen-induced DNA damage in these bacteria since the mutant cells showed decreased survival compared to the wild type controls.

The focus of this study was to determine the role of *B. fragilis* RecA in coping with various stress conditions and DNA damaging agents. The approach used was to monitor the *recA*<sup>-</sup> mutant's response to the cell stress factors hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and temperature, as well as the DNA damaging agents UV and Mtz. Since Mtz causes cell death through generating DNA damage, over-expression of DNA repair proteins could lead to improved survival and Mtz resistance (MtzR). RecA-mediated DNA repair was, therefore, evaluated as a possible resistance mechanism.

## 4.2. MATERIALS AND METHODS

### 4.2.1. BACTERIAL STRAINS AND PLASMIDS

The strains and plasmids used in this study are listed in Table 4.1.

**Table 4.1: Description of bacterial strains and plasmids**

Strain/plasmid	Genotype/phenotype	Source/Reference
<b>Plasmids</b>		
pTZ57R/T	3'-ddT tailed, T7 promoter. Amp <sup>R</sup>	Fermentas
pTrecA	pTZ57R/T derivative containing <i>B. fragilis recA</i>	This study
pLYL01	Mob <sup>-</sup> , Tet <sup>R</sup> Amp <sup>R</sup>	Li <i>et al.</i> , 1995
pLYLrecA	pLYL01 derivative containing <i>B. fragilis recA</i>	This study
<b>Strains</b>		
<i>Bacteroides fragilis</i>		
638R	Clinical strain, Rif <sup>R</sup> Gent <sup>R</sup>	Privitera <i>et al.</i> , 1979
638R <i>recA</i> mutant	638R, <i>recA</i> , Rif <sup>R</sup> Gent <sup>R</sup> Erm <sup>R</sup>	This study
638R (pLYL01)	638R, Rif <sup>R</sup> Gent <sup>R</sup> Tet <sup>R</sup>	This study
638R <i>recA</i> (pLYL01)	638R, <i>recA</i> , Rif <sup>R</sup> Gent <sup>R</sup> Erm <sup>R</sup> Tet <sup>R</sup>	This study
638R <i>recA</i> (pLYLrecA)	638R, <i>recA</i> , Rif <sup>R</sup> Gent <sup>R</sup> Erm <sup>R</sup> Tet <sup>R</sup>	This study
638R (pLYLrecA)	638R, <i>recA</i> overexpressor, Rif <sup>R</sup> Gent <sup>R</sup> Tet <sup>R</sup>	This study
<i>Escherichia coli</i>		
AB1157	RecA <sup>+</sup>	Willets and Clark, 1969
AB2463	AB1157 UV <sup>s</sup> RecA <sup>-</sup>	Willets and Clark, 1969
AB2463 pLYL01	AB1157 UV <sup>s</sup> RecA <sup>-</sup> Amp <sup>R</sup>	This study
AB2463 pTrecA 4	AB1157 UV <sup>s</sup> RecA <sup>-</sup> Amp <sup>R</sup> <i>B. fragilis recA</i>	This study
AB2463 pTrecA 8	AB1157 UV <sup>s</sup> RecA <sup>-</sup> Amp <sup>R</sup> <i>B. fragilis recA</i>	This study
AB2463 pTrecA 12	AB1157 UV <sup>s</sup> RecA <sup>-</sup> Amp <sup>R</sup> <i>B. fragilis recA</i>	This study
S17-1	RP4-2-Tc::Mu <i>aph</i> ::Tn7 <i>recA</i> , Strep <sup>R</sup>	Simon <i>et al.</i> , 1983
S17-1 pLYL01	S17-1 containing pLYL01	This study
S17-1 pLYLrecA	S17-1 containing pLYLrecA	This study

Rif, rifampicin; Gent, gentamicin; Erm, erythromycin; Tet, tetracycline; Amp, ampicillin; R, resistant; S, sensitive.

#### 4.2.2. MEDIA AND GROWTH CONDITIONS

*E. coli* strains were grown in Luria-Bertani (LB) broth or on LB plates under aerobic conditions at 37°C (Maniatis *et al.*, 1982). *E. coli* cells containing plasmids were grown in LB supplemented with 100 µg/ml ampicillin (LB+amp). Brain heart infusion (BHI) agar medium was supplemented with 5 µg/ml haemin-menadione, 0.5 µg/ml cysteine and 1 µg/ml Na<sub>2</sub>CO<sub>3</sub> (BHISA) while BHI broth was supplemented with 5 µg/ml haemin-menadione and 4 µg/ml Na<sub>2</sub>CO<sub>3</sub> (BHISB). *B. fragilis* 638R strains were grown in BHISB or on BHISA at 37°C under anaerobic conditions (Holdeman and Moore, 1972). *B. fragilis* cells containing pLYL01 or pLYrecA were grown on BHISA supplemented with 2 µg/ml tetracycline (BHISA+tet), while *B. fragilis recA* mutants were grown on BHI including erythromycin at a concentration of 10 µg/ml (BHISA+erm).

#### 4.2.3. CELL MORPHOLOGY

Cells were grown on BHISA or BHISB to log phase and then mounted on slides and Gram stained (Gillies and Dodds, 1965). Slides were viewed using a Leitz Diaplan light microscope at a magnification of 1000x. Microscope photographs were captured by a Zeiss AxioCam camera and visualised with Axiovision 2.0.5.3 software.

#### 4.2.4. BACTEROIDES FRAGILIS PLASMID CONJUGATION

The full length *B. fragilis recA* gene was obtained by PCR using primer pair FRA and RRA specific for BF638R1245 (Table 3.2). A GeneAmp PCR System 9700 was used for PCR reactions (Perkin Elmer, Applied Biosystems). The PCR parameters were: initial denaturation of 95°C for 5 min, then 25 cycles of denaturation at 95°C for 30 sec, annealing at 53.8°C for 30 sec and elongation at 72°C for 3 min. A final elongation step was carried out at 72°C for 5 min. PCR products were analysed with agarose electrophoresis using λ DNA (digested with *Pst*I) as a molecular size marker. The PCR fragment of the full length *B. fragilis recA* gene was cloned into pTZ57R/T (Fermentas), according to the manufacturer's instructions, to generate pTrecA (Table 4.1). Electroporation was used to transform electrocompetent *E. coli* AB2463 *recA* cells with pTrecA (Tung and Chow, 1995). Electroporation was carried out with the Gene pulser apparatus (Bio-Rad laboratories, Richmond, Calif.) at 2.5kv, 200Ω and

25µF. The transformed strains were tested for complementation by analysing which clones showed increased survival in the presence of UV. The complementing plasmid was then isolated according to the alkali lysis protocol of Ish-Horowicz and Burke (1981). Plasmids were checked with restriction enzyme digests (Fermentas) and analysed with agarose electrophoresis using λ DNA (digested with *Pst*I) as a molecular size marker. For sub-cloning, the plasmid pTrecA was digested with *Sal*I and *Kpn*I to release *recA* and the gene was cloned into pLYL01, which had also been digested with *Sal*I and *Kpn*I, to generate pLYLrecA (Table 4.1). All cloning reagents (enzymes, T4 polymerase and T4 ligase) were purchased from Fermentas and used according to the manufacturers instructions. Electrocompetent *E. coli* S17-1 cells were transformed with pLYL01 or pLYLrecA using electroporation. Both the *E. coli* donor strain and the relevant *B. fragilis* 638R strain were grown to an OD<sub>600</sub> of 0.2. The cultures were then mixed at a ratio of 3:1 (*B. fragilis*:*E. coli*) and centrifuged. HAWP filters (Millipore) were placed on BHISA lacking cysteine. The pellet was resuspended in 100 µl of LB and inoculated onto the filters. The plates were incubated at 37°C aerobically overnight. Filters were then washed with pre-reduced BHISB and incubated anaerobically for one hour. Aliquots of the rinses were plated onto BHISA containing tetracycline (2 µg/ml) and gentamycin (200 µg/ml). Incubation was carried out at 37°C under anaerobic conditions. Single colonies were analysed using PCR to confirm the presence of the plasmids.

#### 4.2.5. GROWTH CURVES

Cultures were incubated anaerobically at 37°C for sixteen hours in BHISB. The cells were then sub-cultured into BHISB to a starting OD<sub>600</sub>=0.1 and incubated anaerobically at 37°C. Samples were taken periodically for eight hours and the final sample was collected at 24 hours. The growth of the bacteria was measured spectrophotometrically on a WPA Spectrawave spectrophotometer (Biochrom Ltd, England). Growth rate was calculated using the formula:

$$\mu = [2.303(\log OD_2 - \log OD_1)] / (t_1 - t_2)$$

where  $\mu$  = growth rate; OD<sub>1</sub> = optical density of bacteria at t<sub>1</sub> (earlier in log phase); OD<sub>2</sub> = optical density of bacteria at t<sub>2</sub> (later in log phase). Note: the OD values must be chosen from the linear section of a graph where a log scale is used for optical density.

#### 4.2.6. SURVIVAL AGAINST HYDROGEN PEROXIDE

The filter disk experiment was carried out as described by Kikuchi *et al.* (2005) except that 5 µl of a 10 % hydrogen peroxide solution was used.

#### 4.2.7. TEMPERATURE SENSITIVITY

Cultures were spread-plated onto BHISA and incubated anaerobically at either 37°C or 25°C for 48 h.

#### 4.2.8. CELL SURVIVAL IN THE PRESENCE OF ULTRAVIOLET LIGHT

*E. coli* cells were grown for 16 h in LB broth while *B. fragilis* cells were incubated for 16 h in BHISB. A five millilitre aliquot of culture was then diluted 100 fold in water and pipetted into a sterile glass petri-dish for UV exposure. The UV lamp (Cole-Parmer 9815-series lamps, 4 watts, 254nm UV-C bactericidal, Cole-Parmer Instrument Co. Chicago Ill. 60648) was set to deliver 1 J/m<sup>2</sup>/s and the cells were subjected to the required UV range. After exposure, the cells were suitably diluted in water and plated on LA+amp (for *E. coli*) or BHISA (for *B. fragilis*). The plates were covered with foil to prevent photo-reactivation and incubated at 37°C. Colonies were enumerated after two days. All *B. fragilis* work was concluded under strictly anaerobic conditions.

#### 4.2.9. CELL SURVIVAL IN THE PRESENCE OF METRONIDAZOLE

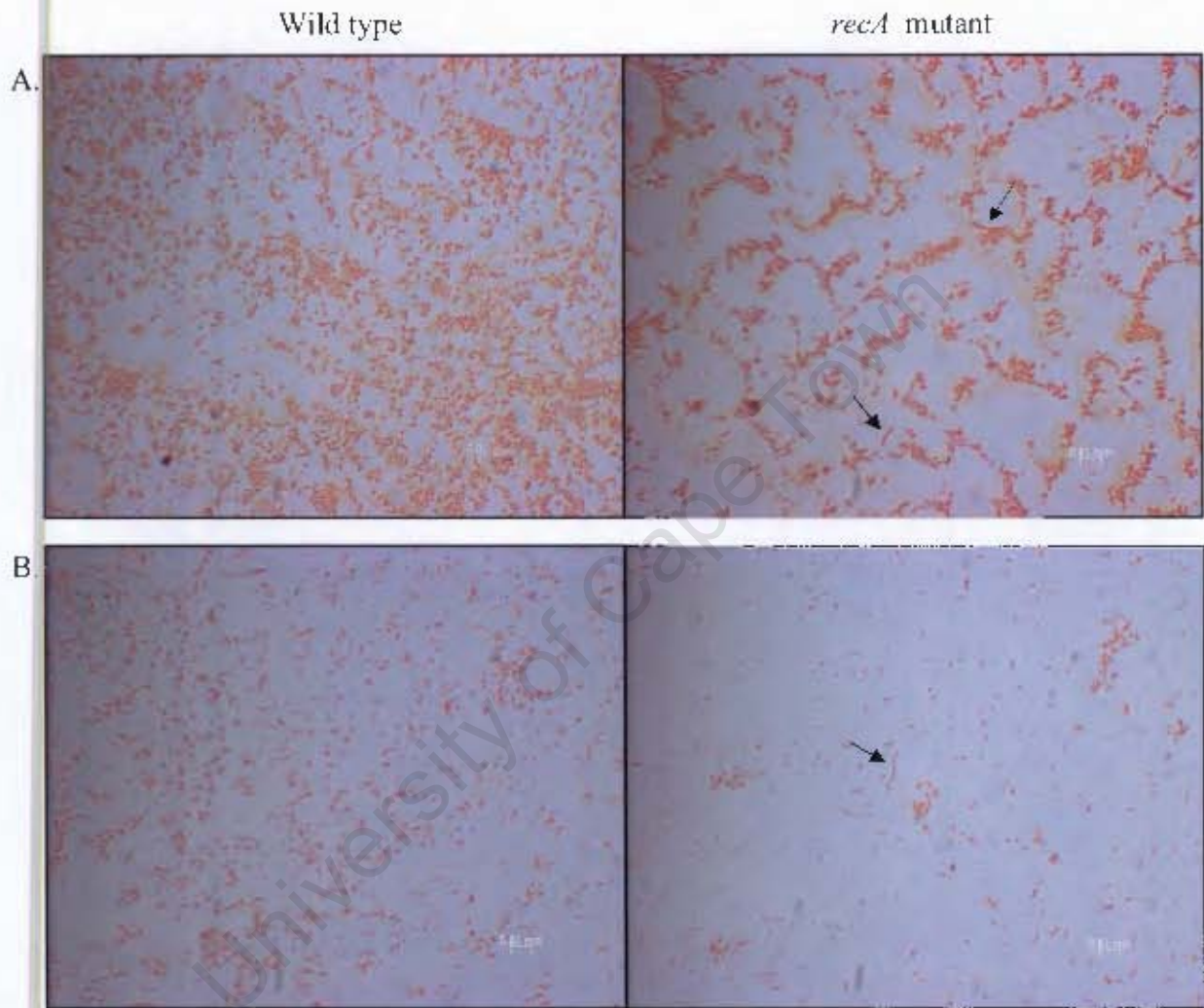
Log-phase BHI cultures were exposed to 5 µg/ml Mtz and incubated anaerobically at 37°C. Cells were collected at fifteen minute intervals, diluted in water and plated on BHISA. The plates were incubated anaerobically at 37°C and colonies were enumerated after 48 h.

### 4.3. RESULTS AND DISCUSSION

#### 4.3.1. ELONGATED CELL MORPHOLOGY

Under normal growth conditions, the *B. fragilis recA*<sup>-</sup> mutant appeared to display a greater degree of cell elongation compared to wild type cells. This was seen when

cells were grown on BHISA and BHISB (Fig. 4.1). Accurate enumeration of the numbers of these cells in a particular field was not possible due to the tendency of the cells to aggregate, thus, this must be regarded as preliminary data.



**Figure 4.1: Microscope pictures comparing wild type and mutant cells grown on BHISA and BHISB**

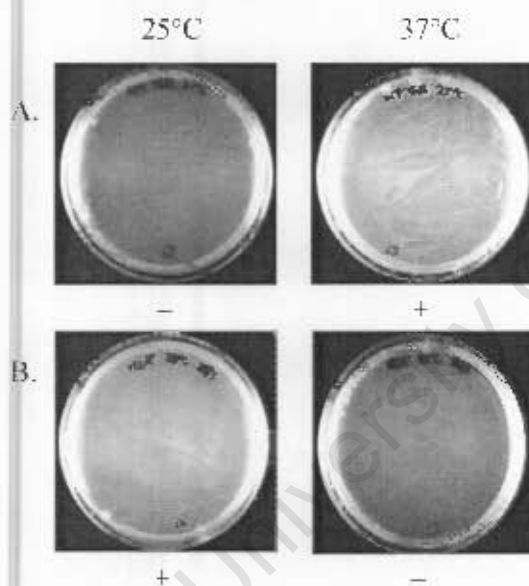
A. BHISA; B. BHISB. Arrows highlight the sub-population of elongated cells found in the mutant culture. The scale bar represents 0.01 mm.

Sciocchetti *et al.* (2001) observed that a sub-population of *B. subtilis* *recA* mutants formed abnormally elongated cells and nucleoids during stationary phase. Further investigation attributed the elongated morphology to cell wall irregularities and partitioning malfunction. A similar phenotype was also seen in *S. thermophilus* *recA* mutants, which formed misshaped cells upon heat shock and nutrient starvation (Giliberti *et al.*, 2002). Scanning electron microscopy of these *S. thermophilus* *recA*

mutants revealed that they had failed to divide leading to the formation of elongated cells. Thus, the phenotype observed for the *B. fragilis* mutants may be a result of unresolved DNA damage, arising during normal cell growth, which leads to partitioning failures. This hypothesis would require further investigation and is discussed in the conclusions.

#### 4.3.2. TEMPERATURE AND HYDROGEN PEROXIDE SENSITIVITY

During the isolation of the *B. fragilis* 638R *recA*<sup>-</sup> mutant, it was noted that plates supporting the growth of transconjugants needed to be incubated at 25°C since the mutated colonies did not grow at 37°C.



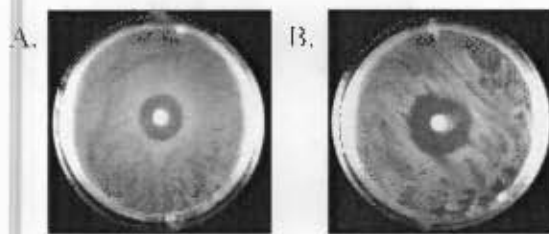
**Figure 4.2: Temperature sensitivity of the *B. fragilis* *recA*<sup>-</sup> mutant immediately after isolation**

A. *B. fragilis* 638R wild type; B. *B. fragilis* 638R *recA*<sup>-</sup> mutant; (-), no growth; (+), growth. Growth was noted as a confluent white lawn of cells.

During the first round of sub-culturing these transconjugants, they were able to grow in BHISB at 37°C but the plates still needed to be retained at 25°C (Fig. 4.2). However, after repeated sub-culturing, the phenotype of the mutant gradually altered and it was eventually able to grow on BHISA at 37°C. It is possible that secondary mutations had entered the genome, due to a decrease in the repair capacity of the cell, allowing for alterations in growth. Increased genetic instability has been reported for

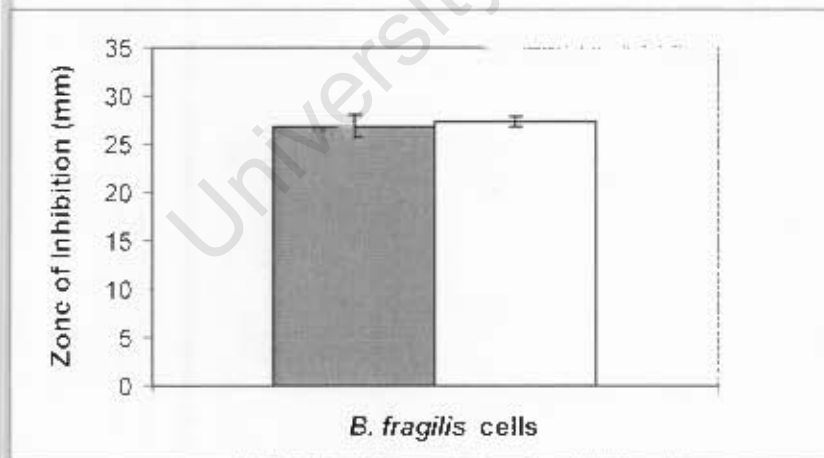
*S. lividans recA* insertion mutants, which segregated 70 times more mutants than the wild type (Volf and Altenbuchner, 1997).

Changes in the mutant's ability to survive hydrogen peroxide ( $H_2O_2$ ) were also noted over time. Preliminary experiments pointed to the *B. fragilis recA* mutant showing decreased survival in the presence of  $H_2O_2$  compared to wild type cells (Fig. 4.3). However, after continuous sub-culturing, repetition of the experiment showed that the mutant and wild type displayed similar survival in the presence  $H_2O_2$  (Fig. 4.4).



**Figure 4.3: Hydrogen peroxide sensitivity**

A. *B. fragilis* 638R wild type; B. *B. fragilis* 638R *recA* mutant. The zone of clearance for the wild type was 19 mm and for the mutant was 24 mm.



**Figure 4.4: Hydrogen peroxide sensitivity reversal**

(■), *B. fragilis* 638R wild type; (□), *B. fragilis* 638R *recA* mutant. Cells were exposed to 10% hydrogen peroxide. The errors bars represent the standard error calculated from three replicates of data.

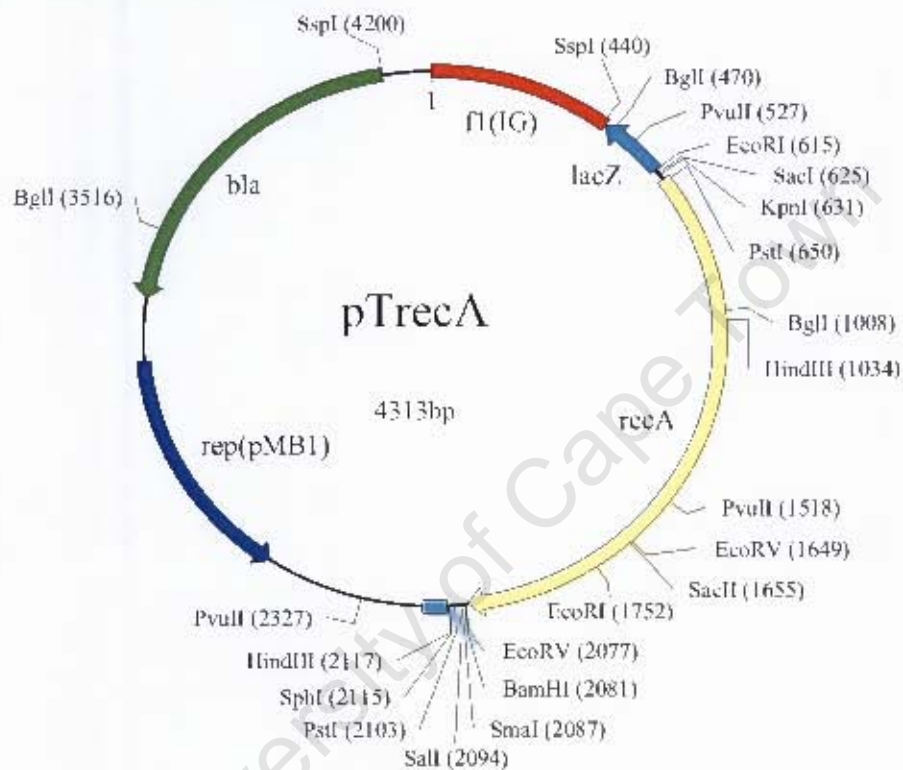
During cell metabolism, oxygen is converted to reactive oxygen species such as H<sub>2</sub>O<sub>2</sub> (Rocha *et al.*, 1996). H<sub>2</sub>O<sub>2</sub> damages proteins by reacting with cysteine residues and it can also produce hydroxyl radicals (OH<sup>•</sup>) that cause DNA strand breaks and base mutations (Miyoshi *et al.*, 2003). The *B. thetaiotaomicron recA*<sup>-</sup> mutant showed decreased survival in the presence of oxygen compared to wild type (Cooper *et al.*, 1997). However, the reason why the *B. fragilis recA*<sup>-</sup> mutant's phenotype changed over time could be due to the mutant upregulating catalase or other OSR proteins to cope with H<sub>2</sub>O<sub>2</sub> exposure, preventing extensive DNA damage and increasing the cell's survival.

In aerobes and facultative bacteria, cell metabolism converts oxygen to the reactive oxygen species (ROS): superoxide anions (O<sub>2</sub><sup>-</sup>) and H<sub>2</sub>O<sub>2</sub> (Rocha *et al.*, 1996). Catalases, peroxidases and superoxide dismutase protect the cell by resolving ROS (Rocha *et al.*, 1996). Anaerobes were originally thought to be oxygen sensitive since the exclusion of oxygen from their environment had precluded the necessity for developing mechanisms that combat ROS. Thus, exposure to oxygen should result in rapid macromolecule damage and cell death. However, different anaerobes display varying levels of aerotolerance, with *B. fragilis* being one of the most oxygen resistant strains (Rocha *et al.*, 1996) and the presence of catalase, superoxide dismutase and an extensive oxidative stress response (OSR) has been identified in this species (Herren *et al.*, 2003). Phagocytic cells generate a respiratory burst of ROS to defend against bacterial invasion (Sander *et al.*, 2001). Consequently, pathogens require an oxidative stress response to cope with ROS damage whilst infecting the host. Since *B. fragilis* is an opportunistic pathogen, it uses its OSR to defend itself against the immune cell ROS attack (Herren *et al.*, 2003).

#### 4.3.3. CLONING OF FUNCTIONAL *recA* GENE

Further experiments to characterise the *B. fragilis recA*<sup>-</sup> mutant involved exposing it to UV and Mtz and comparing its survival to that of the wild type. However, to determine whether the results obtained could be attributed directly to the *recA* mutation alone, it was necessary to complement the *recA*<sup>-</sup> mutant with a functional wild type copy of *B. fragilis recA* to see if it regained the wild type phenotype. PCR errors introduced during cloning of *recA* could, however, result in inactive copies of

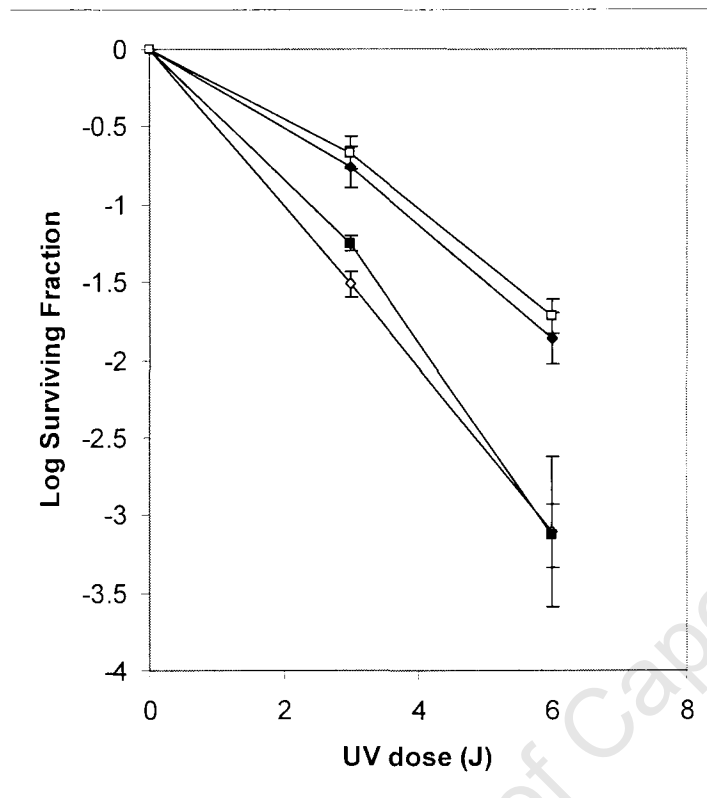
the *recA* gene leading to an unsuccessful complementation. Thus, *recA* copies would need to be screened for functionality in an *E. coli recA* mutant before complementing the *B. fragilis* mutant. The screening was carried out by PCR cloning *B. fragilis recA* into pTZ57R/T to generate pTrecA plasmids (Fig. 4.5); the clones were then used to test for complementation in the *E. coli* AB2463 *recA* mutant prior to the functional gene being introduced into *B. fragilis* 638R *recA*.



**Figure 4.5: Map of pTrecA**

*B. fragilis recA* was cloned into pTZ57R/T. The plasmid contains the Amp<sup>R</sup> gene for screening in *E. coli*.

Three *E. coli* AB2463 transformants containing pTrecA4, pTrecA8 or pTrecA12, were exposed to UV and their survival compared to *E. coli* AB2463 *recA* containing the control plasmid pLYL01 (for the Amp<sup>R</sup> phenotype). Two clones containing pTrecA8 and pTrecA12 showed increased survival compared to *E. coli* AB2463(pLYL01) (Fig. 4.6). These plasmids, therefore, contained active copies of the *recA* gene together with its own promoter region. *E. coli* AB2463(pTrecA4) exhibited the same survival pattern as the uncomplemented *E. coli* AB2463 strain. The plasmid pTrecA4 could have contained a copy of the *recA* gene with PCR errors. The *E. coli* isogenic wild type strain was not included in this experiment since it shows no change in viability over this range.



**Figure 4.6: UV survival curves of *E. coli* AB2463 clones containing the *B. fragilis* 638R *recA* gene**

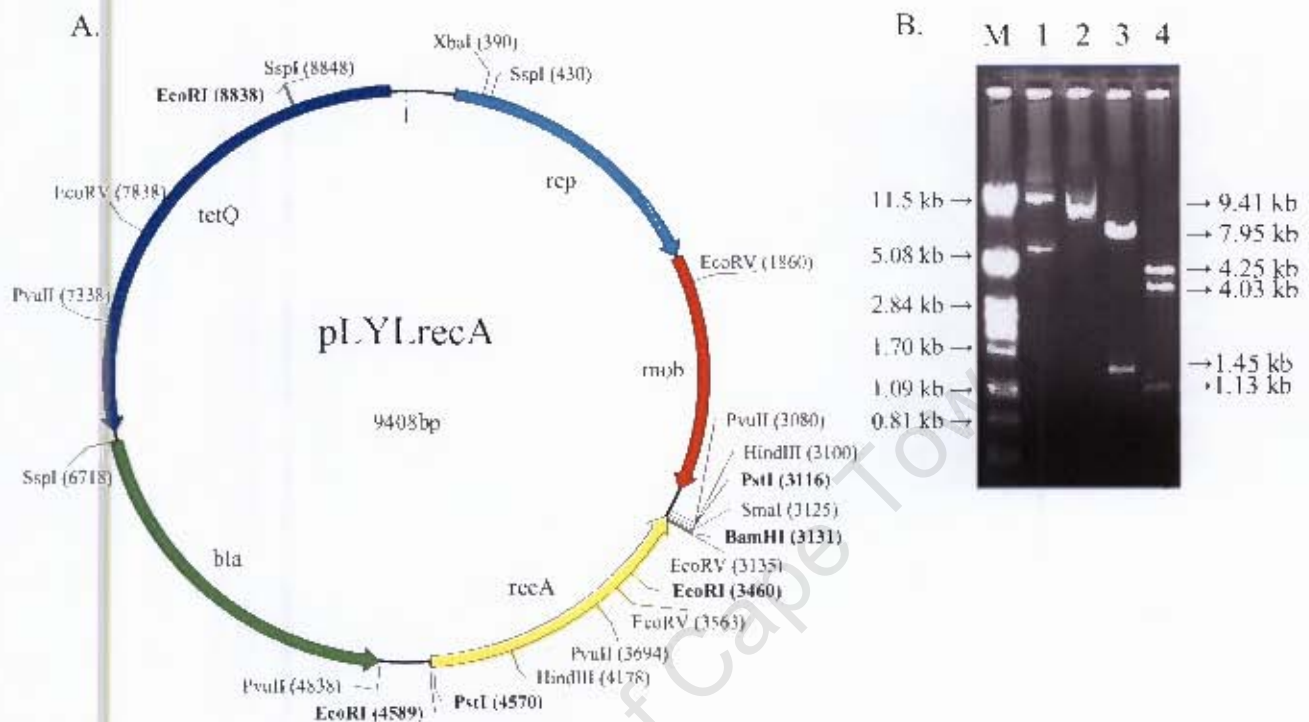
(◇), *E. coli* AB2463(pLYL01); (■), *E. coli* AB2463(pTrecA4);  
 (◆), *E. coli* AB2463(pTrecA8); (□), *E. coli* AB2463(pTrecA12).

The errors bars represent the standard error calculated from three replicates of data.

The *recA* copy in pTrecA12 was chosen for complementation of the *B. fragilis* 638R *recA* mutant. It was cloned into the shuttle vector pLYL01, which has a *Bacteroides* origin of replication, (to generate the plasmid pLYLrecA, Table 4.1) and transformed into *E. coli* S17 for mating into *B. fragilis* 638R *recA*.

A restriction enzyme digest of pLYLrecA was performed to confirm the presence of the insert before conjugating into *B. fragilis* (Fig. 4.7). *Bam*HI, *Pst*I and *Eco*RI gave the predicted restriction enzyme digestion patterns. The plasmid construct was, therefore, correct and *E. coli* S17-pLYLrecA was mated with the *B. fragilis* *recA* mutant (complementation) and wild type (over-expression) recipients. The control

plasmid pLYL01 was also conjugated into the wild type and mutant strains to confer Tet<sup>R</sup> to these strains as well.



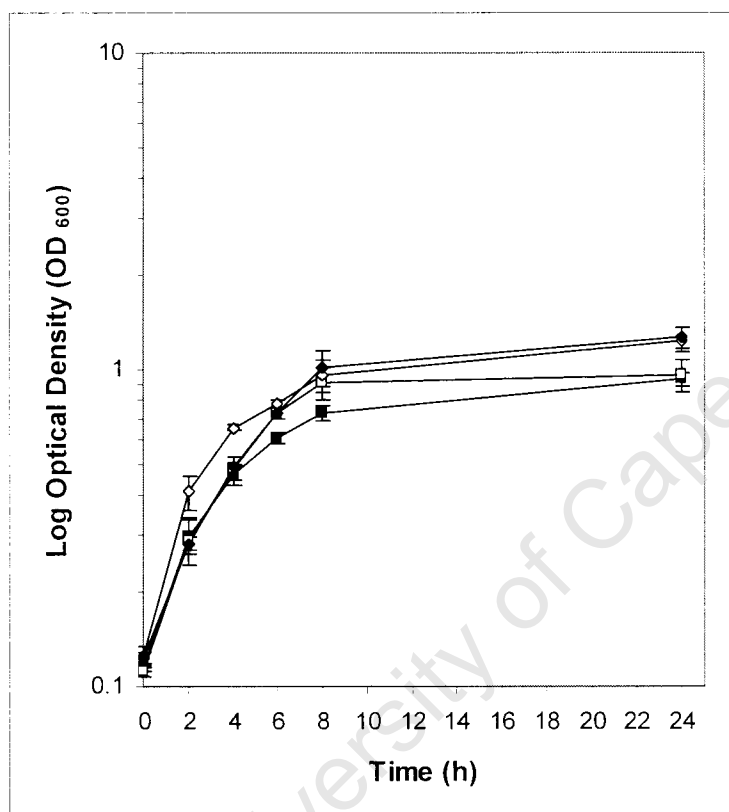
**Figure 4.7: Map and restriction enzyme digest of pLYLrecA**

A. Map of pLYLrecA. The  $\beta$ -lactamase resistance gene (*bla*) allows for screening of the plasmid in *E. coli* (Li *et al.*, 1995). The *mob* region allows for transfer of this plasmid from *E. coli* to *B. fragilis* during conjugation. Tetracycline resistant selection in *B. fragilis* is mediated via the *tetQ* gene product. Relevant restriction sites used for analysis are shown in bold. B. Restriction enzyme digest of pLYLrecA. Lane M, Molecular size marker ( $\lambda$  DNA digested with *Pst*I); 1, pLYLrecA undigested; 2, pLYLrecA *Bam*III (9.41 kb); 3, pLYLrecA *Pst*I (1.45 kb and 7.95 kb); 4, pLYLrecA *Eco*RI (4.25 kb, 4.03 kb and 1.13 kb).

#### 4.3.4. GROWTH CURVES

The growth rate of the *B. fragilis* wild type, *recA*<sup>-</sup> mutant, complemented *recA*<sup>-</sup> mutant and *recA* overexpressing strains was analysed over 24 hours. The wild type and mutant cells showed a similar growth pattern (Fig. 4.8). The growth rate of the wild type was calculated to be 0.22 h<sup>-1</sup> and the mutant growth rate was 0.25 h<sup>-1</sup>, indicating that the mutation had not altered the growth rate of the *recA*<sup>-</sup> mutant cells in comparison to the wild type cells. In contrast, the complemented *recA*<sup>-</sup> mutant and

*recA* over-expressor showed an improved growth pattern compared to the wild type. However, the growth rate of the *recA*<sup>-</sup> complemented strain was calculated to be 0.23 h<sup>-1</sup> while the *recA*<sup>-</sup> over-expressor strain's growth rate was 0.28 h<sup>-1</sup>, thus, all four strains displayed comparable growth rates.



**Figure 4.8: Growth Curve**

(■). *B. fragilis* 638R(pLYL01); (□), *B. fragilis* 638R *recA*<sup>-</sup> mutant(pLYL01);

(◆). *B. fragilis* 638R complemented *recA*<sup>-</sup> mutant(pLYLrecA);

(◇). *B. fragilis* 638R *recA* over-expressor(pLYLrecA).

The errors bars represent the standard error calculated from at least three replicates of data.

The growth rate of bacteria influences the rate at which Mtz uptake occurs since the drug enters the cell through passive diffusion and only once Mtz is reduced intracellularly to active intermediates, does a concentration gradient form which favours the passive diffusion of additional inactivated Mtz into the cell (Lindmark and Müller, 1976). Consequently, to examine the sensitivity of cells to Mtz, the experimental culture needs to be in log phase where it is actively growing and metabolising, which favours the uptake and activation of Mtz. Thus, if the mutation

had increased the growth rate of the cell, the *recA*<sup>-</sup> mutant could have appeared more sensitive compared to the wild type strain but this might have only been due to the growth rate driving an increased uptake and activation of Mtz.

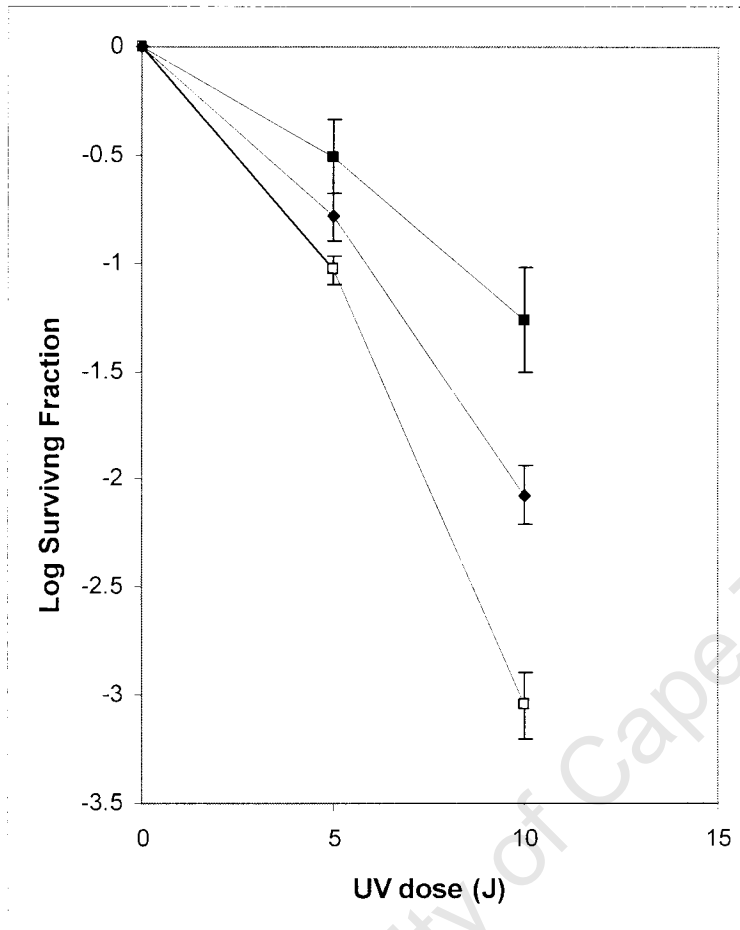
The *B. thetaiotaomicron recA*<sup>-</sup> mutant also showed similar growth to wild type cells (Cooper *et al.*, 1997). However, this was only achieved once cysteine and resazurin were removed from the growth medium and oxygen exposure was minimised. The researchers hypothesised that these factors were generating ROS, which caused irreparable DNA damage in the mutant. The *B. fragilis recA*<sup>-</sup> mutant could have upregulated its OSR, or activated other DNA repair pathways to compensate for ROS damage, since the presence of cysteine and resazurin did not inhibit the mutant's growth. Adaptation of the mutant to media-generated ROS might have attributed to the mutants adaptation to H<sub>2</sub>O<sub>2</sub> (Fig. 4.4). In contrast, *Streptomyces coelicolor recA*<sup>-</sup> mutants exhibited growth defects and produced more anucleate spores than the wild type (Huang and Chen, 2006). This was comparable to the 10% anucleation rate of *E. coli recA*<sup>-</sup> cells. Sciochetti *et al.* (2001) also demonstrated partitioning failures in *B. subtilis recA*<sup>-</sup> mutants which lead to an anucleation rate of 0.16%. They attributed partitioning failures to lack of resolution of stalled replication forks due to the absent RecA. Thus, even though *recA*<sup>-</sup> cells continue dividing, a sub-population loses viability due to anucleation. Further studies into the growth rate of *B. fragilis recA*<sup>-</sup> should include plate counts at the various time points to ascertain the viability of the cells.

#### 4.3.5. ULTRAVIOLET IRRADIATION SURVIVAL STUDIES

The *B. fragilis recA*<sup>-</sup> mutant was exposed to UV to examine whether RecA was required to repair thymine dimers in this organism. The wild type, *recA*<sup>-</sup> mutant and *recA*<sup>-</sup> complemented cells were exposed to 0, 5 and 10 J of UV and samples were plated. The *recA*<sup>-</sup> mutant showed a two log decrease in survival in the presence of UV compared to wild type cells (Fig. 4.9). This result shows that *B. fragilis* RecA is involved in repairing UV-induced damage. These results show the same trend as those found for a *M. bovis recA* mutant which showed 10 000 fold reduction in viability after 30 seconds of UV irradiation compared to the wild type cells which only showed a 25 fold reduction in viability (Sander *et al.*, 2001). An analogous

phenotype was found in a *P. gingivalis recA*<sup>-</sup> mutant which showed 18% survival compared to the wild type's 80% survival after exposure to 1 000 μJ of UV irradiation (Fletcher *et al.*, 1997). For *S. coelicolor*, *recA*<sup>-</sup> spores showed increased UV sensitivity as well. Inactivation of 99% of the spores required 0.3-0.5 of the UV dose used on wild type spores (Huang and Chen, 2006).

The *B. fragilis recA*<sup>-</sup> strain complemented with the functional *recA* gene on a plasmid did not fully regain the wild type phenotype but it did have increased survival compared to the *recA*<sup>-</sup> mutant (Fig. 4.9). Incomplete complementation was also seen for an *E. faecalis recA*<sup>-</sup> mutant containing plasmid pMSP3535 carrying a wild type copy of *recA* (Weaver and Reddy, 2006). They attributed this to secondary mutations or unnatural control of gene expression since the complemented copy was on a plasmid and not integrated into the chromosome. DNA repair mutants are genetically unstable and this can lead to an accumulation of secondary mutations. Thus, the differences in survival between the mutant, complemented and wild type strain could be due to the *recA* mutation along with other unidentified secondary mutations. However, it is also possible that unnatural gene expression is taking place in the *B. fragilis* complemented strain since the wild type copy of *recA* was present on pLYLrecA and not inserted chromosomally. Thus, the lack of full complementation could be a result of *recA* not being induced to its full potential as occurs in the wild type. Alternatively, the complementing plasmid does not contain all three genes of the operon in which *recA* is located (Chapter 3). Thus, incomplete complementation could be due to loss of the upstream promoter. In addition, even though *recA* is downstream of the other two genes in the operon, it has previously been shown that disruption of a downstream operon gene has affected expression of the upstream gene. This was seen for the *H. pylori recA-eno* operon, where disruption of the enolase gene, which is downstream of *recA*, decreased expression of *recA* (Thompson and Blaser, 1995). Thus, it could be that decreased expression of *B. fragilis* BF638R1248 and BF638R126/7 prevents the cell from fully repairing UV damage. This last explanation is unlikely since the BF638R1248 and BF638R126/7 protein products seem to be involved in oxidative stress which generates DNA strand breaks, not thymine dimers. Therefore, these proteins might not be needed for repair of UV.



**Figure 4.9: UV Survival Curve of the *B. fragilis* wild type, *recA*<sup>-</sup> mutant and complemented *recA*<sup>-</sup> mutant**

(■), *B. fragilis* 638R(pLYL01); (□), *B. fragilis* 638R *recA*<sup>-</sup> mutant(pLYL01);

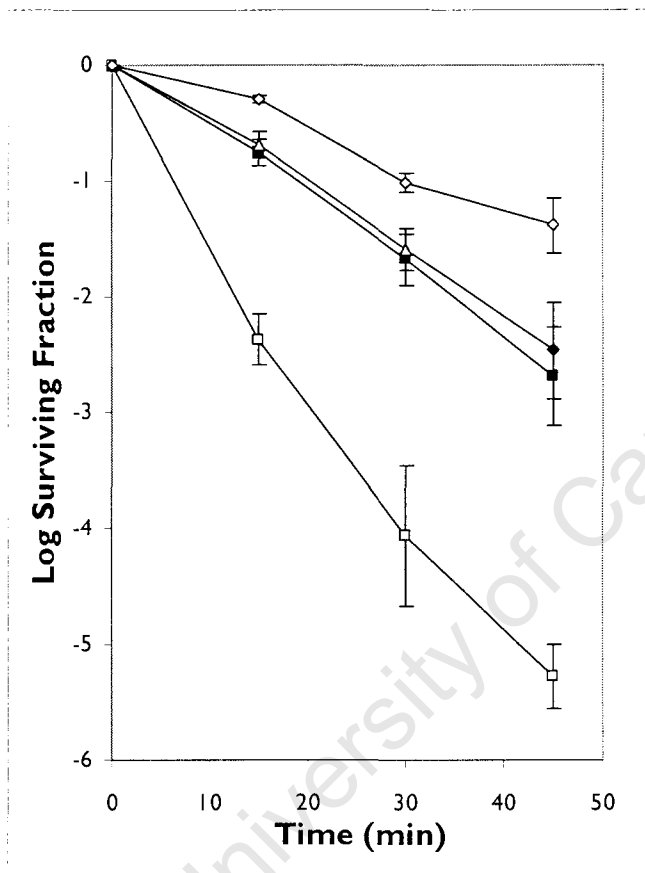
(◆), *B. fragilis* 638R complemented *recA*<sup>-</sup> mutant(pLYLrecA).

The errors bars represent the standard error calculated from at least three replicates of data.

#### 4.3.6. SURVIVAL IN RESPONSE TO METRONIDAZOLE EXPOSURE

*B. fragilis* wild type and *recA*<sup>-</sup> mutant cells were exposed to Mtz (Fig. 4.10). The *B. fragilis* 638R *recA*<sup>-</sup> mutant exhibited a two logs decrease in survival after 45 minutes compared to that of the wild type cells. *B. fragilis* RecA is, therefore, involved in repairing the DNA strand breaks caused by Mtz. Similarly, the *M. bovis* *recA*<sup>-</sup> mutant showed increased sensitivity to Mtz compared to wild type (Sander *et al.*, 2001), as did the *B. thetaiotaomicron* *recA*<sup>-</sup> mutant (Cooper *et al.*, 1997). The complemented *B. fragilis* 638R *recA*<sup>-</sup> mutant regained the full wild type phenotype in the presence of Mtz (Fig. 4.10) unlike the result seen for UV (Fig. 4.9). This suggests that the

regulation of DNA repair for strand breaks (caused by Mtz) and thymine dimers (caused by UV) differs since a plasmid copy of *recA* can allow the *recA*<sup>-</sup> mutant to fully regain its wild type phenotype in the presence of Mtz, but it cannot do the same for UV-damage.



**Figure 4.10: Metronidazole kill curve of the *B. fragilis* wild type, *recA*<sup>-</sup> mutant, complemented *recA*<sup>-</sup> mutant and *recA* over-expressor**

(■), *B. fragilis* 638R(pLYL01); (□), *B. fragilis* 638R *recA*<sup>-</sup> mutant(pLYL01);

(◆), *B. fragilis* 638R complemented *recA*<sup>-</sup> mutant(pLYLrecA);

(◇), *B. fragilis* 638R *recA* over-expressor(pLYLrecA).

The errors bars represent the standard error calculated from at least three replicates of data.

It was very interesting to note that the *B. fragilis* 638R wild type cells that over-expressed RecA displayed increased MtzR compared to wild type cells (Fig. 4.10). Thus, increased expression of *B. fragilis recA* could lead to possible MtzR in a clinical setting. Mtz is itself a mutagen (Trinh and Reysset, 1998) and so the likelihood of *in vivo* mutations allowing for increased expression of *recA* might be

further elevated in the presence of Mtz treatment. Even the mutation of one amino acid in the *B. fragilis* RecA protein could lead to MtzR as has been seen in *H. pylori* (Chang *et al.*, 1997). Also, mutations in the promoter region of *recA* could lead to over-expression of the gene resulting in increased levels of DNA repair. Thus, careful monitoring of treatment is required to prevent the occurrence of MtzR.

#### 4.4. CONCLUSION

The disruption of *recA* in *B. fragilis* resulted in elongated cell morphology and future work could include taking electron micrographs of the *B. fragilis recA*<sup>-</sup> mutant cells to ascertain whether septation has been inhibited. The *B. fragilis recA*<sup>-</sup> mutant also displayed transitory H<sub>2</sub>O<sub>2</sub> and temperature sensitivity. To further investigate H<sub>2</sub>O<sub>2</sub> sensitivity, new *recA* insertional mutants would need to be generated and exposed to this DNA damaging agent. The transitory sensitivity of the mutant may be an indication of decreased genome stability and possibly the occurrence of secondary mutations. Rocha and Smith (1999) showed that *B. fragilis ahpCF*, *ahpF* and *ahpCFkatB* mutants had an increased mutagenesis rate by testing for the frequency of mutation to fusidic acid resistance compared to the parent strain. Therefore, the fusidic acid mutagenicity assay could be used to measure the possible genomic instability of the *B. fragilis recA*<sup>-</sup> mutant. Both the *B. fragilis* wild type, *recA*<sup>-</sup> mutant, complemented mutant and the *recA* over-expressor exhibited comparable growth rates. *B. fragilis* RecA was seen to be involved in repairing thymine dimers and DNA strand breaks since the *recA*<sup>-</sup> mutant was sensitive to UV and Mtz. The possibility of secondary mutations arising in the *recA*<sup>-</sup> mutant genome did not affect the UV and Mtz sensitivity phenotype, which was consistent over time. Complementation restored the wild type phenotype in the presence of Mtz, but for UV, the complemented *B. fragilis recA*<sup>-</sup> mutant only regained partial repair capabilities. This may be attributed to altered gene regulation of the plasmid copy of *recA*. Quantitative or semi-quantitative RT-PCR could be used to compare the transcriptional levels of *recA* in the *B. fragilis* wild type and complemented *recA*<sup>-</sup> mutant to ascertain whether altered gene regulation was affecting complementation. Over-expression of *recA* led to MtzR in *B. fragilis*, thus, over-expression of DNA repair genes could explain the occurrence of MtzR in *B. fragilis* cells that do not display any of the previously described resistance mechanisms.

## CHAPTER 5

### GENERAL CONCLUSIONS

Goodman *et al.* (1987) were the first to identify the *B. fragilis* Bf-1 *recA* gene from a gene bank expressed in *E. coli* HB101. *B. fragilis* RecA increased the ultraviolet (UV) resistance and recombination frequency of the *E. coli* *recA*<sup>-</sup> mutant. The RecA protein was also found to be immunologically conserved since antibodies raised against *E. coli* RecA, cross-reacted with *B. fragilis* RecA. The *B. fragilis* *recA* gene was later sequenced and its transcription start site identified through primer extension (Goodman and Woods, 1990). In more recent years, the genome sequence of *B. fragilis* YCH46 (Kuwahara *et al.*, 2004), NCTC 9343 (Cerdeño-Tárraga *et al.*, 2005) and 638R ([www.sanger.ac.uk](http://www.sanger.ac.uk)) was determined. In the study reported in this thesis, bioinformatic analysis confirmed the presence of *recA* in these strains and showed that the deduced amino acid sequence of the *B. fragilis* RecA protein was highly conserved compared to RecA proteins from a range of bacteria. *B. fragilis* 638R RecA contained a Walker A and Walker B motif for ATP binding and the L1 and L2 loops for ssDNA binding. RecA binds ATP whilst forming nucleoprotein filaments with ssDNA, but ATP hydrolysis is required for filament disassembly (Kuzminov, 1999).

In the study reported in this thesis, analysis of the possible suite of RecA-mediated DNA repair proteins which could be present on the *B. fragilis* genome, confirmed the previous annotation of putative components of the RecFOR and RexAB pathways (Cerdeño-Tárraga *et al.*, 2005 supplementary material; [www.sanger.ac.uk](http://www.sanger.ac.uk)). RecFOR is involved in repairing stalled replication forks (Kuzminov, 1999) and this pathway is highly conserved amongst bacteria (Koroleva *et al.*, 2007). The RecBCD pathway, which initiates the repair of dsDNA breaks, has been replaced by AddAB in *B. subtilis* (Zuñiga-Castillo *et al.*, 2004) and by RexAB in *L. lactis* (Quiberoni *et al.*, 2001b), and this also appears to be the case in *B. fragilis* (Cerdeño-Tárraga *et al.*, 2005 supplementary material; [www.sanger.ac.uk](http://www.sanger.ac.uk)). The substrate for RecFOR is gDNA containing a 5' terminus of a ssDNA-dsDNA junction, this substrate is generated through the helicase activity of RecQ and the exonuclease activity of RecJ (Morimatsu and Kowalczykowski, 2003). The previously annotated *B. fragilis* *recJ* gene was analysed along with three *recQ* genes, one of which might possibly form an operon with *recJ*, and this warrants further transcriptional analysis. SSB has been annotated in the *B. fragilis* genome, and analysis of the protein sequence showed that many residues necessary for

ssDNA binding and dimer or tetramer interaction were conserved between *B. fragilis* SSB and *E. coli* SSB. Although BLAST analysis identified a possible LexA candidate, this protein did not include the DNA binding region common to *E. coli* LexA and *B. subtilis* LexA and it is therefore, unlikely to be functional in *B. fragilis* as an SOS-type regulation protein. Bioinformatic analysis of proteins cannot prove protein function or protein-protein interactions. Therefore, functional characterisation is necessary and to further study the proteins analysed here it would be useful to mutate them in *B. fragilis* and examine the resulting phenotypes with regard to DNA recombination damage repair in particular.

Analysis of the gene arrangement surrounding *B. fragilis* *recA* lead to the hypothesis that *recA* formed an operon with its upstream genes which coded for a hypothetical protein (BF638R1248) and a thiol-specific antioxidant enzyme (TSA) (BF638R1246). A CDD analysis of the hypothetical protein showed that it contained a homospermidine synthase domain, which catalyses the synthesis of polyamine homospermidine from putrescine (Tholl *et al.*, 1996). Polyamines and polyamine synthesis enzymes have been shown to regulate gene expression in response to oxidative stress (Jung and Kim, 2003). TSA peroxidases reduce peroxides to alcohols with the aid of a reduced thiol donor and thus, are important during oxidative stress (Herren *et al.*, 2003). RecA is known to repair DNA damage caused by oxygen radicals and the arrangement of *recA* in an operon with BF638R1248 and BF638R1246 could allow for a co-ordinated response to oxidative stress and the consequent DNA damage. In the study reported here, RT-PCR revealed that during log phase, BF638R1248, BF638R1246 and BF638R245 (*recA*) were co-expressed and shown to form an operon. This confirms the unpublished data of C. J. Smith (personal communication). Further work could involve generating strains with disruptions of BF638R1248 and BF638R1246 and investigating how these strains respond to DNA damage and oxidative stress. These experiments could reveal the function of these genes and clarify the role of the operon in DNA repair and the oxidative stress response. The *recA* gene is included in an operon in a range of bacteria including *D. radiodurans*, where *recA* is co-transcribed with a *cinA* and *ligT* (Bonacossa de Almeida *et al.*, 2002), and *M. smegmatis*, which contains a *recA-recX* arrangement (Vierling *et al.*, 2000). However, the genomic linking of *recA* with oxidative stress response genes is novel and has not previously been reported in the literature.

Chemical mutagens, UV irradiation and transposons were previously used to generate mutants, however, these approaches generated random genetic alterations and painstaking

screening was needed to identify interesting mutants (Salyers *et al.*, 2000). A more recent technique for targeted mutagenesis has been developed which facilitated the production of a *B. fragilis* 638R *recA*<sup>-</sup> mutant for this study. Insertional gene inactivation was used to disrupt the *B. fragilis recA* gene with the suicide vector pGERM (Salyers *et al.*, 2000). PCR, DNA sequencing and Southern hybridisation confirmed the insertion of the vector into the chromosomal copy of *B. fragilis recA*. The mutation was stable in spite of the possibility that pGERM can excise and recircularise (Salyers *et al.*, 2000). However, pGERM excision would require homologous recombination and it has been shown in a *B. thetaiotaomicron recA*<sup>-</sup> mutant that disruption of *recA* leads to an absence of homologous recombination (Cooper *et al.*, 1997). In *B. thetaiotaomicron* the frequency of recombination was tested using the insertional vector pBT-2:ARR. The plasmid was able to insert in the chromosome of the wild type strain but no insertional events could be detected in the *recA*<sup>-</sup> mutant. Therefore, future work could include testing whether homologous recombination has been abolished in the *B. fragilis recA*<sup>-</sup> mutant using the insertional vector pBT-2:ARR.

In *E. coli*, RecA function was first investigated through generating a variety of *recA*<sup>-</sup> point mutations (Kuzminov, 1999). Different mutations in *E. coli* gave rise to various phenotypes allowing for detailed analysis of protein function. RecA730 (Glu38→Lys) mutants displayed a constitutive SOS response which, at a molecular level, was attributed to increased efficiency of ssDNA binding and enhanced stimulation of LexA autocleavage (Lavery *et al.*, 1992; Ennis *et al.*, 1995; Watanube-Akanuma *et al.*, 1997). Thus, it was deduced that ssDNA binding is important for RecA activation and subsequent LexA deactivation. Another important mutant was *recA430*; it contained a Gly204→Ser point mutation in loop L2 and displayed a partial LexA autocleavage deficient phenotype (Larminat *et al.*, 1992 and Sweasy and Witkin, 1991). Since RecA ssDNA binding was important for LexA deactivation, the Gly204 mutation affected a region of the protein important for binding DNA. Two other mutants, *recA659* and *recA611*, exhibited total loss of RecA activity due to mutations in L2 (Larminat *et al.*, 1992). Thus, L2 was important for RecA activation and recombination pointing to a role for this region in ssDNA binding. Analysis of these and many other *E. coli recA* mutants led to a thorough understanding of RecA function. Thus, further investigation into *B. fragilis* RecA function could involve analysis of point mutations to determine which regions are involved in DNA binding.

Insertional mutation of *B. fragilis recA* resulted in morphological changes where a sub-population of cells became filamentous. This has been observed for *B. subtilis* (Sciochetti *et al.*, 2001) and *S. thermophilus* (Giliberti *et al.*, 2002) *recA*<sup>-</sup> mutants. The phenotype was attributed to a disruption in DNA repair which lead to an inhibition of cell division. Since RecA is a major DNA repair protein, mutating it in *B. fragilis* probably resulted in disordered DNA repair and partitioning failures leading to abnormally long cells. Future work should look at DAPI staining of *B. fragilis recA*<sup>-</sup> cells to visualise the nucleoids (Giliberti *et al.*, 2002). If the nucleoids were longer in the mutant cells than the wild type cells, this would show that unresolved DNA damage had prevented replication fork progression, thereby halting cell division. Electron micrographs of the *B. fragilis recA*<sup>-</sup> mutant cells could also be taken to determine whether septation was being inhibited.

Genomic instability in a *recA* insertion mutant was reported for *S. lividans*, which accumulated 70 times more chloramphenicol-sensitive mutants than the wild type (Volf and Altenbuchner, 1997). In this study, the *B. fragilis recA*<sup>-</sup> mutant gradually altered its phenotype with respect to temperature and H<sub>2</sub>O<sub>2</sub> sensitivity after repeated sub-culturing which may have been due to secondary mutations. Since RecA repairs DNA damage, disruption of the *recA* gene could decrease the genomic integrity of the cell, leading to secondary mutations. An increased rate of mutagenesis was observed in *B. fragilis ahpCF*, *ahpF* and *ahpCFkatB* mutants compared to the parent strain, by measuring the frequency at which the cells spontaneously mutated to become fusidic acid resistant (Rocha and Smith, 1999). Thus, this mutagenicity assay could be used to measure the rate at which secondary mutations are occurring in the *B. fragilis recA*<sup>-</sup> mutant and see if it has increased genomic instability compared to the wild type.

The *B. fragilis recA* mutation did not significantly alter the growth rate of the mutant compared to wild type as measured by optical density. This has been observed for other *recA*<sup>-</sup> mutants although the cells have been shown to be anucleate (Huang and Chen, 2006). Thus, further studies would require growth curves where samples are plated to calculate viability. The *B. fragilis recA*<sup>-</sup> complemented mutant and *recA* over-expressing strains displayed similar growth rates to the wild type strain.

The *B. fragilis recA*<sup>-</sup> mutant was highly sensitive to UV and Mtz. Hence, it could be concluded that *B. fragilis* RecA is involved with repairing thymine dimers (generated by UV)

and DNA strand breaks (caused by Mtz). Other *recA* mutants have also been shown to be sensitive to these DNA damaging agents (Cooper *et al.*, 1997; Sander *et al.*, 2001; Weaver and Reddy, 2006). In *L. lactis*, the RexAB complex repairs DNA strand breaks by facilitating RecA-nucleofilament formation for the subsequent homologous recombination repair which is essential to this pathway (Kuzminov, 1999; Quiberoni *et al.*, 2001b). Bioinformatic analysis suggested that *B. fragilis* contained the RexAB proteins, therefore, mutating *recA* would disrupt the repair of DNA strand breaks in *B. fragilis*. In *E. coli*, nucleotide excision repair (NER) proteins repair thymine dimers (Skorvaga *et al.*, 2002) and during the SOS response, RecA promotes expression of the NER proteins to cope with the extensive DNA damage present in the cell (Friedberg *et al.*, 1995). However, since a LexA homologue was not identified in this study, there is as yet no evidence for an SOS response in *B. fragilis*, thus, *B. fragilis* RecA may be directly involved in repairing thymine dimers through homologous recombination.

Since Mtz toxicity is mediated by extensive DNA damage, enhanced repair or over-expression of DNA repair genes could result in MtzR (Chang *et al.*, 1997; Trinh and Reysset, 1998). To test this hypothesis, a *B. fragilis* *recA* over-expressing strain was constructed and exposed to Mtz. The over-expresser showed an increase in Mtz tolerance compared the wild type strain. Thus, over-expressing *recA* can lead to MtzR in *B. fragilis*. Given that Mtz is a mutagen, treatment with this drug could facilitate the introduction of mutations resulting in over-expression of DNA repair proteins in the pathogen (Trinh and Reysset, 1998).

The list of recognized MtzR mechanisms for *B. fragilis* includes over-expression of drug efflux pumps (Pumbwe *et al.*, 2007), decreased activity or inactivation of electron transport chain components (Diniz *et al.*, 2004) and the activity of 5-nitroimidazole nitroreductases (encoded by *nim* genes) which convert Mtz to non-toxic amino derivatives (Diniz *et al.*, 2004, Fang *et al.*, 2002; Jeong *et al.*, 2000). We now add one more possible mechanism of MtzR to this list: over-expression of *recA* which allows the cell to cope with the extensive DNA damage generated by the drug. Further work should include over-expressing other *B. fragilis* DNA repair genes essential for repairing DNA strand breaks, to see if this also leads to MtzR.

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