The molecular characterisation of the *recA* locus in the opportunistic pathogen *Bacteroides fragilis*

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CERTIFICATION OF SUPERVISOR

In terms of paragraph 9 of “General regulations for the degree of Ph.D.”, University of Cape Town, I, as the supervisor of the candidate Samantha A. Nicholson, certify that I approve of the incorporation in this thesis of material that has already been published or submitted for publication

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“Somewhere, something incredible is waiting to be known”

~Carl Sagan

Table of Contents

Abstract

Bacteroides fragilis is a human gut commensal and an opportunistic pathogen causing anaerobic abscesses and bacteraemias which are treated with the drug, metronidazole, a DNA damaging agent. The RecA protein is thought to be involved in the repair of metronidazole damage as well as damage caused by oxidative stress. The ability to survive oxygen stress is a strong indicator in an anaerobic bacterium of pathogenic potential and bacterial persistence in the oxygen rich peritoneal cavity. The aim of this thesis was to characterise the *B. fragilis recA* gene cluster with respect to its genomic context and the transcriptional regulation of the genes in response to metronidazole and oxygen stress. The possible functional roles of the proteins encoded by these genes in protection against these processes would also be evaluated.

The functional characterisation of the RecA protein from *B. fragilis* showed that it was important for survival after exposure to nitrogen (metronidazole) and oxygen (hydrogen peroxide) radicals. RecA was shown to be important for the maintanence of genomic integrity even under normal growth conditions, and overexpression of this protein was shown to be important for increased survival after exposure to metronidazole.

RT-PCR of *B. fragilis* cDNA showed that the *recA* gene was co-transcribed as an operon together with two upstream genes. Bioinformatic analysis revealed that the first ORF, BF638R1248, was a putative saccharopine dehydrogenase gene (*sdh*), encoding the SDH protein which may be involved in lysine degradation. The second ORF, BF638R1246/7 had homology to *bcp* genes, and encoded a putative Bactoferritin co-migratory protein (BCP) belonging to the thiol specific antioxidant superfamily. The functional roles of these proteins suggested that they might also be involved in survival after univalent electron stress.

Quantitative RT-PCR showed that all three genes were transcriptionally regulated, but at different levels, after exposure to either metronidazole or H_2O_2. This suggests that in addition to being expressed as an operon, the genes may also possess independent regulatory elements.

Functional characterisation of *sdh* and *bcp* was done using a gene mutation approach. Both insertional and deletion mutation methods were attempted but neither produced viable mutants in either of the upstream genes, suggesting that they may be critical for the survival of *B. fragilis* under normal growth conditions.

The use of heterologous gene expression was subsequently employed to establish the functional role of the *bcp* gene and the encoded putative BCP. A similar approach for SDH was not successful. Heterologous complementation and protein expression of BCP in *E. coli*, with subsequent biochemical assay, showed that the *B. fragilis bcp* gene encoded a functional bacterioferritin co-migratory protein (BCP), which is a small thiol-specific protein with antioxidant properties. This BCP showed flexibility in its substrate preference with activity against H_2O_2, tet-butyl hydroperoxide and linoleic acid. The peroxidase activity of this TSA protein was dependent on the presence of one or more members of the thioredoxin group and NADPH. The BCP aided protection of the enzymatic activity of the *B. fragilis* redox sensitive Fe-S metalloenzyme Glutamine synthetase (GSIII) during exposure to 100 µM H_2O_2. There was also evidence to suggest that it aided the recovery of the enzymatic activity of GSIII after exposure to 100 µM H_2O_2.

The findings of this research have resulted in the following hypothesis: The *recA* operon of *B. fragilis* acts during host invasion to contribute to the maintenance of DNA integrity and the anaerobic cellular environment until the *oxyR* regulated system and the pathogenicity genes are activated.

Abbreviations

%	percentage	GSIII	Glutamine Synthetase III
µ	micro	H_2O_2	Hydrogen Peroxide
ADP	Adenosine diphosphate	His	Hitadine
AhpC	Alkyl Hydroperoxidase C	IS	Insertion sequence
Amp	Ampicillin	J	Joule(s)
ATP	Adenosine triphosphate	Kan	Kanomycin
BCP	Bactoferritin co-migratory protein	kDa	Kilo dalton(s)
bcp	BCP gene	L	Loop
BfPAI	*B. fragilis* pathogenicity island	l	Litre(s)
BHI	Brain heart infusion	LB	Luria Bertani
BHISA	Supplemented BHI agar	Mg	Magnesium
BHISB	Supplemented BHI broth	mg	Milligram(s)
Bp	Base pairs	MIC	Minimum inhibitory concentration
CDD	Conserved domains database	min	Minute(s)
cDNA	Complementary DNA	ml	Millilitre(s)
CFB	*Flavobacteria-Chlorobium-Bacteroides*	mM	millimolar
Cys	Cysteine	Mob	Mobilisation
DAPI	4',6-diamidino-2-phenylindole	Mtz	Metronidazole
DNA	Deoxyribonucleic acid	MtzR	Mtz Resistant
DNase	deoxyribonuclease	MtzS	Mtz Sensitive
dsDNA	double stranded DNA	NAD(P)H	Nicotinamide adenine dinucleotide phosphate
Erm	Erythromycin	NER	Nucleotide Excision Repair
ETBF	Enterotoxigenic *B. fragilis*	ng	Nano gram(s)
Fe-S	Ferric- Sulpahte	No.	Number
Fig	Figure	OB	Oligomer binding
gDNA	genomic DNA	ºC	degrees celsius

Gent	Gentamycin	OD	Optical density
OD_{545}	Optical density at 545nm	ROS	Reactive oxygen species
OD_{600}	Optical density at 600nm	rpm	Revolutions per minute
ORF	Open reading frame	rRNA	Ribosomal ribonucleic acid
OSR	Oxidative stress response	SDH	Saccaharopine Dehydrogenase protein
PAGE	polyacrylamide gel electrophoresis	*sdh*	SDH gene
PCR	Polymerase chain reaction	SDS	sodium dodecyl sulfate
PCR	Polymerase chain reaction	sec	Second(s)
PFOR	pyruvate:ferredoxin oxidoreductase	ssDNA	single stranded DNA
pH	Potential Hydrogen	Tet	Tetracycline
Pro	Protein	Trn1	Thiredoxin
Prx	Peroxiredoxin	Trx1	Thioredoxin reductase
RecA	Recombinase A protein	TSA	Thiol specific antioxidant
recA	recA gene	UV	Ultraviolet light
Rif	Rifampicin	Volts	voltage
RNA	ribonucleic acid	ʎ	lambda
RNase	Ribonuclease	α	Alpha
RNS	Reactive Nitrogen species	µg	Micro gram(s)

Chapter 1

Literature Review

1.1 General Introduction

The *Bacteroides* genus is one of 5 predominant groups of bacteria in the intestinal microbiome accounting for around 30% of culturable gut microbes (Salyers, 1984, Wexler 2007). *Bacteroides fragilis* is a non-spore forming, gram negative, anaerobic rod (Wexler, 2007). It is a vital component of the commensal gut bacteria and is amongst the earliest colonisers of the gut passed from mother to child during vaginal birth (Reid, 2004). Although *B. fragilis* accounts for only around 0.5% of the *Bacteroides* it is a virulent opportunistic pathogen (Salyers, 1984) and is isolated from the majority of clinical cases of bacterial septicaemia resulting from intestinal ruptures or surgeries forming abscesses in the abdomen, pelvis, lungs and brain (Rocha *et al*., 2003).

This literature review will therefore focus on the multifaceted nature of *B. fragilis*, as both a commensal and opportunistic pathogen. The DNA repair pathways of prokaryotes in general will be reviewed with specific reference to known pathways in *B. fragilis*. The roles of DNA repair as well as oxidative stress responses in the evasion of the innate immune response will also be discussed.

1.2 *Bacteroides fragilis* commensal and opportunistic pathogen

As a commensal *B. fragilis* maintains a complex mutualistic relationship with the human host. It is responsible for the degradation of complex polysaccharides to volatile fatty acids used for metabolism (Hooper, 2002). It is also fundamental to the development of the gut-associated lymphatic tissue (GALT) (Rhee *et al*., 2004). The cell surface antigens of *B. fragilis* are associated with the production of the pre-immune antibody repertoire, and Zwitterionic polysaccharides developed on the cell surface aid in the production of the CD-4 T-cells in

infants (Mazmanian and Kasper, 2006). This bacterial species also assists the immune response in eradicating other intestinal pathogens (Wells *et al.*, 1988).

As a pathogen *B. fragilis* has systems that allow colonisation of the host tissue, evasion of the host immune response and destruction of the host tissues. This bacterium has a number of adhesion proteins (Ferreira *et al*., 2006; Oyston and Handley, 1991; Pauer *et al*., 2009,) that allow the colonisation of the abdominal environment. They produce several proteases including one for fibrinogen (Ferreira *et al*., 2006) and sialic acid residues (Berg *et al*., 1983) which aid in the degradation of the extracellular matrix (Wexler, 2007). Some strains of this species producing enterotoxin, ETBF (Sears, 2009), are extremely virulent and cause extensive tissue damage. *B. fragilis* has a complex system of cell surface lipopolysaccharides, which can be changed through the inversion of promoters in the DNA in an "on/off" manner (Coyne *et al*., 2008; Patrick *et al.*, 2009). This "mosaic" property of the cell-surface antigens is useful for the evasion of the host immune system (Mazmanian and Kasper, 2006; Patrick *et al.*, 2009). A number of virulence associated genes are regulated through DNA inversions in the genomic DNA of *B. fragilis*. This allows the bacterium to tailor its metabolic requirements to the specific cellular environment. This type of metabolic flexibility is important in an opportunistic pathogen as it saves metabolic effort (Sund *et al*., 2008). *B. fragilis* also possesses a comprehensive oxidative stress response which may be required for cellular survival outside of the anaerobic gut environment (Rocha *et al*., 2003) and is useful for the evasion of the host immune response in a number of bacteria (Miller *et al*., 1997). This will be reviewed in depth later in this chapter.

B. fragilis possesses intrinsic resistance to a number of structurally unrelated antibiotic groups (Hecht, 2004), including the β-lactams (Rogers *et al*., 1993). *B. fragilis* isolates from a

number of countries, including South Africa, are showing a trend towards increasing antibiotic resistance to the primary antibiotic of choice, metronidazole (Galvao *et al*., 2011). Metronidazole (Mtz) is the preferred antibiotic for treating anaerobic infections (Haggoud *et al*., 1994), and it exerts a bactericidal effect by generating nitro radicals which cause single-stranded (ss) and double-stranded (ds) DNA breaks (Sisson *et al*., 2000; Trinh and Reysset, 1998). The emergence of Mtz resistance mechanisms is increasingly compromising the effectiveness of treatment (Chang *et al*., 1997; Wareham *et al*., 2005), and a wide range of Mtz resistance mechanisms have been described in *B. fragilis*. These include decreased activity or total inactivation of electron transport chain components (Diniz *et al*., 2004), overexpression of multidrug efflux pumps (Pumbwe *et al*., 2006) and the expression of 5-nitroimidazole nitroreductases (encoded by *nim* genes) that convert Mtz to non-toxic amino derivatives instead of nitro radicals (Diniz *et al*., 2004). In addition, overexpression of the rhamnose regulatory protein RhaR is linked with Mtz resistance in *Bacteroides thetaiotaomicron* (Patel *et al*., 2009) and the *reg* gene (BF3248) of *B*. *fragilis*, a member of the AraC regulator protein family, is also involved in resistance to Mtz and other DNA damaging agents (Casanueva *et al*., 2008). A number of Mtz-resistant clinical isolates, however, do not contain *nim* genes or any of the previously described resistance mechanisms. Since Mtz exerts its bactericidal effect through generating DNA strand breaks, the possible role of DNA repair proteins in the response to treatment with Mtz is of interest (Chang *et al*., 1997). Relevant examples of possible responses are reviewed below.

1.3 General DNA repair systems in prokaryotes

Prokaryotes deal with compromised DNA in one of two ways, namely: through the direct reversal of damage; or by allowing continued cellular metabolism in the presence of compromised DNA integrity to be repaired later. The direct reversal systems are highly

efficient but may be overwhelmed by excessive DNA degradation (Friedberg *et al.*, 1995). Repair mechanisms of this type rely on fewer enzymes and are specific to DNA damage type. Some examples of this are the Ada repair proteins which rely on the reversal of incorrect O_6-methylation of the adenine residue and the photolyase pathway employed to repair pyrimidine dimers resulting from UV exposure (Friedberg *et al.*, 1995; Snyder *et al.*, 2003).

1.3.1 Nucleotide and base excision repair

In the case of more advanced DNA damage i.e. damage resulting from exposure to agents that are not the subject of a specific repair pathway, more generalised responses are initiated. These repair pathways utilise a number of enzymes which work simultaneously on both strands of the DNA. Excision repair systems remove the damaged nucleotides. The intact strand is used as a template to repair the damage. This system employs a polymerase and a ligase to rebuild the removed section and close the nicks (Bichara *et al.,* 2007; Nankano *et al.,* 2007). This type of DNA repair has high fidelity and minimises the number of alterations to the underlying genetic material. These systems are often inducible and their induction is directly proportional to the degree of genomic degradation in the bacterium (Friedberg *et al.*, 1995; Snyder *et al.*, 2003).

1.3.2 Homologous Recombination

Systems that rely on a static undamaged DNA strand as a template for repair are of limited use when a section of DNA is undergoing replication. Replication may stall if the replicative machinery encounters a section of damaged DNA, and this halt may result in cell death. In this case a more complex system of DNA repair is initiated. Homologous recombination uses a series of inter-related pathways to repair damage and re-initiate the replication process. Homologous recombination is also employed in cases of severe DNA degradation allowing trans-lesion synthesis and mismatch repair, allows the metabolic functioning of the cell,

even with limited genomic stability, thus circumventing cell death. This "tolerance" does increase the mutation rate which may be advantageous or detrimental to the cell in the long term. Homologous recombination facilitates DNA repair by pairing damaged DNA to an intact region with high sequence homology within the cell. This acts as the template for DNA replication and thus gap repair. This system is catalysed by the RecA protein (Friedberg *et al.,* 1995; Snyder *et al.*, 2003).

1.3.3 The RecA protein

The RecA protein has amino acid sequence homology of between 40% and 100% in the more than 60 eubacterial species that have been characterised to date (Ahel *et al.,* 2005; Aminov *et al.,* 1998). It contains three regions of functional importance. The central region exhibits the highest degree of conservation amongst species and is responsible for ATP binding through the Walker A and B regions (Ahel *et al.,* 2005). The functional activity of RecA is dependent on the conformational changes associated with binding and hydrolysis of ATP (Chen *et al*., 2007). This region also contains the L-loops L1 and L2 which are vital for ssDNA binding (Ahel *et al*., 2005). The C-terminal, which is more variable than other regions of this protein, is responsible for species specific functional regulation. This is illustrated in both *Streptomycetes* and *Deinococcus radiodurans* (Ahel *et al.,* 2005; Carroll *et al.,* 1996). The C-terminal is also associated with the co-protease activity of RecA in its role as positive regulator of the SOS response in several bacterial species (section 1.3.4.3). The N and C terminal domains interact between monomers to allow the development of the nucleofilament needed for strand invasion and DNA repair (Ahel *et al.,* 2005).

RecA is activated by ATP binding. This activated protein binds damaged DNA and forms polymers with other activated RecA monomers to form a nucleofilament. Extension and

disassembly of this nucleofilament is driven by ATP. Nucleofilament assembly requires the binding of an intact ATP molecule on the 3' overhang. Disassembly is facilitated by ATP hydrolysis and occurs in the opposite direction from assembly (Cox *et al.*, 2005). Once the RecA nucleofilament is extended, the homology search is activated (Campbell *et al.,* 1999). Strand migration and the creation of the Holliday junctions is an energy rich process and relies on the hydrolysis of ATP associated with the disassembly of the RecA filament to facilitate this process (Campbell *et al.,* 1999; Chen *et al*. 2007).

1.3.4 RecA mediated DNA repair

Homologous recombination is the largest system of DNA repair, whose main catalytic component is the RecA protein (Kuzminov, 1999; Smith, 1988; 1987). This system is employed to repair double and single stranded DNA breaks, amongst other types of damage and is therefore, of interest in relation to repair of metronidazole associated damage. It also assists with the recovery of stalled replication forks (Fulconis *et al.,* 2006; Kuzminov, 1999). The search for homology is based on the understandings gained from the *E. coli* model system. The initial steps of this repair system can be catalysed by two separate pathways namely; the RecBCD pathway and the RecFOR pathway (Courcelle *et al.,* 2005) (Fig 1.1). In either pathway the main catalytic component is the RecA protein which is employed to bind DNA, facilitate homology searching as well as the subsequent pairing of the damaged DNA to its undamaged counterpart as template for repair (Kuzminov, 1999; Li *et al.,* 2008). The choice of enzyme complex to facilitate the initialisation of the RecA reaction is dependent on the DNA substrate (Fulconis *et al.,* 2006). RecBCD is usually used for dsDNA damage while RecFOR is employed for ssDNA damage (Courcelle *et al.,* 2005). All of the reactions that result in the restart of replication also require the activity of the PriA protein and its co-

enzymes. These proteins serve to reload the replisome once repair is completed (Ivancić-Bacće *et al.*, 2006).

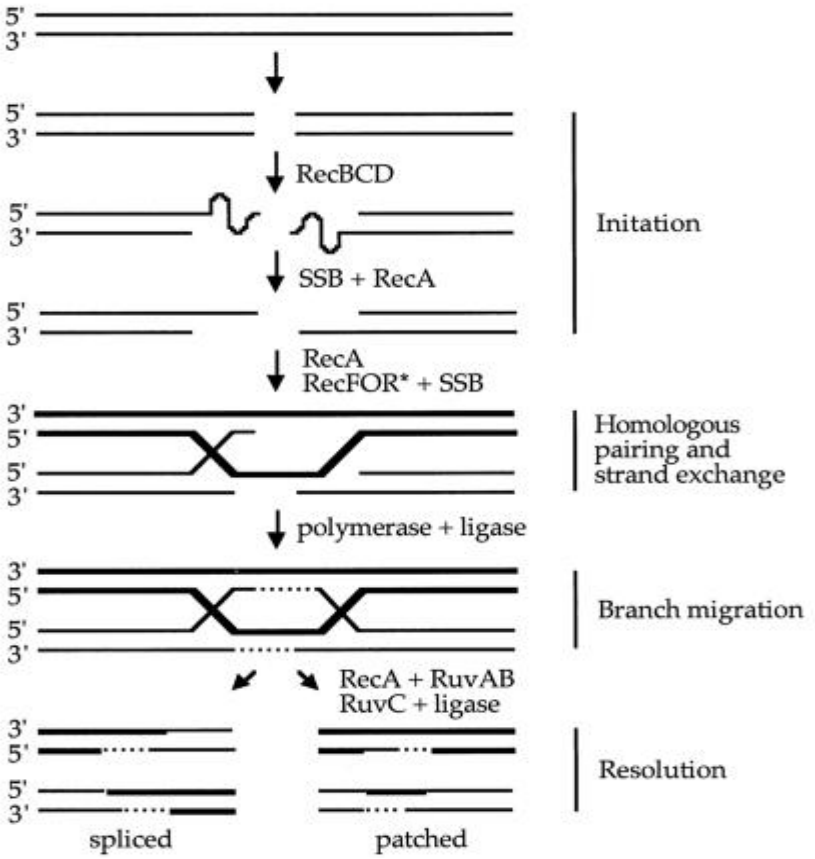


Figure 1.1: Diagram representing the steps common to both pathways of homologous recombination. (McGrew *et al.*, 2003) SSB: single stranded binding protein.

The mode of action for RecA mediated repair is universal (Courcelle *et al.,* 2005; Kuzminov, 1999) (Fig. 1.1). The RecA proteins bind ssDNA, forming filaments, and these filaments perform homology searching by constantly binding to DNA looking for a sequence of 40-50 base pairs of high sequence homology (Kuzminov, 1999; Smith, 1988). The ssDNA is thus paired to the most homologous sequence and the filament is extended by RecA mediated branch extension (Fulconis *et al.,* 2006; Li *et al.,* 2008). This strand migration results in the creation of a four armed dsDNA structure called a Holliday junction (Friedberg *et al.,* 1995; Fulconis *et al.,* 2006; Snyder *et al.,* 2003). Holliday junctions are recognised by the migratory

proteins, RuvAB, and the RecG helicase which catalyses final branch migration. Binding of the RuvC resolvase to the RuvAB complex results in Holliday junction separation, the result being some degree of strand exchange and DNA repair (Fig. 1.1) (Friedberg *et al.,* 1995; Fulconis *et al.,* 2006; Snyder *et al.,* 2003).

1.3.4.1 The RecBCD pathway

The RecBCD pathway is used on DNA templates which have an end generated by a dsDNA break. RecBCD is a linear motor protein that acts as a processive helicase and an ATP-dependent nuclease (Spies *et al.,* 2006). RecBCD activity leads to RecA loading by unwinding the DNA and creating nicks to develop and extended the ssDNA tail which can then act as the substrate for RecA binding (Churchill *et al.*, 2000; Kuzminov, 1999). This filament is then used for homologous recombination (Kuzminov, 1999; Smith, 1988). This system uses the Chi regulatory element which is a highly conserved, asymmetrical, oligomeric sequence (Courcelle *et al.*, 2005; Kuzminov, 1999; Smith, 1987; 1988). This regulatory element leads to a conformational change in the RecBCD complex which allows the RecB subunit to load RecA asymmetrically onto the DNA strand which contains the Chi element (Churchill *et al.,* 2000; Kuzminov, 1999; Spies *et al.,* 2006). This Chi element is recognised by the RecC subunit on its 3' end. When the RecBCD encounters a Chi element the number of nicks decreases and the strand with the Chi element is coated with the RecA protein and converted to a D-loop like structure (Fig. 1.2)(Churchill *et al.* 2000; Spies *et al.,* 2006). This D-loop shares many functional characteristics with the replication fork and allows homologous recombination to be coupled to a replication re-initiation reaction (Kuzminov, 1999). Once RecA is loaded onto the ssDNA template, homologous recombination proceeds as previously described through homology search and strand exchange (Kuzminov, 1999; Smith, 1987;1988).

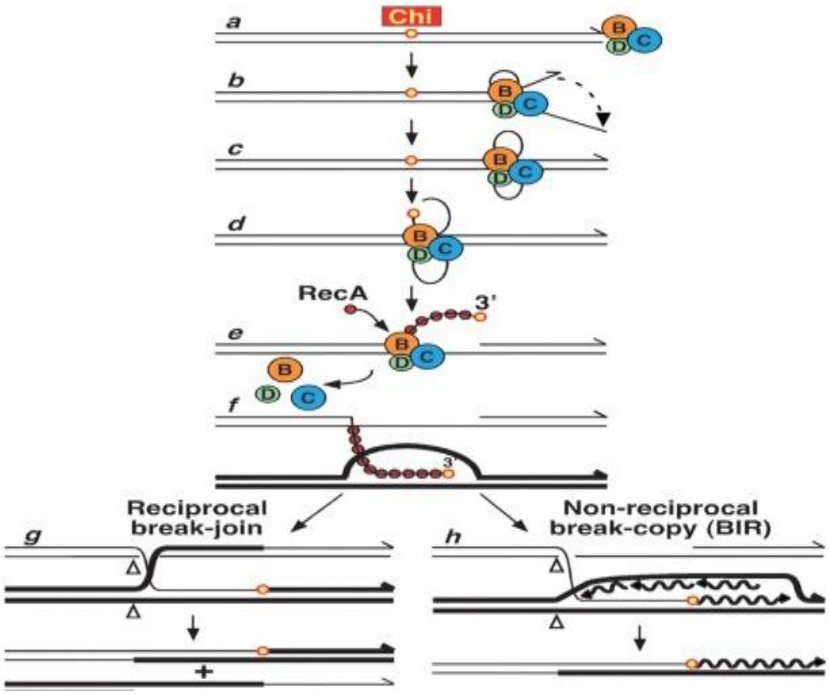


Figure 1.2: DNA unwinding by RecBCD enzyme and structure of the enzyme bound to a dsDNA end. The RecB subunit is orange, RecC is blue and RecD is green. RecBCD binds a duplex DNA end (a) and unwinds the DNA with the formation of a loop–tail structure (b). The loop and tails enlarge as RecBCD unwinds; the tails can anneal to form a twin-loop structure (c). Upon encountering Chi, the enzyme cuts the top strand (d) and loads RecA onto the 3'-ended strand (e). The RecA-ssDNA filament forms a D-loop with a homologous duplex (f). The DNA loop can be cut, with the formation of a Holliday junction, which can be resolved into crossover-type recombinants (g). Alternatively, the D-loop can prime DNA synthesis, with the formation of a replication fork and a break-induced recombinant (BIR) (h). (Amundsen *et al*., 2007)

1.3.4.2 The RecFOR pathway

RecFOR is often described as an alternative pathway to RecBCD and has been characterised in many bacteria that do not possess a RecBCD pathway. (Courcelle *et al.,* 2005) The RecFOR pathway is primarily employed for the repair of single-stranded gaps often formed as the result of stalled replication (Maisnier-Patin *et al.,* 2001; Michel *et al.*, 2001). It also functions to protect the replication fork from collapse (Michel *et al.*, 2001).

The first step in this process is the removal of single strand binding proteins (SSB) from the ssDNA. RecO has a higher affinity for ssDNA than SSB and facilitates the interaction of RecA and ssDNA (Courcelle *et al.,* 2005; Inoue *et al.,* 2008). The Rec O, R and F proteins form a

complex that acts as a structure specific mediator of homologous repair and targets ss-dsDNA junctions (Fig 1.3) (Morimatsu *et al.,* 2003). RecFOR limits the extension of the RecA filament beyond the gap in the DNA (Courcelle *et al.,* 2005; Morimatsu *et al.,* 2003). RecA loading by this protein complex is dependent on the presence of a 5' junction terminus produced by RecJ (Morimatsu *et al.,* 2003). RecFR binds the gapped DNA (gDNA) and the RecOR complex binds the gDNA-RecFR complex displacing the bound SSB protein. This leads to RecA loading onto the DNA (Courcelle *et al.,* 2005; Kuzminov 1999; Morimatsu *et al.,* 2003). RecJ and RecQ facilitate the processing of the DNA break and the formation of an ssDNA tail (Morimatsu *et al.* 2003). RecA facilitates homologous pairing and strand exchange (Kuzminov, 1999; Smith, 1987; 1988)

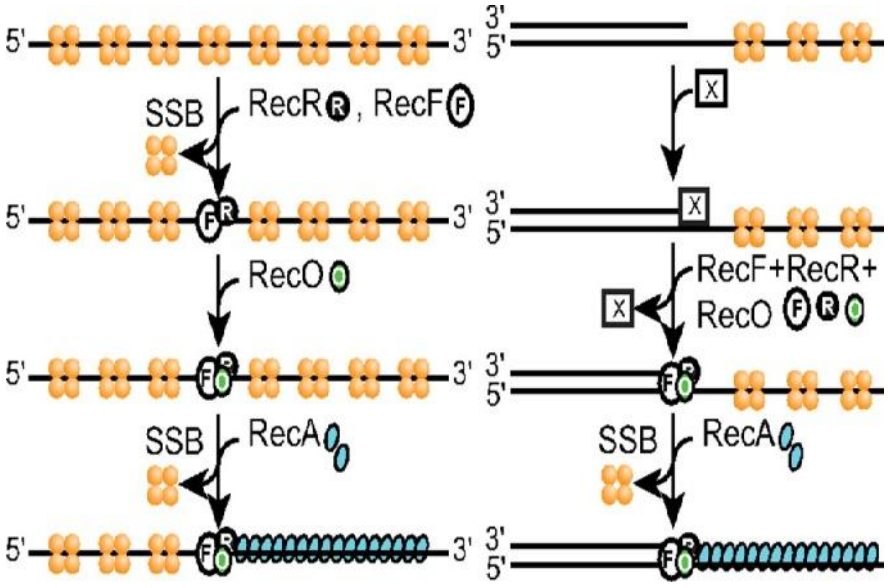


Figure 1.3: Model for RecFOR function. RecFOR functions on both SSB-coated ssDNA (left) and gapped DNA (right) are shown. On the first of these DNA substrates, the RecOR pathway is more facile as long as the SSB C terminus is present. On gapped DNA, the RecFOR pathway is effective only if the ReFOR complex (or staged sets of subcomplexes) is bound near the end of the duplex region near the gap. An unknown factor (X) must be present to guide RecF(R) to the ss-dsDNA junctions. (Sakai and Cox, 2009)

1.3.4.3 The SOS response

The SOS response, first described in *Escherichia coli*, is a global transcriptional response induced by DNA damage (Janion, 2001; Kuzminov, 1999; Smith, 1987; 1988; Yasbin *et al.* 1991). Cells that have undergone severe stress exhibit an enhanced capability for

recombinatorial and excision repair as well as a heightened rate of mutation (Bhattacharya *et al.*, 2002; Janion, 2001; Maul *et al.*, 2005). The SOS regulon is highly specific, and genes that are included in this response must contain a palindromic LexA binding sequence that overlaps the promoter. The activation of this response is RecA dependent (Friedberg *et al,* 1995; Janion, 2001; Snyder *et al.,* 2003; Yasbin *et al.*, 1991). RecA induces the auto-cleavage of LexA relieving the transcriptional repression experienced by the SOS response genes (Yasbin *et al.*, 1991).

Both nucleotide excision repair and homologous recombination are transcriptionally influenced by the SOS response. The genes of both of these systems have a LexA binding site within their promoters (Bichara *et al.*, 2007). If homologous recombination and nucleotide excision base repair fail, the polymerase V mediated trans lesion synthesis system is initiated (Maul *et al.*, 2005). Polymerase V is a complex of the *umuC* and *umuD* gene products (Schlacher *et al.,* 2006). This polymerase allows for the error-prone replication of severely damaged DNA in the absence of a template strand. It is highly mutagenic (Friedberg *et al.*, 1995; Snyder *et al.,* 2003). The activity of this polymerase is catalysed by RecA. RecA cleaves the UmuD subunit of the UmuC-UmuD complex leading to the active form of Polymerase V (Simon *et al.*, 2008). The SOS response is characterised by a filamentous morphology (Janion, 2001). These characteristics are used to define SOS type responses in other bacteria.

There are SOS responses described in both Gram negative (Bhattacharya *et al.*, 2002) and Gram positive bacteria, including *E. coli* and *Bacillus subtilis*. The *B. subtilis* SOS response comprises 33 genes and uses a LexA homologue as well as RecA in an auto regulatory loop to allow for induction (Yasbin *et al.*, 1991). There are, however, a number of bacteria that

exhibit a heightened DNA repair response and cell filamentation not regulated by the LexA protein, for example *D. radiodurans* (Bonacossa de Almeida *et al.*, 2002).

1.4 DNA repair in *B*. fragilis

The mechanisms for DNA damage repair and tolerance are not, as yet, well characterised in *B. fragilis*. However, progress has been made in the elucidation of some of the main pathways known to be employed by model systems like *E. coli* and *B. subtilis.*

Goodman *et al*. (1987) complemented a RecA deficient *E. coli* strain using a putative *recA* gene product from *B. fragilis*. This protein showed amino acid sequence homology and antibody cross reactivity to the *E. coli* RecA protein (Goodman *et al.*, 1990). This suggested that there was an active homologous repair system in *B. fragilis*. Other investigations of the repair systems in *B. fragilis* produced evidence that excision repair pathways exist in response to mytomycin C and UV damage (Abratt *et al.*, 1986), and bioinformatic analysis carried out by Steffens *et al*. (2010) showed the presence of putative nucleotide excision repair genes as well as RecF, RecO and RecR homologues but no clear RecBCD proteins. Work by Reuter *et al*. (2010) showed an active AddAB helicase, with mutants being sensitive to UV light, in *B. fragilis*, an enzyme which is employed in a number of bacterial groups lacking the two helicase RecBCD complex (Reuter *et al*., 2010). Further, the identification of 3 putative *recQ* helicases in *B. fragilis* (Paul *et al*., 2011) lends support to the presence of a functional homologous repair pathway in *B. fragilis* with a classical RecA protein as the central component (Steffens *et al*., 2010). Work by Dachs *et al*. (1995) showed increased sensitivity to metronidazole in *recA* deficient *E.coli* strains when compared to wild type suggesting a link between DNA repair and survival during and after Mtz exposure.

The SOS response system, under its strictest definition, is not present in *B. fragilis* species as there is no sequence homologue to the LexA protein in the *Bacteroides* genome (Goodman

et al., 1990; Steffens *et al.*, 2010). Experiments have revealed some filamentation of *B. fragilis* cells in response to DNA damage and an enhanced repair capability which suggests that this bacterium may have a global stress response independent of a LexA type regulator (Steffens *et al.*, 2010). The RecA protein from *Bacteroides thetaiotaomicron* is linked to the filamentation of cells under stress and survival after oxygen exposure, but this bacterium also has no LexA sequence homologue (Cooper *et al.*, 1997).

1.5 RecA and Virulence

The repair of damaged DNA is a vital component in establishing a successful infection within a hostile host environment (Kuzminov 1999). In *Salmonella enterica* typhimurium DNA repair protects the cells from phagocyte oxidative burst during intracellular invasion (Mertens *et al*. 2008). It also plays an integral role in the genomic rearrangement which has downstream effects on the expression of virulence factors in many pathogenic *E. coli* strains (Fuchs *et al.* 1999). RecA has been associated with the reversible integration of plasmid and phage DNA into the chromosome. This often results in the inclusion of new virulence factors from other bacteria. Genomic rearrangements allowing for the alteration of cell surface polysaccharides and proteins to avoid host defence detection have also been attributed to RecA function (Fuchs *et al.* 1999). The pathogens, *Erwina carotovara* and *Klebsiella spp.*, use RecA to directly regulate the virulence genes (Fuchs *et al.* 1999). *Neisseria gonorrhoea* uses RecA-mediated recombination to generate diversity in their pili which are used to transport the bacteria through the extracellular environment of the host (Fuchs *et al.* 1999). In *Vibrio cholerae* and *Staphylococcus aureus*, there is evidence to suggest attenuated adhesion in the absence of RecA (Fuchs *et al.* 1999), although this has yet to be molecularly characterised. The *B. fragilis* ETBF (Enterotoxigenic *B. fragilis*) strains use the translocation and activation of the pathogenicity islands to improve and alter the virulence status of the

bacteria and avoid host immune detection (Wexler, 2007). These genomic rearrangements may also be a partially RecA dependent process.

1.6 The Immune Response

The human response to infection is primarily reliant on both physical and chemical barriers. Should these fail to recognise foreign material, the neutralisation and elimination of infectious agents are of the highest priority (Modlin and Cheng, 2004). This is mediated by the 2 arms of the host immune response, namely, the humoral (innate) immune response and the adaptive immune response (Modlin, 2011). Both are reliant on the recognition of cell surface antigens and the labelling of invaders as non-self (Modlin, 2011). The adaptive immune response is vast and depends on specific antibodies. Bacterial evasion of this type of response is well characterised in the opportunistic pathogen *B. fragilis* (Coyne *et al.*, 2008; Patrick *et al*., 2009). The adaptive immune response is, therefore, not explored in this research and thus will not be discussed further in the literature review.

The innate or inflammatory response is mediated by a number of cells and is a non-specific response reliant on phagocytosis and oxidative burst (Modlin, 2011). The inflammatory response depends on the cytokine burst and subsequent chemotaxic gradient. Cytokines recruit the granulocyte (neutrophils) and agranulocyte (macrophages) cells to the site of inflammation. Neutrophils respond to sterile injury as well as pathogen invasion (Phillipson and Kubes, 2011). In the case of sterile injury, they are recruited by the damage-associated molecular pattern molecules (DAMPs) produced by injured tissue (Tanaka *et al*., 2012). The presence of neutrophils at sites of sterile injury is important for the opportunistic pathogen *B. fragilis* as it uses a wound in the endothelial layer of the gut to escape from the gut lumen into the abdominal environment. The establishment of an immune response prior to

pathogen invasion of the site has implications for cellular survival (Miller *et al*., 1997; Sund *et al*., 2008). Once in the area, neutrophils recruit other immune response cells including monocyte derived macrophages and lymphocytes. These cells are then responsible for the engulfment and destruction of foreign cells (Miller *et al*., 1997).

Macrophages and neutrophils are responsible for phagocytosis of invading pathogens (Modlin, 2011). When the cells encounter the bacterial pathogen, they produce the phagosome. This structure is the primary source of exogenous oxidative stress for invading pathogens. It has a much higher consumption of oxygen then the rest of the neutrophil and thus a specialised NADPH-oxidase complex in the cellular membrane is induced. This complex is responsible for the reduction of O_2 to O_2^-, and these oxygen radicals are then secreted into the phagosome where they are broken down into any number of oxygen radicals including H_2O_2. The iNOS pathway, a biochemical pathway that produces nitric oxide, is also active in these cells (Spiro, 2007). The phagosome eventually fuses with cytoplasmic granular molecules that contain hydrolases that breakdown the dead bacterial cells (Miller *et al.*, 1997).

1.7 Bacterial evasion of the innate immune response

Bacterial pathogens have developed a multitude of ways to avoid the innate immune response (Fig 1.4) (Miller *et al*., 1997). Examples of these are the avoidance of phagocytosis by the production of streptolysins by some *Streptococcus spp.* (Bernheimer *et al*., 1960), the production of hydrophobic cellular capsules by *N. meningitides* (James and Swanson, 1977) or alginate by *Pseudomonas aeruginosa* (Learn *et al*., 1987; Simpson *et al*., 1989). *Legionella pneumophila* reduces the acidification of the phagosome (Horwitz and Maxfield, 1984), and other bacteria are not damaged by the phagosome but produce enzymes that counteract

the hydrolytic enzymes that cause cellular breakdown (Hof, 1991). A number of bacteria including most *Yersinia spp* produce tyrosine phosphatases that disrupt cellular signalling allowing the escape of pathogens from the phagocyte (Guan and Dixon, 1993).

Intracellular pathogens like *Leishmania major* (a eukaryotic organism) are dependent on phagocytosis for survival and they circumvent the oxidative burst by non-enzymatic means, either through the inhibition of the NADPH-oxidases or proteinase K (Brandonisio *et al*., 1994; Frankenburg *et al*., 1990).

1.7.1 DNA repair and the evasion of the innate immune response

In *Porphyromonas gingivalis*, an oral pathogenic bacterium showing similarities to *B. fragilis*, the *mutY* gene has been shown to be important for survival during oxygen radical stress. If replication occurs after H_2O_2 exposure without the repair of 8-oxoguanine residues MutY removes the adenine that has been mispaired with these residues allowing for maintanence of genomic integrity after mismatch repair (Robles *et al*., 2011). In *Acinetobacter baumanni*, RecA is associated with genomic integrity and consequently its survival after exposure to oxidising agents (Aranda *et al*., 2011). The extreme oxidative resistance of *D. radiodurans* is imparted by the synergistic activities of its antioxidant pathways and DNA repair enzymes (Slade and Radman, 2011). *H. pylori* RecO/RecR double mutants deficient in ssDNA break repair are also extremely sensitive to oxidative stress (Wang *et al.*, 2011) while *Listeria monocytogenes* uses radical induced DNA breaks and RecA facilitated DNA repair to increase genetic variation in biofilms (Van der Veen and Abee, 2011). These observations suggest that the RecA protein from *B. fragilis* may also be important for surviving the oxygen radicals associated with the innate immune response.

1.7.2 The role of oxidative defence in bacterial survival of the innate immune response

The majority of pathogens caught in the oxidative burst associated with the innate immune response use organised enzymatic systems of oxidative defence (Miller *et al*., 1997). *E. coli* and *B. subtilis* both encode multiple super-oxide dismutase enzymes and catalases that are differentially expressed in altering oxidising environments (Switala *et al*., 1990). The induction of the oxidative stress response to reduce radicals by specific antioxidant proteins is an efficient and wide spread system of defence within the human pathogens. This system is particularly well developed in those pathogens that rely on injury to the host system for the establishment of infection (Modlin, 2011).

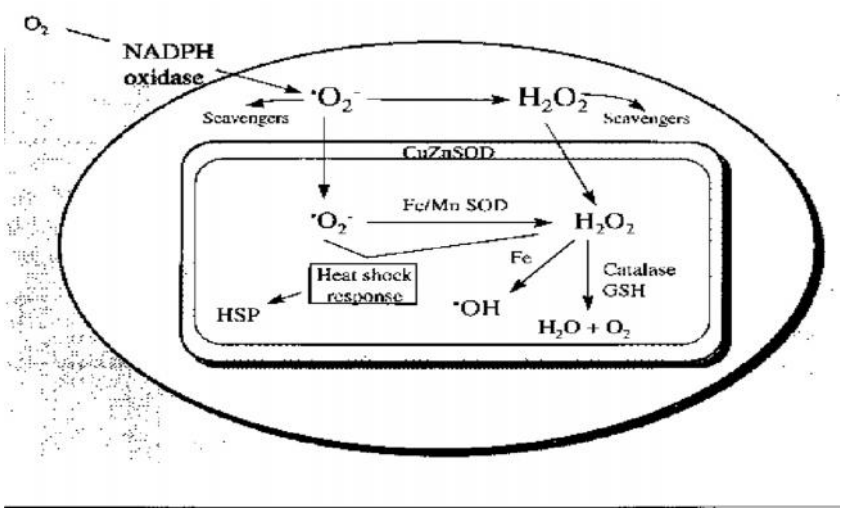


Figure 1.4: Representative diagram of the mechanisms of avoidance used by pathogens after exposure to phagocytes. (Miller *et* al., 1997). SOD: Superoxide Dismutase; HSP: heat shock protein and GSH: glutathione peroxidase/reductase.

1.8 The oxidative stress response (OSR) of *B. fragilis.*

Despite its classification as an obligate anaerobe, *B. fragilis* has been described as being able to remain viable in atmospheric oxygen for up to 48 hours (Rocha *et al.*, 2003). This bacterium has an extensive, complex, co-ordinated response to oxidative stress that involves at least 3 independent regulons, 28 proteins and alterations to physiology at the metabolic level (Baughn and Malamy, 2004; Meehan and Malamy 2012 ; Rocha *et al*., 2003).

These genes may be responsive to oxygen, hydrogen peroxide or both (Rocha *et al*., 2003) and the transcriptional response to these oxygen species includes up to 45% of the transcriptome (Sund *et al*., 2008). The oxygen adaptive response of *B. fragilis* can be divided into 3 main mechanisms namely, the detoxification of oxygen radicals; the protection of macromolecules; and the alteration of the metabolic pathways (Rocha *et al.,* 2003; Reott *et al.*, 2009; Meehan and Malamy, 2012).

1.8.1 The detoxification of oxygen radicals

During acute exposure to oxygen radicals *B. fragilis* transcription shows a bias towards detoxification of oxygen radicals as a survival strategy (Sund *et al*., 2006; 2008). This is clearly illustrated by the number of redundant peroxidases present (Rocha *et al*., 2003; Sund *et al.*, 2006). *B. fragilis* has an active super-oxide dismutase (SOD) protein (Gregory, 1985), catalase (KatB) (Rocha *et al*., 1997), cytochrome c peroxidase (Ccp) (Rocha *et al*., 2003), thioredoxin peroxidase (Tpx) (Rocha *et al*., 2003), 6 putative thioredoxins (Trx) (Reott *et al*., 2009), the thiol peroxide scavengase (Tps) (Sund *et al*., 2006), and the alkyl hydroperoxidases AhpC and AhpF (Rocha *et al*., 1999). These proteins all work co-operatively with some functional redundancy within the bacterium. They all show activity against the organic peroxides which suggests that these are the compounds that pose the largest problem during acute oxidative stress within the anaerobic environment (Reeves *et al*., 2011; Johnson *et al.,*2011; Reott *et al*., 2009; Wei *et al*., 2012). A number of these proteins are regulated by more than one promoter (Rocha *et al.*, 1997; 2003; 2007) and have roles outside of the oxidative stress response associated with normal anaerobic growth. An example of this is the *trxA* gene which is essential for growth even in the anaerobic environment (Reott *et al*., 2009). The presence of the *trxB* gene has been shown to be fundamental for the formation of abscesses by this bacterium in mice (Rocha *et al*.,

2007) and the thioredoxin proteins are associated with the maintenance of the thiol/disulfide homeostasis in *B. fragilis* (Reott *et al*., 2009; Rocha *et al*., 2007).

1.8.2 The protection of macromolecules

Oxygen radicals damage lipids, proteins and nucleic acids (Imlay, 2003). Protection of these macromolecules is, therefore, an important consideration within the OSR. *B. fragilis* induces a number of protective proteins targeted to these macromolecules. An RNA binding protein is produced from a bicistronic mRNA along with the thioredoxin peroxidase enzyme (Rocha *et al*., 2003). This protein may function to stabilise the mRNA transcripts of the anti-oxidant protein and maybe other additional transcripts associated with the global OSR regulons (Rocha *et al.*, 2003). *B. fragilis* carries an *ftnA* gene which encodes a ferritin protein. The transcriptional induction of this gene is iron independent under anaerobic conditions but is sharply up regulated in iron-rich aerobic environments (Rocha *et al.*, 2004). The role of this protein is to sequester free iron to prevent Fenton reactions within the bacterial cells and the generation of more hydroxyl radicals (Rocha *et al.*, 2004). *B. fragilis* also produces a novel class of miniferritin proteins that are closely related to the Dps-like proteins of the archea (Gauss *et al*., 2012). These proteins have been demonstrated to produce cage-like structure around the DNA (Gauss *et al*., 2012) preventing hydroxyl radical cleavage. In addition *B. fragilis* produces a DNA binding protein that protects genomic integrity (Rocha *et al.*, 2003) and a lipoprotein specific chaperone with unknown function from the *lolA* gene under regulation by oxidative stress (Rocha *et al*., 2007).

1.8.3 Alterations to metabolism

B. fragilis exhibits a change in its metabolism under oxidative stress. During the acute stages of exposure, the bacterium decreases the activity of its energy generating enzymes but over

time a broad metabolic response can be observed (Sund *et al*., 2008). The bacterium experiences a shift in its metabolism that is similar to that occurring when cells move from log to stationary phase in the growth cycle (Sund *et al*., 2008). Baughn and Malamy (2004) showed that *B. fragilis* processes a cytochrome bd oxidase which is essential for growth in nanomolar concentrations of oxygen. This protein is encoded by the *cydAB* operon and assists in the depletion of oxygen and enhances NADH-oxidase activity. This metabolic component acts as the terminal component of the respiratory pathway and has a redundant metabolic function to fumarate reductase (Baughn and Malamy, 2004; Meehan and Malamy, 2012 a). In some aerobic bacteria, this operon has been described as essential for the maintenance of cytoplasmic redox potential. However, it is not essential under anaerobic conditions in *B. fragilis* (Baughn and Malamy, 2004). Smalley *et al*. (2002) showed that 3 genes associated with metabolism experience a change after exposure to oxygen. The *osu* operon, which is described in *B. thetaiotaomicron* as being involved in starch utilisation (Cho *et al*., 2001), aspartate decarboxylase (Smalley *et al*., 2002) and *nrdA*, encoding an aerobic ribonucleotide reductase, are all transcriptionally up regulated in response to oxygen stress (Smalley *et al*., 2002). The *nrdA* gene product is used to produce a pool of undamaged deoxyribonucleotides needed in DNA repair during recovery and its presence suggests a strong selective pressure for oxygen adaptation within *B. fragilis* (Smalley *et al*., 2002). The *oxe* gene has been described as important in limiting microaerophilic growth of this organism and deletion of this gene has allowed growth in up to 2% atmospheric oxygen. This is facilitated by an improved ability to scavenge H_2O_2 (Meehan and Malamy, 2012 b).

1.8.4 Transcriptional Regulation of the OSR

The complex nature of the OSR in *B. fragilis* suggests a co-ordinated response to oxygen radicals. In other bacteria, this has been reported to be facilitated by a number of different

regulators (Wei *et al*., 2012). To date, only the redox sensitive OxyR transcriptional activator has been described in *B. fragilis*. This regulon, controlled by OxyR, includes no fewer than 6 peroxidases (Rocha *et al.,* 2003; 2007) and is extremely sensitive to oxygen stress (Rocha *et al.*, 1998; 2000). OxyR activation is observable from as little as 10 µM H_2O_2 or 0.5% oxygen, and experiences maximal induction at 2% oxygen (Sund *et al.,* 2008). There are alterations to the induction profiles of the different genes in this regulon which are indicative of a more complex system of interrelated pathways affecting expression and induction (Rocha *et al*., 2000; 2003; 2007; Wei *et al*., 2012). The complex and multifaceted nature of the OSR within *B. fragilis* is mirrored in its regulation.

The extent of the oxidative stress response in *B. fragilis* is surprising given the limited number of pathways that produce radicals within the normal metabolism of this organism (Meehan and Malamy, 2012 a). The maintenance of metabolic and cellular integrity under OSR is possibly more important in the opportunistic pathogen form of *B. fragilis* (Herren *et al.*, 2003; Renvall and Niinikoski, 1975). Sund *et al*. (2008) suggest that the importance of the oxidative stress response in the bacteria can be seen in the ability of *B. fragilis* to tailor its metabolism to altering environments associated with the extra-intestinal environment and survival in opportunistic infection.

1.9 Aims and objectives

B. fragilis has devolped a number of systems that allow it to change from being a commensal to a highly efficient opportunistic pathogen of the human host. The molecular systems of capsule formation (Coyne *et al*., 2008; Patrick *et al*., 2009), toxin production (Sears, 2009), oxidative stress (Meehan and Malamy., 2012; Rocha *et al,* 2003;2007; Reott *et al*., 2009) and tissue invasion (Ferreira *et al*., 2006) are all being actively researched within

this bacterium by various research groups. Our interest is in DNA repair systems and the possible overlap between these fundamental bacterial pathways, the initial stages of pathogenicity and the associated oxygen stress. This project seeks to elucidate the molecular functions of the RecA protein in *B. fragilis*. It aims to define the *recA* locus, and explore potential links between the homologous repair pathway of *B. fragilis* and the environmental changes associated with pathogenicity. The ultimate aim of this project is to characterise the *recA* locus of *B. fragilis* and develop future hypotheses regarding the ability of this normal gut commensal to become an opportunistic pathogen with specific regard to the roles of the integral DNA repair protein RecA.

The research approach will include functional characterisation of the role of the RecA with regard to maintaining genomic integrity and in supporting cell survival after exposure to different environmental stressors (Mtz, H_2O_2, bile salt and UV). Transcriptional studies using RT-PCR and quantitative RT-PCR methods will be used to elucidate the genomic context of the *recA* locus and the transcriptional regulation of *recA* and its associated genes after exposure to Mtz and H_2O_2. The functional characterisation of the proteins associated with RecA expression will be done using direct gene mutation of the genes as well as heterologous protein expression and biochemical assay of the gene products.

Chapter 2

Functional Characterisation of the *recA* gene of *Bacteroides fragilis*

Abstract

Bacteroides fragilis is a human gut commensal and an opportunistic pathogen causing anaerobic abscesses and bacteraemias which are treated with metronidazole, a DNA damaging agent. This study examined the role of the DNA repair protein, RecA, in maintaining endogenous DNA stability and its contribution to resistance to metronidazole and other DNA damaging agents. Fluorescence microscopy of the *B. fragilis recA* mutant using DAPI staining showed an aberrant cell division phenotype. Alkaline gel electrophoresis of the *recA* mutant DNA showed increased amounts of strand breaks under normal growth conditions. The *recA* mutant was more sensitive to metronidazole, ultraviolet light and hydrogen peroxide than the wild type strain but exhibited no increased sensitivity to bile salts at physiologically relevant concentrations. A *B. fragilis* strain over-expressing the RecA protein exhibited increased resistance to metronidazole compared to the wild type. This chapter provides evidence for a link between the RecA protein and nitrogen and oxygen radical survival. This suggests that the RecA protein may have an important role in the evasion of the host immune response in the early steps of host invasion.

2.1 Introduction

RecA is a major DNA repair protein which carries out homologous recombination repair and regulates the expression of many other DNA repair proteins in certain bacterial species through the SOS response (Janion, 2001; Kuzminov, 1999; Yasbin *et al.*, 1991). In both *E. coli* and *B. subtilis* species, this response has been shown to be regulated by a negative transcriptional regulator and the *recA* gene product (Janion, 2001; Kuzminov, 1999; Yasbin *et al.*, 1991). Bioinformatic analysis of the *B. fragilis* genome has been carried out to determine whether there may be an analogous system operating in this organism.

The *B. fragilis* Recombinase A (BF638R1245; Accession number FQ312004) has been recognised as a functional homologue of the *E. coli* RecA protein since Goodman *et al.* (1987) classified its function by performing heterologous complementation of an *E. coli recA* deficient strain with a gene from a *B. fragilis* gene library. Little was done to further characterise the gene's functional roles within *B.fragilis* itself, however, until the genetic tools became available and Steffens *et al.* (2010) produced a stable insertional *recA* mutation in *B. fragilis*. A *recA* internal fragment was generated by PCR and cloned into the suicide vector pGerm (Shoemaker *et al*., 2000) and transformed into *B. fragilis* crafting a stable *B. fragilis recA*$^{-}$ mutant (Steffens *et al*., 2010).

A search to identify a possible transcriptional regulation site upstream of the *recA* gene as well as a homologue to the LexA regulatory protein was undertaken (Steffens, 2008). To date no genetic or functional homologue of the *E. coli* LexA regulator or its putative DNA binding domain has been identified in *B. fragilis*. Studies in other bacteria such as *D. radiodurans* however have shown that the transcriptional regulators in these species are totally unrelated to the *E. coli* and *B. subtilis* repressor proteins (Earl *et al.*, 2002). It is, therefore, hypothesised that this may also be the case in *B. fragilis*.

In order to aid further characterisation of the RecA mediated repair response and the way it is regulated, it is important to perform an analysis of the molecular roles of the *B. fragilis recA* gene product and the other DNA repair/survival systems with which it might interact in this bacterium.

Extensive studies using DNA damaging agents have been used in other bacteria in an effort to characterise the molecular roles of the *recA* gene product (Kuzminov, 1999; Vollmer *et al.*, 1997). Exposure to different cellular stress conditions that are either associated with RecA function, or those that are RecA independent (Vollmer *et al.*, 1997), have also proven useful. Fluorescence microscopy has been used to study cellular morphology in order to investigate a connection between the RecA protein, DNA integrity within the cell, and cell division in bacteria such as *B. subtilis* (Cooper *et al.*, 1997; Lovett *et al.*, 1985; Paul *et al.*, 2011; Yasbin *et al.*, 1991).

B. fragilis is an effective opportunistic pathogen. The ability of an anaerobic bacterium to survive the oxidative burst associated with transition from the anaerobic environment into the aerobic sterile abdominal environment through a wound to the endothelial layer is an important factor in the pathogenesis of anaerobic sepsis (Hasset *et al.*, 1989; Rocha *et al.*, 1997). A major component of the inflammatory response is the production of oxygen radicals which damage proteins, cellular membranes and DNA/RNA complexes (Pan and Imlay, 2001). The use of hydrogen peroxide to mimic this response has been documented in other opportunistic pathogens like *H. pylori* (Hazell *et al.*, 1991). Since H_2O_2 produces single stranded (ss) and double stranded (ds) DNA breaks makes it an ideal tool for elucidating the function of bacterial *recA* in response to this type of stress. Similarly, investigating the response of this gene to nitrogen radicals is important in order to generate a full picture of the roles of this protein in *B. fragilis.* The drug metronidazole (Mtz) acts by creating nitrogen

radicals which create single and double stranded DNA breaks (Dachs *et al.*, 1995). The ability to withstand DNA damage that doesn't cause break formation is also fundamental to surviving as a commensal and pathogen within the host environment (Dachs *et al.*, 1995; Paul *et al.*, 2011; Steffens *et al.*, 2010) and is studied by exposure to UV (an intrastrand cross-linking agent). The ability to withstand other stresses to the system such as acid stress (Jeong *et al.*, 2008), which alters the electron flux within the cell, is also important. Bile salts are an integral part of the intestine in the human host and can fluctuate greatly in concentration. Therefore, they can be used to assess bacterial response to acid stress. The survival mechanism in response to bile salts is well characterised in *B. fragilis* (Pumbwe *et al.*, 2007), but the possible involvement of the *recA* gene has not been determined.

This Chapter investigates the possible role of the *B. fragilis* RecA protein in response to various cellular stressors that may have physiological significance during gut survival and pathogenesis.

2.2 Materials and Methods

2.2.1 Bacterial strains and plasmids, media and growth conditions

The strains and plasmids used are described in Table 2.1. *B. fragilis* 638R strains were grown in supplemented brain heart infusion broth (BHISB) or on plates (BHISA) at 37°C under anaerobic conditions (Holdeman and Moore, 1972). *B. fragilis* mutants were grown on BHISA including erythromycin (Erm; 10 mg/L), while *B. fragilis* cells containing pLYL01 or pLYrecA were grown on BHISA supplemented with tetracycline (Tet; 2 mg/L).

2.2.2 Strain specific PCR

In order to ensure that all of the strains used in the study were correctly genetically classified, with respect to plasmids and chromosome mutations, before use in all experiments, a series of colony PCRs were done using the M13 primers and the

complimentary internal RecA primer. All PCR primers used are described in Table 2.1. PCR was carried out using 30 cycles with a denaturation step of 30 seconds at 94°C, an annealing temperature of 52°C, and a 30 second extension step at 72°C, followed by a final 5 minute elongation step at 72°C. All reactions used Kapa Ready Mix (Lasec) with 2.5 mM $MgCl_2$. Appropriate antibiotic selection was used to maintain strain integrity.

Table 2.1: Strains, Primers and Plasmids used in this study

Strain/Plasmid	***Relevant Characteristics or use****	**Source/ Reference**
Bacteroides fragilis		
638R	Clinical strain, *recA*$^+$, Rif^{R}Gent^R	Privitera *et al.*, 1979
638R *recA*	*recA*$^-$ Rif^{R}Gent^{R}Erm^R	Steffens, 2008
638R (pLYL01)	*recA*$^+$ Rif^{R}Gent^{R}Tet^R	Steffens, 2008
638R *recA* (pLYL01)	*recA*$^-$ Rif^{R}Gent^{R}Erm^{R}Tet^R	Steffens, 2008
638R *recA* (pLYLrecA)	*recA*$^+$ Rif^{R}Gent^{R}Erm^{R}Tet^R Complemented strain	Steffens, 2008
638R (pLYLrecA)	*recA*$^+$ Rif^R Gent^R Tet^R Overexpressor strain	Steffens, 2008
Plasmids		
pLYL01	Mob$^+$, Tet^{R}Amp^R	Li *et al.*, 1995
pLYLrecA	pLYL01 containing *B. fragilis recA*	Steffens 2008
Primers		
M13F 5'-CGC CAG GGT TTT CCC AGT CAC GAC-3'	M13F and M13R in combination with RIF or RIR allow verification of mutation in *B. fragilis* 638R *recA*.	Yanisch-Perron *et al.*, 1985
M13R 5'-GAG CGG ATA ACA ATT TCA CAC AGG-3'	M13F and M13R in combination with RIF or RIR allow verification of mutation in *B. fragilis* 638R *recA*.	Yanisch-Perron *et al.*, 1985
RIF 5'-CAGGTTCGATAGCACTGAATGCTGC-3'	RIF and RIR amplify an internal fragment of *B. fragilis recA* BF638R1245.	Steffens, 2008
RIR 5'-CGG ATT ACC GAA CAT TAC ACC G-3'	RIF and RIR amplify an internal fragment of *B. fragilis recA* BF638R1245.	Steffens, 2008

***Rif=Rifampicin, Erm=Erythromycin, Gent=Gentamycin, Tet=Tetracycline, R=Resistant, S=Sensitive**

2.2.3 Cell morphology

Cell morphology was investigated using a method developed by Sciochetti *et al.* (2001) with several strain specific modifications. *B. fragilis* 638R and *B. fragilis* 638R*recA*$^-$ were subcultured on BHISA and a single colony was resuspended and then washed in 200 µl of

phosphate buffered saline (PBS) buffer (pH 7.4). For nuclear staining, 4'6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich) at 1 mg/L was used, and for membrane staining FM-4-64 (Sigma-Aldrich) was applied at 1 mM/ml. The stains were added directly to the 200 µl cell suspensions which were incubated on ice for 15 minutes, washed and then resuspended in 200 µl of PBS. The resuspended cells were placed on acid-washed slides, dried at 65°C, and covered with Mowiol pre-treated with n-propylgallate (Sigma-Aldrich). A glass cover slip was placed over the sample and the slides were visualised using fluorescence microscopy at 1000X magnification on the Zeiss Axiovert 200 and photographed using Zeiss Axiocam HR and Axiovision 4.6 software. Images were separated into quadrants, the cell numbers exhibiting atypical DAPI staining (reduced blue signal) were counted, and the percentage occurrence of these, with reference to the total number of cells, was calculated for 30 quadrants for each strain. Gram staining and conventional light microscopy (Leitz Diaplan light microscope at a magnification of 1000X) were also used. Microscope photographs were captured by a Zeiss Axiocam camera and visualised with Axiovision 2.0.5.3. This experiment was carried out in technical duplicate and biological triplicate, and a standard student two-tailed t-test was used to determine the statistical significance. The confidence interval was 95% and only $p < 0.05$ was classified as significant.

2.2.4 DNA strand break analysis

Sixteen hour cultures of *B. fragilis* 638R and 638R*recA*$^-$ were used for standard genomic extraction (Campbell and Yasbin, 1984). The denaturing gel was prepared by making a 0.5% agarose gel with neutral buffer (50 mM NaCl, 1 mM EDTA) and then equilibrating in an alkaline running buffer (30 mM NaOH, 2 mM EDTA) for a minimum of 2 hours. Genomic DNA (8 µg) was loaded on the gel using an alkaline tracking dye (50 mM NaOH, 25% glycerol, 0.12% Bromocresol green) and then run at 16 volts for 20 hours in the alkaline running

buffer at 4°C (Abratt *et al.*, 1986). After electrophoresis the gel was first neutralised in neutral buffer (50 mM NaCl, 1 mM EDTA) for 1 hour, stained using ethidium bromide (50 µg/ml) for one and a half hours, and destained for 15 minutes in neutral buffer. The gel was visualised with short wavelength UV light using a GelDoc imager (BioRad) and photographed.

2.2.5 Cell survival in the presence of DNA damaging agents

Cultures of *B. fragilis* 638R(pLYL01), 638R*recA*$^-$ (pLYL01), 638R(pLYLrecA) and 638R*recA*$^-$ (pLYLrecA) were incubated anaerobically for 16 hours at 37°C in BHISB and then exposed to three different DNA damaging agents as well as 1.5% (w/v) bile salt under strict anaerobic conditions. Appropriate concentrations for each agent were established in pilot experiments using cell dilution and plating for a range of concentrations over a range of time periods.

For exposure to UV light (254 nm), the 16 hour culture was diluted 100-fold in water, 5ml was placed in a glass petri dish, and exposed to varying doses of UV radiation (measured as $J.m^{-2}.s^{-1}$), using a UV lamp. A dilution series from 10^{-1} – 10^{-6} was made in anaerobically reduced water and the cells plated on BHISA. For Mtz exposure, the 16 hour culture was grown to log phase in BHISB (OD_{600}=0.6) and exposed to 5 mg/L Mtz. Cell samples were collected at 30lm / min intervals, diluted as before and plated on BHISA. For hydrogen peroxide exposure, the 16 hour culture was similarly grown to log phase in BHISB (OD_{600} = 0.6). One millilitre of the culture was removed, centrifuged and the pellet resuspended in pre-reduced PBS (pH 7.4). Hydrogen peroxide (Sigma-Aldrich) was then added to a final concentration of 73 µM. Cells were sampled at 5 minute intervals for 15 minutes and plated on BHISA (without L-cysteine). For bile salts exposure, cells were allowed to grow to log phase (OD_{600} =0.6) and the bile salts added to a final concentration of 1.5% using LP0055 Complex Bile Salts (Oxoid) which includes both sodium glycocholate and sodium

taurocholate. Cells were sampled at 5 minute intervals for 15 minutes and plated on BHISA (without L-cysteine). For all treatments, the plates were incubated anaerobically at 37°C for 3 days and the surviving fraction of cells was calculated for each time point. All experiments were done in biological triplicate and technical duplicate. Statistical significance was determined using the student t-test with a p-value for statistical significance at $p < 0.05$.

2.2.6 Determination of the minimum inhibitory concentrations of the RecA strains for metronidazole

The Mtz susceptibility of the strains was also determined by measuring the minimum inhibitory concentration (MIC) on BHISA plates using E-strips according to the manufacturer's instructions (AB Biodisk).

2.3 Results and Discussion

2.3.1 *B. fragilis* recA mutant cell morphology

The cellular morphologies of *B. fragilis* wild type and the *recA* mutant strains were investigated using fluorescence microscopy coupled with a nucleophilic dye (DAPI) and a lipophilic dye (FM4-64) which allowed for evaluation of the nucleiod material and membrane structure, respectively. A representative segment from one such image is shown in Fig. 1 from the *B. fragilis* BF638R and BF638R*recA*$^-$ strains. These images show the nuclear DAPI stain (blue) and the Lipophilic stain FM4-64 (red).

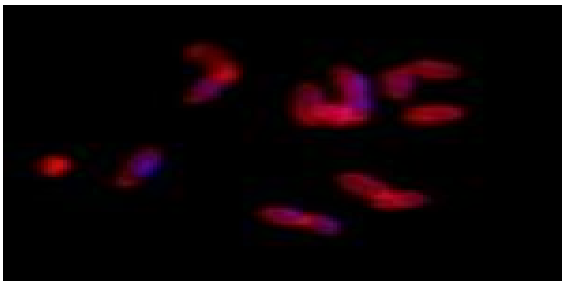
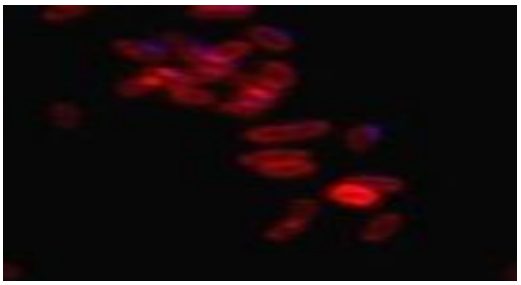
(a)  (b) 

Fig 2.1: Fluorescence microscopy images of representative cells from 1000X field of view for (a) BF638R and (b)BF638R*recA*$^-$ with nucleophilic staining with DAPI (blue) and lipophilic staining by FM4-64 (red).

No significant elongation of the *B. fragilis* typical cell morphology was observed in the BF638R *recA*$^-$ as has previously been reported in *recA* deficient *E. coli* (Hill *et al.*, 1997). The *recA* mutant strain did however, exhibit a statistically significant increase (p = 0.00009) in the proportion of aberrant DAPI stained cells when compared to the wild type. The proportion of cells exhibiting a reduced DAPI signal in the *recA*$^-$ mutant was 43.95% as compared to 2.33% in the wild type. DAPI binds to double stranded DNA fragments and fluoresces in a concentration dependent manner. The reduction of the DAPI signal may, therefore, be attributed to a decrease in the amount of intact double stranded DNA within the *recA*$^-$ strain; this indicates a reduction in the DNA integrity in the RecA deficient strain of *B. fragilis.*

In both Gram positive and Gram negative bacteria, there is a well characterised link between the DNA integrity of a cell and cellular division (O'Reiley *et al.*, 2004). A change in the integrity of the nucleiod material of the cell is usually indicated by cellular elongation due to a halt in cell division (Hill *et al.*, 1997; O'Reiley *et al.*, 2004). In both *E. coli* and *B. subtilis,* this elongation and halt in cell division have been described as both RecA dependent and independent processes (Goranov *et al.*, 2005; Hill *et al.*, 1997). The lack of cellular elongation seen in the *B. fragilis recA* mutant, despite the serious genomic degradation, may indicate that the RecA protein is a potential contributing factor in cellular elongation. This would need to be evaluated under environmental stress.

2.3.2 DNA strand break analysis

The reduction of DAPI signal exhibited in the fluorescent microscope images led to the hypothesis of that there was inherent reduced DNA integrity within the *B. fragilis* RecA deficient strain. In order to test this, denaturing gel electrophoresis was undertaken using

equivalent quantities of DNA (8 µg) from both the wild type and the *recA* mutant strains (Fig. 2.2)

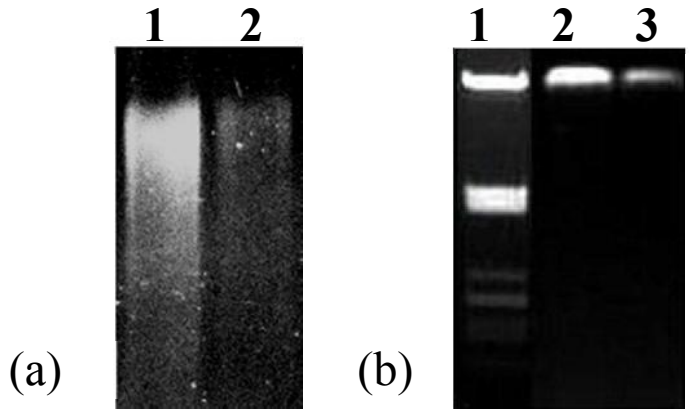


Fig. 2.2: **Determination of DNA breaks** (a) Alkaline denaturing agarose gel electrophoresis (0.5%) of *B. fragilis* 638R (Lane 1) and the *recA* mutant (lane 2). (b) Agarose gel electrophoresis (0.8%) of molecular size marker (λ DNA digested with *Pst*I, lane 1); *B. fragilis* 638R (lane 2) and *recA* mutant (lane 3). All lanes contain 8 µg of *B. fragilis* DNA

The difference between the two strains was very marked under denaturing conditions (Fig. 2.2a). The wild type strain exhibited more high molecular ss DNA and very limited smearing indicating only a small number of ssDNA breaks and thus a high level of DNA integrity. In contrast, the mutant strain exhibited reduced high molecular mass DNA and considerable DNA degradation, indicating the presence of single strand DNA breaks. This supports the hypothesis that there was an accumulation of DNA strand breaks in the *recA* mutant. When DNA from both strains was evaluated using standard agarose gel electrophoresis (Fig. 2.2b) it was evident that both strains contained high molecular weight DNA. However, despite equivalent amounts of DNA being loaded on the gel in both lanes, the ethidium bromide signal was slightly weaker in the mutant strain indicating the possibility of low levels of double stranded DNA breaks within this strain. This difference was not marked enough to be considered a quantifiable observation. The DNA extraction method employed had been optimised by previous investigators to reduce DNA strand degradation and allow the

maximum extraction of high molecular weight DNA. The method has with a minimum of centrifugation steps and exposure to salts and solvents. When taken in conjunction with the fluorescence microscopy results, the denaturing gel result supports the hypothesis that RecA is involved in the maintenance of DNA integrity under normal growth conditions, and in the absence of RecA the cell continues to divide even with diminished DNA integrity, suggesting that cell cycle arrest in *B. fragilis* might be RecA dependent (Goranov *et al.*, 2005; Hill *et al.*, 1997).

The replicative status of the cell has been closely linked to the presence of a functional RecA protein. The induction of the SOS response and the repair of ssDNA breaks have also been linked to the replicative status of the cell in *E. coli* and *B. subtilis* (Goranov *et al.*, 2005; Hill *et al.*, 1997). The role of the replisome is undefined in this process, although the redistribution of the RecA protein to ssDNA breaks seems to require the presence of the replisome in both of these model systems (Sciochetti *et al.*, 2001). This type of atypical cell division has been linked to RecA in *B. subtilis* amongst others. In these cases RecA mutant cells have been shown to inherit no nucleiod material after division (Sciochetti *et al.*, 2001). This type of cell division results in daughter cells filled with fragmented or no DNA and mother cells with damaged DNA. These findings lead to the hypothesis that, in the RecA deficient system of the *B. fragilis recA*$^-$ mutant, replication is undertaken to facilitate the redistribution of the absent RecA protein. The *B. fragilis recA*$^-$ strain exhibited an atypical distribution of its DNA when compared to the wild type; this may be the direct result of an aberrant cell cycle arrest switch. The decrease in the number of these cells in the wild type suggests that, in *B. fragilis,* functional RecA plays a role in maintaining the nucleiod material and may restart or halt cellular division in response to the damage status of the DNA (Goranov *et al.*, 2005;

Hill *et al.*, 1997; O'Reiley *et al*., 2004). This replication leads to cell division and explains the atypical DAPI staining as well as the increased occurrence of ssDNA in the *recA* mutant.

2.3.4. Cell survival in response to environmental stressors

The effects of UV, MTZ, H_2O_2 and bile salts on the viability of the *B. fragilis* strains 638R *recA* (pLYL01), 638R *recA* (pLYLrecA) and 638R (pLYLrecA) were examined. The RecA overexpressing and complemented strains were constructed by Steffens (2008) as follows. Complementation of the *recA* mutant was accomplished by transforming a *B. fragilis recA*$^-$ strain with an expression plasmid pLYL01 (Li *et al*., 1995) with a full length copy of the *B. fragilis recA gene* (pLYLrecA). Overexpression of the RecA protein in B*. fragilis* cells was accomplished by transformation of the wild type strain with the pLYLrecA plasmid.

A two $\log_{10}$ reduction in the survival of viable cells after exposure to UV was observed in the *recA* mutant strain compared to wild type cells (Fig. 2.3a), indicating *B. fragilis* RecA involvement in repairing UV-induced thymine dimers. When the *recA* mutant strain was complemented using the pLYLrecA plasmid, the cells did not fully regain their wild type phenotype but they did show a significant increase in survival when compared to the *recA* mutant strain (Fig. 2.3a). In *B. fragilis* 638R, over-expression of the RecA protein, using the same plasmid as that used for complementation, resulted in an increased survival of the transconjugant strain compared to the wild type strain carrying the pLYL01 plasmid (empty vector). This increase was not, however, statistically significant.

The partial recovery of the complemented strain under UV stress may possibly be attributed to alterations of the transcriptional regulation between the chromosomal *recA* and the full length *recA* gene on the pLYL01 plasmid. This alteration may be the result of a change in the

genomic context. It is possible that the gene on its own on the plasmid was not entirely equivalent to its endogenous form. *B. fragilis* has an active excision repair system which is involved in the repair of single nucleotide repair like that associated with pyrimidine dimers created by UV exposure (Abratt, 1987; Janion, 2001; Kuzminov, 1999). The presence of this system suggests that the *B. fragilis* strain is not entirely reliant on the RecA-mediated repair process alone when dealing with this type of DNA damage.

B. fragilis wild type and *recA* mutant cells were exposed to 5 mg/L Mtz (Fig. 2.3b). After a 45 minute exposure the *B.fragilis recA*$^-$ strain exhibited a three $\log_{10}$ decrease in survival when compared to that of the wild type cells and the *B. fragilis* RecA protein is clearly associated with this. Mtz exposure results in ss and dsDNA breaks (Dachs *et al*., 1995). A similar result was observed in the *recA* mutants of *B. thetaiotaomicron* (Cooper *et al.*, 1997). The complemented *B. fragilis* 638R *recA* mutant regained the full wild type phenotype in the presence of Mtz (Fig. 2.3b) and over-expression of RecA in the wild type *B. fragilis* cells caused a statistically significant (p=0.03) improvement in survival when they were challenged with Mtz under the same conditions.

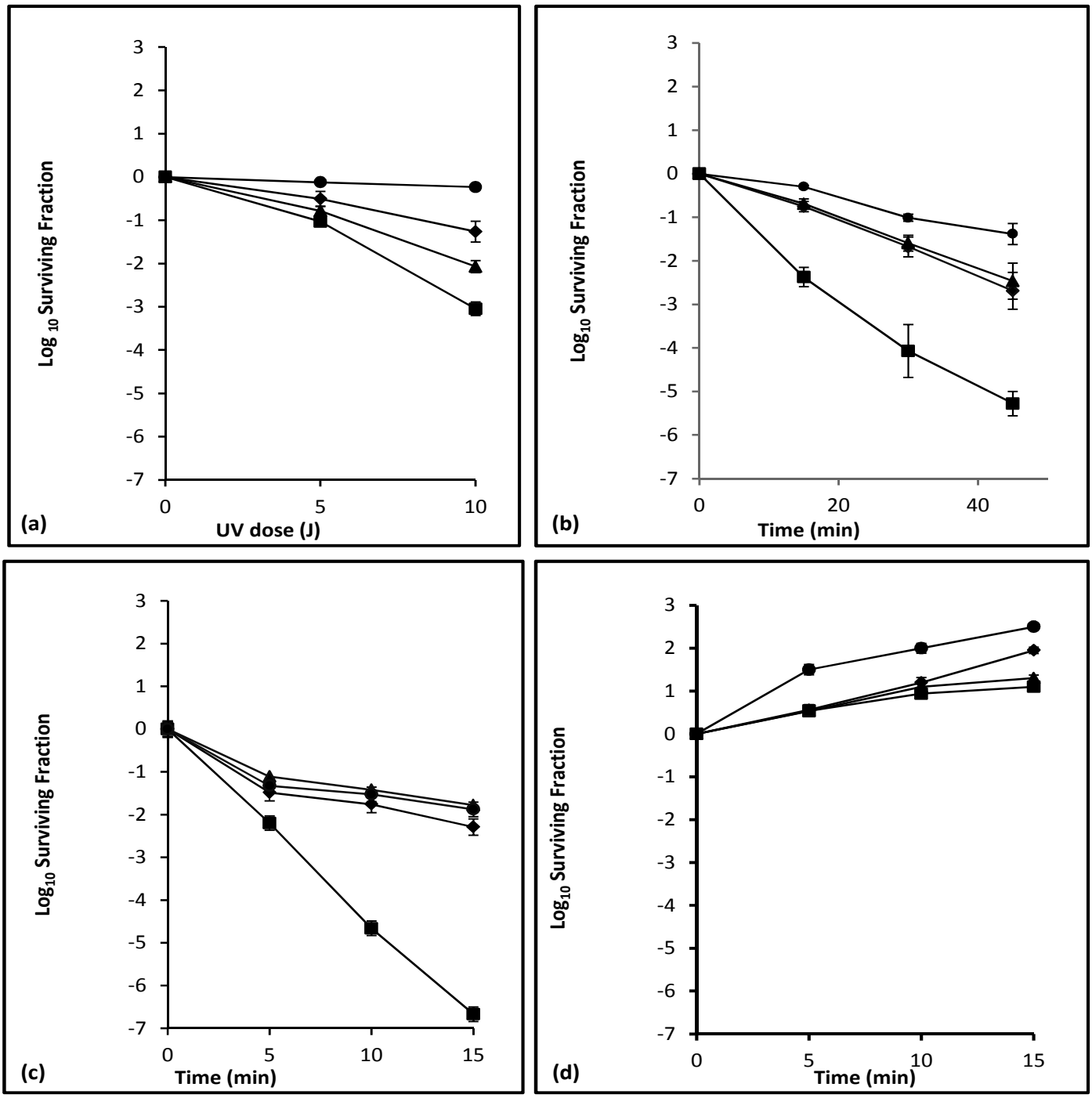


Fig. 2.3: **Survival curves of the *B. fragilis* strains in response to DNA damage with** (a) UV, (b) Mtz (5 mg/L), (c) H_2O_2 (73 µM) and (d) Complex Bile Salts (1.5%). Diamonds, *B. fragilis* 638R(pLYL01); Squares, *B. fragilis* 638R *recA*$^-$ mutant(pLYL01); Triangles, *B. fragilis* 638R *recA*$^-$ mutant complemented with pLYLrecA; Circles, *B. fragilis* 638R *recA* over-expressor (pLYLrecA). The errors bars represent the standard error calculated from at least three replicates of data.

E-test experimentation showed that there was a statistically significant difference in the MIC value in the mutant strain (0.016 mg/L) when compared to the wild type (0.125 mg/L) ($p <$ 0.05) but no change in MIC in the overexpressor strain (0.125 mg/L) (Table 2.2) relative to

the wild type. The alteration in regulation between the chromosomal and plasmid copies of the *recA* gene is again evident in the failure of the complemented strain to regain the same MIC value as that of the wild type strain (0.094 mg/L). The differences between the kill curves and the E-test MIC values may be the result of the differences in exposure time and growth phase of cells between the 2 methods, and highlights the difficulties in comparing Mtz resistance between two different methods (Glupczynski *et al*., 2002)

Table 2.2 Mtz susceptibility (MIC) of the B. fragilis strains.

***B. fragilis* Strain**	**MIC (mg/L)**
638R	0.125
638R (pLYL01)	0.125
638R *recA*$^-$	0.016
638R *recA*$^-$ (pLYL01)	0.016
638R *recA*$^-$ (pLYL *recA*)	0.094
638R (pLYL*recA*)	0.125

Mtz is activated in anaerobic bacteria (Chapter 1), releasing a nitrogenous radical, this radical damages the phosphate backbone of the DNA molecule causing both single and double stranded breaks (Dachs *et al*., 1995). The ability of a cell to survive nitrogenous radicals can affect the efficacy of Mtz treatment as well as assist the bacterium to evade the human immune system (Mydel *et al*., 2006). Metronidazole therefore, serves an important duel experimental function as a stressor. It is both an important antibiotic, and it also mimics the nitrogen stress associated with neutrophil attack during bacterial invasion of the human host. This makes the increased survival of the RecA overexpressor strain an important discovery since it gives insights into mechanisms that may be used for bacterial survival during Mtz treatment, and also suggests a potential link between RecA and survival in the human abdominal cavity during early host-pathogen interactions.

The *B. fragilis* wild type and *recA* mutant strains were exposed to 73 µM hydrogen peroxide for 15 minutes (Fig. 2.3c). Over this time, the *recA* mutant exhibited a five $\log_{10}$ decrease in survival compared to the wild type, and complementation with pLYLrecA lead to a full recovery of wild type phenotype (Fig. 2.3c). Over-expression of the RecA protein did not cause an increase in the ability of the cells to recover from lethal doses of hydrogen peroxide. The decreased survival of the *recA* deficient strain indicated that one of the pathways needed to allow the cell to persist in the face of oxidative stress is the DNA repair pathway catalysed by the RecA protein in *B. fragilis*.

The ability of a cell to survive oxidative stress is imperative for the aerotolerance required for the successful pathogenicity of *B. fragilis* in the invasion of the abdominal cavity. This is not due solely to the increase oxygen content of the abdomen but also to the innate immune response where neutrophil attack uses nitrogen and oxygen radicals to kill invading bacteria (Chapter 1). *B. fragilis* has a well-defined and extensive oxidative stress response (Chapter 1) that is vital to the efficacy of *B. fragilis* in its role as an opportunistic pathogen. Hydrogen peroxide acts by altering the redox potential in the cell (Imlay, 2003) and damages lipids, proteins, the cell membrane and DNA as well as causing metabolic breakdown by oxidising the Fe-S clusters needed for catalysis in many of the vital metabolic enzymes in *B. fragilis* (Imlay, 2003; Rocha *et al.,* 1997; Sund *et al.*, 2006). The oxidative stress response acts to prevent the damage by directly reducing the oxygen radicals causing the damage as well as by influencing pathways to repair the damage that is associated with this type of stress (Sund *et al.*, 2006; 2008). This is facilitated by breaking down lipids and proteins and reducing the damaged catalytic centres of metabolic enzymes (Herren *et al.*, 2003; Imlay, 2003; Sund *et al.*, 2008). The observation that the RecA protein plays an important role in *B. fragilis* survival following exposure to H_2O_2 draws a strong link between the oxidative stress

response and the DNA repair process, which is exhibited universally in described SOS type bacterial responses (Kuzminov, 1999). The OxyR regulator for the oxidative stress system (Herren *et al.*, 2003; Rocha *et al.*, 2000; Sund *et al.*, 2008) brings about its regulation through binding to the *oxyR* "box" ahead of the stress responsive genes. It does not, however appear to be a direct regulator in the RecA mediated stress response in *B. fragilis* since bioinformatic analysis has not revealed any *oxyR* binding motif upstream of the *recA* gene (Chapter 3). There is a possibility that the OxyR transcriptional regulator may regulate a distal promoter for this gene.

In order to establish whether the RecA protein was involved in the Bile stress response in *B. fragilis*, the *B. fragilis* wild type and *recA* mutant strains were exposed to 1.5% bile salts for 15 minutes (Fig. 2.3d). Survival of all the strains was unaffected by the bile salts and they continued to grow in a similar way. This suggested that the survival and adaptation of *B. fragilis* to bile salts is a RecA independent process. This is consistent with the literature about this type of response (Hylemon *et al*., 1976; Pumbwe *et al.*, 2007).

Bile salts act on the bacterial cell to permeabilise the cell membrane acting as a detergent against the lipid cell membrane. *B. fragilis,* a gut anaerobe, has a well-documented high tolerance to bile salts due to several resistance mechanisms. Exposure from as little as 0.15% bile salt results in the increased expression of the RND-type efflux pump genes (Pumbwe *et al.*, 2007) allowing the active export of the damaging H^+ ions associated with acid stress before they can damage the cellular structure. In addition, Hylemon *et al.* (1976) identified and categorised a BSH (bile salt hydrolase) from *B. fragilis* which allows the breakdown of complex bile salts by the bacterium. Pumbwe *et al*. (2007) also identified two more putative BSH genes. The literature suggests that bile salt acts as a potential adhesion

signal in bacteria and may cause vesicle and pilli formation (Pumbwe *et al*., 2007). All of these mechanisms are RecA independent and support the finding in this study that bile salt resistance in *B. fragilis* is in no way dependent on the RecA protein.

2.4 Conclusions

The use of a mutational strategy to evaluate the functional roles of a *recA* gene has support in the literature (Galan *et al*., 1992; Murphy *et al*., 1990; Winzeler *et al*., 1999,). In this study cell morphology, genomic integrity and cellular survival under various environmental stressors have been evaluated. This approach has proven useful in answering the initial questions for this study. It has highlighted the areas in which RecA is involved in cellular function and the data generated using this method has led to the development of novel research questions regarding the *recA* regulation and genomic context which will be further investigated in Chapter 3.

The findings of the fluorescence microscopy experiment were highly reproducible and strongly support a link between the RecA protein and the cell cycle machinery in *B. fragilis*. It would be advantageous to do further morphology studies of the wild type and *recA* mutant *B. fragilis* strains under stress conditions to confirm the link between the RecA protein and cellular elongation. This could be done in conjunction with a comparison of the phenotypes of this strain against phenotypes associated with cell cycle mutants in *B. fragilis*. Overlapping phenotypes may allow an investigator to pinpoint the interactions between the cell division machinery and the RecA protein. The use of a confocal or immuno-staining methodology might be advantageous in future work to reduce the intra and inter-slide variability associated with this method and would greatly reduce the number of repeats required for statistically robust data sets to be collected.

The kill curves have allowed the evaluation of cellular survival in the presence of a number of different DNA damaging agents and stressors. This allowed analysis following exposure to several of the major types of DNA damaging agents, including direct damage to the purine/pyrimidine dimer (UV), damage to the phosphate backbone (Mtz) and alteration to the hydroxyl ion concentration within the system (H_2O_2). Both double stranded and single stranded DNA break inducing agents have been analysed. All analysis was done during the active phase of growth to ensure that no stationary phase resistance mechanisms have been induced altering the outcomes of the kill curves. A set of experiments on the stationary growth phase might be beneficial in future for establishing a connection between the RecA protein and the altered transcriptome of *B. fragilis* in the final phases of growth.

Experiments linking RecA to the accumulation of advantageous mutations might possibly be useful, but due to the extreme sensitivity of the BF638R*recA*$^-$ strain to the damaging agents tested, finding a suitable agent may prove difficult. In addition, a large number of advantageous mutations are single point mutations and frequently RecA independent.

The results of the research described in Chapter 2 have established connections between RecA and cellular responses involved in maintaining genomic integrity, cell cycle regulation, UV, oxidative stress and Mtz resistance. They have shown that there is a potential link between the RecA protein and the bacterium's survival during the initial stages of infection and have provided a fuller appreciation of the molecular roles of this protein within *B. fragilis*. The results of these experiments have pointed to a number of pathways that could be co-regulated. More in depth analysis of these would be the first step in

determining whether the RecA protein of *B. fragilis* is involved with a more global stress response in this bacterium, like the SOS type responses reported in other bacteria. One approach to answering these questions could be the use of bioinformatic analysis of the genomic context of the *recA* gene in *B. fragilis*. This will be discussed in detail in Chapter 3 along with findings regarding its transcriptional activation and possible functional link to the redox and nitrogen radical stress systems in *B. fragilis*.

Chapter 3

The *recA* operon of *Bacteroides fragilis*

Abstract

Physiological studies showed that cell survival following metronidazole and hydrogen peroxide (H_2O_2) exposure was decreased in recombinase negative *B. fragilis recA* mutants, while overexpression of the RecA protein in *B. fragilis* resulted in increased survival of cells exposed to metronidazole. RT-PCR of *B. fragilis* cDNA showed that the *recA* gene was co-transcribed as an operon together with two upstream genes which are possibly involved in repairing oxygen damage. Quantitative RT-PCR was done using RNA extracted from untreated cells as well as after metronidazole and H_2O_2 treatment. A transcriptionally regulated response to both sets of treatment conditions was seen, with all three genes being up-regulated as a group as well as individually in response to these stress conditions. These results suggest that the *recA* gene of *B. fragilis* is a member of a conditional 3 gene operon responsive to nitrosative and oxidative stress.

3.1 Introduction

The ability of the strictly anaerobic opportunistic pathogen *B. fragilis* to colonise the abdominal cavity is an interesting phenomenon. Not only does the cell have to survive high oxygen levels and activate its pathogenicity-associated genes, but it also needs to survive the initial host immune onslaught. Data presented in chapter 2 on the functional characterisation of the *recA* gene indicated a link to the oxidative stress response as well as the cell's survival of nitrogenous radicals. The link to these stress responses may be the presence of transcriptional regulatory sequences or an operon linking proteins with a number of different functions. This suggests that further exploration of the genomic context of the *recA* gene might be of interest to determine whether it is associated with other stress response genes.

The *recA* gene is found to be in an operon in a number of bacterial species including *P. gingivalis* which is closely related to *B. fragilis* (Johnson *et al*., 2011). There is clearly a conferred evolutionary advantage in all instances of *recA* being part of an operon. *P. ginigivalis* has a large *recA* operon that includes a Bacterioferritin co-migratory protein as well as *vimA, E* and *F*. This operon is crucial for the survival of this oral pathogen in the oral cavity especially in incidences of oxidative stress and the establishment of infection after the initiation of the inflammatory response (Johnson *et al*., 2011). In both M*ycobacterium smegmatis* and *Streptomyces lividans*, *recA* and *recX* are co-transcribed as an operon (Forse *et al*., 2011). In *S. lividans*, the operon is only transcribed in the presence of DNA damage while *recA* is also constitutively expressed at basal levels under non-inducing conditions (Vierling *et al*., 2000). This differs from *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 during recombination, thus functioning as a negative

regulator of RecA activity. Consequently, RecX protects the cell from RecA over-expression toxicity (Papavinasasundaram *et al*., 1997). 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 (Steffens *et al*., 2010). The *recA* gene in *D. radiodurans* forms an operon with *cinA* and *ligT* (Bonacossa de Almeida *et al*., 2002), while in *Streptococcus pneumoniae*, *recA* forms an operon with *cinA*, *dinF* and *lytA*. The *cinA* gene is a competence-induced gene and might encode a recombination accessory protein. The *ligT* gene encodes a 3'-5' DNA ligase, *dinF* codes for a multidrug efflux pump in *Ralstonia solancearum* and *lytA* codes for the pneumococcal autolysin (Mortier-Barrière *et al*., 1998). In the case of *S. pneumoniae* the *recA* gene clusters with the *lytA* gene that confers a potential survival advantage during infection.

The literature, therefore, supports the possibility that the *recA* gene from *B. fragilis* may cluster with genes that are associated with nitrosative and oxidative stress response. Experimentation including bioinformatics, RT-PCR and qRT-PCR was undertaken with the aim of elucidating the genomic context and transcriptional profile of the *recA* gene cluster.

3.2 Methods and Materials

3.2.1 Bacterial Strains, Plasmids and Growth conditions

B. fragilis 638R was grown in supplemented brain heart infusion broth (BHISB) or on plates (BHISA) at 37°C under anaerobic conditions as described in Chapter 2.

3.2.2 Bioinformatic Analysis

Protein and DNA sequences were obtained from the National Centre for Biotechnology Information (www.ncbi.nih.gov). BLAST 2.2.17 was used to calculate the predicted

percentage identity between protein sequences for the CDS from *B. fragilis* 638R (NC_016776.1) for the 3 ORFs, BF638R1245, BF638R1246/7, BF638R1248, that make up this putative 3 gene cluster. Conserved domains database (CDD) searches were used to identify conserved domains in the protein sequences. Due to the previously undescribed role in bacteria for the BF638R1248 ORF KEGG analysis was undertaken to establish whether the other enzymes in its metabolic pathway were present in *B.* fragilis. In addition the nucleotide sequence of *B. fragilis* 638R from 1497145 bp to 1493100 bp was analysed using BLAST. Sequence alignment was done using DNAman software 4.13 (Lynnon BioSoft) in order to evaluate the occurrence of this gene cluster in other bacteria in the database

3.2.3 RNA Extraction and Integrity Studies

RNA was extracted using the hot phenol method of Aiba *et al*. (1981) with the following modifications: after 16 hour precipitation of the RNA at -20°C, a DNase1 treatment was performed at 37°C for 3 hours. Purification and final RNA precipitation was done using the Qiagen Total RNA kit (WhiteSci). DNA contamination of the RNA was evaluated by PCR of the 16S rRNA gene using the universal F27/R5 primer pair combination and the RNA preparation as template (Table 3.1). The products were electrophoresed on a 1% agarose gel. RNA quantity and quality were determined using the nanodrop spectrophotometer (Nanodrop Technologies) and denaturing agarose gel electrophoresis (2% Formaldehyde, MOPS running buffer), respectively.

3.2.4 Reverse-Transcription PCR

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. Conversion of RNA to cDNA was evaluated using the 16S rRNA PCR primer pair (F27/R5). The cDNA (2 µl) was then used as template for the amplification of specific target genes using gene specific primers. The GeneAmp PCR System 9700 was used for the cycling reactions (Perkin Elmer, Applied Biosystems). The PCR was done using Kapa Ready Mix (Lasec). 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, BF638R1246/7 and BF638R1245 respectively (Table 3.1, Fig 3.1). To generate PCR fragments of intergenic regions, primers pairs FBRT-RBRT were used for BF638R1248 and BF638R1246/7, and FRA-RART were used for BF638R1246/7 and BF638R1245. Primers 11F and 11R were used to evaluate the intergenic region between BF638R1245 and BF638R1243. PCR products were analysed with agarose electrophoresis gel using λ DNA (digested with *Pst*I) as a molecular size marker.

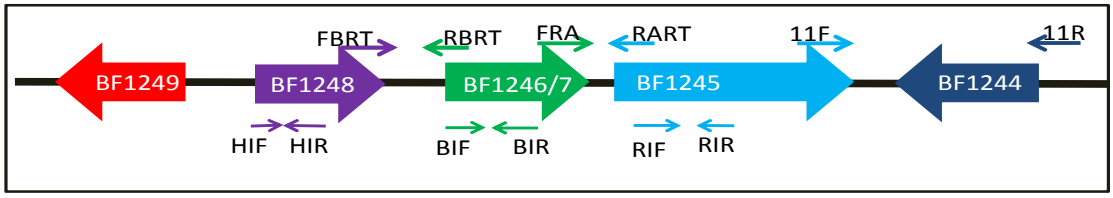


Fig 3.1: Schematic Representation of the *recA* gene cluster and the relative positions of the primer pairs used in RT-PCR. A region of the chromosome from nucleotide positions 1497145bp – 1493100bp is shown with annotated ORFs from *B. fragilis* 638R BF638R1249-BF638R1244. The *recA* gene is shown as BF1245. Primers are reported in Table 3.1.

Table 3.1 Primer Sequence and descriptions for all primers used in this study

Primer	Sequence	Characteristics/Use	Source
F27	5'-AGAGTTTGATCITGGCTCAG-3'	16S rRNA Published Primers	Chèneby *et al.*, 2000
R5	5'-ACGGITACCTTGTTACGACTT-3'	16S rRNA Published Primers	Chèneby *et al.*, 2000
M13F	5'-GAG CGG ATA ACA ATT TCA CAC AGG-3'	Plasmid Confirmation	Yanisch-Perron *et al.*, 1985
M13R	5'-CGC CAG GGT TTT CCC AGT CAC GAC-3'	Plasmid Confirmation	Yanisch-Perron *et al.*, 1985
HIF	5'-GTG AAA GCC ATC GGC AAT CCC-3'	SDH Internal Fragment	Steffens *et al*., 2010
HIR	5'-CGA ATG TCA TCC AGA AAC GTG C-3'	SDH Internal Fragment	Steffens *et al*., 2010
BIF	5'GAG ATA AAG CCC CAG AAC TGC-3'	BCP Internal Fragment	Steffens *et al*., 2010
BIR	5'-GAT GAT CCG TTC GAT AAC TCC C-3'	BCP Internal Fragment	Steffens *et al*., 2010
RIF	5'-CAG GTT CGA TAG CAC TGA ATG CTG C-3'	RecA Internal Fragment	Steffens *et al*., 2010
RIR	5'-CGG ATT ACC GAA CAT TAC ACC G-3'	RecA Internal Fragment	Steffens *et al*., 2010
FBRT	5'-CCG GCT ATG ATC GGT GCC-3'	1248/1247 Intergenic fragment	Steffens *et al*., 2010
RBRT	5'-CGG CTT TAC GTA GCT CTG CG-3'	1248/1247 Intergenic fragment	Steffens *et al*., 2010
FRA	5'-GTA AAG CTG CAG ATG AAG TGA TCG-3	1247/1245 Intergenic fragment	Steffens *et al*., 2010
RART	5'-CGT GGA TGG CCA GTG TCG-3'	1247/1245 Intergenic fragment	Steffens *et al*., 2010
11F	5’-CGG AAT TGC GTA CAG GCG A-3’	1245/1243 Intergenic fragment	This Study
11 R	5’- GCG TTG CGA ATG GAA CGG TGC-3’	1245/1243 Intergenic fragment	This Study
12F	5’-ATATGCCTCTTCGTGACTAC-3’	qPCR SDH Primer	This Study
12R	5’-GGATTAGGTAACACGGCTT-3’	qPCR SDH Primer	This Study
13F	5’-AGCGTGTTCTTTGGTCTTTAC-3’	qPCR BCP primer	This Study
13R	5’-CCATACTCCAAATTGCTCTAC-3’	qPCR BCP primer	This Study
14F	5’-CAGTCAAGGCGGCTACAGAG-3’	qPCR RecA primer	This Study
14R	5’-CAGTTTAGCCGCATAGAAGC-3’	qPCR RecA primer	This Study
15F	5’-ACACGGTCCAAACTCCTAC-3’	qPCR 16S rRNA primer	This Study
15R	5’-GTGAAGGATGAAGGCTCTAT-3’	qPCR 16S rRNA primer	This Study

3.2.5 Quantitative RT-PCR

Sample preparation, storage and Primer design

B. fragilis 638R was grown to mid-log phase OD_{600}=0.6 and then half of the culture was exposed to 100 µM H_2O_2 or 1 mg/ml metronidazole. The other half of the culture was used as the uninduced control. Samples of 100 ml were taken for each treatment at time points 0, 15, 30 and 60 minutes. Three biological repeats were performed and each separated into 3 technical repeats for RNA extraction. RNA was extracted from all samples as described in 3.2.4, and assessed for integrity and DNA contamination as described above. All RNA samples were then frozen using liquid nitrogen and stored at -70°C. cDNA conversion was undertaken within 1 day of the completion of the RNA integrity studies. RNA (1 mg) was converted to cDNA using the first strand synthesis kit (Fermentas) as described earlier according to the manufacturer's instructions. cDNA conversion was tested using the 16S rRNA gene PCR method employed in the RT-PCR experiment using the F27/R5 primer pairs. cDNA was stored for a maximum of 16 hours at -20°C before use in any experiment. Each primer set was designed to the 5' region of each of the three genes (Table 3.1). Each amplicon was 100 bp in size to correct for any error associated with SYBR green intercalation of products with different lengths (Fig 3.2.). The primers were designed using the Beacon Designer program (Premier Biosoft). They were synthesised and purified using HPLC methods (University of Cape Town Oligo Synthesis Service) and their site specificity was tested using BLAST (Altschul *et al*., 1990) as well as by standard PCR methods. This method was also used to determine the annealing temperature and $MgCl_2$ concentrations required for each primer set under standard PCR conditions. These criteria were then used in the qRT-PCR optimisation experiments as a starting point.

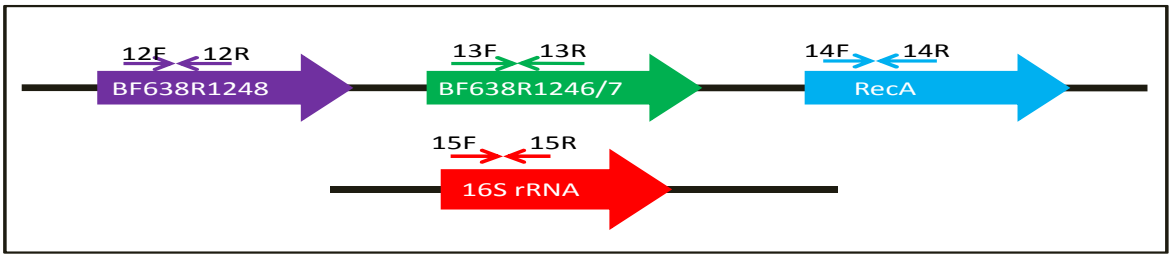


Fig 3.2.:Figure showing the relative positions of the primers designed for the qRT-PCR experiment for all three genes in the *recA* cluster and for the 16S rRNA reference gene

qRT-PCR reaction conditions, controls and optimisation

The design and implementation of the quantitative RT-PCR experiment was done according to the relevant MIQE guidelines (Bustin *et al.*, 2009) using the Rotogene 6000 (Corbet). All runs were done using the 96 tube rotor and all qPCR tips and tubes and belonged to the gene target system (GTS) range (Celtic Diagnostics). All tubes and tips were gas sterilised and maintained in an RNase/DNase free environment. Optimisation was first undertaken for the PCR reaction mixture using gDNA extracted according to the Dachs method (1995) (Chapter 2), a known concentration for each primer pair, and using Kapa Ready Mix (Lasec). The reaction was optimised for the final PCR reactions using Sensimix (Celtic Diagnostics). The $MgCl_2$ concentration, annealing temperature, annealing time, elongation time and cycle number for each primer pair were tested and optimised. Once the optimal conditions for the primers had been established, the optimum amount of cDNA added in the reaction mix was tested (Melt curves and raw cycle data in Appendix B, Fig S4).

The final cycle conditions were as follows: 95°C for 10 min, 95°C for 15 sec, 50°C for 20 sec, and 72°C for 20 sec for 45 cycles. The final reaction mix was as follows: 0.25 µl SYBR green,

1 µl cDNA template, 0.1 µl forward primer, 0.1 µl reverse primer, 6.2 µl Sensimix (Celtic Diagnostics), and 4.85 µl MilliQ water for a final reaction volume of 12.5 µl .

Three biological samples of each set of conditions were tested, with each biological sample being analysed in technical triplicate. The standard curves were created by mixing cDNA for each technical repeat at each time point under each condition and then a dilution series from 10^{0}-10^{-8} was made with sterile MilliQ water in technical duplicate for each primer pair. Each run had its own standard curve for each primer pair including the reference gene to allow for differences in cDNA and PCR efficiency. All R^2 values above the 0.95 threshold and all efficiency values between 0.7 and 0.95 for each primer pair were used in the data analysis and the validation of the qRT-PCR experiment (Bustin *et al*., 2009). The controls for each run included a no template control (to determine the baseline of SYBR green fluorescence), an RNA template control (to confirm no DNA contamination), and a gDNA control (to ensure that the PCR was successful).

Data Analysis

The Corbet Rotogene machine internal specialised software, Rotor-gene 6000 series software 1.7 , was used for the primary portions of data analysis i.e. the standard curves, melt curves and cycle threshold (CT) determination, and the data was exported to an excel spread sheet. The Pfaffl method was then employed (Pfaffl,2001) for data analysis. Using the standard curve, the relative cDNA concentration was determined for each sample, at each time point, and with each primer pair. Each of the technical repeats was used to obtain an average value for the biological run under each condition at each time point. Any of the values that had a CT value discrepancy of more than 4 cycles was discarded as an outlier. The 16S rRNA gene was used as the reference gene. Each run was evaluated for statistical significance and reproducibility by comparing the CT values and the efficiency values for each primer pair under each condition. Any experimentally statistically significant divergent values were excluded from the data analysis (Bustin *et al*., 2009). A mean value for each

biological repeat was established for each time point. These values were then calibrated using their primer and run specific efficiency values. All values were then normalised against the calibrated 16S rRNA value (Pfaffl, 2001). The values were averaged by using all 3 biological repeats at each time point. The relative abundance of each gene at each time point was calculated and compared to the uninduced (T0) value. The relative increases in these values were then evaluated for statistical significance (Pfaffl, 2001).

3.3 Results and Discussion

This study aimed to establish whether there were genes within the 1497145bp – 1493100bp region of the *B. fragilis* genome, which contains the *recA* locus, that might be associated with the cellular response to oxidative or nitrogen stress. It was also interesting to ascertain the degree of conservation of this region of the chromosome within the *Bacteroides* genus.

3.3.1 Genomic Context

The sequenced *B. fragilis* genomes 638R, NCTC9343 and YCH46 confirmed the presence of the *recA* gene. The ORFs within the nucleotide sequence of the 1497145bp – 1493100bp region were shown to be identical in relation to each other within the 3 sequenced *B. fragilis* genomes although the number of intergenic bases varied slightly. There were 2 ORFs (BF638R1248 and BF638R1246/7) upstream of the *recA* gene that showed potential for co-transcription with the *recA*. In *B. fragilis* 638R, BF638R1246/7 and BF638R1248 had only 82bp between their putative start and stop positions, while BF638R1246/7 and BF638R1245 had 11bp between them. These 3 putative ORFS as well as the other putative ORFS in this region that are encoded on the negative strand within this region are shown in Fig 3.1 and Table 3.2.

Table 3.2.: The hypothetical functions of genes located on the negative strand within the region 1497145bp – 1493100bp of the *B. fragilis* 638R genome.

Gene Tag	Annotated function
BF638R1249	Hypothetical function with signal polypeptide, no significant database matches, negative strand, 3'-5' orientation
BF638R1248	Saccharopine Dehydrogenase (*sdh*), negative strand, 5'-3' orientation
BF638R1246/7	Bacterioferritin Co-Migratory Protein (*bcp*), negative strand, 5'-3' orientation
BF638R1245	Recombinase A (*recA*) negative strand, 5'-3' orientation
BF638R1244	Only 150 bp, negative strand, 3'-5' orientation
BF638R1243	Hypothetical protein with similarity to rpoE, negative strand, 5'-3' orientation

The bioinformatic analysis showed that ORFs BF638R1245-BF638R1248, encoding the *recA, bcp* and *sdh* genes respectively, were all transcribed in the same direction. The analysis did not support the inclusion of BF638R1249, BF638R1244 and BF638R1243 in this putative cluster (Table 3.2., Fig 3.1), since these ORFs are transcribed in the opposite orientation (BF638R1244, BF638R1249) or read from the positive strand. Thus all further investigation of transcriptional regulation was conducted on ORFs BF638R1245, BF638R1246/7, and BF638R1248.

3.3.1.1Genomic conservation of this region across the *Bacteroides* genus

The genomic context of this cluster was evaluated by comparing the nucleotide sequence of the 3 putative ORFs and their intergenic regions from *B.fragilis* 638R to those of all annotated bacterial genomes on the NCBI database. While the *recA/bcp* gene cluster is common to a number of genera closely related to *Bacteroides*, including *Porphyromonas* (Johnson *et al.*, 2011) the presence of the full three gene cluster including the putative

saccharopine dehydrogenase gene (*sdh*; BF638R1248) is found only in the 4 *Bacteroides* species shown in Fig 3.3. The annotation for *B. xylanisolvens* is incomplete and thus appears to be slightly different to the annotation in the other 3 species. However, carboxynorspermidine synthetase is the protein superfamily which includes saccharopine dehydrogenase. Peroxiredoxin is the superfamily of the putative BCP seen in the other bacterial species.

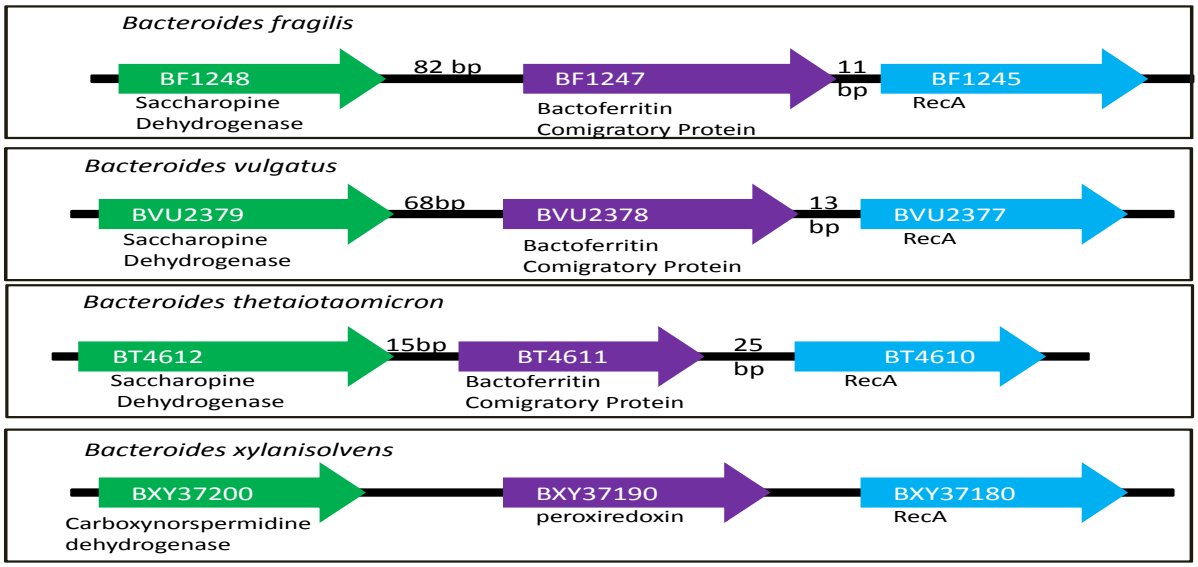


Fig 3.3: The three gene cluster arrangements found within the *Bacteroides* genus involving the *recA* gene

The functional role of *B. fragilis* RecA has been discussed in Chapters 1 and 2 of this thesis. Thus, the focus of the further bioinformatic analysis of this region was on the two upstream genes (BF638R1248 and BF638R1246/7) with the aim of identifying their possible functional roles.

3.3.1.2 The BCP Protein

A putative Bacterioferritin comigratory protein (BCP) is encoded by ORF BF638R1246/7. BCP belongs to the thiol specific antioxidant (TSA) protein family (Comtois *et al*., 2003). These proteins are fairly ubiquitous and catalyse the reduction of H_2O_2 and organic hydro

peroxides (Comtois *et al*., 2003; Lai *et al*., 2006). This reduction prevents free radical formation and radical damage to metabolic processes, cellular machinery and DNA. *B. fragilis* has a number of TSA proteins including AhpC and 2 thiol peroxiredoxin genes. The putative BCP encoded by BF638R1246/7 appears to be the sole BCP protein in this bacterium.

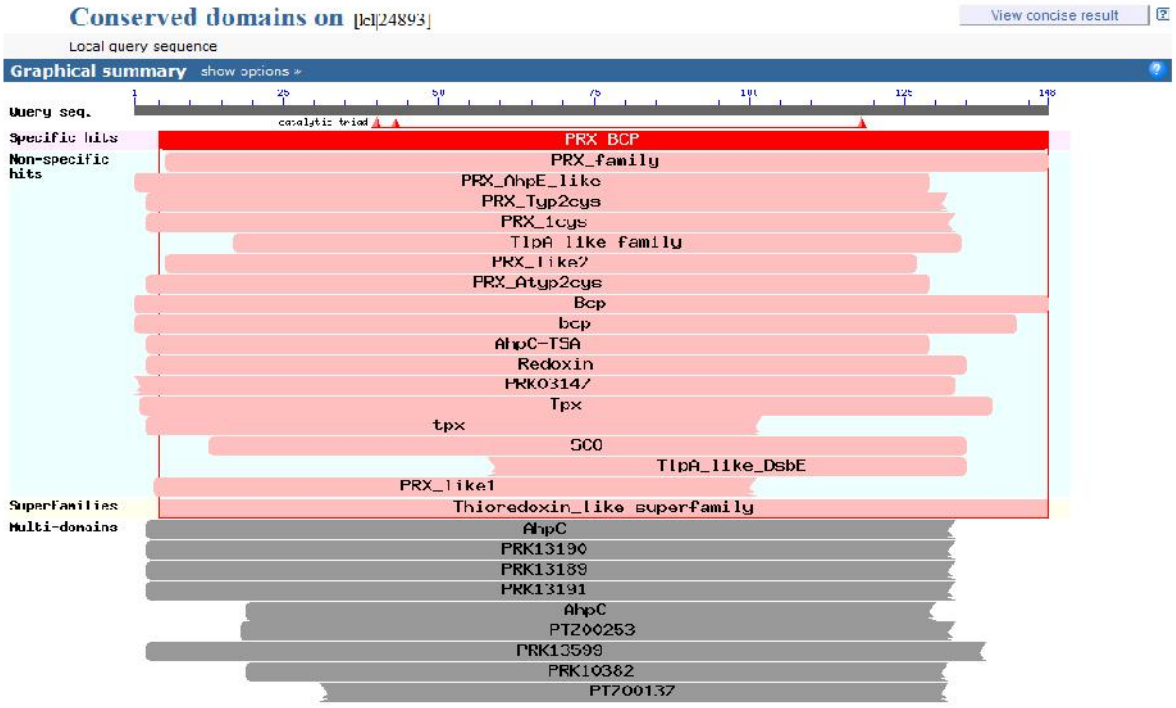


Fig 3.4: Conserved domain database search data for BF638R1246/7.

Protein BLAST of the BF638R1246/7 CDS showed homology to a large number of proteins belonging to the Thioredoxin-like superfamily suggesting some functional domain conservation (Appendix, Fig S1). Conserved domain database searches (Fig 3.4) showed a conserved PRX-BCP domain amongst other Thioredoxin domains. This protein shows conserved domains corresponding to all the major subgroups of the TSA peroxiredoxin superfamily of proteins, suggesting a conservation of the functional role of this protein to those predicted by the presence of the PRX catalytic site. This specific BCP protein shows extremely high protein homology to the described BCP proteins of other anaerobic bacteria,

including *Prevotella* and *Porphyromonas spp* (Appendix, Fig S2). Protein homology to the AhpC protein domains and the Alkylhydroperoxide reductase supports a similar overlapping role for the *B. fragilis* BCP as that of the *Helicobacter pylori* BCP (Wang *et al*., 2005).

3.3.1.3 The Saccharopine Dehydrogenase Protein

The saccharopine dehydrogenase gene (*sdh*) was first identified and its product characterised, in the eukaryote *Saccharomyces cerevisiae* (Xu *et al*., 2006). The SDH protein catalyses the final step in the α-aminoadipate pathway for lysine biosynthesis (Xu *et al*., 2007). SDH catalyses the reversible pyridine-dependent oxidative deamination of saccharopine to generate α-ketoglutarate and lysine using NAD^+ as the oxidising agent (Xu *et al*., 2006). This is an ordered mechanism that produces lysine, α-ketoglutarate and NADH (Xu *et al.*, 2006; 2007). SDH has recently been described in the bacterium *Silicibacter pomeroyi* where it has a role in lysine degradation through the saccharopine pathway (de Mello Serrano *et al*., 2012).

Protein BLAST (Appendix, Fig S3) and conserved domain database searches (Fig 3.5) showed a high protein homology to the saccharopine dehydrogenases, lysine α-ketogluterate reductases and carboxynorspermidine synthase proteins of other anaerobic bacteria, including a number of *Prevotella, Porphyromonas, Clostridium* and *Bacteroides* species. KEGG Pathway analysis has revealed a number of genes that encode putative enzymes associated with lysine metabolism throughout the amino adipate pathway Bfr0300 and Bfr01110. A gene cluster has been found in *T. thermophilus* that exhibits lysine auxotrophy when the genes are disrupted. This pathway also shows homology to the eukaryote amino-adipate pathway for biosynthesis (Nishida *et al*., 1999). The analysis of the literature around the function of SDH (BF638R1248) does not immediately suggest a possible functional link

to the two other ORFs in this putative cluster. However the proximity of the end of the BF638R1248 ORF to the BF638R1246/7 ORF start site suggested that they may be transcriptionally linked.

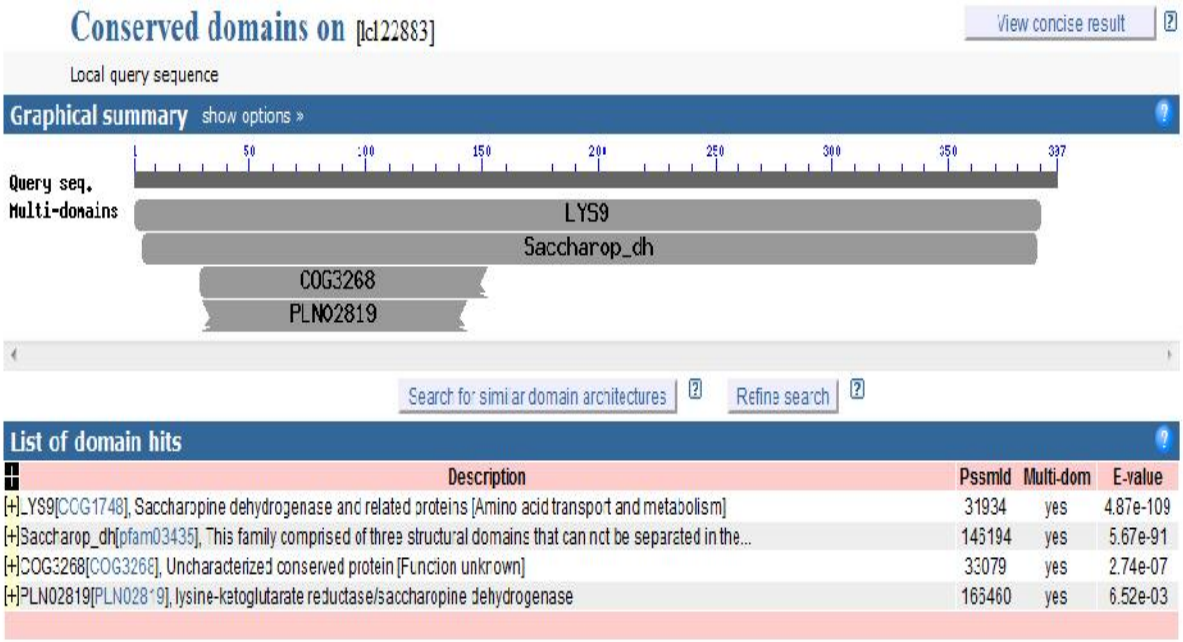


Fig 3.5: Conserved domain database search data for BF638R1248

3.3.2 Reverse Transcription PCR

RT-PCR was done in order to determine whether the putative *sdh* and bcp genes were transcriptionally linked to recA. A cDNA conversion was carried out on DNA-free RNA extracted from exponential phase *B. fragilis* 638R cultures under normal growth conditions. Primer pairs to the intergenic regions produced PCR products from the cDNA template

(Fig 3.6), indicating that the three gene cluster BF638R1245, BF638R1246/7 and BF638R1248 was transcribed as an operon. A set of primers that recognised the intergenic region between BF1245 and BF1243 (11F/11R, Table 3.1) was also included in the initial RT-PCRs but no product was seen in the cDNA (results not shown). An internal control of each of the three genes independently tested for cDNA transcript was also included (Fig 3.6).

Wild type genomic DNA was used as a positive control, while no product was obtained when RNA was used as the template as a negative control.

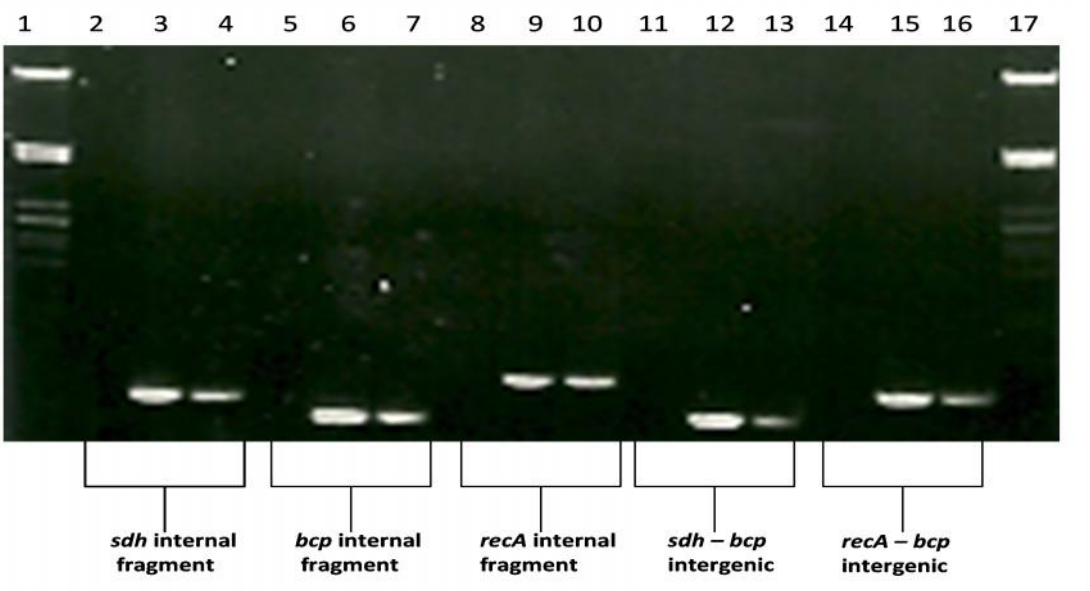


Fig 3.6: RT-PCR analysis of the BF638R1245, BF1246/7 and BF638R1248 gene cluster. Agarose gel (2%); Lanes 1 and 17 are Molecular weight Marker *λPstI*. Lanes 2, 5, 8, 11, and 14, RNA template; Lanes 3, 6, 9, 12 and 15, gDNA template; Lanes 4, 7, 10, 13 and 16 cDNA template. Lanes 2, 3, and 4, *sdh* internal fragment; Lanes 5, 6, and 7, *bcp* internal fragment; Lanes 8, 9 and 10, *recA* internal fragment; Lanes 11, 12 and 13, *sdh-bcp* intergenic region; Lanes 14, 15 and 16, *bcp-recA* intergenic region.

The RT-PCR confirmed that the 3 gene cluster was transcribed under normal growth conditions as a single transcript.

The *recA* gene had been shown to be involved in cellular responses to stressors associated with univalent electron exchange (Chapter 2). The transcriptional response of the *recA* and the associated genes of this operon following H_2O_2 and Mtz treatment was, therefore, examined.

3.3.4 Quantitative Real Time Reverse Transcription PCR

qRT-PCR was performed on cDNA created by the reverse transcription of RNA from induced and uninduced cell cultures. Induction conditions were exposure to either 1 mg/ml metronidazole for up to 60 minutes or 50 µM H_2O_2 for up to 30 minutes. RNA was extracted

at interim time points 15 and 30 min for H_2O_2 and 30 and 60 minutes for metronidazole. The data generated was analysed using the Pfaffl method of statistical normalisation (Pfaffl, 2001).

Optimisation for qRT-PCR

In order to validate the experimental data a number of controls and optimisation experiments were undertaken. The RNA integrity was evaluated as well as any possible DNA contamination of the RNA template. The RNA was found to be intact (Fig 3.7) and of high quality with no DNA contamination (Fig 3.8). cDNA conversion was, therefore undertaken. The success of the cDNA conversion was evaluated by 16S rRNA gene PCR.

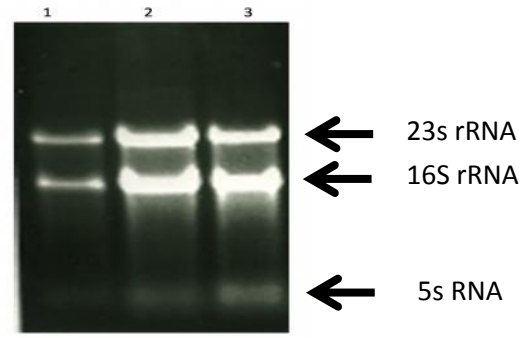


Fig 3.7: Determination of RNA Integrity. Representative 2% Formaldehyde gel of RNA following Mtz induction. Lanes 1, T0; 2, 30 min; and 3, 60 min (All the RNA integrity gels all looked similar)

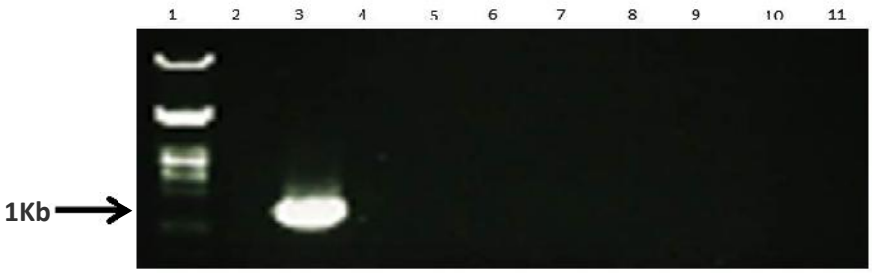


Fig 3.8: 16S rRNA gene PCR using F27/R5 primers to check for DNA contamination. Lane 1, λ*PstI* Molecular weight marker, Lane 2, No template negative control; Lane 3, gDNA positive control; Lanes 4,5,6, Mtz Treatments T0,T1, T2 respectively; Lanes 7,8,9, H_2O_2 treatment T0, T1, T2 respectively; Lanes 10 and 11, uninduced cells at 30 and 60 min respectively.

Optimised PCR conditions were then applied to experiments in the Corbet Rotogene using gDNA as the template. The optimum amount of cDNA to be added was evaluated (see

Appendix, Fig S4 A-D). Runs were then undertaken and analysed. A melt curve was done for each run from 72°C to 95°C and a representative of each curve for each primer set can be seen in Fig 3.9. A standard curve was included in each run for each primer pair. A representative for each standard curve can be seen in Fig 3.10.

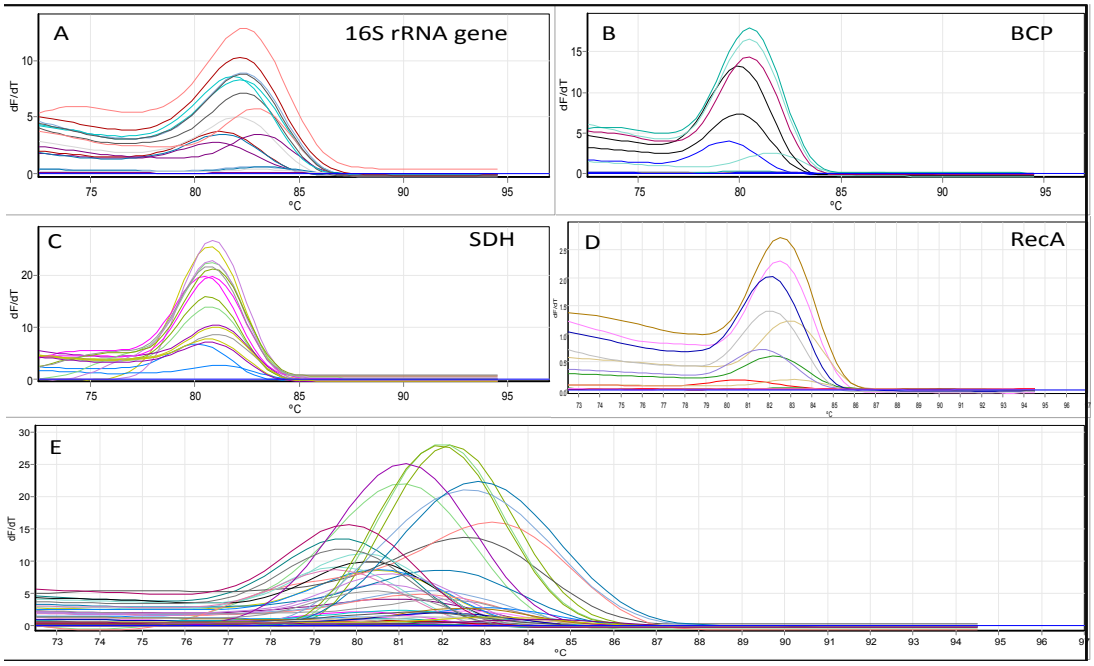


Fig 3.9: Representative Melt Curves for each Primer pair and all tubes in a single biological run. Melt curves for A: 16S rRNA gene primers; B: BCP gene primers; C: SDH gene primers; D: RecA gene primers and E: All 4 primer sets.

The 16S rRNA gene was used as the reference gene for this experiment, since it is a well-characterised component of the cellular system and is not subject to variability in concentration under these inducing conditions. The CT values for this gene under all conditions at all-time points for every run were within 4 cycles.

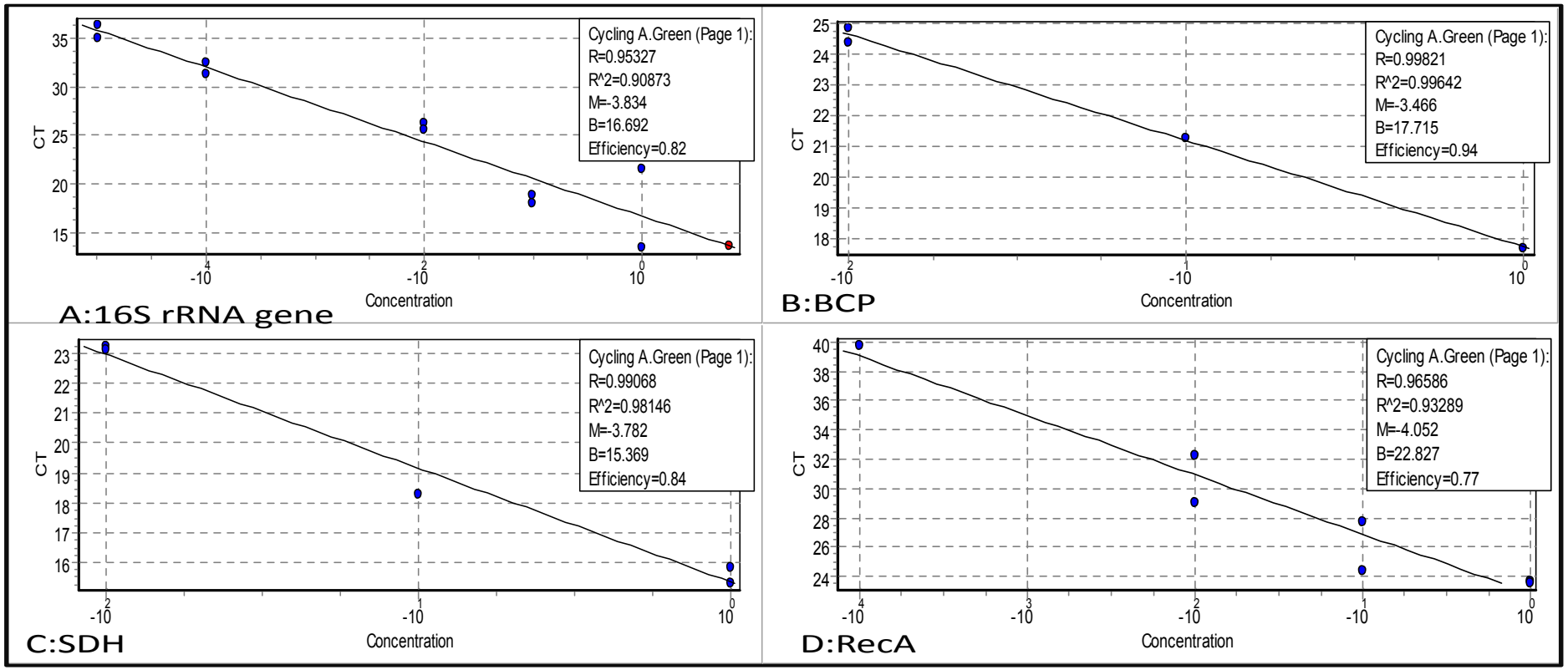


Fig 3.10: Representative standard curves for each primer pair showing R^2 and efficiency values as well as the M gradient value. Standard curves for A: 16S rRNA gene primers; B: BCP gene primers; C: SDH gene primers; D: RecA gene primers.

Analysis using the Pfaffl criteria (2001) revealed a statistically significant change (more than 2 fold) in the transcriptional levels of all 3 genes under both inducing conditions when compared to the uninduced state of cell growth. After Mtz induction, all three genes were up regulated after 30 or 60 minutes of exposure as follows: *sdh* 8.08, 6.47; *bcp* 2.07, 4.28 and *recA* 2.42, 16.43 fold increase (Fig 3.11, Table 3.3). Only the *recA* gene demonstrated an approximate 9-fold increase in transcript abundance between 30 and 60 minutes of exposure to Mtz. After H_2O_2 induction, a similar pattern of induction was observed. All 3 genes were transcriptionally up-regulated at 15 and 30 min of exposure as follows: *sdh* 4.47, 8.99; *bcp* 3.18, 8.44 and *recA* 3.47, 27.1 fold, respectively. While all three genes experienced an increase in transcript abundance at both time points compared to the uninduced conditions, only *recA* experienced a 9-fold increase in transcription between 15 and 30 min exposure to H_2O_2.

The co-induction of the transcription of the 3 genes confirms the RT-PCR finding that they function as an operon. Genes forming an operon usually share a functional purpose. Both Mtz and H_2O_2 introduce single and double stranded DNA breaks (Imlay, 2008; Sharp *et al*., 1977) which are specifically repaired by the RecA protein. An increase in transcription would result in an increase in the amount of RecA available for this purpose (Kuzminov, 1999).

Table 3.3: CT values and relative increase in transcript following metronidazole or H_2O_2 inductions.

	Metronidazole						**Hydrogen Peroxide**					
GENE ID	**0 Minutes**		**30 Minutes**		**60 Minutes**		**0 Minutes**		**15 Minutes**		**30 Minutes**	
	CT	**Fold**	**CT**	**Fold**	**CT**	**Fold**	**CT**	**Fold**	**CT**	**Fold**	**CT**	**Fold**
BF1248 (*sdh*)	**29**	**1**	**18**	**8.08**	**21**	**6.47**	**36**	**1**	**33**	**4.47**	**29**	**8.99**
BF1246/7 (*bcp*)	**22**	**1**	**16**	**2.073**	**13**	**4.288**	**37**	**1**	**28**	**3.18**	**25**	**8.44**
BF1245 (*recA*)	**31**	**1**	**28**	**2.421**	**20**	**16.42**	**39**	**1**	**36**	**3.47**	**21**	**27.1**

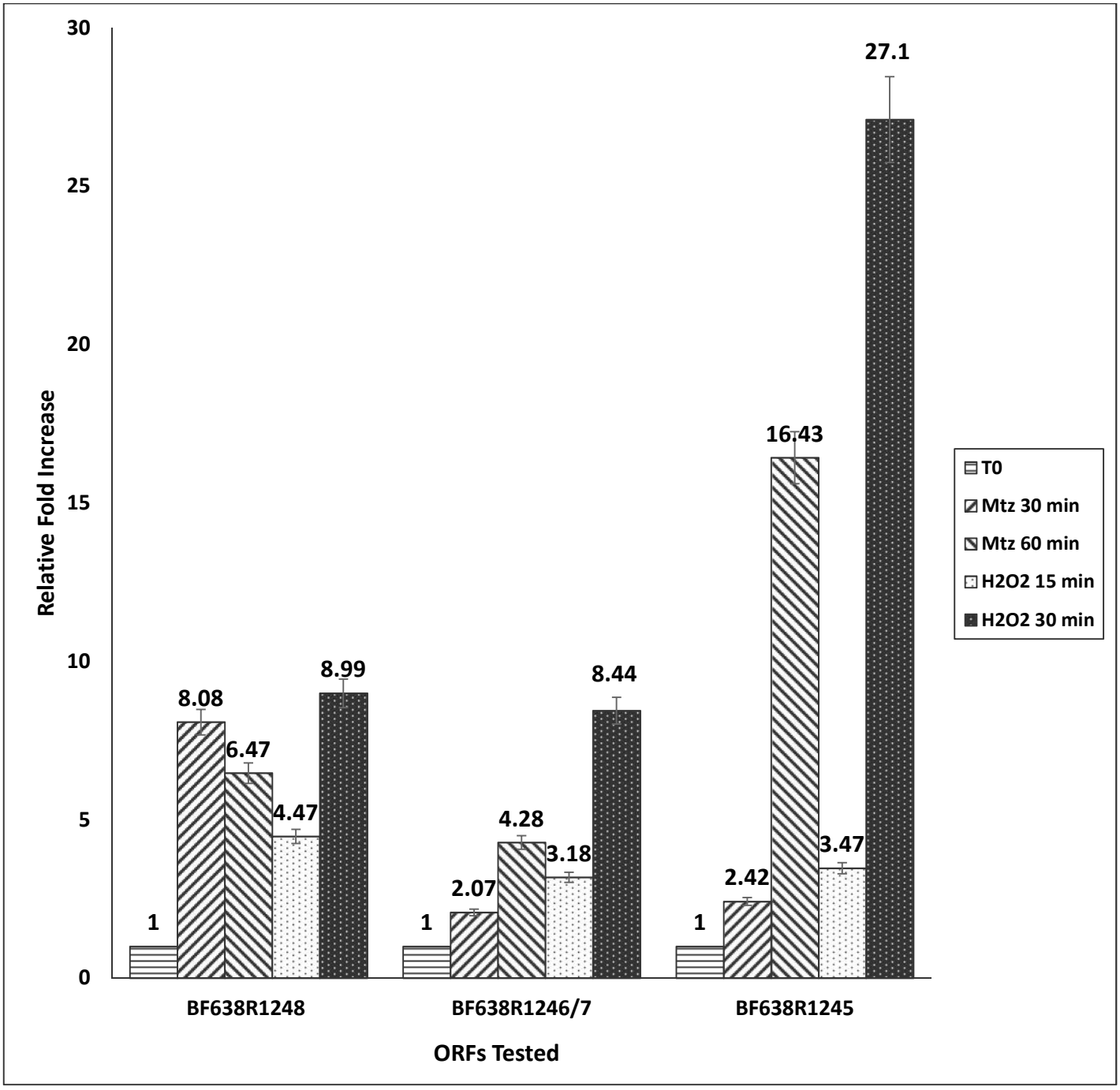


Fig 3.11: **Relative fold increase in transcription of the three genes in the *recA* operon of *B. fragilis*** BF638R1245*;* BF638R1246/7 and BF638R1248 were evaluated over time after the addition of 1 µg/ml of Metronidazole or after exposure to 100 µM H_2O_2 Error bars represent the standard error of the mean.

The BCP protein is associated with the removal of ferric ions produced after univalent electron challenge. This type of stress is experienced after exposure to both Mtz and H_2O_2 (Reott *et al*., 2009). The possible involvement of SDH protein in survival of *B. fragilis* following stress is less clear. A possible role is that the SDH may act within the bacteria to

degrade damaged nitrogen sugars and support the synthesis of new undamaged amino acids and nucleotide sugars (Xu *et al*., 2007). This type of role could be envisioned during the univalent damage associated with both of these stresses. Repair of damage to the DNA backbone and the synthesis of new undamaged DNA template in strand repair, represent possible overlaps between redox stress responses and the described role of SDH in characterised systems like *Cryptococcus neoformans*. (Kingsbury *et al*., 2004; Valdes-Santiago *et al*., 2009). This gene has also been described by De Mello Serrano *et al.* (2012) as being a member of a putative operon in a number of bacterial groups consisting of oxidative stress response genes.

Despite the evidence for their co-transcription supplied by the qRT-PCR experiment, the relative transcriptional differences between the genes, suggests that there may be a number of promoters within the region allowing for multiple points of origin for transcription. The different transcriptional profiles associated with differing agents of stress suggest that the transcription of these three genes as a single transcriptional unit may be a conditional event and that the degree to which each of these genes is transcribed with the others is dependent on the inducing agent (Rodriguez *et al*., 2008). The qPCR supports the hypothesis that the *recA* gene can be regulated independently of the two upstream genes. BF638R1246/7 (*bcp*) also appears to be independent of BF638R1248 since the transcript induction levels are different. This arrangement would allow the benefits of an operon for induction under stress conditions but allows the induction of an important cellular maintenance gene, *recA*, to be tightly regulated under normal cellular conditions without producing unnecessary gene products. This phenomenon has been described in *Mycobacterium bovis* BCG in the *Rv3134c/devR/devS* operon where at least 3 different

promoters have been identified for controlling expression under different conditions. *Rv3134c* and *devR* are only co-transcribed under hypoxia while *devR* and *devS* are co-transcribed under hypoxic and starvation conditions (Rodriguez *et al*., 2008). A similar conditional transcription event is seen in the *S. lividans recA* operon where *recX* is only transcribed under conditions where it becomes necessary to limit the activity of RecA or to limit the possibility of RecA toxicity (Vierling *et al*., 2000). Using precedents set in the literature in conjunction with bioinformatic analysis and the transcriptional profiles generated in this study it seems likely that the *B. fragilis recA* gene may belong to a similar conditional operon, although discrepancies in mRNA transcript stability and cDNA conversion can't be discounted. Further experimentation would need to be done in reference to transcript mapping to make this finding more conclusive. It is also important to note that this dramatic increase in *recA* transcription was not seen in the study carried out by Sund *et al*. (2008). This may be due to discrepancies in growth phase, the Sund study looked at cultures that had been growing for 16 hours compared to the early log in this experiment. Sund *et al.* (2008) used oxygen as the inducing condition with pre-induction with H_2O_2 where this study used only H_2O_2. The media was also different and the lengths of exposure to the inducing agents are not comparable. It is difficult to compare the two findings but the discrepancy is noteworthy and should be investigated in future work.

3.4 Conclusions

Bioinformatic analysis led to the identification of a putative 3 gene operon in *B. fragilis* that was conserved only in the more closely related *Bacteroides spp.* This cluster appeared to include the *recA* gene and 2 upstream open reading frames encoding a putative Bacterioferritin Co-migratory Protein (BCP) and a putative Saccharopine Dehydrogenase (SDH). RT-PCR showed that these three genes were co-transcribed under normal growth

conditions. Published literature suggests that the BCP gene is associated strongly with oxidative stress survival and was well conserved within the *Eubacteria* (Reeves *et al*., 2011; Steffens *et al*., 2010). There is also evidence in the literature that provides a possible link between the BF638R1248 ORF, encoding SDH, and nitrosative stress and damaged amino acid degradation and lysine biosynthesis, although the mechanism of this association is still unclear (de Mello Serrano *et al.*, 2012; Valdes-Santiago *et al*., 2009,). The SDH protein has not been described in many bacterial systems and its role in *B. fragilis* remains unclear. The putative roles of these proteins could be associated with the repair of damage instigated in the cell after exposure to a univalent electron stress like Mtz or H_2O_2.

qRT-PCR analysis showed increases in transcription of all three genes after exposure to Mtz and H_2O_2. These inducing agents generated similar but not identical profiles of transcriptional induction of the genes, with each having a different degree of induction. This suggests that this is a conditional operon with alternative promoters in the intergenic or even coding regions. Transcript mapping experiments like 5'RACE-PCR or northern blot may prove useful for identifying the putative promoters associated with each transcript. This would allow a better understanding of the regulation of this transcriptional unit in response to different types of stress.

Further investigation into the transcriptional responses of the 3 genes is necessary to show a definitive link between all 3 genes to additional nitrogen associated damage, nitrosative stresses and molecular oxygen. Metronidazole generates a different type of nitrogen radical from those associated with oxidative burst and is reliant on anaerobic metabolism for activation which may result in a differential response from those associated with Nitric oxide stress. Thus new qPCR experiments using the nitric oxide generating Deanonoate as a

stressing agent would be ideal for the exploration of the RNS response of these genes. If a similar pattern of transcriptional response is observed, the SDH, BCP and RecA proteins may be definitively linked to DNA repair, oxidative and nitrosative stress response in the obligate anaerobe *B. fragilis*. This may be the starting point in establishing a connection between the DNA repair system, oxidative/nitrosative stress response and the transition of the opportunistic pathogen through the wounded endothelium to the primary infection sites in anaerobic sepsis.

The qPCR experiment described in this work was done using SYBR green. This technique was the more established and frequently published method associated with this kind of experiment at the time the research was performed. However, the use of a multiplex probe based approach should perhaps be considered in future work since it is reported to reduce run and sample variation and is more sensitive compared to the SYBR green method.

The findings in this chapter suggest a role for the two genes upstream of the *recA* in the functional stress responses of *B. fragilis* when exposed to nitrogen and oxygen radicals. In order to further investigate the functional roles of the *sdh* and *bcp* genes, a two pronged approach was designed. The first was a physiological approach using gene specific mutation followed by cell survival analysis. The second was an *in vitro* approach using protein purification and functional biochemical assays. This research is described in Chapter 4.

Chapter 4

Functional Characterisation of the *bcp* and *sdh* genes of *Bacteroides fragilis*

Abstract

The genes of the *B. fragilis recA* operon respond transcriptionally to in *vitro* exposure to metronidazole and reactive oxygen species similar to those associated with the host immune system during infection. The functions of the two upstream genes of the operon, BF638R1248 and BF638R1246/7, were unknown and further experimentation was undertaken in order to identify their possible roles in protection against oxidative stress and/or DNA damage. A mutational approach and heterologous expression were used as well as biochemical analysis. The generation of stable mutations within the 2 upstream genes, BF638R1248 (*sdh*) and BF638R1246/7 (*bcp*) proved impossible, possibly due to the essential nature of their products. While there was little success with the heterologous expression and purification of the protein encoded by the first gene in this operon, SDH: BF638R1248, the second gene (BCP) was characterised in this manner. The *B. fragilis* BCP encoded by ORF BF638R1246/7 was able to complement an *E. coli* BCP mutant strain (KD2301) and protect it after exposure to H_2O_2. Biochemical assay of this protein showed that it had peroxidase activity against H_2O_2, t-butyl hydroperoxide and linoleic acid and this activity was shown to be thioredoxin reductase and thioredoxin peroxidase dependent. *B. fragilis* BCP was able to protect the function of the *B. fragilis* glutamine synthetase (GSIII), an Fe-S metalloenzyme, during H_2O_2 exposure in an *in vitro* assay. BCP also aided the recovery of GSIII enzymatic activity *in vitro* after pre-exposure to H_2O_2. The second gene in the *recA* operon is, therefore, a functional bacterioferritin co-migratory protein (BCP), a small thiol-specific protein with antioxidant properties. The *in vitro* characterisation of the *bcp* gene and its product supports the hypothesis that the genes of the *recA* operon are involved in protection of the bacteria from the oxidative burst during tissue invasion and may play a significant role in bacterial survival during *B. fragilis* infections.

4.1 Introduction

Open reading frames upstream stream of the *recA* gene were identified as encoding a putative saccharopine dehydrogenase (SDH) and a putative Bacterioferritin comigratory protein (BCP) (Chapter 3). All genes of the operon showed a statistically significant up-regulation in response to univalent electron stress, induced by metronidazole and H_2O_2.

Bacterioferritin comigratory proteins belong to the thiol specific antioxidant (TSA) protein family (Comtois *et al.*, 2003). These proteins are fairly ubiquitous and catalyse the reduction of hydrogen peroxide and organic hydroperoxides (Comtois *et al.*, 2003; Lai *et al.*, 2006). This reduction prevents free radical formation and the resultant radical damage to metabolic processes, cellular machinery, and DNA. *H. pylori* has three members of this protein family, AhpC, Tpx and BCP (Comtois *et al.*, 2003; Wang *et al.*, 2005). *B. fragilis* NCTC 9343 has AhpC and six other Tpx proteins (Reott *et al*., 2009) as well as the annotated *bcp* gene within the *recA* operon. This appears to be the only BCP protein within the *B. fragilis* genome as evaluated by bioinformatic analysis. In *H. pylori*, *BCP* has been shown to be a thiol peroxidase which is dependent on the reducing activity of thiol specific peroxiredoxin and thioredoxin reductase, as well as having linoleic acid as a preferential substrate (Lai *et al.*, 2006). The BCP protein has been shown to be less specific in its substrate choice and less prevalent within the cell than its TSA counterpart, AhpC (Comtois *et al.*, 2003) and seems to act only in situations where the oxidative exposure is acute (Comtois *et al.*, 2003; Lai *et al.*, 2006). BCP acts to protect pools of reduced iron by reducing potential oxidants before they can facilitate any damage, allowing substitution of oxidised iron in Fe-S clusters. This prevents widespread distribution of ferric iron which can be used in damaging Fenton reactions (Imlay, 2003; 2008). The BCP protein also acts as an electron acceptor/donor

accessory protein in the Thioredoxin reduction of sulphur to remove oxygen radicals from the bacterial species (Imlay, 2003; 2008).

The first gene of the operon showed homology to saccharopine dehydrogenase (*sdh*). The SDH protein was first identified and characterised in the eukaryote *Saccharomyces cerevisiae* (Xu *et al*., 2006) and catalyses the final step in the α-aminoadipate pathway for lysine biosynthesis (Xu *et al.*, 2007). Fungal species have been reported to become avirulent in the absence of the *sdh* gene (Kingsbury *et al*., 2004). In *Cryptococcus neoformans*, the mutation of the *lys*9 gene, the central enzyme in the aminoadipate pathway of *C. neoformans*, resulted in a slower growth rate, reduced melanin production and a reduction in the capsule polysaccharides needed for cell survival within the host environment. This mutation also led to lysine auxotrophy and cell death in the face of lysine starvation (Kingsbury *et al.*, 2004). *Fusarium graminearum*, a plant pathogen, has reduced virulence when infected with *Fusarium graminearum Dk-12* virus. One of the proteins down regulated in this attenuated strain is saccharopine dehydrogenase (Kwon *et al*., 2009). In *S. pomeroyi* a putative *sdh* gene has been characterised, and is hypothesised to be involved with lysine degradation within this marine bacterium (de Mello Serrano *et al*., 2012). During analysis of the *S. pomeroyi* SDH, bioinformatics revealed the presence of the *sdh* gene in a number of closely related *Cyanobacteria* and *Bacteroidetes*. In these bacteria *sdh* often clusters with genes associated with oxidative stress response (de Mello Serrano *et al*., 2012). However, the exact role of the SDH protein in this stress response remains unclear.

This chapter describes the use of several approaches to functionally characterise the two upstream open reading frames of the unique *recA* operon of *B.* fragilis. These were a genetic

approach (mutation and heterologous complementation) and an *in vitro* approach using protein purification and biochemical analysis.

4.2 Methods and Materials

4.2.1 Bacterial Strains, Plasmids and Growth conditions

Bacteroides fragilis strains were grown as described in Chapter 2. *B. fragilis* strains with pGB910 or pGerm chromosomal integrations were grown on BHISA with 10 µg/ml erythromycin (Patrick *et al*., 2009; Shoemaker *et al*., 2000), and *B. fragilis* pGB920 strains also had 12.5 µg/ml tetracycline added to the media to confer selective pressure and maintain the plasmid/chromosomal integrations. The strains and plasmids used are described in Table 4.1. *E. coli* KD2301 was grown as described in the literature with kanamycin (10 µg/ml) used as the selective antibiotic (Jeong *et al*., 2000). *E. coli* BL21DE3 was grown on Luria Agar or in Luria Broth with no selection. All *E.coli* growth was under aerobic conditions at 30°C. *E. coli* BL21DE3 and KD2301 strains expressing the pET22b1247pro or pET22b1248pro plasmid were grown with ampicillin (100 µg/ml). *E. coli* S17-1 strains with pGB910 constructs were grown under aerobic conditions using 30 µg/ml chloramphenicol supplemented Luria agar (LA), while *E.coli* S17-1 strains carrying the pGerm constructs were grown on LA supplemented with 100 µg/ml ampicillin (Bonheyo *et al*., 2001; Patrick *et al*., 2009)

Table 4.1 Strains and Plasmids used in this study

Strain/ Plasmid[1,2]	Relevant Characteristic/ Use*	Source/ Reference
B. fragilis		
638R	Clinical strain, $Rif^R Gent^R$	Privetella *et al.*, 1979
638R(pGB91247)	638R derivative pGB91247 integration $Rif^R Gent^R Erm^R$	This Study
638R(pGB91248)	638R derivative pGB91248 integration $Rif^R Gent^R Erm^R$	This Study
638R(pGB920)	638R derivative with pGB920 Rif^R $Gent^R$ Tet^R	This Study
638R(pGB91247/pGB920)	628RpGB91247 derivative with pGB920 $Rif^R Gent^R Erm^R Tet^R$	This Study
638R1247ins	638R derivative with pGerm1247ins integrated $Rif^R Gent^R Erm^R$	This Study
638R1248ins	638R derivative with pGerm1248ins integrated $Rif^R Gent^R Erm^R$	This Study
E. coli		
S17-1	*RP4-2-Tc::Mu aph::Tn7 recA* $Strep^R$	Simon *et al.*, 1983
S17λpir	*TpR SmR recA, thi, pro, hsdR-M$^+$RP4: 2-Tc:Mu: Km Tn7 λpir*	Simon *et al.*, 1983
BL21DE3	*E. coli* B strain with DE3, a λ prophage carrying the T7 RNA polymerase gene and $lacI^q$	Studier and Moffatt, 1986
BL21DE3(pLysS)	*E. coli* BL21DE3 strain expressing T7 lysozyme	Novagen
BL21DE3(pET22b(+))	BL21DE3 with empty pET22b(+) vector	This Study
BL21DE3(pET22b1247pro)	BL21DE3 derivative with pET vector expressing full length *B. fragilis* BCP	This Study
BL21DE3(pET22b1248pro)	BL21DE3 derivative with pET vector expressing full length *B. fragilis* SDH	This Study
KD2301	BCP deficient *E.coli* mutant derived from BL21DE3	Jeong *et al.*, 2000
KD2301(pET22b1247pro)	KD2301 derivative expressing full length *B. fragilis* BCP	This Study
Plasmids		
pGB910	Plasmid with Erm^R and the *SceI* recognition site; no *B. fragilis* oriT	Patrick *et al.*, 2009
pGB920	pLYL01 derived plasmid with Tet^R and encoding the full length SceI restriction enzyme from a fucose dependent promoter	Patrick *et al.*, 2009
pGerm	pUC19-based suicide vector, Erm^R	Shoemaker *et al.*, 2000
pGB91247	pGB910 with 1247 crossover fragment	This Study
pGB91248	pGB910 with 1248 crossover fragment	This Study
pGerm1247ins	pGerm with 1247 insertion sequence	This Study
pGerm1248ins	pGerm with 1248 insertion sequence	This Study
pET22b(+)	*pelB* coding sequence, His-tag coding C-terminal,	Novagen
pET22b1247pro	pET22b(+) plasmid with full length BCP protein under IPTG induction	This Study
pET22b1248pro	pET22b(+) plasmid with full length SDH protein under IPTG induction	This Study
pColdTF	N-terminal His tag and expressing trigger factor (TF) as a solubilisation fusion tag	Takara

1. All plasmid and strain names are derived from *B.fragilis* 638R ORF numbers
2. Rif=Rifampicin, Erm=Erythromycin, Gent=Gentamycin, Tet=Tetracycline, Strep= Streptomycin R=Resistant, S=Sensitive,

4.2.2 Mutagenesis

4.2.2.1 Insertional Mutagenesis of ORFs BF638R1248 and BF638R1246/7

B. fragilis genomic DNA was extracted according to Dachs *et al.* (1995). All cloning reagents and restriction enzymes were purchased from Fermentas. Plasmids were transformed into electro-competent *E. coli* cells (Rocha *et al*., 1999) using electroporation parameters of 2.5 kV, 200 Ω and 25 µF (BioRad Gene Pulser). A *B. fragilis* internal fragment was obtained by PCR for open reading frames BF638R1248 and BF638R1246/7 using primer pairs HIF/HIR and BIF/ BIR respectively (Table 4. 2). The PCR parameters were as described previously (Chapter 2). The internal fragments were cloned into the pGerm *Sma*I site to generate pG1247 or pG1248, which were then transformed into *E. coli* S17-1. Mating of *E. coli* S17-1 and *B. fragilis* was performed (Bonheyo *et al*., 2001) and single colonies were analysed to confirm the mutation using PCR and primer pairs 1F/2F-M13R and 1R/2R-M13F (Chapter 2) as described previously. The PCR products were sequenced to confirm their identity.

4.2.2.2 Unmarked Deletion Mutants

In order to create an unmarked deletion for open reading frames BF638R1248 and BF638R1246/7, the Patrick *et al.* (2009) method was employed as described with the following modifications; crossover PCR was undertaken using the 7F-7R and 6F-6R primer pairs for BF638R1248 and the 8F-8R and 9F-9R for BF638R1246/7 using Phusion taq (Fermentas) and cycling conditions as described before with an annealing temperature of 55°C. These were then digested as described with *NotI* and ligated into pGB910 using the Ficoll method (Patrick *et al*., 2009). Plasmid constructs were isolated using the Ficoll method and then confirmed by PCR and sequencing. The remainder of the method was according to Patrick *et al.* (2009). Trans-conjugant *E. coli* with recombinant pGB910 plasmids were isolated by plating *E. coli* S17-λpir on Luria agar with chloramphenicol and conjugation was

undertaken as described. *B. fragilis* strains were then screened on BHISA with Erm for integration and the constructs were confirmed by PCR methods using primers 7F and 6R for BF638R1248 and 9F and 8R for BF638R1246/7. PGB920 plasmid carrying the novel restriction enzyme *SceI* was then introduced into the cell using electroporation. These colonies were plated on minimal media (van Tassell and Wilkins, 1978) enriched with 1% fucose to induce *SceI* expression. Colonies were then picked and plated in duplicate on to BHIS agar in the presence and absence of Erm. Colonies that were present on both media were discarded and those present only on the BHIS without Erm (i.e. those that had undergone resolution) were investigated further. Isolation of deletion mutants was then done and reversion to wild type was evaluated using HIF/HIR or BIF/BIR primers as well as 7F-6R and 9F-8R primer pairs (Patrick *et al*., 2009)

Table 4.2. Primers used in this study

Primer	Description	Relevant Characteristic/Use	Source
BF1247proF BF1247proR	5'-CATCAACCATGGATGTAGGAGATAAAGC-3' *NcoI* 5'-CGATCTCGAGAATTTGTAGAGCG-3' *XhoI*	Primers for directional cloning of full length *B. fragilis* BCP	This Study
BF1248proF BF1248proR	5'-TCTTAATTAAAGCCATGGGTAGAGTTCTTATTATCGG-3' *NcoI* 5'-GTGCACTCGAGTTCCAAATTTCCATCG-3' *XhoI*	Primers for directional cloning of full length *B. fragilis* SDH	This Study
7F 7R	5'-CTTCGTGCCAAGGTACCTACACCGCCTGCACC-3' 5'-AATTGCGGCCGCGAGTTATTACGTTATACCCC-3' *NotI*	Right flanking region for BF638R1248 deletion	This Study
6R 6F	5'-GGTGTAGGTACCTTGGCACGAAGTATTCGATGG-3' 5'-AATTGCGGCCGCGGTCCGATGATCCGTTCGATAAC-3' *NotI*	Left flanking region for BF638R1248 deletion	This Study
9F 9R	5'-CTTGGGTCCGCAGTTCTGGGGCTTTATCTCC-3' 5'-GCGCGGTAAGGCTATAAGACTGCGGCCGCTTAATCTAAT-3' *NotI*	Right flanking region for BF638R124866/7 deletion	This Study
8F 8R	5'-ATCTTCAATTGCGGCCGCTCAAGGCGGCTACAGAGTC-3' *NotI* 5'-CCAGAAATGAAGAAACCCAGGCGTCAAGACCCCG-3'	Left flanking region for BF638R1248 deletion	This Study
HIF HIR	5'-GTGAAAGCCATCGGCAATCCC-3' 5'-CGAATGTCATCCAGAAACGTGC-3'	SDH Internal Fragment	Steffens *et al.*, 2010
BIF BIR	5'GAGATAAAGCCCCAGAACTGC-3' 5'-GATGATCCGTTCGATAACTCCC-3'	BCP Internal Fragment	Steffens *et al.*, 2010

4.2.3 Protein Expression and Purification

Protein expression was undertaken using the pET expression system (Novagen) and plasmids expressed in *E.coli* BL21DE3. Full length sequences from both BF638R1248 and BF638R1246/7 were obtained by PCR using BF1247proF and BF1247proR for the *bcp* gene and BF1248proF and BF1248proR for the *sdh* gene. The details of these primers can be seen in Table 4.2. PCR was undertaken on gDNA extracted as described above (4.2.2.1). The PCR was carried out using Kappa Ready Mix and the parameters were: initial denaturation of 95°C for 5 min, then 25 cycles of denaturation at 95°C for 30 sec, annealing at 55°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 purified using the BioSpin PCR purification kit (BioFlux) and cloned using the instaclone kit (Fermentas) as per the manufacturer's instructions. The recombinant plasmids were sequenced (Macrogen) and those carrying the correct full length sequences of the *sdh* or *bcp* gene were digested using the *NcoI* and *XhoI* restriction enzymes. The full length *bcp* or *sdh* genes were then directionally cloned into the pET22b (+) (Novagen) vector. The sequences were analysed to ensure that the reading frame was correct and that the 6xHis residue tag was present on the C-terminus of each protein.

Each construct was then transformed separately into *E.coli* BL21DE3 and successful transformation was confirmed by PCR using 1248proF/1248proR (for *sdh*) or 1247proF/1247proR (for *bcp*) primers. The cells containing the correct plasmid constructs were then grown for 16 hours in LB Broth with ampicillin (LB-Amp) and subcultured for log phase growth in LB-Amp. Once growth reached an OD_{600} of 0.4, 1 mM IPTG was added and samples were taken at 2, 4, 6, 8, 12, and 24 hours. These cells were sonicated (Sonix 5000) and the insoluble and soluble fractions were run on a 15% SDS-PAGE gel. These gels were

then visualised using Coomassie staining and novel bands were assessed to determine optimum induction time.

For the BCP, all cells were grown to an OD_{600} of 0.4 and induced with IPTG for 3 hours. The protein fractions were analysed by western blot using Anti-His antibodies (Sigma) and visualised using Pierce ECL (Thermo Scientific). The soluble cell free extract was passed through a Nickel-Affinity Chromatography Column (His-Select Nickel Affinity gel Sigma) which was prepared according to the manufacturer's instructions and stored at 4°C before use. The column with bound protein was sequentially washed with 30 ml of 10 mM imidazole wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole), and then 30 mM imidazole (50 mM NaH_2PO_4, 300 mM NaCl, 30 mM imidazole). The bound purified protein was eluted using 250 mM imidazole (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole) and the purity of the protein was assessed using both SDS-PAGE and His-tag Western Blots. The purified protein was used in subsequent biochemical assays.

SDH protein expression was induced using 1 mM IPTG initially and samples were taken at 1 hour intervals for 6 hours. These samples were then sonicated and separated into soluble and insoluble fractions and analysed on 10% SDS-PAGE and Anti-His western blot.

4.2.4 Heterologous Complementation of an *E. coli bcp*$^-$ mutant with *B. fragilis bcp*.

An *E. coli* KD2301 *bcp*$^-$ strain (Jeong *et al.*, 2000) was complemented with the pET22b1247pro construct (section 4.2.3) and the protein was induced as described before. The KD2301 (pET22b (+)) (mutant), BL21DE3 (pET22b (+)) (wild type) and KD2301 (pET22b1247pro) (complemented) strains were used. The resistance of the strains to H_2O_2 was determined using a disk diffusion method (Johnson *et al.*, 2011). The survival of strains

in liquid culture in the presence of H_2O_2 was also determined. Cells were grown in Luria broth with ampicillin to an OD_{600} of 0.4 and 1 mM IPTG was added to all cultures, cells were then grown for a further 3 hours and only cultures with OD_{600} of 0.8 were used. One millilitre of the culture was removed, centrifuged and the pellet resuspended in PBS pH 7.4. H_2O_2 (Sigma-Aldrich) was then added to a final concentration of 100 µM. Cells were sampled at 5 min intervals for 15 minutes, diluted in sterile distilled water (10^{-1}-10^{-8}) and plated on Luria Agar. The plates were incubated aerobically at 30°C for 1 day and the surviving fraction of cells was calculated for each time point. All experiments were done in biological triplicate and technical duplicate. Statistical significance was determined using the student t-test for statistical significance at p <0.05.

4.2.5 Biochemical Assay

Purified BCP was used for all of the assays. After purification, the imidazole-inactivated BCP was activated using a buffer exchange column (Peirce Zeba Spin desalting columns, Thermo Scientific) which exchanged the 250 mM imidazole elution buffer for the assay buffer of 50 mM Hepes (Sigma).

4.2.5.1 Thiol-dependent peroxidase activity of BCP

The thiol-dependent peroxidase activity of BCP was evaluated using the method of Jeong *et al.* (2000), (thioredoxin + thioredoxin reductase + NADPH+ BCP + substrate) with the following modifications: 8 µM of purified BCP was used in all experiments, 150 µM t-butyl hydroperoxide, 100 µM H_2O_2, and 100 µM linoleic acid were used as substrate. Thioredoxin 1 (Trn1) and Thioredoxin reductase 1 (Trx1) (Sigma) were added at published concentrations (Jeong *et al.*, 2000). Activity of BCP was observed as a decrease in total peroxide concentration. Fox 1, a redox sensitive indicator, was used to measure the total peroxide in each reaction (Jeong *et al*., 2000; Kim *et al*., 1988). Twenty microliters of each reaction mix

was added to the Fox1 reagent every minute for 30 min. Colour development was complete after 30 min incubation at room temperature and the colour change was measured by OD readings at 560 nm. The OD_{560} readings were used to calculate the total peroxide concentration at each time point using a Fox1 peroxide standard curve.

4.2.5.2 Antioxidant Characteristics of the *B. fragilis* BCP

The antioxidant properties of the BCP were tested using partially purified glutamine synthetase III (GSIII) from *B. fragilis* as the target enzyme. Purification of GSIII was done according to the method described by Van Rooyen *et al*. (2010). The design of the antioxidant assay was based on the work of Jeong *et al*. (2000) with the following modifications: 8 µM of purified BCP protein was used in all experiments, 100 µM H_2O_2, Trn1 (Sigma), and Trx1 (Sigma) were added at published concentrations (Jeong *et al*., 2000). GSIII was exposed to H_2O_2 in the presence of BCP alone (with NADPH), BCP with both thioredoxin and thioredoxin reductase (with NADPH), or with just thioredoxin and thioredoxin reductase (with NADPH) and assayed for activity which was compared to GSII activity in the absence of H_2O_2. The activity of GSIII was assayed every 10 min for 30 min using the γ-glutamyltransferase assay (Bender *et al*., 1977). The ability of BCP to allow the recovery of GSIII activity following oxidative damage was tested. In this experiment the BCP and/or Trx1 and Trn1 were added after 10 min exposure of GSIII to the oxidising agent (100 µM H_2O_2) and the GSIII activity was assayed as before over a 30 min time period.

4.3. Results and Discussion

4.3.1 Insertional Mutations of BF638R1248 and BF638R1246/7

An insertional mutation method using the Bonheyo *et al*., (2001) pGerm plasmid was undertaken. pGerm constructs containing 500 bp sequences corresponding to the 5' region of both BF638R1246/7 and BF638R1248 were created (Fig 4.1).

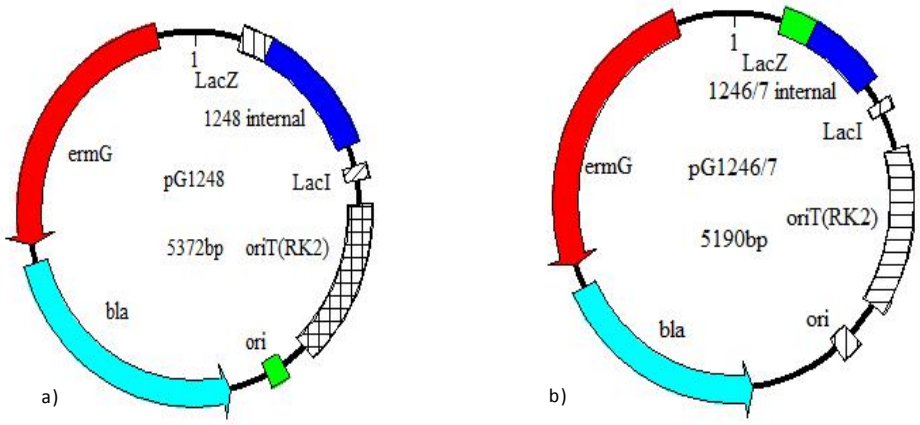


Fig 4.1: Plasmid maps showing important features for pGerm-based insertional mutation plasmids.
(a) pG1248 (*sdh*) and (b) pG1246/7 (*bcp*)

The plasmid containing the insertion sequence for the BF638R1248 ORF (sdh) was not stable for more than a single generation in *E. coli* and thus the method of conjugation using *E. coli* S17-1 could not be undertaken. The plasmid was therefore, purified and electroporated into the *B. fragilis* cells. No insertional mutants were detected. A number of different *E. coli* strains were used for plasmid preparation including methalyse negative (HB110) and RecA deficient (HB101) strains but these also did not generate any stable *B. fragilis* 638R 1248 mutants. The pG1246/7 (*bcp*) plasmid was successfully maintained in *E. coli* S17-1, and *B. fragilis* trans-conjugants carrying this insertion were observed after the initial round of erythromycin selection. These trans-conjugants, however, were not stable and reverted to wild type on subculture. This was monitored through PCR screening for the chromosomal presence of the insertion. No stable integration of the insertion was ever seen for the BF638R1246/7 ORF, despite repeated attempts and culturing the putative *B. fragilis* trans-conjugants at 30°C instead at 37°C.

The lack of stability of the BF638R1248 (*sdh*) construct in *E.coli* may indicate that the cloned portion of the gene may be producing a truncated lethal protein that is disruptive to the

E. coli (Baneyx, 1999). The instability of the BF638R1246/7 (*bcp*) insertion in *B. fragilis* suggests that interruption of the whole operon by inserting large sections of DNA into the middle gene is detrimental to cellular function. For this reason it was decided that a gene deletion method for removing the *sdh* and *bcp* genes would be attempted. Such a method should not disrupt the function of any of the other genes in the operon.

4.3.2 Deletion Mutagenesis of BF638R1248 and BF638R1246/7

The marked deletion method employed was described by Patrick *et al.* (2009) and is shown in Fig 4.2.

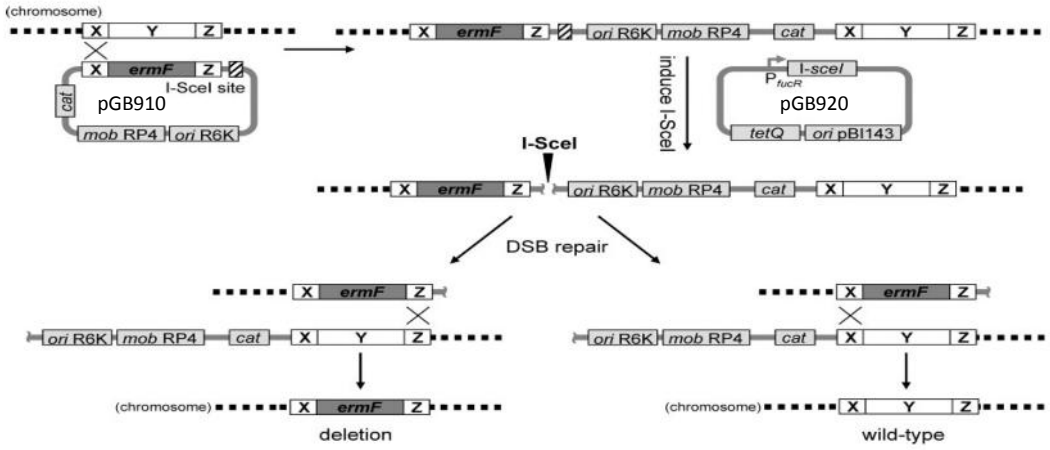


Fig 4.2: Generation of marked chromosomal deletions in *B. fragilis*. A suicide plasmid (pGB910), containing an SceI recognition site (hatched box) and sequences homologous to chromosomal DNA (X and Z) flanking an ermF cassette and replacing the gene to be deleted (Y), is introduced into *B. fragilis* by conjugation. Plasmid integrants are selected for resistance to erythromycin. Diploids are transformed with a plasmid expressing *SceI* (pGB920) under the control of the fucose-inducible promoter PfucR. Growth on defined medium in the presence of fucose leads to a *SceI*-mediated double-strand break and resolution of the diploid by generation of either the deletion or wild-type genotypes by homologous recombination. Chromosomal DNA is indicated as dashed black lines; plasmid DNA is indicated as solid grey lines. (Patrick *et al*., 2009)

Primers for crossover PCR were developed for each gene with as little of the BF638R1248 coding sequence as possible included in the final product (Table 4.2, Fig 4.3). These were then used to construct plasmids using pGB910 for each of the upstream ORFs in this operon (Fig 4.4).

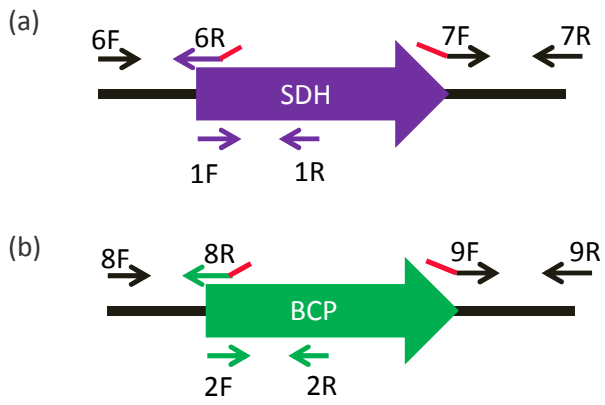


Fig 4.3: Diagram representing the position of primers used in both insertional and deletion mutation methods for (a) BF638R1248 (SDH) (b) BF638R1246/7 (BCP). The red lines represent the regions of homology used in crossover PCR. Primer sequences are in Table 4.2.

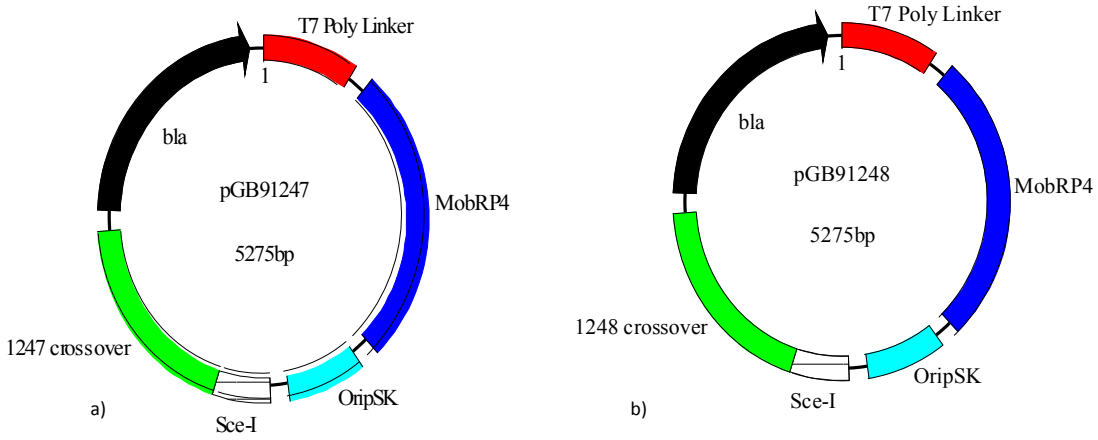


Fig 4.4: Plasmid Maps of pGB910 derived constructs. (a) pGB91247 and (b) pGB91248 used in the first step of the deletion method (Patrick *et al*., 2009).

Stable pGB910 derived constructs for either BF638R1246/7 or BF638R1248 were isolated and confirmed by PCR. Sequenced plasmids were then used to create trans-conjugate *B. fragilis* strains (integrant strain). These trans-conjugates were electroporated with pGB920 encoding the *Sce*-I restriction enzyme. Thirty colonies containing pGB920 were identified for BF638R1246/7 integrants and 20 for BF638R1248 integrant strains. These

were then plated on fucose enriched minimal media. Six hundred colonies for each ORF were screened for deletion of the *sdh* or *bcp* genes. All screened colonies had resolved to wild type and no viable deletion mutants were isolated. These results suggest that any disruption to either the *sdh* or *bcp* genes may be lethal and that the mutation of them may be impossible.

The failure to create mutants in *sdh* or *bcp* using either a disruption or deletion method suggests that they are vital to normal cellular growth even under unstressed conditions. This might also be attributed to slower growth rates in the mutants making them harder to isolate on the defined medium. Functional characterisation of the roles of these genes was, therefore, undertaken using an *in vitro* approach by heterologous complementation in *E. coli*, as well as protein expression and the use of biochemical assays.

4.3.3 Protein Expression and Purification

4.3.3.1 The Saccharopine Dehydrogenase Protein

E. coli BL21DE3 was used to express the *sdh* gene from *B. fragilis* from a pET22b (+) vector. There was very little protein expression and all of the protein was expressed in the insoluble fraction (Fig 4.5a). This was confirmed by His-tag Western (Fig 4.5b).

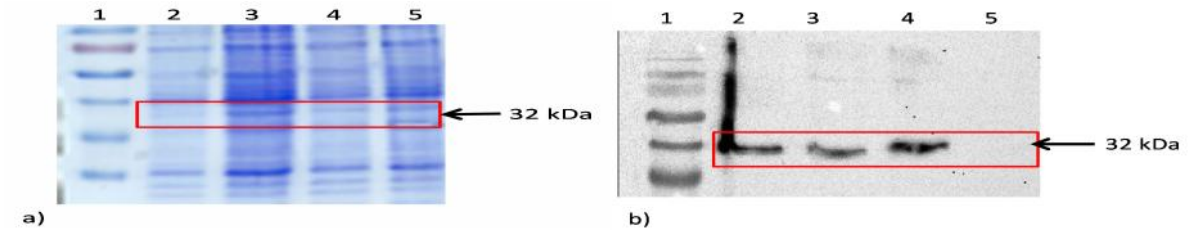


Fig 4.5: Expression of the *B. fragilis sdh* encoded protein in *E. coli* BL21DE3. (a) 10% SDS-PAGE Gel showing expression of pET22b1248pro plasmid in BL21DE3. Lane 1: MW Marker Protein Ladder (Fermentas), Lane 2 and 3: BL21DE3 pET22b1248pro soluble and insoluble fraction, Lane 4 and 5: BL21DE3 pEt22b (+) soluble and insoluble fraction. (b) His-Tag Western showing expression of pET22b1248pro plasmid in BL21DE3 Lane 1: MW Marker Protein Ladder (Fermentas), Lanes 2, 3, 4, BL21DE3 pET22b1248pro insoluble fractions 2,4 and 6 hours respectively, Lane 5, BL21DE3 pEt22b1248pro soluble fraction.

Attempts to improve expression and solubility by changing the temperature or modifying the IPTG concentration were not successful (results not shown). The findings of the insertional mutation experiment suggested that there may be toxicity in *E. coli* associated with overexpression of the SDH protein. A more stringent expression system for controlling *sdh* expression was, therefore, employed (*E. coli* BL21DE3 pLysS). No change to the amount of protein in the soluble fraction or in the overall protein expression was seen (results not shown).

Attempts to solubilise the protein that was being expressed were undertaken. These included the addition of chloramphenicol to disrupt inclusion bodies (Carrió *et al*., 2001) as well as homogenization of the cell free extract to mechanically disrupt inclusion bodies (Middelburg, 1995). There was no change to the solubility of the protein (results not shown). The use of a different expression system was explored. The pColdTF plasmid was considered as an alternative (Takara), however no stable integration of the full length *sdh* gene into the plasmid could be isolated (results not shown). The heterologous expression of the SDH protein using an *E. coli* expression system thus does not seem like a feasible option and alternatives that include cell free expression systems would have to be evaluated in the future. The SDH protein expression and functional characterisation was designated as being appropriate for future work and the focus turned to the 2nd gene of the operon, encoding a putative BCP protein.

4.3.3.2 The BCP protein

E. coli BL21DE3 was used to express the *bcp* gene from *B. fragilis* from a pET22b (+) vector. There was a large amount of protein expressed in both the soluble and insoluble fractions (Fig 4.6a). This was confirmed to be the recombinant BCP protein by His-tag western

analysis (Fig 4.6b). The soluble protein fraction was purified by Nickel-Affinity Chromatography (Fig 4.7) and used in subsequent experiments.

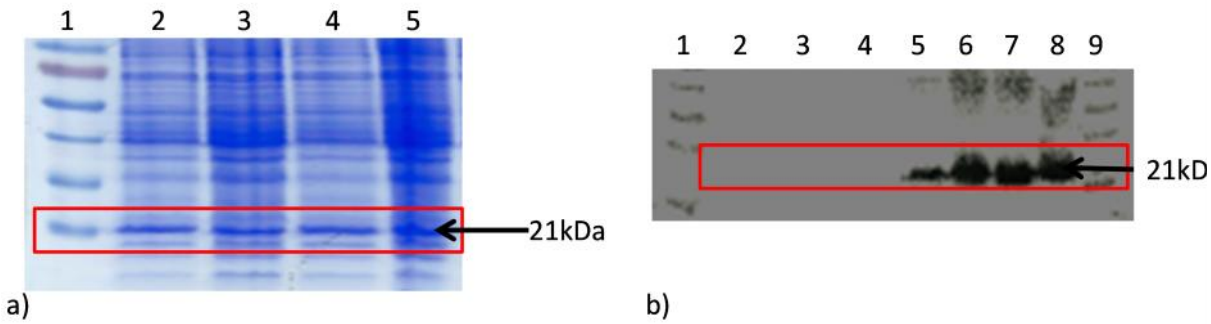


Fig 4.6: Expression of *B. fragilis* BCP in *E.coli* BL21DE3. (a) 15% SDS-PAGE Gel stained with Coomassie showing expression of BL21DE3 pET22b1247pro Lane 1, MW Marker Protein Ladder (Fermentas); Lane 2, 2hr IPTG expression soluble fraction; Lane 3, 2hr IPTG expression insoluble fraction; Lane 4, 4hr IPTG expression soluble fraction; Lane 5, 4hr IPTG expression insoluble fraction. (b) His-Tag Western showing expression of BL21DE3 pET22b1247pro Lanes 1, 9, MW Marker Protein Ladder (Fermentas), Lanes 2,3,4, pET22b(+) soluble fraction 2-4 hours after IPTG induction; Lanes 5, 6, pET22b1247pro, 2hr , IPTG expression soluble and insoluble fraction; Lanes 7, 8, 4hr IPTG expression soluble and insoluble fraction.

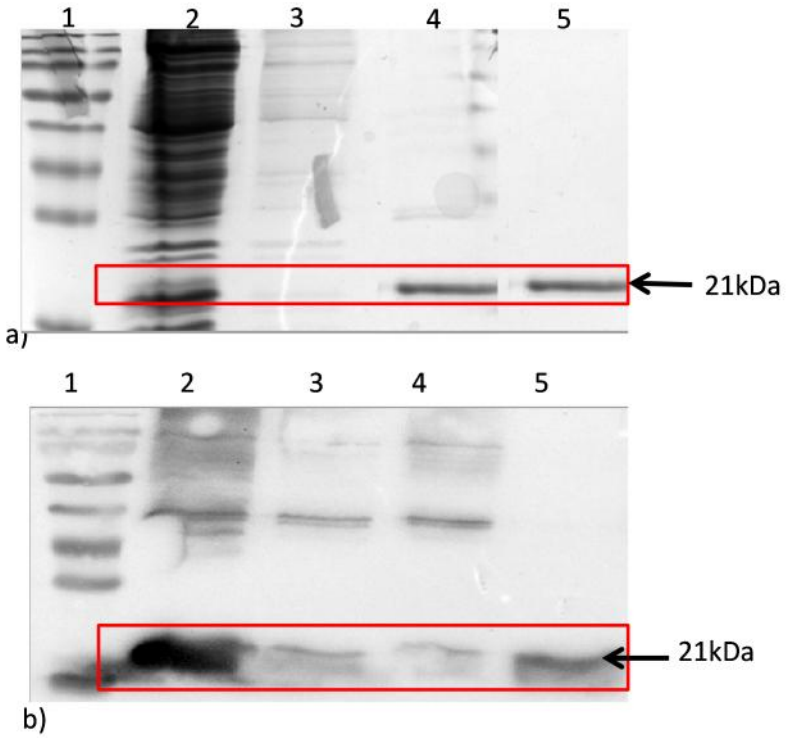


Fig 4.7: Purification of the *B. fragilis* BCP expressed in *E. coli* BL21DE3. a) 15% SDS-PAGE Gel stained with Coomassie showing purification of *B. fragilis* BCP Lane 1, MW Marker Protein Ladder (Fermentas); Lane 2, Crude fraction; Lane 3, 10 mM Imidazole wash; Lane 4, 30 mM Imidazole wash; Lane 5, Purified BCP. (b) His-Tag Western showing purification of *B. fragilis* BCP Lane 1, MW Marker Protein Ladder (Fermentas); Lane 2, Crude fraction; Lane 3, 10 mM Imidazole wash; Lane 4, 30 mM Imidazole wash; Lane 5, Purified BCP.

4.3.4 Heterologous Complementation of an *E. coli* BCP mutant

The *E. coli* KD2301 *bcp*$^-$ strain was created in a study by Jeong *et al*. (2000). This mutant has subsequently been used to characterise a number of BCP proteins from other bacteria including *P. gingivalis* (Johnson *et al.*, 2011). This mutant is derived from the *E.coli* BL21DE3 parental strain in which the expression of the *B. fragilis* BCP was optimised. For this reason, it was possible to attempt complementation of the BCP-deficient KD2301 strain using the pET22b1247pro construct, expressing the *B fragilis* BCP.

The *E.coli bcp*$^-$ mutant has been shown in previous studies to have an increased sensitivity to H_2O_2 by disk diffusion assay (Jeong *et al*., 2000; Johnson *et al*., 2011). A disk diffusion experiment was done in this study to see if the *B. fragilis bcp* gene could complement the *E. coli bcp*$^-$ mutant. No significant difference in survival was seen between the wild-type and mutant using this method and a more sensitive method using survival curves was, therefore, used.

The BCP deficient *E. coli* KD2301 showed a high degree of sensitivity to H_2O_2 during the first 15 minutes of exposure (Fig 4.8) but prolonged exposure resulted in increased survival. The complemented strain exhibited a phenotype that was identical to that of the wild-type *E. coli* strain (Fig 4.8).

The improved survival of the mutant after prolonged exposure could be the result of the initiation of redundant oxidative stress response systems in the *E. coli* (Jeong *et al*., 2000). These could include the catalase system as well as the alkyl hydroperoxidases which respond to the damage associated with H_2O_2. This experiment, however, conclusively demonstrated the capacity for the putative *bcp* gene from *B. fragilis* to complement an *E. coli bcp*$^-$ mutant strain and restore the phenotype to that associated with the wild-type

strain. Investigations into the properties of the BCP protein with respect to substrate specificity and preference as well as antioxidant properties were, therefore, undertaken.

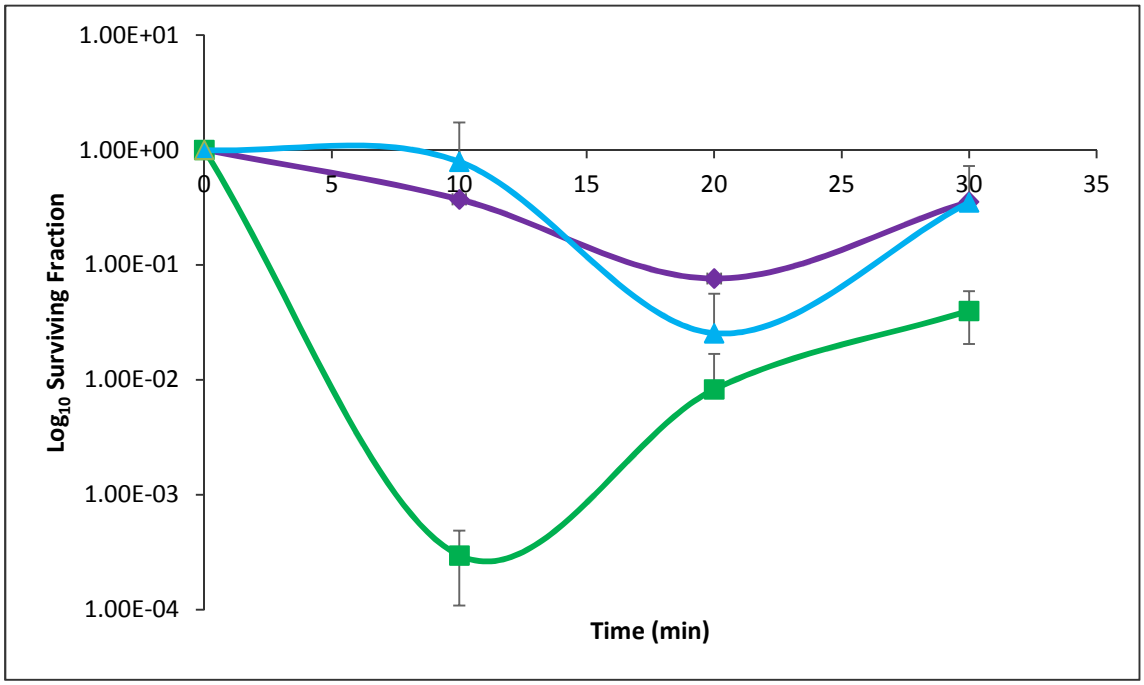


Fig 4.8: Heterologous complementation of an *E. coli* BCP mutant with BCP from *B. fragilis* after exposure to 100 µM hydrogen peroxide. *E. coli* strains: diamonds BL21DE3 (wild type); triangles KD2301 with pET22b1247pro (complement); squares KD2301 (*bcp* mutant)

4.3.5 Thiol-dependent peroxidase activity of *B. fragilis* BCP

The BCP genes from *H. pylori*, *E. coli, P. gingivalis* and *C. jejuni* have all been described as thiol dependent peroxidases (Atack *et al*., 2008; Comtois *et al.,* 2003; Jeong *et al.*, 2000; Johnson *et al.*, 2011). They all exhibit a reliance on NADPH and require the presence of thioredoxin reductase (Trx1) and thiol specific peroxiredoxin (Trn1) (Reeves *et al*., 2011) in order to achieve catalysis. This is a defining characteristic of BCP proteins along with the presence of the cysteine catalytic centre of the PRX catalytic domains (Chapter 3). In order to test whether the *B. fragilis* BCP met these criteria, an experiment was undertaken using 3 different substrates namely H_2O_2, t-butyl hydroperoxide and linoleic acid. BCP activity was assayed by measuring the OH^- (peroxide radical) concentration of the reaction mix using the

redox sensitive Fox1 reagent. BCP activity was measured in the presence and absence of the thioredoxins and NADPHthioredoxins (Fig 4.9). The *B. fragilis* BCP showed activity against all 3 substrates and no preference for a single substrate could be determined. In the absence of only one of the thiol-reductases, the activity against H_2O_2 and t-butyl hydroperoxide was greatly reduced. However, the activity against the linoleic acid is not significantly affected in the absence of either one of the thiol-reductases; only a single reductase was required (results not shown). This suggests that there may be a substrate preference for the organic peroxides over the more complex inorganic compounds. The *B. fragilis* BCP showed no activity against any of the peroxide compounds in the absence of NADPH or both of the thiol reductases. Auto-oxidation/reduction in the presence of NADPH was also evaluated, with no significant decrease in peroxide concentration observed over the 30 minute time period. The spontaneous degradation of the substrate was also observed during this time period with no significant differences in peroxide concentration being seen for any of the substrates. It may, therefore, be concluded that this protein is acting in the same way as previously described BCPs and is thus a thiol-reductase dependent peroxidase enzyme.

Peroxiredoxins (Prx) proteins are strongly expressed ubiquitous Cys-dependent peroxidases (Jeong *et al*., 2000; Reeves *et al.*, 2011). These proteins have been described as having oligomeric kinetics and substrate preferences (Comtois *et al*., 2003; Reeves *et al.*, 2011; Wang *et al*., 2005). They have the ability to interact with a wide variety of substrates in terms of hydroperoxide and its reducing equivalent. This allows the BCP to be active and retain efficacy under a wide variety of cellular conditions (Reeves *et al*., 2011). Prx proteins have been described as being more flexible in their reducing pathways than many other

classes of antioxidant proteins. This flexibility allows for the high reducing capacity of this protein family even in very unfavourable conditions (Reeves *et al.*, 2011).

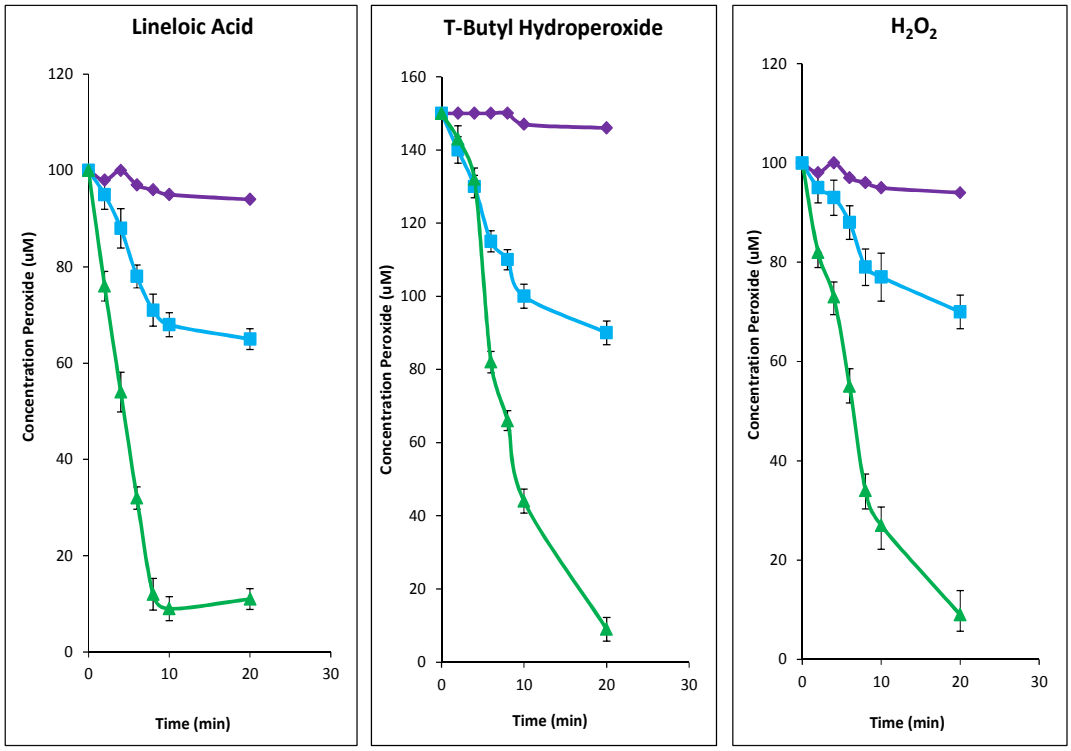


Fig 4.9 : Activity of BCP protein from *B. fragilis* using various substrates (a) Linoleic Acid 100 µM; (b) t-butyl hydroperoxide 150 µM (c) Hydrogen Peroxide 100 µM. Diamonds *B. fragilis* BCP alone; squares Trn1 and Trx1 alone; triangles Trn1, Trx1 and BCP. Trn1: Thioredoxin 1; Trx1: Thioredoxin reductase 1.

The ability of BCP to recognise and act on a variety of compounds is advantageous. Its small size (21kDa), and rapid transcriptional up-regulation after exposure to an oxidising agent (chapter 3), make it an ideal candidate to act as an electron sink in cases of acute cellular exposure to oxygen stress.

Given the substrate flexibility shown in this experiment and the thiol-reductase dependency of the protein it was possible to classify the *B. fragilis* BCP in the family of working Prx proteins to be found within this bacterium. It was, therefore, important to evaluate the possible protective role this antioxidant protein might have in an *in vitro* system that would mirror a stress response associated with this type of protein.

4.3. 6 The Antioxidant potential of *B. fragilis* BCP

The glutamine synthetase is a key catalytic enzyme of anaerobic metabolism as it is responsible for the synthesis of glutamine from glutamate making it vital to cellular survival (van Rooyen *et al*., 2010). This enzyme relies on a Fe-S cluster at its catalytic centre to facilitate its metabolic function and is thus highly sensitive to oxidative damage (Fucci *et al.*, 1983). The oxidation of the Fe-S clusters of many anaerobic enzymes is the direct result of the introduction of partially reduced oxygen species to the anaerobic environment (Imlay, 2003; 2008). This oxidation results in the release of a ferric iron ion. This ion is then available for interaction with hydrogen peroxide at the surface of biomolecules for Fenton reactions that create the hydroxyl radical which causes lesions throughout the bacterial system (Imlay, 2003; 2008). In order to prevent this, ferritin-like proteins act as sinks for ferric iron preventing its availability to act as the reducing metal in Fenton reactions with hydrogen peroxide (Imlay, 2003; 2008). Proteins like BCP protect pools of reduced iron by reducing peroxides before they can damage these clusters.

The *glnN* gene, encoding the *B. fragilis* glutamine synthetase is well characterised. It has been previously purified *in vitro*, its activity quantified, and its crystal structure resolved. This makes it an ideal target for evaluating the potential of the BCP to act as an antioxidant protein. GSIII activity was assayed after exposure to 100 µM H_2O_2 in the presence and absence of BCP. The results of this experiment can be seen in Fig 4.10.

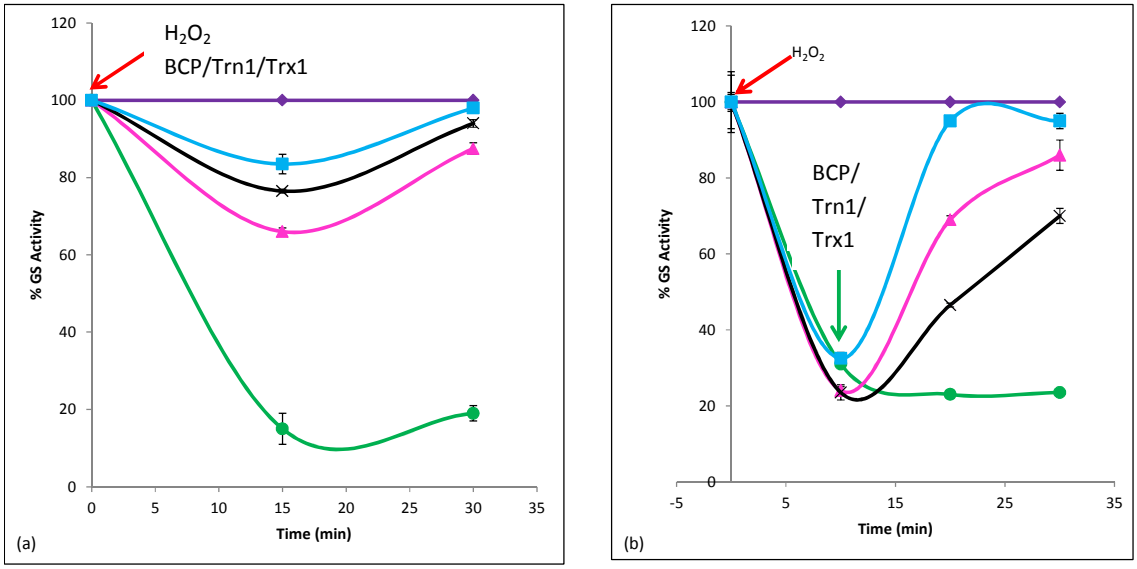


Fig 4.10: Relative Activity of *B. fragilis* GSIII after exposure to hydrogen peroxide (a) Protective properties of BCP for FeS enzymes during exposure to hydrogen peroxide. Diamonds GS without peroxide; squares GS exposed to H_2O_2 in the presence of Trn1, Trx1 and BCP, crosses GS exposed to H_2O_2 in the presence of Trn1 and Trx1; triangles GS exposed to H_2O_2 in the presence of BCP and circles GS and H_2O_2 only. Trn1: Thioredoxin 1; Trx1: Thioredoxin reductase 1 (Sigma). (b) Recovery properties of BCP for FeS enzymes after exposure to hydrogen peroxide demonstrated using the relative percentage activity of the *B. fragilis* GSIII protein. Diamonds GS without peroxide; squares GS exposed to H_2O_2 and the subsequent addition of Trn1, Trx1 and BCP, crosses GS exposed to H_2O_2 and the subsequent addition of Trn1 and Trx1; triangles GS exposed to H_2O_2 and the subsequent addition of BCP and circles GS and H_2O_2 only. Trn1: Thiol specific Peroxiredoxin 1; Trx1: Thioredoxin reductase 1 (Sigma).Error bars represent standard deviation. Arrows indicate the time of addition of various components were relevant.

The BCP protein, in combination with its thiol-reductases, Trn1 and Trx1, was able to protect the activity of GSIII during exposure to 100 µM H_2O_2 allowing between 80% and 100% of activity to be maintained (Fig 4.10a). The contribution of the thioredoxins without BCP was also evaluated and 80-90% of activity was maintained. BCP without the addition of the other thiol-peroxidases maintained approximately 70-80% of the GSIII's wild type activity (Fig 4.10a). In the absence of any of the proteins, over 80% of the GSIII activity was lost following H_2O_2 treatment. When the BCP, Trn1 and Trx1 combination was added to H_2O_2 inactivated GSIII, full GSIII enzymatic function was restored within ten minutes (Fig 4.10b). When the BCP protein was added to inactivated GSIII without the thioredoxins, 90% of the GSIII enzymatic function was recovered after 20 minutes. In the absence of BCP, the Trn1 and

Trx1 proteins were able to recover only 60-70% of GSIII enzymatic function after 20 min (Fig 4.10b). The ability of BCP to protect the GSIII activity can be attributed to the peroxidase activity exhibited by the BCP protein in earlier experiments.. A similar effect of H_2O_2 on GS activity was previously seen in *E. coli* (Jeong *et al*., 2000). The ability of BCP to aid in the recovery of the Fe-S enzyme activity of GSII may be potentiated indirectly by protecting pools of reduced iron ions that can be substituted for damaged iron, or prevent the damage to the Fe-S clusters from occurring. The evaluation of the contribution of BCP to the recovery of the ferric centre and restore GSIII activity following H_2O_2 damage is, to the best of our knowledge, unique to this study. The mechanism of this activity is unknown and further experiments to evaluate a more direct role in reduction of ferric iron would need to be undertaken.

The antioxidant activity of BCP suggests its probable function within the *in vivo* environment of *B. fragili*s and could possibly include other Fe-S metalloenzymes such as PFOR (Pan and Imlay, 2001). This would help in the maintanence of anaerobic metabolism in the face of the oxidative burst resulting either from fluctuations within the gut microcosm or from exposure to the inflammatory response (Mazmanian and Kasper, 2008). The ability of a cell to maintain metabolic function, even under severe oxidative stress, is vital for its continued survival (Imlay, 2008; Sund *et al.*, 2008). The roles and preferences of the *B. fragilis* BCP elucidated in this chapter definitively link this gene and thus its operon to the oxidative stress response of *B. fragilis*.

4.4 Conclusions

A multifaceted approach to the functional characterisation of the 2 upstream genes of the *recA* operon of *B. fragilis* was used in this study. A directed gene mutation approach failed

to produce mutants of either gene. This failure may be attributed to either a toxigenic effect of the products in the *E.coli* intermediates, or lethality in the case of the mutations in *B. fragilis*.

The *in vitro* functional characterisation of the *sdh* gene was further complicated by the fact that the encoded protein remained insoluble when expressed in *E. coli*. The use of chemical denaturation and dissolution of inclusion bodies and the refolding of proteins has previously been evaluated (Middelburg, 2002) but due to the lack of information around the protein structure of SDH from a bacterial system this method was not attempted in the current research. Recent studies in *S. pomeroyi* suggest that purification and assay of this protein directly from *B. fragilis* may be possible, either by specific affinity chromatography (de Mello Serrano *et al*., 2012) or by overexpression using a *B. fragilis* specific protein expression system which incorporates a His-tag on the C-terminal of the protein (Parker *et al*., 2012).

The functional characterisation of the *bcp* gene product proved to be more successful. The heterologous complementation of the *E.coli bcp*$^{-}$ mutant by the BF638R1246/7 gene product confirmed its functional identity as a BCP protein and allowed the development of further experimentation regarding its substrate specificity, thiol-reductase reliance and reducing equivalent. These experiments confirmed that the *B. fragilis* BCP belongs to the thiol-dependent peroxidase family with variable substrate specificity, reducing equivalent requirements and reduction pathway. The use of the GSIII protein allowed for the evaluation of the BCP protein as an antioxidant both in the prevention of and recovery from hydroperoxide-induced damage allowing for a hypothesis regarding the *in vivo* functionality of BCP. The BCP of *B. fragilis* may be acting in times of oxidative stress to facilitate the reduction of hydroperoxides and the recovery of ferric centres to prevent Fenton reactions,

lipid oxidation and DNA damage. This action may, in some way, facilitate the survival of *B. fragilis* either during translocation through the wounded epithelium or after exposure to the high oxygen environment of the abdominal cavity. In either case, BCP would confer an advantage to the cell in oxidative stress survival.

The further analysis of the SDH and BCP proteins within *B. fragilis* itself is essential for a fuller understanding of their roles *in vivo*. One method that should be investigated in future work is the use of antisense RNA knock-down as an alternative to mutagenesis. This system does not rely on any alteration to the genomic context of the target but rather reduces the number of available mRNA transcripts for the expression of proteins (Edwards et al., 2001; Nakashima and Tamura, 2009). It might, therefore, be possible to avoid the deleterious effects of both insertional and deletion methods of mutation and would also allow modulation of the protein levels where complete elimination might be lethal. It would be possible to evaluate each individual gene's contribution to the cellular environment without altering the efficacy of either of the other genes. These knock-down strains could then be characterised in the same way as the *recA* deficient strain in Chapter 2 and used in a murine model to investigate the potential roles of these three genes in terms of persistence and establishment of infection (Johnson *et al*., 2011; Wang *et al*., 2005)

Chapter 5

General Conclusions

5.1 Aim and Hypothesis

The aim of this thesis was to characterise the *recA* operon of the opportunistic pathogen *B. fragilis*. This characterisation included ascertaining the functional roles of the RecA, BCP and SDH proteins within this bacterium as well as evaluating their genomic context and transcriptional regulation.

The findings of this research have resulted in the following hypothesis: The *recA* operon of *B. fragilis* aids bacterial survival during acute oxidative stress, which may be associated with the innate immune response, through maintenance of genomic integrity and cellular metabolism.

5.2 Functional characterisation of the *B. fragilis* RecA

This thesis sought to characterise the functional role(s) of the *B. fragilis* RecA protein both under normal growth conditions and conditions associated with pathogenicity, oxidative burst and metronidazole treatment. The study linked the *B. fragilis* RecA to the regulation of the cell cycle in this bacterium. Future work is necessary to evaluate the direct role of RecA in this system. Experimentation to elucidate this function could include confocal microscopy using strains that are *recA*$^-$ as well as deficient in some of the key proteins associated with cell division and replicative halt. Cellular filamentation in response to Mtz or H_2O_2 stress should also be evaluated in this way. Physiological characterisation of the *B. fragilis recA*$^-$ mutant provided evidence for a link between the RecA protein and genomic integrity, survival after exposure to UV, H_2O_2, and metronidazole exposure. Overexpression of the RecA protein resulted in an improved survival of *B. fragilis* cells after exposure to metronidazole.

The need for bacteria to persist in the face of antibiotic treatment is well documented in bacterial pathogens like *H. pylori* and *M. tuberculosis* (Spiro, 2007; Wang *et al.,* 2005). The RecA protein in *B. fragilis* may be functioning to protect the bacterium during this time. This has implications for its roles in virulence aside from those discussed with reference to genome rearrangement in Chapter 1. It may suggest that there is a more direct role for RecA in infection and survival than previously assumed. This type of role is not uncommon to RecA as discussed in Chapter 1 and opens a novel series of pathways for exploration in terms of their mutual regulation with the *recA* gene in *B. fragilis*. While the link to metronidazole resistance is not a new idea, the publication of this work (Steffens *et al.* 2010) was the first to provide evidence of a direct link between the overexpression of a DNA repair protein and the increased *in vitro* ability of *B. fragilis* to withstand exposure to this antibiotic.

5.3 The genomic context of the *B. fragilis recA*

The requirement for an active RecA protein in cell survival following H_2O_2 challenge revealed a link between RecA and the oxidative stress response. The *recA* locus was, therefore, evaluated. Bioinformatic analysis revealed a putative 3 gene cluster, consisting of the *recA* gene, a putative *sdh* gene and a putative *bcp* gene, and suggested that the two other genes in this cluster were associated with oxidative stress (*bcp*) and lysine degradation (*sdh*). RT-PCR confirmed that all 3 ORFs were co-transcribed under normal growth conditions.

5.4 Transcriptional Regulation of the *recA* operon

A qRT-PCR experiment was done to investigate the transcriptional regulation of this three gene operon after H_2O_2 and metronidazole exposure. This experiment showed that all three genes were up regulated in response to both of these stressors, but that the extent of

induction was different for different genes. These findings suggested that there was a role for each of these genes when confronted with univalent electron stress with either oxygen or nitrogen radicals and that these responses are differentially regulated. The differing degree of induction for each individual gene also suggested that there may be a number of regulatory regions within this operon allowing its conditional expression.

Transcriptional mapping experiments such as northern blot or 5'RACE-PCR should be done in future work to further describe the regulation of this operon. The up-regulation of this operon after metronidazole exposure suggests that this operon may have a role in evading the nitrogenous radicals produced during neutrophil attack. However, metronidazole is not the ideal quantitative reagent for identifying this property since it requires *in vivo* activation which may be variable. For this reason an extension of the qPCR experiment using the nitric oxide donor DEANONOate should be done (Meehan and Malamy, 2012). This experiment would evaluate the transcriptional response of this operon to a quantifiable physiological mimic.

The results of the transcriptional experimentation and the bioinformatic analysis of the RecA operon suggested that both of the upstream genes were expressing proteins vital to cellular survival. Thus the functional characterisation of these genes became necessary to fully understand the *recA* locus of *B. fragilis* and its possible roles in pathogenicity and cellular survival.

5.5 Mutagenesis of BF638R1248 and BF638R1246/7

A direct gene mutation approach was applied to the characterisation of the *sdh* and *bcp* genes. Both deletion and insertional mutation could not be achieved. This suggests that disruption of these genes may be lethal to this bacterium. This may be the result of loss of

crucial functions or a disruption to some of the promoter/regulatory regions associated with the conditional expression of the *recA* operon. Deletion of a single gene may remove a vital regulatory region within the gene needed for expression of its downstream counterparts. In future, further attempts at mutation should be undertaken in a *B. fragilis recA*$^-$ background. This may facilitate a stable *bcp*$^-$ mutation and prevent reversion of this genotype to wild type. The mutation of the *sdh* gene is more complex due to the instability of mutational constructs in the *E. coli* intermediate host, but perhaps the use of a different type of intermediate host might be the solution.

In addition the use of antisense RNA knock-down might be advantageous in future work. This system does not disrupt the genomic context of the genes in the operon and thus does not adversely affect possible regulatory regions on the chromosome. It allows the retention of some of the gene function allowing cellular survival where complete elimination of a gene might result in death (Edwards et al., 2001; Nakashima and Tamura, 2009). This system allows the evaluation of a single gene's contribution to the cellular environment without adversely affecting the other genes but also allows for the elimination of more than one gene at a time. The flexibility of this system makes it ideal for evaluating the roles of this conditional operon under a number of different environmental conditions, as well as the individual roles of each protein within the cellular system.

5.6 *In vitro* functional Characterisation of the *B. fragilis bcp* gene

In the absence of a *B. fragilis bcp*$^-$ mutant, alternative methods for functional characterisation of the gene product were employed. An *E. coli bcp*$^-$ mutant (Jeong *et al*., 2000) was acquired and complemented using the *B. fragilis bcp* gene. The expressed BCP

protein was able to fully restore the wild-type *E. coli* phenotype after exposure to 100 µM H_2O_2. This experiment confirmed that this gene encoded a functional BCP protein.

Biochemical assays developed from the work of Jeong *et al*. (2000) confirmed that the *B. fragilis* BCP is a thiol dependent peroxidase protein and has flexible substrate specificity with activity against H_2O_2, t-butyl hydroperoxide and linoleic acid. This substrate flexibility is a classical characteristic of the BCP proteins in *H. pylori* (Wang *et al*., 2005) and *E. coli* (Jeong *et al.*, 2000; Reeves *et al*., 2011).

These proteins have also been described as having antioxidant properties. The evaluation of the *B. fragilis* BCP antioxidant properties was evaluated by assessing the loss of enzymatic function of the metalloenzyme Glutamine Synthetase (GSIII) from *B. fragilis*. The BCP was able to protect the enzymatic activity of GSIII during exposure to H_2O_2. In this study we also observed the ability of *B. fragilis* BCP to recover the enzymatic function of GSIII after exposure to 100 µM H_2O_2. This function of BCP may be unique to this protein, and needs to be evaluated against other metalloenzymes under other oxidising conditions. The activity of BCP should also be evaluated against the nitric oxide donor DEANONOate in future work.

5.6.1 BCP and Virulence

The host immune system produces macrophage and neutrophil cells that act as the first line of defence against a pathogen attack (Miller *et al*., 1997). Both of these cells produce oxidative or nitrosative radicals in an attempt to cause cell damage and reduce the pathogenicity of invading bacteria (Comstock and Kasper, 2006). Long term colonisation and persistence in the host environment is linked to the microbes' ability to maintain anaerobic metabolism in an oxygen enriched environment (Comstock and Kasper, 2006). This is directly associated with the efficacy of the oxidative defence proteins (Sund *et al.,* 2008). The long term persistence of *H. pylori* was explored, and the absence of a functional

BCP protein resulted in a diminished capacity for long term persistence in a mouse model (Wang *et al*., 2005). In *P. gingivalis* the *bcp* gene was shown to be important for oxidative stress survival and the establishment of infection although it was not vital for pathogenicity (Johnson *et* al., 2011). *C. jejuni* has been shown to rely on the BCP to facilitate growth after exposure to increased amounts of molecular oxygen (Atack *et al*., 2008). In *M. tuberculosis* peroxiredoxins are responsible for the reduction of ·NO to nitrate to prevent nitric oxide mediated cellular respiration damage (Bogdan, 2001). In *B. fragilis,* Rocha *et al.* (2007) showed that the formation of abdominal abscesses and cellular homeostasis after oxidative stress was dependent on the putative thioredoxin proteins (Tpx) found in this bacterium. The reliance of this BCP protein on the presence of these proteins suggests that it may also be a vital component in this protective response. This represents a possible model for the functional role of the *B. fragilis* BCP protein during pathogenesis and the early evasion of the immune system.

5.7 *In vitro* functional characterisation of the *B. fragilis sdh* gene

The failure to produce mutations in the *sdh* gene resulted in the evaluation of *in vitro* methods of characterisation. There are no published bacterial mutations of the *sdh* gene and thus the heterologous complementation of this gene was not possible. Heterologous expression using an *E. coli* expression system was explored in depth but no soluble protein was obtained and thus functional analysis using this method is not possible. In future, the evaluation of this protein may need to be undertaken in a cell free system or by using the *B. fragilis* IPTG-inducible plasmid constructs developed by Parker *et al*. (2012). Recently published work by de Mello Serrano *et al*. (2012) has described a bacterial *sdh* gene in *S. pomeroyi*. They used cell free extract for assay and purified the SDH protein directly from this marine bacterium. In future, modifications to their method might prove useful in

describing the roles of the SDH protein in *B. fragilis*. The *in vivo* characterisation of this gene is also important and should be undertaken using the antisense RNA knockdown method described earlier. The transcriptional analysis of this gene does suggest that it has some unknown function in reversing/preventing the damage associated with oxygen and nitrogen radicals and thus may have a role in virulence and pathogenicity within this opportunistic pathogen.

5.7.1 SDH and Virulence

Fungal species have been reported to become avirulent in the absence of the SDH encoding gene (Kingsbury *et al.,* 2004). In *Cryptococcus neoformans* the mutation of the *lys*9 gene, an *sdh* homologue, resulted in a slower growth rate, reduced melanin production and a reduction in the capsule polysaccharides needed for cell survival within the host environment (Kingsbury *et al,.* 2004). *Fusarium graminearum,* a plant pathogen, has reduced virulence when infected with *Fusarium graminearum Dk-12* virus (Kwon *et al.,* 2009). One of the proteins down regulated in this attenuated strain is saccharopine dehydrogenase. De Mello Serrano *et al*. (2012) have published evidence of the *sdh* and *lkr* genes in *S. pomeroyi* amongst other *Cytophaga/Flexibacter/Bacteroides* (CFB) group bacteria. A number of these gene homologues appear in gene clusters that include oxidative stress response proteins. This finding suggests a possible link between the SDH protein and evasion of oxidative stress.

5.8 A potential model for the roles of the proteins in the *B. fragilis recA* operon

The ability of the strictly anaerobic, opportunistic pathogen *B. fragilis* to colonise the abdominal cavity is an interesting conundrum. The invading cells need to survive transient

oxidative burst, the higher oxygen content of the abdominal cavity and activate their virulence genes.

The transcriptional regulation of these three genes has shown they are an operon that is responsive to oxygen and nitrogen radicals which are associated with the inflammatory response in the human host (Miller *et al*., 1997). This response is present even at sterile wound sites in the endothelium (Phillipson and Kubes, 2011). *B. fragilis* infects the sterile abdomen through wounds in the endothelial layer (Wexler, 2007). Thus this bacterium must be able to survive a transient oxidative burst while traversing the endothelial wound and then survive in the oxygen rich abdominal cavity before forming abscess to generate an anoxic environment (Rocha *et al*., 2007; Sund *et al*., 2008; Wexler, 2007).

We have developed a hypothesis in which we ascribe a transient role for the *recA* operon to facilitate metabolic continuity and genomic integrity during short-lived oxidative stress. This would allow time for the induction of more complex and specific responses within the bacterial system to take place. Fig 5.1 describes a possible model for this.

In summary RecA repairs the ss and ds DNA breaks associated with nitrogen and oxygen radicals. BCP acts with its thioredoxins to directly reduce oxygen radicals preventing damage to the ferric centres of metalloenzymes like GSIII vital for cellular metabolism. SDH facilitates the degradation of lysine to the glutamine glutamate pathway degrading and recycling damaged amino-acids and providing a pool of undamaged amino acids and substrates like glutamine and saccharopine for the production of antioxidant compounds by other pathways.

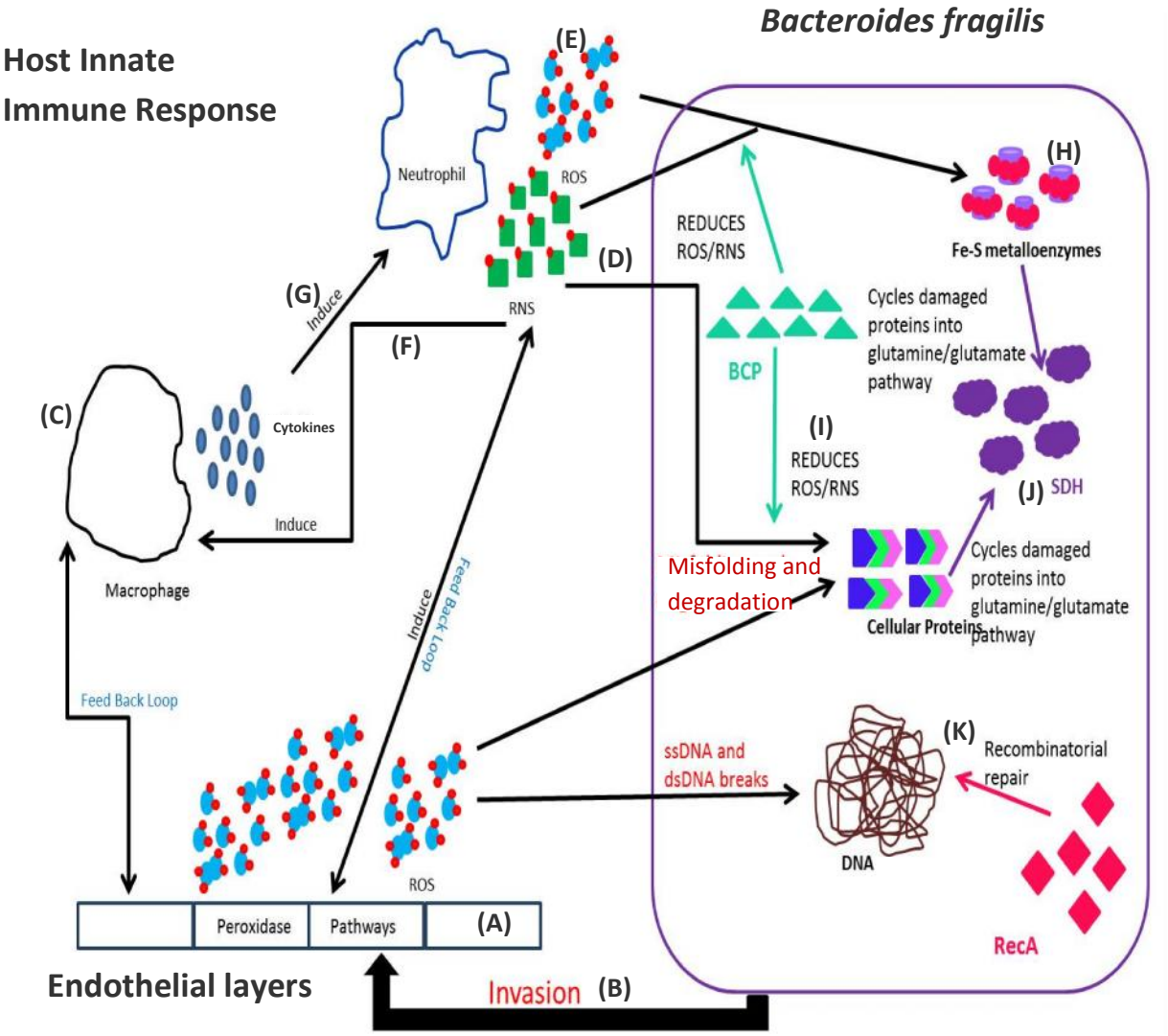


Fig 5.1: Model for protective mechanism of *recA* operon in opportunistic pathogen *B. fragilis*.

A wound to the intestinal mucosa (A) allows the invasion of *B. fragilis* (B) into the sterile abdominal cavity. This wound triggers the innate immune response. The macrophages (C) produce cytokines that induce the production of radical nitrogen species (RNS) (D) and reactive oxygen species (ROS) (E). The RNS induce the peroxidase pathways of the endothelia, macrophage (F) and neutrophil (G) cells creating even more ROS. The RNS/ROS species travel through the bacterial membrane into the cell where they target the Fe^{2+} ions of the metalloenzymes (H) rendering the Fe-S clusters non-functional, damaging cellular proteins causing misfolding and degradation as well as damaging the DNA. The BCP acts to reduce the ROS before it damages the Fe-S cluster or other proteins (I). The putative role of SDH is to cycle the damaged amino acids into the degradation pathway and the glutamine/glutamate synthesis pathway (J) to prevent any damaged amino acids being incorporated into newly synthesized proteins and producing a pool of substrates for the production of other antioxidant proteins. The RecA protein acts to counteract the effects of the RNS/ROS on the DNA integrity of the cell, by repairing any ssDNA/dsDNA breaks K) and removing any damaged nucleotide bases. These three genes working together allow the cell to maintain its metabolic function and DNA integrity, affording a greater opportunity for the cell to activate all of its pathogenicity and survival genes.

5.9 Future Work

In order to support the model presented in this thesis, a number of experiments should be undertaken. The first is to establish the transcriptional response of these genes to nitric oxide the major nitrogen radical of the innate immune response. It is important to establish SDH function and evaluate its necessity to the production of antioxidant products within the bacterial cell. It would be useful to test the survival of *B. fragilis* antisense RNA knockdown strains in cell culture and subsequently during colonisation and persistence of these cells in a murine model. These experiments would allow for the fuller understanding of this operon in pathogenicity, cell survival and pathogenic persistence and test the major hypotheses of the model.

5.10 Conclusions

This thesis has served to characterise the functional role of two of the three genes in the *B. fragilis recA* operon. It has evaluated the transcriptional response of this operon to a number of stressors and revealed the possibility that it may be a conditional operon. It has allowed the development of a model and hypothesis to better understand the ability of this strict anaerobe to survive oxidative stress and establish infection. The main objectives of this research have been met and the findings have revealed a number of novel avenues of exploration in *B. fragilis*.

Appendix

List of domain hits

Description	Pssmid	Multi-dom	E-value
[+]PRX_BCP[cd03017], Peroxiredoxin (PRX) family, Bacterioferritin comigratory protein (BCP) subfamily;...	48566	no	1.14e-49
[+]PRX_family[cd02971], Peroxiredoxin (PRX) family; composed of the different classes of PRXs including many...	48520	no	7.23e-37
[+]PRX_AhpE_like[cd03018], Peroxiredoxin (PRX) family, AhpE-like subfamily; composed of proteins similar to...	48567	no	6.85e-24
[+]PRX_Typ2cys[cd03015], Peroxiredoxin (PRX) family, Typical 2-Cys PRX subfamily; PRXs are thiol-specific...	48564	no	2.60e-21
[+]PRX_1cys[cd03016], Peroxiredoxin (PRX) family, 1-cys PRX subfamily; composed of PRXs containing only one...	48565	yes	1.20e-14
[+]TlpA_like_family[cd02966], TlpA-like family; composed of TlpA, ResA, DsbE and similar proteins. TlpA, ResA and...	48515	no	4.73e-11
[+]PRX_like2[cd02970], Peroxiredoxin (PRX)-like 2 family; hypothetical proteins that show sequence similarity...	48519	no	1.65e-08
[+]PRX_Atyp2cys[cd03014], Peroxiredoxin (PRX) family, Atypical 2-cys PRX subfamily; composed of PRXs containing...	48563	no	6.94e-08
[+]Bcp[COG1225], Peroxiredoxin [Posttranslational modification, protein turnover, chaperones]	31418	no	2.03e-49
[+]bcp[PRK09437], thioredoxin-dependent thiol peroxidase; Reviewed	169871	no	2.41e-43
[+]AhpC-TSA[pfam00578], This family contains proteins related to alkyl hydroperoxide reductase (AhpC) and thiol...	144244	no	8.39e-41
[+]Redoxin[pfam08534], This family of redoxins includes peroxiredoxin, thioredoxin and glutaredoxin proteins.	149549	no	1.02e-19
[+]PRK03147[PRK03147], thiol-disulfide oxidoreductase; Provisional	167501	no	2.75e-11
[+]Tpx[COG2077], Peroxiredoxin [Posttranslational modification, protein turnover, chaperones]	32260	no	2.09e-07
[+]tpx[PRK00522], thiol peroxidase; Provisional	166995	no	2.74e-03
[+]SCO[cd02968], SCO (an acronym for Synthesis of Cytochrome c Oxidase) family; composed of proteins...	48517	no	2.80e-03
[+]TlpA_like_DsbE[cd03010], TlpA-like family, DsbE (also known as CcmG and CycY) subfamily; DsbE is a membrane-...	48559	no	4.24e-03
[+]PRX_like1[cd02969], Peroxiredoxin (PRX)-like 1 family; hypothetical proteins that show sequence similarity...	48518	no	8.47e-03
[+]AhpC[COG0450], Peroxiredoxin [Posttranslational modification, protein turnover, chaperones]	30799	yes	9.55e-22
[+]PRK13190[PRK13190], putative peroxiredoxin; Provisional	106159	yes	2.48e-13
[+]PRK13189[PRK13189], peroxiredoxin; Provisional	171890	yes	4.78e-13
[+]PRK13191[PRK13191], putative peroxiredoxin; Provisional	171891	yes	7.39e-13
[+]AhpC[TIGR03137], This gene contains two invariant cysteine residues, one near the N-terminus and one...	163149	yes	2.36e-12
[+]PTZ00253[PTZ00253], tryparedoxin peroxidase; Provisional	140280	yes	2.00e-11
[+]PRK13599[PRK13599], putative peroxiredoxin; Provisional	106544	yes	4.01e-11
[+]PRK10382[PRK10382], alkyl hydroperoxide reductase subunit C; Provisional	170403	yes	1.26e-08
[+]PTZ00137[PTZ00137], 2-Cys peroxiredoxin; Provisional	173427	yes	5.84e-05

Blast search parameters

Data Source: Live blast search **RID** = 7RNB3H9G01N

User Options: **Database:** cdsearch/cdd **Low complexity filter:** yes **E-value threshold:** 0.01 **Maximum number of hits:** 500

Fig S1: **Protein Domain Hits for BF638R1246/7**

Accession	Description	Max score	Total score	Query coverage	E value	Links
YP_210850.1	putative bacterioferritin comigratory protein [Bacteroides fragilis NCTC 9343]	305	305	100%	1e-81	G
YP_098458.1	putative bacterioferritin co-migratory protein [Bacteroides fragilis YCH46] >ref|ZP	303	303	100%	3e-81	G
ZP_06091237.1	conserved hypothetical protein [Bacteroides sp. 2_1_16]	300	300	100%	2e-80	
ZP_05230109.1	putative bacterioferritin co-migratory protein [Bacteroides fragilis 3_1_12]	299	299	100%	7e-80	
ZP_05757736.1	putative bacterioferritin co-migratory protein [Bacteroides sp. D2]	283	283	100%	3e-75	
ZP_01950445.1	hypothetical protein BACCAC_02060 [Bacteroides caccae ATCC 43185] >ref|ZP_	283	283	100%	4e-75	
ZP_02058387.1	hypothetical protein BACOVA_05403 [Bacteroides ovatus ATCC 8483] >ref|ZP_0	282	282	100%	6e-75	
NP_813522.1	putative bacterioferritin co-migratory protein [Bacteroides thetaiotaomicron VPI-5	282	282	99%	8e-75	G
ZP_05416467.1	bacterioferritin comigratory protein [Bacteroides finegoldii DSM 17565]	281	281	100%	9e-75	
ZP_02434702.1	hypothetical protein BACSTE_00931 [Bacteroides stercoris ATCC 43183]	278	278	100%	3e-74	
ZP_03459355.1	hypothetical protein BACEGG_02140 [Bacteroides eggerthii DSM 20697]	276	276	100%	5e-73	
ZP_02073130.1	hypothetical protein BACUNI_04590 [Bacteroides uniformis ATCC 8492] >ref|ZP_	273	273	100%	3e-72	
ZP_03016265.1	hypothetical protein BACINT_03869 [Bacteroides intestinalis DSM 17393]	273	273	100%	3e-72	
ZP_03679502.1	hypothetical protein BACCELL_03860 [Bacteroides cellulosilyticus DSM 14838]	273	273	100%	3e-72	
YP_001299559.1	putative bacterioferritin co-migratory protein [Bacteroides vulgatus ATCC 8482] >	270	270	100%	3e-71	G
ZP_03239176.1	hypothetical protein BACPLE_02641 [Bacteroides plebeius DSM 17135]	265	266	100%	4e-70	
ZP_03302301.1	hypothetical protein BACDOR_03695 [Bacteroides dorei DSM 17855] >ref ZP_04	264	264	100%	2e-69	
ZP_03644592.1	hypothetical protein BACCOPRO_02982 [Bacteroides coprophilus DSM 18228]	263	263	100%	3e-69	
ZP_03011525.1	hypothetical protein BACCOP_03437 [Bacteroides coprocola DSM 17136]	262	262	100%	7e-69	
ZP_06437033.1	bacterioferritin comigratory protein [Prevotella melaninogenica D18]	244	244	100%	2e-63	
ZP_05857186.1	bacterioferritin comigratory protein [Prevotella veroralis F0319]	243	243	100%	4e-63	
YP_003813351.1	antioxidant, AhpC/TSA family [Prevotella melaninogenica ATCC 25845]	243	243	100%	4e-63	G
ZP_06422406.1	bacterioferritin comigratory protein [Prevotella sp. oral taxon 317 str. F0108]	234	234	100%	2e-60	
ZP_05919117.1	bacterioferritin comigratory protein [Prevotella sp. oral taxon 472 str. F0295]	233	233	100%	3e-60	
ZP_06257619.1	antioxidant, AhpC/TSA family [Prevotella bivia JCVIHMP010]	233	233	100%	3e-60	
ZP_02031055.1	hypothetical protein PARMER_01036 [Parabacteroides merdae ATCC 43184]	232	232	100%	5e-60	
ZP_03475283.1	hypothetical protein PRABACTJOHN_00942 [Parabacteroides johnsonii DSM 18315	231	231	100%	1e-59	
ZP_05546242.1	conserved hypothetical protein [Parabacteroides sp. D13]	230	230	100%	3e-59	
YP_001302330.1	putative bacterioferritin co-migratory protein [Parabacteroides distasonis ATCC 8	230	230	100%	3e-59	G
ZP_06932644.1	bacterioferritin comigratory protein [Bacteroidetes oral taxon 274 str. F0056]	229	229	100%	5e-59	
ZP_07215755.1	bacterioferritin comigratory protein [Bacteroides sp. 20_3]	225	226	100%	5e-58	
ZP_06074477.1	conserved hypothetical protein [Bacteroides sp. 2_1_33B]	225	225	100%	8e-58	
ZP_07323386.1	antioxidant, AhpC/TSA family [Prevotella disiens FB035-09AN]	221	221	100%	2e-56	
ZP_06239527.1	antioxidant, AhpC/TSA family [Prevotella timonensis CRIS 5C-B1]	220	220	100%	4e-56	
ZP_06405127.1	bacterioferritin comigratory protein [Prevotella sp. oral taxon 299 str. F0039]	217	217	100%	2e-55	
YP_003575562.1	thioredoxin-dependent thiol peroxidase Bcp [Prevotella ruminicola 23]	217	217	100%	3e-55	G
ZP_06236586.1	antioxidant, AhpC/TSA family [Prevotella buccalis ATCC 35310]	215	216	100%	4e-55	
YP_003365327.1	Peroxiredoxin [Spirosoma linguale DSM 74]	212	212	100%	1e-53	G
YP_001929174.1	putative bacterioferritin comigratory protein [Porphyromonas gingivalis ATCC 332	205	205	100%	3e-52	G
ZP_06007227.1	bacterioferritin comigratory protein [Prevotella bergensis DSM 17361]	205	205	100%	9e-52	

Fig S2: Protein BLAST Hits for BF638R1246/7

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Links
YP_098459.1	hypothetical protein BF1215 [Bacteroides fragilis YCH46] >ref|YP_21C851.1| hypc	831	831	100%	0.0	G
NP_813523.1	hypothetical protein BT_4612 [Bacteroides thetaiotaom cron VPI-5482] >ref ZP_C	813	813	100%	0.0	G
ZP_05280110.1	hypothetical protein Bfra3_02518 [Bacteroides fragilis 3_1_12]	811	811	98%	0.0	
ZP_05416466.1	saccharopine dehydrogenase [Bacteroides finegoldii DSM 17565]	808	808	100%	0.0	
ZP_01960446.1	hypothetical protein BACCAC_02061 [Bacteroides caccae ATCC 43185]	808	808	100%	0.0	
ZP_02068368.1	hypothetical protein BACOVA_05404 [Bacteroides ovatus ATCC 8483] >ref|ZP_0	806	806	100%	0.0	
ZP_04543965.1	saccharopine dehydrogenase [Bacteroides sp. D1] >ref|ZP_04553116.1| saccharo	801	801	100%	0.0	
ZP_03679503.1	hypothetical protein BACCELL_03661 [Bacteroides cellulosilyticus DSM 14833]	784	784	100%	0.0	
ZP_03016264.1	hypothetical protein BACINT_03868 [Bacteroides intestinalis DSM 17393]	780	780	100%	0.0	
YP_001259660.1	hypothetical protein BVU_2379 [Bacteroides vulgatus ATCC 8482] >ref|ZP_05255	779	779	100%	0.0	G
ZP_04540672.1	conserved hypothetical protein [Bacteroides sp. 9_1_42FAA]	779	779	100%	0.0	
ZP_02073128.1	hypothetical protein BACUNI_04568 [Bacteroides uniformis ATCC 8492] >ref ZP_	778	778	100%	0.0	
ZP_04558174.1	conserved hypothetical protein [Bacteroides sp. D4] >ref|ZP_06037062.1| saccha	778	778	100%	0.0	
ZP_03302302.1	hypothetical protein BACDOR_03703 [Bacteroides dorei DSM 17855]	778	778	100%	0.0	
ZP_03459356.1	hypothetical protein BACEGG_02141 [Bacteroides eggerthii DSM 20697]	777	777	100%	0.0	
ZP_02434701.1	hypothetical protein BACSTE_00930 [Bacteroides stercoris ATCC 43183]	777	777	100%	0.0	
ZP_03644593.1	hypothetical protein BACCOPRO_02983 [Bacteroides coprophilus DSM 18228]	756	756	100%	0.0	
ZP_03209175.1	hypothetical protein BACPLE_02840 [Bacteroides plebeius DSM 17135]	754	754	100%	0.0	
ZP_03011524.1	hypothetical protein BACCOP_03436 [Bacteroides coprocola DSM 17136]	753	753	100%	0.0	
ZP_06405131.1	saccharopine dehydrogenase [Prevotella sp. oral taxon 299 str. F0039]	714	714	100%	0.0	
ZP_07365811.1	saccharopine dehydrogenase [Prevotella marshii DSM 16973]	711	711	100%	0.0	
ZP_05735015.1	saccharopine dehydrogenase [Prevotella tannerae ATCC 51259]	705	705	100%	0.0	
ZP_07035632.1	saccharopine dehydrogenase [Prevotella oris C735]	690	690	100%	0.0	
ZP_06418530.1	saccharopine dehydrogenase [Prevotella buccae D17]	689	689	100%	0.0	
ZP_06254236.1	saccharopine dehydrogenase [Prevotella oris F0302]	688	688	100%	0.0	
ZP_07324640.1	saccharopine dehydrogenase [Prevotella disiens FB035-09AN]	686	686	100%	0.0	
ZP_06289105.1	saccharopine dehydrogenase [Prevotella timonensis CRIS 5C-B1]	685	685	100%	0.0	
NP_904953.1	saccharopine dehydrogenase [Porphyromonas gingivalis W83]	683	683	100%	0.0	G
ZP_06287203.1	saccharopine dehydrogenase [Prevotella buccalis ATCC 35310]	681	681	98%	0.0	
YP_001928829.1	saccharopine dehydrogenase [Porphyromonas gingivalis ATCC 33277]	678	678	100%	0.0	G
YP_001302829.1	saccharopine dehydrogenase [Parabacteroides distasonis ATCC 8503] >ref|ZP_0	675	675	100%	0.0	G
ZP_02031057.1	hypothetical protein PARMER_01038 [Parabacteroides merdae ATCC 43184]	674	674	100%	0.0	
YP_003573471.1	saccharopine dehydrogenase [Prevotella ruminicola 23]	674	674	100%	0.0	G
ZP_05856754.1	saccharopine dehydrogenase [Prevotella veroralis F0319]	674	674	100%	0.0	
YP_003613664.1	saccharopine dehydrogenase [Prevotella maninogerica ATCC 25845]	670	670	100%	0.0	G

Fig S3: Protein BLAST hits for BF638R1248

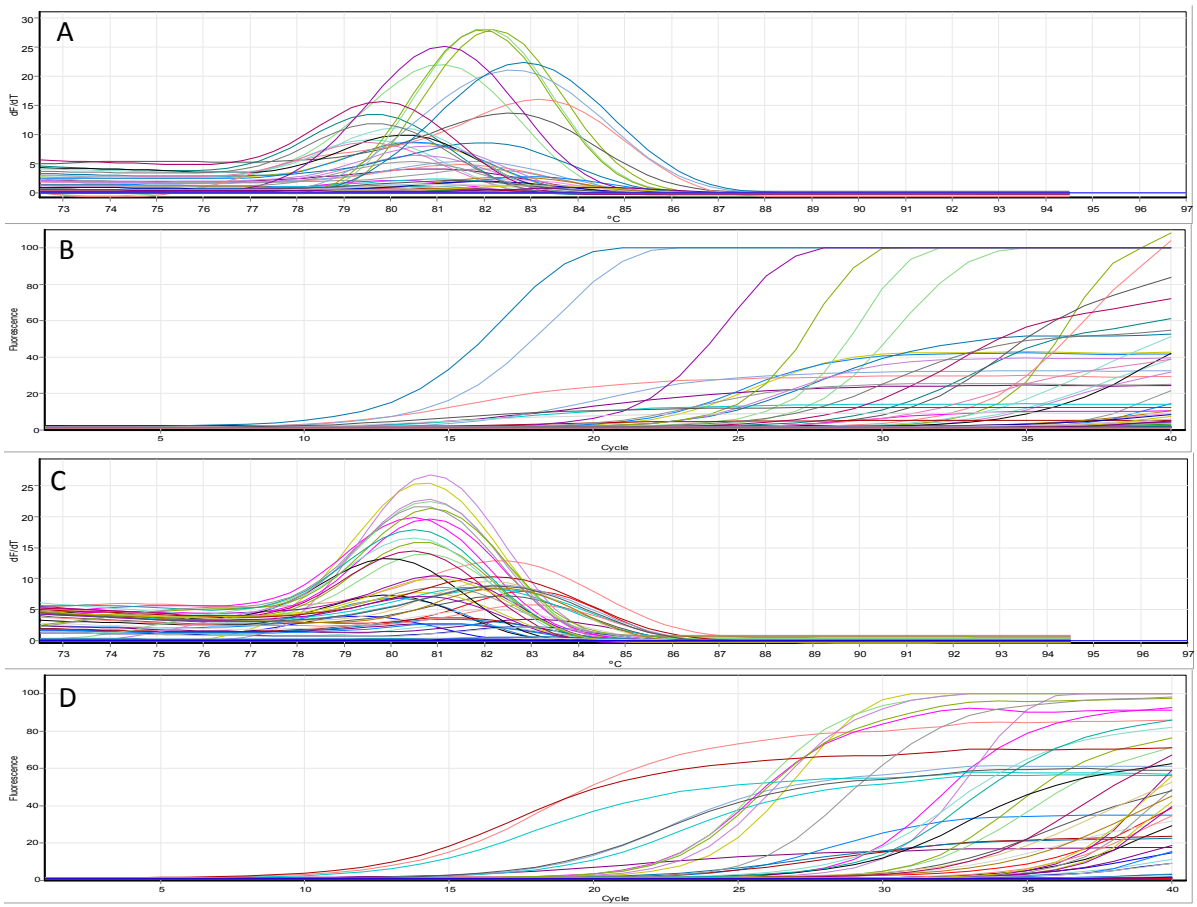


Fig S4: Optimisation for amount of cDNA added to each qPCR run. A: Melt curve analysis for cDNA optimisation 2ul of cDNA template, B: Run curves for cDNA optimisation with 2ul of cDNA template, C: Melt curve analysis for cDNA optimisation 1ul of cDNA template, D: Run curves for cDNA optimisation with 1ul of cDNA template

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