Rhamnose Metabolism and Metronidazole Resistance in *B. thetaiotaomicron* VPI-5482

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Thesis Presented for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Molecular and Cell Biology

UNIVERSITY OF CAPE TOWN

February 2004-September 2006

ACKNOWLEDGMENTS

I would like to thank my supervisor Associate Professor Dr. Valerie Abratt for giving me this scholastic opportunity. Dr. A, thank you for all your support and guidance throughout this project and especially for all your time and efforts in helping me edit this thesis. This is an experience I will never forget. I have gained more knowledge and skills than I had bargained for so thank you for your excellent supervision and scientific insight. The pushing and pulling of doing that "one more experiment" was all worth it.

I would also like to thank Dr. Lynthia Paul, Dr. Ana Casanueva and Di James for all their scientific insight as well as my laboratory peers for their endless entertainment.

Very special thanks to some dear relatives and friends in the USA, UK, Canada, Kenya and S. Africa for your extremely encouraging emails and conversations. Last but not least, I would like to thank my parents and brother. Dad and mum, thank you very much for your unswerving support and confidence in me throughout these years. I am grateful for it everyday.

I would like to thank the Head of Department of Molecular and Cell Biology, Dean of Science Faculty, University of Cape Town and the Wellcome Trust for their financial support.

CONTENTS

ABSTRACT

Bacteroides thetaiotaomicron is an important human gut commensal organism that facilitates polysaccharide utilization, but can act as an opportunistic pathogen outside of this environment causing anaerobic bacteraemia and abscess formation. There is a growing resistance by *B. thetaiotaomicron* and other opportunistic pathogens to metronidazole, the leading drug of choice. This dissertation aimed to use physiological, molecular and biochemical analyses to elucidate the possible mechanism/s of metronidazole resistance in *B. thetaiotaomicron*, in order to extend the understanding of metronidazole resistance at a genetic level. A *B. thetaiotaomicron* VPI-5482 transposon-generated metronidazole resistant mutant, named *B. thetaiotaomicron* Tn Met^R, that displayed a MIC = 8 μg/ml was isolated and characterized. Southern hybridization was used to identify the insertion of a single copy of the transposon in an intergenic region of a gene cluster involved in the uptake and catabolism of $_L$-rhamnose.

RNA hybridization studies confirmed that the genes in *B. thetaiotaomicron* Tn Met^R were transcribed in the presence and absence of the substrate, $_L$-rhamnose. In addition, five of the catabolic genes were expressed as an operon, *rhaKIPAO*. A monocistronic gene, *rhaR*, located downstream of the transposon insertion site had predicted amino acid sequence similarity to a group of AraC/XylS transcriptional regulators. Insertional inactivation of the coding sequence of this gene, using the pGERM suicide vector rendered this mutant, *B. thetaiotaomicron rhaR*$^-$, unable to utilize $_L$-rhamnose. Introducing RhaR in this strain, on a plasmid, restored growth in the medium supplemented with $_L$-rhamnose as the sole carbon source, confirming that *rhaR* is a positive regulator of the rhamnose operon. The transcriptional regulation of the rhamnose pathway was further examined by primer extension and two promoter sites, $P_{rhaKIPAO}$ and P_{rhaR} were

identified. The link between increased $_{L}$-rhamnose metabolism and metronidazole resistance was further investigated by creating an overexpressor of the positive transcriptional regulator, named *B. thetaiotaomicron* (pLYLrhaR). The *B. thetaiotaomicron* Tn Met^{R} and *B. thetaiotaomicron* (pLYLrhaR) exhibited resistance to metronidazole in medium with $_{L}$-rhamnose as the sole carbon source, and displayed elevated levels of lactate dehydrogenase and decreased levels of pyruvate oxidoreductase activity. These two enzymes have been linked to the electron flux required for the intracellular anaerobic activation of metronidazole and this phenotype has previously been described in metronidazole resistant *B. fragilis* clinical isolates. This study demonstrated that the overexpression of the rhamnose pathway in *B. thetaiotaomicron* VPI-5482 resulted in metronidazole resistance and provides the first data to support this link.

ABBREVIATIONS

A	adenosine
aa	amino acids
ATCC	American Type Culture Collection
ATP	adenosine 5'-triphosphate
bp	base pair (s)
C	cytosine
C-	carboxy-(terminal)
CFE	cell free extract
CFU	colonies forming units
DIG	digoxygenin
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetra acetic acid
G	guanosine
GDH	glutamate dehydrogenase
GOGAT	glutamate synthase
GS	glutamine synthetase
h	hour(s)
kb	kilobase pairs
kDa	kilodalton
LB	Luria-Bertani medium
LDH	lactate dehydrogenase
Mb	megabase pair
Met	metronidazole
Met^R	metronidazole resistant
MIC	minimum inhibitory concentration
min	minute(s)
mM	millimolar
mRNA	messenger RNA
MW	molecular weight
ng	nanogram
nm	nanometer
N-	amino-terminal
nt	nucleotides
NCBI	National Center for Biotechnology Information
OD	optical density
ORF	open reading frame
ori	origin of replication
p	plasmid
PCR	polymerase chain reaction
PFOR	pyruvate ferredoxin/flavodoxin oxidoreductase
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
T	thymidine
TAE	tris-acetate-EDTA electrophoresis buffer
UV	ultraviolet light
w/v	weight per volume
α	alpha
λ	lambda
µg	microgram
µl	microliter
µM	micromolar

CHAPTER 1

General Introduction

1.1 INTRODUCTION

The human microbiota found in the gut consists of approximately 500-1000 bacterial species and over 99.9 % of the culturable bacterial population are obligate anaerobes (Xu and Gordon, 2003). The genera found include *Bacteroides, Clostridium, Fusobacterium, Veillonella* and *Bifidobacterium* (Xu and Gordon, 2003; Xu *et al;* 2004, Suau *et al.,* 1999) of which the *Bacteroides* spp. account for 25 % of the colonic bacteria isolated from faecal samples (Salyers *et al.,* 2004; Shoemaker *et al.,* 2001; Xu and Gordon, 2003). *Bacteroides* spp. are the most abundant of the normal gut microbiota (Kuwahara *et al.,* 2004) and are Gram-negative, anaerobic opportunistic pathogens (Spence *et al.,* 2006). As commensal organisms, *Bacteroides* spp. assist in breaking down host dietary products and polysaccharides, and this provides nutrients and vitamins that the host is unable to produce. However, *B. fragilis* and *B. thetaiotaomicron* are also opportunistic pathogens if they escape from the colon as a result of abdominal trauma or surgery, and are capable of causing abscesses in the abdomen, the female genital tract and the brain, as well as resulting in life-threatening septicaemia (Parker and Smith, 2004; Shoemaker *et al.,* 2001; Spence *et al.,* 2006). *B. fragilis* accounts for 0.5 % - 1 % of total the *Bacteroides* spp. in the human intestine (Robertson *et al.,* 2006) whereas *B. thetaiotaomicron* accounts for 12 % of total the *Bacteroides* spp. (Sonnenburg *et al.,* 2006). *B. fragilis* is the organism most commonly isolated from anaerobic infections (50 – 70 %) followed by *B. thetaiotaomicron* (17 %) (Robertson *et al.,* 2006; Cerdeno-Tarraga *et al.,* 2005). One of the main drugs used to treat these anaerobic infections is metronidazole.

Metronidazole [1-(2-hydroxyethyl-2-methyl-5-nitroimidazole] has been used in the treatment of anaerobic microbial and protozoan infections, as well as in yeast infections (Upcroft *et al.,* 2006). However, metronidazole resistant clinical isolates of bacteria and protozoa have been reported (Dunne *et al.,* 2003; Upcroft *et al.,* 2006).

This chapter will review the literature on *Bacteroides* spp., with the focus on *B. fragilis* and *B. thetaiotaomicron*, and will consider their metabolic characteristics, virulence and pathogenicity, and their mechanisms of resistance to metronidazole. Other well studied microbial systems that display resistance to metronidazole will also be addressed. Finally, this thesis will address a novel connection between carbohydrate metabolism and antibiotic resistance, possibly at the regulatory level.

1.2 Phylogeny and General Characteristics of *Bacteroides*

Originally, the *Bacteroides* spp., were classified as Gram-negative, anaerobic, pleomorphic rods that did not share similar characteristics to any other known genera (Holdeman *et al.*, 1984). Investigators then redefined this group of bacteria by using physiological characteristics, bacteriophage typing, and 5S-16S rRNA gene sequence comparisons (Johnson and Alut, 1978; Paster *et al.*, 1994; Weisburg *et al.*, 1985). This information led to the current grouping of these bacteria in three genera: *Bacteroides*, *Prevotella,* and *Porphyromonas* (Shah and Collins, 1989). The latter two genera are more closely related to members of the *Bacteroides* genus than any other bacterial genera and together form the Bacteroidacea family. While *Bacteroides* spp. are commonly found in the large intestine, *Prevotella* and *Porphyromonas* spp. are commonly associated with the rumen and oral cavity, respectively. *Bacteroides* spp. belong to the order Bacteroidales, class Bacteroides and phylum Bacteroidetes, which are known as the *Cytophaga-Flavobacteria-Bacteroides* group (Xu and Gordon, 2003; Kuwahara *et al.*, 2004).

The genus *Bacteroides* consists of approximately 22 different species including *B. fragilis*, *B. thetaiotaomicron, B. vulgatus, B. ovatus, B. distasonis,* and *B. uniformis* (Kuwahara *et al.*, 2004). These species are non-spore forming bacilli which are sacchrolytic, obtaining their

carbon and energy sources by hydrolyzing host dietary carbohydrate molecules. Their additional characteristics include having an average G + C DNA-base composition of 40-43 % (Kuwahara *et al.*, 2004), and their membranes contain a mixture of long chain fatty acids, sphingolipids, and straight chain saturated anteiso-methyl and iso-methyl branched acids (Shah, 1992).

1.3 *Bacteroides* metabolism

The human colon lacks oxygen, making it a very competitive environment for diverse anaerobic organisms. In order for *Bacteroides* spp. to maintain their high numbers, they need to compete favourably with other organisms for nutrients. They benefit from the host by obtaining carbon and energy sources from fermentation of carbohydrates, utilizing host nitrogenous substances as well as recirculating enterohepatic bile acid and facilitating bile acid transformation (Hylemon *et al.*, 1983).

1.3.1. Carbohydrate metabolism

The majority of sugars are absorbed in the human small intestine and are not generally available to the microbiota in the colon. *Bacteroides* spp. utilize simple sugars like glucose (Varel and Bryant, 1974), however, this is not their sole carbon and energy source, as these species are able to utilize more complex polysaccharides that the host cannot digest (Salyers *et al.*, 1977a; Salyers *et al.*, 1977b). For example, degraded plant cell wall polysaccharides (cellulose and xylan) can be used by *B. thetaiotaomicron* and *B. ovatus* (Anderson and Salyers, 1989). Starch, in the form of amylose, pullulan, amylopectin and their component maltooligosaccharides, is used by *B. thetaiotaomicron* (Reeves *et al.*, 1997; Reeves *et al.*, 1996; D'Elia and Salyers, 1996a; D'Elia and Salyers 1996b). Polysaccharide degrading enzymes such as xylanase, xylosidase and arabinosidase have been found in *B. ovatus* (Weaver

et al., 1992). The major end products of the *Bacteroides* glucose metabolism are acetate and succinate, whereas propionate, lactate and acetol are the lesser products (Pan and Imlay, 2001). *Bacteroides* spp. produce several enzymes such as chondriotinase (Linn *et al.*, 1983) and glycosidase (McFarlane and Gibson, 1991) that facilitate the digestion of the carbohydrate moieties of compounds, such as chondroitin sulphate, hyaluronic acid, mucin and other muco-polysaccharides (Xu and Gordon, 2003; Comstock and Coyne, 2003; Cheng *et al.*, 1995).

In silico genomic analyses of *B. fragilis* YCH46 (Kuwahara *et al.*, 2004) and *B. thetaiotaomicron* VPI-5482 (ATCC 29148) showed that both these organisms contained genes with the potential for encoding numerous substrate specific polysaccharide degrading enzymes: 132 in *B. fragilis* and 259 in *B. thetaiotaomicron* (Kuwahara *et al.*, 2004). The *B. thetaiotaomicron* VPI-5482 (ATCC 29148) genome has revealed unprecedented information showing that this organism may be predominately dedicated to harvesting host polysaccharides and sugars. Its glycobiome consists of 226 genes that encode for glycoside hydrolases, 24 sugar specific transporters with 8 fucose permeases (Sonnenburg *et al.*, 2006; Xu *et al.*, 2003). Approximately, 106 genes are homologous to the SusC family of outer membrane proteins, of which half are located near glycohydrolases, and 57 genes are homologous to SusD (Xu *et al.*, 2003, Xu and Gordon, 2003).

Analysis of the *B. thetaiotaomicron sus* system revealed a complex eight component starch utilization system (*sus*) (D'Elia and Salyers, 1996b; Reeves *et al.*, 1996; Reeves *et al.*, 1997; Cho *et al.*, 2001). This system allows polysaccharides to bind to the bacterial cell surface, after which they are brought into the periplasm where they are hydrolyzed into mono- or disaccharides (Reeves *et al.*, 1997). The *sus* system consists of degradative enzymes and outer membrane proteins (OMP). The degradative enzymes are cell-associated as opposed to

extracellular. Examples of these are, SusA (a neopullulanase) and SusB (an α-glucosidase), both of which can hydrolyze α-1,4 bonds. The OMP include SusC and SusD, where SusC was reported to be required for growth on starch and maltooligosaccharide (D'Elia and Salyers, 1996b; Reeves *et al.*, 1996; Reeves *et al.*, 1997). All the *sus* genes are transcribed in two transcriptional units, SusA and SusB-G, in the same direction. Regulation of the structural genes was found to be mediated by SusR, encoded by the *susR* gene that is located upstream of the *susA* gene. SusR binds to maltose or oligosaccharides and activates the expression of SusB-G (D'Elia and Salyers, 1996b). Later, a second *sus* regulator, MalR was also identified in *B. thetaiotaomicron* (Cho *et al.*, 2001).

In a recent study, Spence *et al.* (2006) characterized the starch utilization system in *B. fragilis*, which is encoded by a four-gene *osu* operon. This system contained two proteins that were similar to the SusC and SusD proteins of the *sus* system in *B. thetaiotaomicron*. Both the *sus* and *osu* systems were reportedly induced by starch as well as maltose, which is produced when α-amylase cleaves starch and glycogen. In *B. fragilis*, only one transcriptional regulator, OsuR, regulates anaerobic expression of the *osu* genes as compared to the two transcriptional regulators, MalR and SusR, found in *B. thetaiotaomicron* (Spence *et al.*, 2006; D'Elia and Salyers, 1996; Cho *et al.*, 2001).

Hooper *et al.* (1999) used a mouse model to show that *B. thetaiotaomicron* VPI-5482 has the ability to instruct the host to produce hydrolyzable fucosylated glycoproteins or glycolipids. The organism cleaves the moieties of L-fucose, a methyl pentose sugar, from the host cell surface during the weaning period and uses this as energy source. The study also analyzed the reportedly novel fucose (*fuc*) system found in *B. thetaiotaomicron*, revealing a five gene operon, under the regulation of fucose and a negative transcriptional regulator, FucR a member of the gluconate repressor family (GntR). The genes *fucR*, *fucK*, *fucP*, *fucA* and *fucI* code for a

transcriptional regulator, a kinase, a permease, an aldolase and an isomerase, respectively. Four of these genes are transcribed as an operon, *fucRIAK*. However, the permease gene, *fucP*, was found to be regulated by an independent promoter (Hooper *et al.*, 1999). It was reported that L-fucose induced the expression of the fucose enzymes, through binding of the substrate to the FucR repressor protein and reducing its interactions with the promoter, P_{RIAK}. In addition to the presence of the fucose gene cluster in *B. thetaiotaomicron,* there is also a putative gene cluster, which might be involved in the utilization of rhamnose, a similar methyl pentose sugar to fucose. However, the genome of *B. fragilis* only shows the presence of a putative fucose utilizing gene cluster and lacks the genes for rhamnose catabolism. The ability of these organisms to utilize the L-fucose moieties of the host, as well as instruct the host to produce hydrolysable fucosylated glycans, serves as a competitive colonization advantage under nutrient limiting conditions.

1.3.2 Nitrogen Metabolism

In addition to carbohydrate utilization, the gut microbiota can also metabolize nitrogenous substances entering the large intestine (Fuller and Reeds, 1998). Nitrogen sources in the gut include proteins, peptides or ammonia (Cummings *et al.*, 1984). The protein sources obtained from the host include collagen, albumin and trypsin, and these can serve as substrates for the growth of colonic bacteria. Degradation of proteins to peptides, and in turn into amino acids, is facilitated by bacterial proteases (MacFarlane *et al.*, 1986). In the large intestine, *Bacteroides* spp. are the predominant proteolytic bacteria, and play an important role in protein degradation (Gibson *et al.*, 1989; McFarlane *et al.*, 1986). *B. fragilis* has been shown to produce three proteases (P_1, P_2 and P_3) which have specific activities against proteins such as fibrinogen, trypsin and casein (Chen *et al.*, 1995; Gibson and McFarlane, 1988a; Gibson and McFarlane 1988b). P_1 is a 73 kDa serine protease, known as an exopeptidase, located in the periplasm

and functions to further break down peptides (Gibson and McFarlane 1988b), whereas P_2 and P_3 are endopeptidase, associated with cell surface and initiate proteolysis at this location. P_2 is a 52 kDa metalloprotease whereas P_3 is a 34 kDa cysteine protease (Gibson and McFarlane, 1988b). Ammonia is assimilated relatively easily as it exists in a more reduced form than other forms of inorganic nitrogen and, therefore, serves as the preferred source of nitrogen for growth of many bacteria (Merrick and Edwards, 1995). In addition to ammonia, glutamate and glutamine also serve as good nitrogen sources. Glutamate serves as an amino group donor for biosynthesis of approximately 88 % of cells nitrogen requirements, while glutamine is an essential precursor for the biosynthesis of approximately 12 % of nitrogen compounds in the cells (Reitzer, 1996). Most of the knowledge about nitrogen metabolism and its regulation comes from studies of enteric bacteria such as *E. coli*. However, *Bacteroides* spp. do not share some aspects of these metabolic characteristics (Merrick and Edwards, 1995).

E. coli uses nitrogen sources such as amino acids and ammonia, which are assimilated by the metabolic processes involving glutamine synthetase/glutamate synthase (GS/GOGAT), or glutamate dehydrogenase (GDH) (Merrick and Edwards, 1995). These enzymes function according to the amount of nitrogen source available. Under low ammonia, GS/GOGAT sequentially catalyzes ammonia and glutamate into glutamine (GS), or converts glutamine to glutamate (GOGAT), whereas under high ammonia conditions, GDH functions to catalyze ammonia to glutamate (Merrick and Edwards, 1995). *Bacteroides* spp., however, are incapable of utilizing amino acids as nitrogen sources and, therefore, rely on ammonia assimilation (Baggio and Morrison, 1996). The GS superfamily is comprised of four families, GSI, GSII GSIII and GlnT (van Rooyen *et al.*, 2006). While the GS found in *E. coli* is GSI, encoded by *glnA*, the glutamine synthetase in *B. fragilis* and *B. thetaiotaomicron* is a GSIII, encoded by *glnN* (van Rooyen *et al.*, 2006). In addition to the presence of this GSIII, *B. fragilis* and *B. thetaiotaomicron* also contain the genes *gdhA* and *gdhB*, encoding two GDH enzymes that

are postulated to play a major role in nitrogen assimilation in this organism. The *gdhA* gene encodes a NAD(P)H-glutamate dehydrogenase that catalyzes assimilation of ammonia by reductative amination of α-ketogluterate to form L-glutamate, whereas *gdhB* encodes a NADH dependent glutamate dehydrogenase that is thought to catalyze the reverse reaction (Abrahams and Abratt, 1998). In *B. thetaiotaomicron*, it was reported that GdhA functions under low ammonia (Baggio and Morrison, 1996), whereas in *B. fragilis*, it was reported that GdhB activity was the highest when the organism was grown in the presence of high levels of ammonia or organic nitrogen. Under these same conditions, GdhA activity in *B. fragilis* was repressed (Abrahams and Abratt, 1998).

1.4 *Bacteroides* virulence and pathogenicity

B. fragilis occurs at low levels in the normal human gut microbiota, but it is one the most commonly isolated micro-organism from human intraperitoneal and intra-abdominal infections, causing up to 80 % of anaerobic infections (Cerdeno-Tarraga *et al.*, 2005). *B. thetaiotaomicron* accounts for 29 % of the faecal microbiota and is associated with 17 % of infection cases (Cerdeno-Tarraga *et al.*, 2005). Therefore, as mentioned earlier, *Bacteroides* spp. are opportunistic pathogens having various virulence factors which assist them in thriving outside of the gut. Some of these factors include the presence of a bacterial capsule, aerotolerance, and enzymatic activity (Gibson *et al.*, 1998).

1.4.1 *Bacteroides* capsule

Abscess formation associated with intra-abdominal sepsis is the pathological host response of the immune system to the presence of the *B. fragilis* capsular polysaccharide (Tzianabos *et al.*, 1994b). The ability of this capsular complex to induce abscess is dependent on the presence of $CD4^+$ and $CD8^+$ T-cells within the host (Tzianabos *et al.*, 1994a). The capsular polysaccharide

is thought to assist in adhesion of *B. fragilis* to epithelial cells (Gibson *et al*, 1998) and interferes with phagocytic killing of cells (Onderdonk *et al.*, 1990). Capsulated *B. fragilis* strains were shown to survive better than non-capsulated strain under aerobic conditions, while there was no difference under anaerobic conditions (Patrick *et al.*, 1984). Different populations of *B. fragilis* strains have different capsule complexes, comprised of small or large capsules or an electron dense layer. Patrick *et al.* (1995) conducted studies in a mouse model which showed that the capsular populations of certain *Bacteroides* spp. could modify their capsular complexes going from large capsules to non-capsulated. Variation in capsular complexes has also been found in *B. thetaiotaomicron* populations, where some populations formed non-encapsulated or encapsulated strains (Burt *et al.*, 1978).

The structure of the capsular polysaccharide elucidated in *B. fragilis* NCTC 9343 is unusual as it is composed of two distinct high molecular weight components PS A and PS B, which are expressed on the surface of cells (Tzianabos *et al.*, 1994a). PS A and PS B have repeating subunits with free amino, carboxyl and/or phosphonate groups (Tzianabos *et al.*, 1994a; Comstock *et al.*, 1999b). PS A and PS B both have positive and negative charged groups on each repeating unit (Comstock *et al.*, 1999a). PS A is a tetrasaccharide repeating unit having one positive charged amino group and one negative charged carboxyl group, whereas PS B is a hexasaccharide repeating unit with a negative charged carboxyl group (Tzianabos *et al.*, 1994a). These oppositely charged side chains found in *B. fragilis* are essential for abscess formation (Comstock *et al.*, 1999b). Krinos *et al.* (2001) identified a total of 8 loci involved in capsular polysaccharide biosynthesis (PS A-PS H).

Comstock *et al.* (1999b) identified the genetic locus encoding the capsular polysaccharide in *B. fragilis* 638R and *B. fragilis* NCTC 9343 using transposon mutagenesis. Sixteen ORFs coding for proteins involved in polysaccharide biosynthesis were identified in *B. fragilis*

NCTC 9343, and formed a new polysaccharide locus, PS C1. A similar locus, PS C2, was identified in *B. fragilis* 638R (Comstock *et al.,* 1999a). Wang *et al.* (2000) reported that one of the capsular polysaccharides, PS A2, in *B. fragilis* 638R was similar to PS A as it was found to have zwitterionic pentasaccharide repeats (Tzianabos *et al.,* 1992) possessing both negatively and positively charged side chains and was also able to cause abscesses. The PS A1 and PS B1 loci from *B. fragilis* were reported to contain ORFs showing identity to polysaccharide biosynthesis proteins (Coyne *et al.,* 2000; Coyne *et al.,* 2001). Deletion of PS A1 decreased the ability of *B. fragilis* to cause abscesses, reflecting the importance of the capsule as a virulence factor (Coyne *et al.,* 2001).

The PS loci in *Bacteroides* share a common genetic factor, two genes *upxY* and *upxZ*, that are always located upstream of a set of genes involved in polysaccharide biosynthesis and transport. Expression of PS loci in *B. fragilis* is unique in that they under go phase variation and are regulated in an on-off manner (Kuwahara *et al.,* 2004; Krinos *et al.,* 2001). An invertible region of the *B. fragilis* NCTC 9343 genome, *fin*, was found upstream of *upxY* in the polysaccharide biosynthesis loci PS 1-7 except for PS-2 and PS-7 and was shown to have inverted repeats at each end (Patrick *et al.*, 2003; Cerdeno-Tarraga *et al.,* 2005). All of the inverted repeats were reported to contain a consensus promoter, thought to control expression of genes downstream (Cerdeno-Tarraga *et al.,* 2005; Kuwahara *et al.,* 2004). These inverted repeats, designated *fixL* and *fixR*, contained a functional promoter region between the two genes (Krinos *et al.*, 2001; Patrick *et al.*, 2003). A chromosomally-encoded site specific-serine recombinase, Mpi, was shown to be involved in inverting these 7 polysaccharide biosynthesis loci (Coyne *et al.*, 2003; Kuwahara *et al.,* 2004).

The published sequence of the *B. thetaiotaomicron* VPI-5482 genome identified 7 capsular polysaccharide biosynthesis loci, each having 1 or 2 regulatory *upxY* and *upxZ* homologs, and

an invertible promoter was found in four of these loci (Xu *et al.*, 2003; Kuwahara *et al.*, 2004). The presence of the invertible promoters allows certain populations to express certain genes, allowing for interchangeable cell surface structures. This serves as an advantage to *Bacteroides* spp., allowing them to colonize highly competitive environments, and their interchangeable cell surface also gives them an advantage in avoiding the immune response.

1.4.2 Aerotolerance

During the initial stages of infections, oxidative shock is inevitable when anaerobic bacteria shift from the anaerobic gut environment to oxidative conditions (Rocha and Smith, 1997). The oxygen tension in the peritoneal cavity is approximately 6-7 %. Therefore, whilst the capsule is an exceptional virulence factor, aerotolerance is also thought to contribute to the organisms' pathogenicity (Rocha *et al.*, 2003).

It was reported that *B. fragilis* can withstand oxygen for 48-72 h and, within the first 30 min of exposure to oxygen, over 28 new proteins are synthesized which are involved in neutralizing oxygen radicals (Rocha *et al.*, 2003). It was also reported, that growth was terminated when *B. fragilis* anaerobic growth cultures were exposed oxygen (Rocha *et al.*, 2003). Several studies have been conducted that aimed at elucidating the genes and regulons that responded to oxidative stress. It was found that the major stress response genes were regulated by the redox sensitive transcriptional regulator, OxyR (Rocha *et al.*, 2000; Rocha *et al.*, 2003). The OxyR dependent genes, encoding proteins that are responsible for neutralizing oxygen radicals and protecting the cellular components, are *katB* (catalase), *ahpCF* (alkyl hydroperoxidase), *dps* (DNA binding protein), *tpx* (thioredox peroxidase) and *rbpA* (the RNA binding protein) (Rocha *et al.*, 2003; Rocha and Smith, 1995; Rocha and Smith 1999). The OxyR independent genes include *sod, osu* and *czcd*, coding for superoxide dismutase that eliminates superoxide

anions (Rocha and Smith, 1997), oxygen induced starch utilization (Spence *et al.,* 2006) and a cation efflux pump, respectively (Rocha *et al.,* 2003). A *katB* mutant was reported to be more sensitive to hydrogen peroxide compared to the parent strain, confirming the role of catalase in protection of the cell against hydrogen peroxide toxicity. It was later found that KatB directly removed hydrogen peroxide by interacting with the transition metal and prevented the formation of hydroxyl radicals (Rocha and Smith, 1997; Rocha *et al.,* 2003).

Pan and Imlay (2001) conducted studies in *B. thetaiotaomicron* and showed that two of the central metabolic enzymes, pyruvate oxidoreductase and fumerase were inactivated by exposure to oxygen. It was also reported that these enzymatic activities, from the cultures exposed to 2 h of aeration, were reactivated within 40 min following anaerobiosis in the presence of a protein inhibitor. However, after 12 h of aeration, only 40 % of fumarase was reactivated, but pyruvate oxidoreductase activity could not be restored. These results highlight the ability of the organism to recover from transient oxygen exposure during pathogenesis.

B. fragilis aerotolerance is assisted by the 6.6 kb *Batl* operon (Tang *et al.,* 1999). DNA sequencing showed five ORFs corresponding to the *bat* genes (***B****acteroides* **a**ero**t**olerance) from *Batl* operon. The five proteins, batA-E, form the *Batl* operon are reportedly involved in the reduction of periplasmic proteins allowing the organism to withstand oxygen (Tang *et al.,* 1999).

1.4.3 Other virulence factors

Several bacterial enzymes have been suggested as potential virulence determinants. Sialidases, formerly known as neuraminidases, have been implicated as virulence factors in pathogenic organisms including *Vibrio cholerae, Streptococcus pneumoniae, Prevotella* and *Bacteroides*

spp. (Briselden *et al.*, 1992). Sialidases are trypsin like proteases that are cell associated and cleave α-ketosidic links between sialic acid and glycosyl residues of glycoproteins. *Bacteroides* spp. obtained from pathological specimens were reported to have higher levels of sialidase activity than non-pathogenic strains (Ishikura *et al.*, 2003). Akimoto *et al.* (1994) identified a *nanH* gene coding for neuraminidase from *B. fragilis* YCH46 that contained an Asp box with conserved residues found amongst neuraminidases. The high neuraminidases activity in *B. fragilis* is thought to play a role in the attachment of bacteria to animal cell walls and may explain possible entry mechanisms into the cells (Godoy *et al*, 1993).

The bacteria also protect themselves from the host's immune system by displaying surface molecules similar to that of the host and are, therefore, immunologically inert. For example, a study conducted by Coyne *et al.* (2005) showed that *B. fragilis* converts acquired $_{L}$-fucose to GDP-$_{L}$-fucose to incorporate it into multiple surface capsular polysaccharides.

Although *Bacteroides* spp. in the gut are generally commensal, some strains have been reported to cause diarrhoea in humans and animals under certain circumstances (Moncreif *et al.*, 1998). These strains were later named enterotoxigenic *B. fragilis* strains (ETBF). The toxin secreted by them was named fragilysin, and caused proteolytic degradation by targeting cell surface protein E-cadherin (Fulon *et al.*, 2003). ETBF have the *bft* gene that encodes a preprotoxin of 44 kDa that is processed to yield an active toxin (20 kDa) (Franco *et al.*, 1999). Studies showed that specific inhibitors of metalloprotease inhibited cytotoxicity, and prevented fluid secretions and tissue damage (Moncrief *et al.*, 1998). Franco *et al.* (1999) also reported that *bft* was located in a 6 kb region of the genome found only in ETBF strains, and later reported that, in addition to *bft*, the region contained other metalloproteases indicating that *bft* is located in a pathogenicity island. Carlos *et al.* (2006) further characterized the pathogenicity

island in *B. fragilis* isolated from human blood cultures and reported that prevalence of ETBF is between 9-25% of all *B. fragilis* isolates from skin and soft tissue infections.

1.5 Antibiotic resistance

Members of the *Bacteroides* genus are inherently resistant, or can become resistant, to a broad range of antibiotics including aminogylcosides, β-lactams, tetracycline, erythromycin, and clindamycin. Resistance to metronidazole is also becoming increasingly evident. Their resistance can generally be categorized by four different mechanisms namely, 1) Production of an enzyme that hydrolyzes the antimicrobial agent, 2) alteration of the antibiotic target site, 3) decreased permeability of the bacterial cells (Neu, 1995) and 4) active drug efflux pumps (Pumbwe *et al.*, 2006).

Resistance to certain antibiotics can be spread by conjugative transposons (CTns), mobilizing transposons (MTns) and plasmids, and nearly 80 % of natural isolates from *Bacteroides* spp. carry these elements (Shoemaker *et al.*, 2001). *Bacteroides* spp. have been found to carry large self-transmissible elements called CTns (Cheng *et al.*, 2000). CTns are elements that are integrated into the chromosome of a donor cell and can be excised to form a circular intermediate that can transfer into a recipient cell via conjugation (Cheng *et al.*, 2000). They are 50-100 kb in size and responsible for over 75 % of *Bacteroides* tetracycline resistance (Shoemaker *et al.*, 2001). MTns are smaller, 5-12 kb in size, and are a newly recognized class of transmissible elements. They consist of transposons and Non-replicating *Bacteroides* Units (NBU) that are not self-transmissible, relying on CTns for transfer (Rajeev *et al.*, 2006; Cheng *et al.*, 2000; Parker and Smith, 2004; Schmidt *et al.*, 2006; Shoemaker *et al.*, 2000).

CTnDOT is a *Bacteroides* conjugative transposon that has two antibiotic resistance genes, namely, *tetQ* and *ermF* (Wang *et al.*, 2005; Cheng *et al.*, 2000). The *tetQ* gene is responsible for mediating tetracycline resistance whereas the *ermF* gene is responsible in causing erythromycin resistance in *Bacteroides* spp. (Cheng *et al.*, 2000). Clinical isolates showed that *tetQ* in *Bacteroides* spp. was carried on two types of CTns, CTnDOT and CTn*7853*. The *tetQ* gene encodes a protein which is able to modify the ribosomal target site of the tetracycline class of inhibitors (Nikolich *et al.*, 1992). The *tetQ* resistance gene is transferable and inducible. However, unless the organism is exposed to tetracycline, the transfer of resistance is low (Jenkins, 2001). The transfer of CTnDOT is regulated by tetracycline and it was reported that exposure of cells to tetracycline resulted in 1000–10,000 fold increase in transfer frequency (Salyers *et al.*, 2004). The transfer is mediated by three regulatory genes, *rteA, rteB* and *rteC*. Tetracycline in the cell culture causes the expression of the *tetQ-rteA-rteB* operon, and subsequently RteA and RteB activate the expression of *rteC* (Cheng *et al.*, 2001).

One of the first erythromycin genes to be found in *Bacteroides* spp. was *ermF*, which was found to be located on a self-transmissible plasmid (Rasmussen *et al.*, 1986; Gupta *et al.*, 2003). The resistance genes *ermF* and *ermG*, are members of the macrolide-lincosaminde-streptrogramin (MLS) family (Shoemaker *et al.*, 2001). The genes code for an rRNA methylase that acts on the ribosomal target sites (23S rRNA) causing a reduction in the affinity between the 50S subunit and the antibiotic (Arthur *et al.*, 1987; Macrina and Smith, 1992). Both *ermF* and *ermG* genes have also been found on integrated self-transmissible CTns. It was reported that *ermF* was found on several CTns from the CTnDOT group as well as on three plasmids, whereas *ermG* was only found on CTn*7853* (Shoemaker *et al.*, 2001).

Recently, there has been a further interesting development in erythromycin resistance in *Bacteroides* spp. An *ermB* gene, which is normally found in Gram-positive organisms such as

Clostridium perfringens, has also been found in *Bacteroides* spp. (Gupta *et al.,* 2003; Shoemaker *et al.,* 2001). Gupta *et al.,* (2003) showed that a *B. uniformis* strain carrying the *ermB* gene could transfer it to a *B. thetaiotaomicron* BT4001 strain via conjugation, which supported their hypothesis that genes coding for antibiotic resistance in the *Bacteroides* spp. are carried on conjugative transposons. These findings also highlighted the importance of investigating the occurrence of horizontal gene transfer events amongst bacteria in the human colon.

β-lactam antibiotics are the most widely used group of antimicrobials, however, resistance to this drug is high amongst the *Bacteroides* spp. The mechanism of resistance involves production of β-lactamase, reduced penetration of β-lactam antibiotic and alteration of the penicillin binding protein (PBP) (Fang *et al.,* 2002a; Fang *et al.,* 1999). Most of the β-lactamases produced are reported to be chromosomally mediated and are produced constitutively (Jenkins, 2001). *Bacteroides* spp., use the β-lactamase to hydrolyze β-lactams causing resistance to these antibiotics. For example, the carbapenem-hydrolyzing β-lactamase that is produced by *B. fragilis*, (encoded by the *cfiA* and *ccrA* genes) confers resistance to practically all β-lactams (Podglagen *et al.,* 2001). These genes in *B. fragilis* are metallo-β lactamase enzymes and have been found to inactivate β-lactam antibiotics including penicillin-G (Rasmussen *et al.,* 1990; Rasmussen *et al.,* 1991). The PBP are targets of β-lactam antibiotics and these are essential for bacterial growth. There are 3-5 PBP in *Bacteroides* spp. and they function in cell wall synthesis (Jenkins, 2001). The outer membrane proteins of the Gram-negative bacteria also make them intrinsically more resistant to β-lactam antibiotics compared to the Gram-positive bacteria, since the outer membrane protein limits the antibiotic access to the cytoplasmic membrane (Fang *et al.,* 2002b). In Gram-negative organisms including *Bacteroides* spp. the interplay between the outer membrane permeability barriers,

ubiquitous periplasmic β-lactamase and recently recognized multidrug resistance (MDR) efflux pumps all contribute to antibiotic resistance.

Efflux pumps are known to be substrate specific and work in a manner to expel the antimicrobial from the cells into the surrounding spaces, the antimicrobial then have to pass through the outer membrane barrier to regain entry into the bacterial cells (Ueda *et al.*, 2005). *B. fragilis* and *B. thetaiotaomicron* have a variety of pump genes, these efflux pumps belong to the resistance-nodulation-division (RND) as well as the multidrug and toxic compound extrusion class (MATE). In *B. fragilis*, 16 RND-type pumps, named (BmeB1-Bme16) were identified in its genome. *B. fragilis* mutants that were resistant to at least three antimicrobial classes, including metronidazole, were reported to display overexpression of one or more of the *bmeB* genes (Pumbwe *et al.*, 2006a). Constitutive *bmeB* expression is prevalent in *B. fragilis* and 7 BmeB efflux pumps are functional in transporting antimicrobials in this organism (Pumbwe *et al.*, 2006b). *B. thetaiotaomicron* contains 60 proteins predicted to be components of drug efflux systems (Ueda *et al.*, 2005). The BexA pump, a member of MATE was characterized in *B. thetaiotaomicron*, and the inactivation of the *bexA* gene was reported to decrease the norfloxacin MIC from 128 μg/ml to 32 μg/ml (Miyamae *et al.*, 2001).

The presence of antibiotic resistance genes in *Bacteroides* spp. makes it difficult to treat infections with certain drugs. One of the drugs that can still be used successfully is metronidazole. However, resistance to this drug is also increasingly being reported.

1.6 Introduction to Metronidazole

Metronidazole is a nitroimidazole antibiotic that was introduced in the 1960s as the drug of choice to treat anaerobic infections caused by pathogenic organisms such as *B. fragilis* (Müller,

1983). Surveys carried out in the 1970s on metronidazole activity against anaerobic bacteria revealed that it had a very broad spectrum of antibacterial activity. In addition, it has also been used to treat infections caused by *Trichomonas vaginalis*, and is effective against parasitic infections caused by *Gardia lamblia* (Upcroft *et al.*, 2006). It is also one of the drugs used in the triple therapy treatment for gastrointestinal infections caused by *Helicobacter pylori* (Marais *et al.*, 2003). More recently, this class of drug has begun to be explored in the treatment of *Mycobacterium tuberculosis* under anaerobic conditions (Manjunatha *et al.*, 2006). The broad-spectrum activity of metronidazole is achieved by the low molecular weight of the drug, which allows it to penetrate all cell membranes, and its requirement for activation under anaerobic conditions to form the active drug. The specificity of the nitroimidazole derivatives, therefore, cuts across the line between prokaryotes and eukaryotes, since, nitroimidazole derivatives interact with biochemical systems and/or pathways in all anaerobes (Müller and Lindmark, 1976). The structure of the drug consists of an imidazole ring with a nitro-group on the fifth position (Fig 1.1).

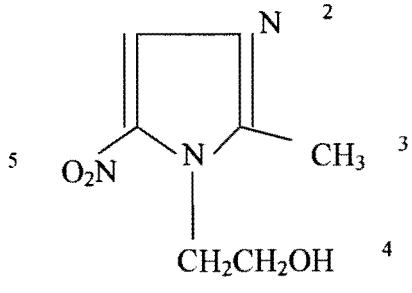


Figure 1.1: Structure of metronidazole. Reduction of the nitro-group (on the 5th position) activates the drug (Derived from Kulda *et al.*, 1993)

1.6.1 Mode of Action

Metronidazole is an inert prodrug which needs to be activated intracellularly by the reduction of its nitro group (Fig. 1.1) resulting in a toxic derivative which causes single and double strand DNA breaks (Leiros *et al.*, 2004). The reduction of the nitro group on the fifth position

of the imidazole ring structure requires a single electron (Müller, 1983). Under anaerobic conditions, the reduction of the RNO_2 (where "R" represents CH_2CH_2OH for metronidazole) is thought to generate short lived free radical anions ($RNO_2^{\cdot-}$). Reduction of the free radical generates the nitroso derivative, and the cytotoxic species, which include a nitroso-free radical and a hydroxylamine derivative. In the presence of molecular oxygen, free radicals generate the nitroso derivative (RNO) and nitroso radical ($RNO^{\cdot}$). The presence of oxygen can also re-oxidize the free radical anion ($RNO_2^{\cdot-}$) and produce the parent drug with the production of the superoxide anion ($O^{\cdot-}$) in a process called "futile cycling" (Edwards, 1993). The short lived reduction products oxidize DNA causing strand breakage and, in turn, cell death.

Rustia and Shubick (1972) and Voodg *et al.* (1974), reported that metronidazole caused carcinogenicity in mice and mutagenicity in bacteria, which suggested that metronidazole was targeting DNA. Although metronidazole is still very effective as an antimicrobial agent, clinical isolates have been reported to show resistance to the drug. There are three possible areas where resistance to metronidazole may be occurring. These are at the levels of 1) cell entry and uptake, 2) activation of the drug and 3) Repair of the DNA damage caused by the drug (Fig. 1.2).

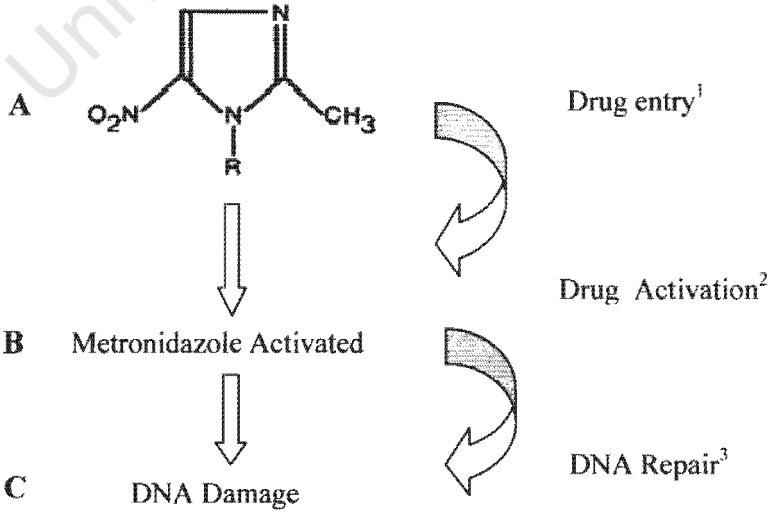


Figure 1.2: Schematic representation of the nitroimidazole ring structure and the levels of possible resistance mechanisms. "R" represents CH_2CH_2OH group for metronidazole. Drug entry[1], Drug Activation [2] and DNA repair[3] are the 3 levels at which metronidazole resistance can occur.

1.6.2 Entry and Uptake

Metronidazole is well absorbed after oral administration and indirect evidence suggests that it enters the cells via passive diffusion (Müller, 1983). Studies conducted by Müller and Lindmark (1976) in *Trichomonas* using [^{14}C] labelled metronidazole, showed that the formation of the toxic form of the drug decreased the intracellular concentration of the prodrug and generated a concentration gradient that caused the further uptake of metronidazole. As mentioned earlier, metronidazole has a low molecular weight, which facilitates its penetration through cell membrane. It also has a redox potential of -415mV, which makes it an efficient electron acceptor (Müller, 1983). There is no direct evidence for active transport of the drug into the cells, although Pumbwe *et al.* (2006a), reported that efflux pumps inhibitors reduced the MICs of the antimicrobials tested, including metronidazole, in the MDR *B. fragilis* mutants, suggesting that an energy-dependent efflux was a major mechanism of resistance. In an alternate study, it was reported that several imidazole derivatives competitively inhibited metronidazole uptake in *H. pylori,* indicating a possible active transport system for specific types of structures (Moore *et al.,* 1995). Not much is reported on uptake of metronidazole specifically in *Bacteroides* spp. However, there have been reports of clinical isolates of metronidazole resistant *B. fragilis* which have shown decreased uptake of the drug. This, however, was interpreted as decreased activation of metronidazole resulting in a slower diffusion rate of the drug (Britz and Wilkinson, 1979; Ingham *et al.*, 1978; Ingham *et al.*, 1980).

1.6.3 Activation

Activation of metronidazole involves a low redox potential process that reduces the 5-nitro group forming the transient toxic radicals (Upcroft *et al.,* 2006). The imidazole ring structure is believed to fragment producing two short-lived cytotoxic intermediates, acetamide and N-(2-

hyroxyethyl)-oxamic acid (Müller, 1983, Koch and Goldman, 1979). Activation of the drug is thought to occur through interaction with a pyruvate oxidoreductase complex in an ATP phosphoroclastic reaction (Diniz *et al.*, 2004; Upcroft *et al.*, 2006). Alternatively, activation of the drug can occur via the nitroreductase activities encoded by several genes, namely, *rdx*, *frx* and *fdx*, coding for an oxygen insensitive NADPH nitroreductase, an oxygen insensitive flavodoxin NADPH nitroreductase and a ferredoxin-linked protein, respectively (Sisson *et al.*, 2002).

1.6.3.1 Activation of metronidazole via the pyruvate oxidoreductase complex

The pyruvate oxidoreductase complex is formed by a metabolic reaction between pyruvate and the oxidoreductase enzyme to facilitate pyruvate decarboxylation to form acetyl CoA. This reaction generates electrons that are accepted by ferredoxins or flavodoxins that have electron efficiency of -430mV or -460mV (Edward, 1980). Ferredoxins and flavodoxins are proteins that consist of iron-sulphur clusters and mediate electron transfer in a range of metabolic reactions (Valentine, 1964). They are the most electro-negative electron carriers in oxidation-reduction reactions and serve as oxido-reductase catalysts by transferring electrons from low potential donors to electron accepting compounds like metronidazole (Valentine, 1964). Metronidazole acts as an electron sink in the pyruvate oxidoreductase complex capturing electrons from reduced ferredoxins or flavodoxins thereby reducing the nitro group on the drug (Müller, 1983). Once pyruvate is produced in the bacterial cells, it is enzymatically oxidized to acetyl phosphate and CO_2, which causes reduction of ferredoxins (Valentine, 1964). In turn, the donation of electrons to metronidazole activates the drug, releasing CO_2 and H_2 as by-products of the pyruvate oxidoreductase complex (Mortenson *et al.*, 1963). Church and Laishley (1995) showed that *Clostridium pasteurianum* used the hydrogenase I enzyme to reduce metronidazole by oxidizing reduced ferredoxins. Sindar *et al.* (1982) proposed that the site at which metronidazole is reduced is also at the pyruvate phosphoroclastic reaction in

C. perfringens. Kaihovaara *et al.* (1998), showed that the drug's activation process in the *H. pylori* system is also thought to be mediated by pyruvate oxidoreductase complex, where flavodoxins are substituted for ferredoxins and metronidazole can be transformed into a bactericidal agent by the electrons generated from the oxidation of pyruvate (Fig 1.3).

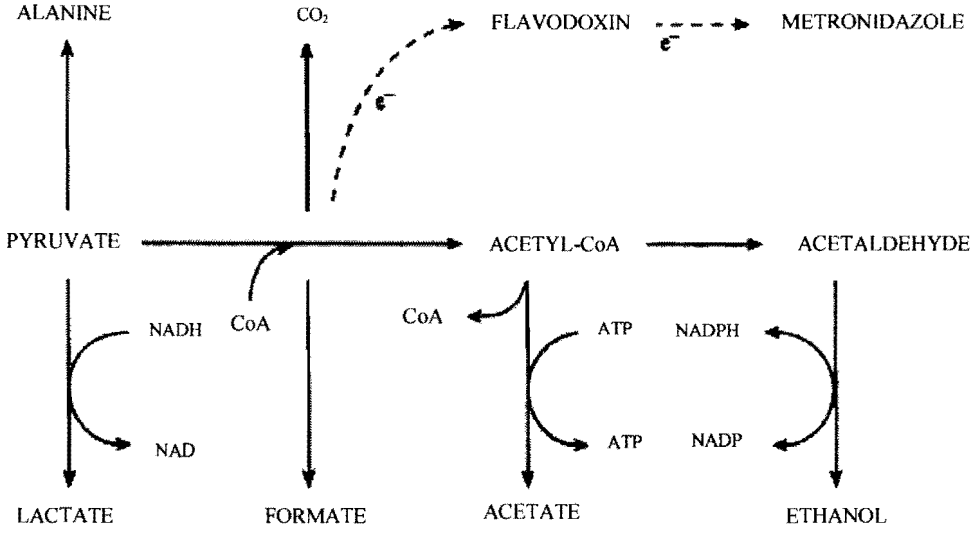


Figure 1.3: Schematic representation of the pyruvate oxidoreductase complex in *H. pylori*. Pyruvate gets decarboxylated into Acetyl-CoA, electron donation to flavodoxin (dashed line) and subsequently to metronidazole to activate the drug. Kaihovaara *et al.* (1998).

A pyruvate oxidoreductase enzyme was extracted from *B. fragilis* NCTC 11295 by Tabaqchali *et al.* (1983), however, ferredoxins have not been extracted from *Bacteroides* spp. Instead the presence of a flavodoxin gene, *fldA*, was reported by Diniz *et al.* (2004) in the *B. fragilis* 638R strain. The genome sequence of *B. thetaiotaomicron* VPI-5482 (ATCC 29148) also has a gene, with a locus tag BT1747, which codes for a putative pyruvate flavodoxin oxidoreductase. Recent studies in *B. fragilis* 638R report the possibility of metronidazole activation occurrence at a similar pyruvate oxidoreductase complex (Diniz *et al.*, 2004) as did former studies conducted by Narikawa *et al.* (1991) and Britz and Wilkison (1979) on other *B. fragilis* strains.

There have been numerous reports of resistance to metronidazole implicating the site of activation. For example, *C. perfringens* mutants showing resistance to metronidazole were reported to have diminished levels of pyruvate dehydrogenase and very high levels of lactic acid (Sindar *et al.*, 1982). Narikawa *et al.* (1991) used a *B. fragilis* NCTC 11295 metronidazole resistant strain to test if lactate dehydrogenase (LDH) was compensating for decreased pyruvate oxidoreductase activity. The activity of pyruvate oxidoreductase and LDH in this strain was also tested in the presence of increasing concentration of metronidazole. Their results indicated that LDH activity was detectable up to 48 mg/L metronidazole; however, pyruvate oxidoreductase was undetectable after exposure to 2 mg/L of metronidazole. In another study, Britz and Wilkinson (1979) used a *B. fragilis* clinical isolate from a urinary tract infection to show that metronidazole resistant mutants from this strain had less pyruvate oxidoreductase activity. Diniz *et al.* (2004) obtained *B. fragilis* resistant strains by passaging the strains in the presence of metronidazole using gradient plates. Using a combination of proteomics, as well as biochemical assays, they identified that flavodoxin was down-regulated. It was also reported that no pyruvate oxidoreductase was detected in the resistant strains and that analysis of the fermentation products showed high levels of lactate (Diniz *et al.*, 2004).

Diniz *et al.* (2004) created a single cross-over insertion mutant in *B. fragilis* 638R, by inactivating the *porA* gene, coding for pyruvate-ferredoxin oxidoreductase. They also inactivated the *rdx* and *fldA* genes coding for nitroreductase and flavodoxin, respectively and found that all three of these mutants had an increase in metronidazole resistance. When double mutants were created, by inactivating the *porA* and *rdx* genes, as well as *porA* and *fldA* genes, these mutants were more resistant to metronidazole than the single mutants.

1.6.3.2 Metronidazole activation via nitroreductase activity

Genes encoding nitroreductases are found in *H. pylori* and *B. fragilis* (Sisson *et al.*, 2002; Diniz *et al.*, 2004). As mentioned earlier, an oxygen insensitive NADPH nitroreductase is encoded by *rdxA*, whereas *frxA* encodes an oxygen insensitive flavodoxin NADPH nitroreductase. These enzymes reduce 5-nitroimidozles to mutagenic products that cause DNA damage (Sisson *et al.*, 2002). It was reported that mutations in the *H. pylori rdxA* gene resulted in metronidazole resistant strains (Kwon *et al.*, 2000; Sisson *et al.*, 2002). The single *frxA* mutation resulted in a moderate level of resistance. However, inactivation of both *rdxA* and *frxA*, produced levels of resistance reaching MIC larger than 100 μg/ml (Sisson *et al.*, 2002). In addition, inactivation of the *frxA* together with *fdxB*, a gene coding for a ferredoxin-linked protein, doubled the MIC to 64 μg/ml.

The reduction of metronidazole via nitroreductase activity does not, however, always result in the formation of the toxic intermediate. One of the major metronidazole resistance mechanisms in *Bacteroides* spp. has also been found due to the presence of *nim* genes (Urban *et al.*, 2002; Gal and Brazier; 2004; Stubbs *et al.*, 2000). These genes encode a nitroimidazole reductase, which converts 4- or 5- nitroimidazole to 4- or 5-aminoimidazole, preventing the formation of the toxic derivatives that are responsible for causing the bactericidal activity of metronidazole (Gal and Brazier, 2004). The six reported *nim* genes, namely, *nimA, nimB, nimC, nimD, nimE* and *nimF* are carried on plasmids or located in the chromosomes (Schapiro *et al.*, 2004; Gal and Brazier, 2004; Diniz *et al.*, 2004). Of the six *nim* genes, *nimA, nimC* and *nimD,* were located on a low copy number mobilization plasmids, pIP417 (7.7 kB), pIP419 (10 kB) and pIP421 (4.3 kB), respectively from *B. fragilis* (Haggoud *et al.*, 1995; Urban *et al.*, 2002; Trinh *et al.*, 1995). Despite the transferable nature of the plasmids, epidemiologists suggested that 75 % of resistant isolates contain the chromosomally encoded resistance mechanisms (Urban *et al.*, 2002; Reysset *et al.*, 1992; Trinh and Reysset, 1996).

Studies conducted by Gal and Brazier (2004) showed that out of 206 *Bacteroides* spp. clinical isolates tested, 50 had *nim* genes, of which *nimA* was the most prevalent, followed by *nimB* and *nimE.* The *nimC, nimD*, and the putative novel gene, *nimG*, were the least prevalent. The *nim* genes are not always active, and expression has been shown to be promoted by insertion sequence elements (IS*1168*, IS*1169*, IS*1170*) (Haggoud *et al.,* 1995; Gal and Brazier, 2004). Homologues of *nim* have also been found in other bacteria, including *Deinococcus radiodurans, Helicobacter hepaticus, Clostridium* spp. as well as archaea *Methanosarcina* (Leiros *et al.,* 2004). In a recent study, Leiros *et al.* (2004) presented the first available structure of a *nim* family protein, drNimA from *D. radiodurans*. Based on the native structure of the protein, they reported that the Nim protein does function as a reductase and leads to non-toxic intermediates of metronidazole being formed.

1.6.4 DNA damage and repair

The primary DNA damage is caused by short-lived metronidazole intermediates which destabilize and unwind the helix causing strand breakage. Mendez *et al.* (2001) used a single cell gel electrophoresis (SCGE) assay to demonstrate DNA damage by metronidazole in lymphocytes obtained from individuals on a 10 day treatment of metronidazole, and reported that there was an increase in the frequency of chromosomal aberrations. Reitz *et al.* (1991) analyzed DNA unwinding to measure DNA damage, and showed that metronidazole produced DNA single strand breaks in human lymphocytes from patients treated with 4800 mg of the drug. This damage was repaired seven days after ending metronidazole therapy. Diniz *et al.* (2000) also reported DNA damage in *B. fragilis* which had been treated with metronidazole. Plant and Edwards (1976) showed that metronidazole caused *in vivo* degradation of DNA in *Clostridium bifermentas* through inhibiting DNA synthesis. *In vitro* experiments carried out by Knight *et al.* (1979) showed that single and double stranded DNA breaks were caused by the

reduced drug and Sisson *et al.* (2000) reported high level of DNA breakage and fragmentation in *H. pylori.* Metronidazole preferentially causes DNA damage at high A+T % rather than low A+T % regions (Edward, 1993). A mixture of thymine and thymidine phosphates are released from DNA by 2- and 5- nitroimidazoles, making the "T" site the more probable targets (Edward, 1993).

The effectiveness of metronidazole as an antibacterial agent can be influenced by the efficiency of DNA the repair systems which respond to the damage. Therefore, some of the possible mechanisms of DNA repair and their link to metronidazole resistance or sensitivity will be reviewed here. The well-studied *E. coli* repair systems that have been linked to dealing with DNA damage by metronidazole will be discussed and similar systems in *Bacteroides* spp. and *H. pylori* will also be reviewed.

In nucleotide excision repair (NER), the regions of DNA with damaged nucleotides are excised and gaps are filled using the complementary strands of DNA as template. NER requires the UvrABCD protein complex which is comprised of excinucleases and helicase II gene products (Friedberg *et al.*, 1995). *E. coli* lacking NER was found to be more sensitive to metronidazole (Jackson *et al.*, 1984; Yeung *et al.*, 1984). Not much is known about the NER systems in *Bacteroides* spp., however, examination of the published genomes of *B. fragilis* YCH46 (Kuwahara *et al.*, 2004) and *B. thetaiotaomicron* VPI-5482 (Xu *et al.*, 2003) confirm that putative DNA NER genes are present. Earlier work by Abratt *et al.* (1986, 1990) found that *B. fragilis* had a thymine dimer excision repair system operating under anaerobic and aerobic conditions. Additional analysis suggested that the excision repair system in *B. fragilis* also recognized Mitomycin C damage, in a linked but not identical pathway (Abratt *et al.*, 1985). The genes involved in this system have not yet been functionally identified.

The plasmid borne *metA* gene of *B. fragilis* was found to confer resistance to metronidazole (Dachs *et al.*, 1995). The MetA protein reduced DNA breakage caused by metronidazole, but it did not inactivate metronidazole. This suggested that its mode of action could be by repairing the damage.

One of the major ways in which DNA strand breaks are repaired in *E. coli* is via recombination repair. This involves the activities of the RecA protein and results in the regulation of the SOS response to DNA damage. The SOS system is under the regulation of two key proteins, LexA and RecA and is induced by the presence of single stranded DNA (ss-DNA) (Ivančić-Baće *et al.*, 2006; Ivančić-Baće *et al.*, 2005). LexA acts as a repressor of the genes of the SOS system and binds to their promoter regions. RecA acts as a depressor of the SOS, inducing it via the auto-cleavage of the LexA repressor (Kuzminov, 1999). The SOS system is induced when replication is inhibited. The accumulation of ss-DNA allows the RecA protein to form filaments on the ss-DNA in the presence of ATP, thus activating RecA (RecA-ss-DNA).

The role of RecA-ss-DNA is to identify DNA duplex homologs and facilitate strand recombination. While the mechanism of identifying the DNA duplex homolog is unknown, it has been suggested that RecA may have two binding sites allowing for both the ss-DNA as well as the duplex homolog to bind to it (Cox, 1995). Upon identification of the DNA duplex homologs, the ss-DNA forms hydrogen bonds with the complementary strand of the DNA duplex. RecBCD is responsible for the repair of double stranded DNA breaks via promoting RecA binding to the SSB-covered ssDNA. RecA is then involved homologous recombination. RecFOR complex replaces the SSB that is complexed with the ss-DNA and the RecA protein and processes the single strand gaps that occur in the daughter strands after reinitiation of DNA replication (Ivančić-Baće *et al.*, 2006).

E. coli RecA mutants were 30-fold less resistant to metronidazole showing that RecA protein is an important component of the repair of DNA damage caused by metronidazole (Dachs *et al.*, 1995). It was reported that mutations in the *recA* gene of *H. pylori* enhanced the sensitivity of that strain to metronidazole, and were also found to be 10 fold more sensitive than the parent strain (Thompson and Blaser, 1995). Chang *et al.*, (1997) have also reported that certain other mutations in recA were associated with metronidazole resistance, possibly through enhanced expression of the protein. Both *B. fragilis* and *B. thetaiotaomicron* have a RecA protein (Goodman *et al.*, 1987; Cooper *et al.*, 1997) which may be involved in DNA repair since *B. thetaiotaomicron recA* mutants were reportedly more sensitive to DNA damaging agents, including metronidazole (Cooper *et al.*, 1997). Genome analysis can assist in identifying other possible genes that are linked to DNA repair systems.

1.7 Genetic tools for analyzing genes involved in metronidazole resistance in *Bacteroides*

Genetic tools for the analysis of *Bacteroides* spp. have, in the past, been limited. However, in recent years, progress in the field of *Bacteroides* genetics has been significant and the application of these tools has allowed for further analysis of *Bacteroides* genes (Salyers *et al.*, 2000). For example, conjugation of mobilizing plasmids into *Bacteroides* spp. from *E. coli* relies on plasmids such as RK23 and R751 from the broad host range IncP plasmids, serving to provide conjugation functions in a range of *E. coli-Bacteroides* shuttle vectors (Guiney *et al.*, 1984; Salyers *et al.*, 2000). These shuttle vectors carry *E. coli* and *Bacteroides* spp. resistance marker genes for each host and the transfer of origin (*oriT*) for *E. coli.*

Additional genetic tools, such as transposons, assist in creating random single gene disruptions. These genetic elements also carry drug resistance genes that are unrelated to the transposition functions. Transposons are flanked by copies of insertion sequence (IS) elements in a direct or

indirect repeat orientation and insert at random into the bacterial chromosome. Once the mutant of interest is identified, plasmid rescue can be used to determine the transposon insertion site, and the transposon itself can be used as a marker for cloning and studying the specific gene that has been interrupted. In addition, hybridization methods can be used to check if the mutant has more than one insertion within the chromosome (Salyers *et al.*, 2000).

There are three well characterized compound transposons that are involved in antibiotic resistance (Smith *et al.*, 1992). These are Tn*4551* and Tn*4351* that are flanked by the IS element, IS*4351*, and Tn*4400* that is flanked by IS*4400*. All of these mediate MLS antibiotic resistance because of expression of *ermF* (Smith *et al.*, 1992). Tn*4351* is 5092 bp and carries an *ermF* gene that is expressed in *Bacteroides* spp. It also carried a *tetX* gene, that does not confer resistance on *Bacteroides* spp. but it does so in *E. coli* when grown aerobically (Salyers *et al.*, 2000). In a recent study, the 4.6 kb Tn*5520* isolated from *B. fragilis* was analyzed (Vedantam *et al.*, 2006). It was found that Tn*5520* had only two genes, one that encoded an integrase, BipH and the other encoded a single Mob protein, BmpH. The *oriT* region was a 17 bp sequence upstream of the *bmpH* gene.

Other genetic tools allow for specific genes to be disrupted or deleted. For example, gene specific mutagenesis has been proven to be a successful tool in disrupting specific genes in *Bacteroides* spp. (Parker and Smith, 2004; Spence *et al.*, 2006). Alternatively, an in-frame deletion of a specific gene has also been proven to be a valuable genetic tool for mutational analysis and to provide insight into how specific genes function (Baughn and Malamy, 2002). The use of genetic tools and methodologies in *Bacteroides* spp. assists in identifying genes may be responsible in drug resistance mechanisms.

1.8 Dissertation Aims

Very little is known about metronidazole resistance in *Bacteroides* spp. or the mechanisms involved in this. In previous work by Casanueva (2004), *B. thetaiotaomicron* mutants were created by random transposon mutagenesis using the method of Tang and Malamy (2000). Mutant banks were screened for metronidazole resistant strains and a mutant was isolated that displayed resistance to 8 μg/ml metronidazole in comparison to the parent strain that was only resistant to 0.8 μg/ml of metronidazole.

The aims of this project were, therefore, to characterize the transposon mutant, by identifying the location of the transposon insertion and confirm that the interrupted locus was responsible for the metronidazole resistance phenotype. Further, the thesis aimed to study the mechanism of metronidazole resistance in the mutant using physiological, genetic and biochemical analysis.

CHAPTER 2

Isolation and molecular characterization of a *B. thetaiotaomicron* metronidazole resistant mutant

2.1 ABSTRACT

Bacteroides thetaiotaomicron VPI-5482 metronidazole resistant mutants were created using a modified transposon mutagenesis method outlined by Tang and Malamy (2000). Mutant libraries were screened on solid medium for metronidazole resistance. One of the mutants isolated, named *B. thetaiotaomicron* Tn Met^R, was resistant to at least 8 µg/ml metronidazole in comparison to *B. thetaiotaomicron* VPI-5482, the parent strain (MIC= 0.8 µg/ml) and was selected for further study. This chapter addresses the analysis of the region around the transposon insertion through plasmid rescue and DNA nucleotide sequencing. To further confirm the site of insertion, PCR of the genomic DNA from the mutant was carried out using primers derived from the insertion sequences IS*4400*R and IS*4400*L, in combination with primers designed from the genes flanking the sites of the transposon insertion. These fragments were amplified and sequenced. The exact site of the transposon insertion was located in an intergenic region between two genes of the putative rhamnose gene cluster, provisionally annotated as BT3767 and BT3768 in the *B. thetaiotaomicron* VPI-5482 (ATCC 29148) published genome sequence. Southern blot hybridization confirmed that a single copy of the transposon had inserted into the chromosome. Physiological analysis showed that *B. thetaiotaomicron* Tn Met^R grew slowly in comparison to *B. thetaiotaomicron* VPI-5482 in complete medium. Metronidazole survival studies in complete broth medium confirmed that the *B. thetaiotaomicron* Tn Met^R mutant displayed significant resistance to metronidazole compared to the *B. thetaiotaomicron* VPI-5482 parent strain.

2.2 INTRODUCTION

The aim of this study was to determine possible metronidazole resistance mechanisms in *B. thetaiotaomicron* using random transposon mutagenesis, an approach that has proven to be a successful and invaluable tool for genetic studies (Chen *et al.*, 2000; Tang and Malamy, 2000). Transposons are genetic elements containing additional genes unrelated to the transposon function. They encode drug resistance genes that are flanked by copies of insertion sequence elements (IS) in direct or indirect repeats. The construction of a *B. thetaiotaomicron* VPI-5482 transposon mutant bank was carried out using a modified transposon mutagenesis method outlined by Tang and Malamy (2000) as described by Casanueva (2004). The transposon library was constructed using an *E. coli – Bacteroides* conjugation system whereby *E. coli* HB101 cells carrying the mobilizing IncP plasmid RK23 (Guiney *et al.*, 1984) and the transposon delivery vector pYT646B (Fig 2.1) (Chen *et al.*, 2000) were used as donor cells and mated with recipient *B. thetaiotaomicron* cells.

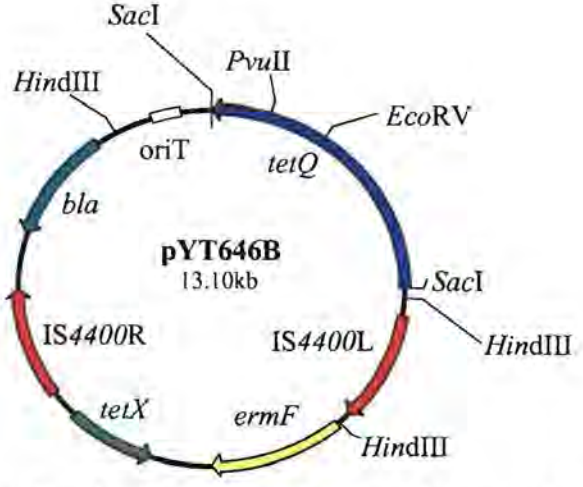


Figure 2.1: Partial restriction map of pYT646B (Chen *et al.*, 2000).

Plasmid pYT646B (Fig 2.1) contains the Tn*4400* transposon element, flanked by insertional sequence elements, IS*4400L* and IS*4400R*, the *bla* gene and *oriT* region functional in *E. coli*. The tetracycline genes, *tetX* and *tetQ* on pYT646B are expressed aerobically in *E. coli*, and

anaerobically in *B. thetaiotaomicron*, respectively, and the erythromycin gene, *ermF*, is expressed anaerobically in *B. thetaiotaomicron*.

Plasmid, pYT646B is capable of facilitating three possible transposon insertion events in *Bacteroides* species. Firstly, direct transposition of Tn*4400'* could occur, conferring erythromycin resistance. Alternatively, an inverse transposition event could occur if the entire IS*4400*R, IS*4400*L, as well as the *tetQ* gene, integrated into the chromosome conferring tetracycline resistance (Fig. 2.2). In the third event, erythromycin and tetracycline resistance could occur if the whole plasmid integrated into the chromosome, forming a co-integrate (Tang and Malamy, 2000).

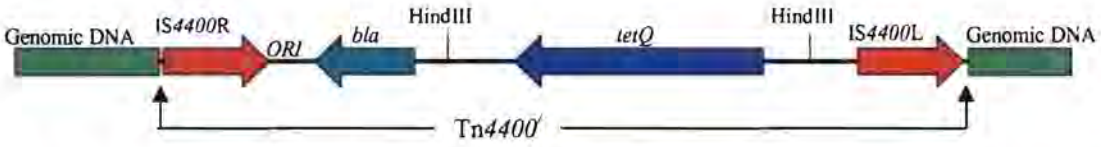


Figure 2.2: Inverse transposon insertion into genomic DNA of *Bacteroides* species. Transposon insertion shown as indicated with IS elements (red arrows), ORI region, *bla* gene (light blue arrow), *tetQ* gene (dark blue arrow) within genomic DNA (green bands flanking the transposon).

Of the *B. thetaiotaomicron* transposon mutants generated in the library (Casanueva, 2004), one displayed resistance to at least 8 μg/ml of metronidazole as compared to the parent strain (0.8 μg/ml). The mutant, named *B. thetaiotaomicron* Tn Met^R, was selected for further physiological, biochemical and genetic analysis. This chapter describes the growth of the mutant in complete medium, confirms its metronidazole resistance phenotype, and evaluates whether a single transposon insertion has occurred within the genomic DNA. In addition, it identifies the genetic locus of the transposon insertion by plasmid rescue and analyses it using DNA sequencing and bioinformatics.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial Strains, Plasmids and Growth Conditions

B. thetaiotaomicron VPI-5482 is a plasmid-free strain and has been described previously (ATCC 29148). *B. thetaiotaomicron* was grown in Difco brain heart infusion, supplemented with haemin, menadione and cysteine (BHIS) broth or agar (1.5% w/v) (Holdeman and Moore, 1972). All *B. thetaiotaomicron* cultures were grown at 37°C, in an anaerobic chamber (Model 1024, Forma Scientific Inc., Marietta, Ohio) containing an atmosphere of oxygen free N_2, CO_2 and H_2 (85:10:5 by volume). *E. coli* DH5α (Rasmussen *et al.*, 1994) strains were grown aerobically on Luria-Bertani (LB) broth or agar (1.5% w/v) at 37°C. The transposon delivery vector pYT646B (Tang and Malamy, 2000) was used as a positive control for Southern hybridization. The antibiotics gentamicin (200 µg/ml), ampicillin (100 µg/ml), and tetracycline (2 µg/ml) were added to BHIS and/or LB whenever necessary.

2.3.2 General Recombinant DNA Procedures

B. thetaiotaomicron genomic DNA was prepared according to the method outlined by Wehnert *et al.* (1992). *E. coli* plasmid DNA was prepared using the QIAGEN® Plasmid Midi Kit (25) (QIAGEN). Restriction enzyme digestions were carried out according to the manufacturer's instructions.

2.3.3 Growth studies in complete medium

B. thetaiotaomicron VPI-5482 and the *B. thetaiotaomicron* Tn Met^R mutant were grown separately under anaerobic conditions at 37°C, in 10 ml volume of BHIS broth for 16 h. Cultures were then diluted (1:50) in 100 ml of BHIS broth and the further growth of these

cultures was monitored at hourly intervals by recording optical density (OD_{600}) using a Beckman DU®530 Lifescience UV/Vis Spectrophotometer.

2.3.4 Metronidazole survival studies in complete medium

Mid-logarithmic phase cultures of *B. thetaiotaomicron* VPI-5482 and *B. thetaiotaomicron* Tn Met^R were exposed separately to 20 μg/ml of metronidazole in BHIS broth. Cultures were incubated anaerobically at 37°C. Samples were removed at different time intervals, diluted appropriately in BHIS broth and plated onto BHIS agar to determine the surviving fraction from viable counts.

2.3.5 Identification of the transposon insertion site in the *B. thetaiotaomicron* Tn Met^R mutant

2.3.5.1 Plasmid Rescue

Genomic DNA from the mutant was digested using the restriction enzyme *Hin*dIII. The fragment adjacent to the transposon was isolated by plasmid rescue (Tang and Malamy, 2000) and the junction region on the resulting ampicillin resistant plasmid, containing the *bla* gene was sequenced using primer L58, derived from IS*4400*R (5'-CAA TAA TGG ACC TCG TAA AAG-3') (Chen *et al.*, 2000) (Fig 2.3.).

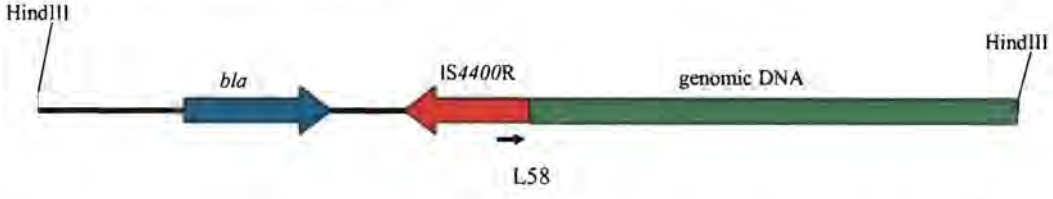


Figure 2.3: Plasmid rescue of *B. thetaiotaomicron* Tn Met^R genomic DNA flanking the transposon insertion region. The green band represents *B. thetaiotaomicron* Tn Met^R chromosomal DNA, the black line represents pYT646B plasmid DNA where the blue arrow represents the *bla* gene, and the red arrow represents the IS element (IS*4400*R). The black arrow represents the L58 primer. (Derived from Chen *et al.*, 2000).

2.3.5.2 PCR of the transposon insertion site in *B. thetaiotaomicron* Tn Met^R genomic DNA

A forward primer, TnOF (5′-AAT CGT ACA TTC CAT GGC-3′) was designed to anneal within BT3767 approximately 400 bp upstream of the putative site of transposon insertion. The reverse primer L58 (See section 2.3.5.1) was used with the forward primer to amplify the region of interest. In addition, a reverse primer, TnRR (5′-ACC GAA TTT GCG GTC TCC AT-3′) was designed to anneal within BT3768 approximately 200 bp downstream of the site of transposon insertion and a forward primer, named primer *c* (5′-TAG CAA ACT TTATCC ATT CAG-3′) (Chen *et al.*, 2000) annealing within the IS*4400*L region, was used to amplify the region downstream of the transposon junction. The PCR fragments obtained were purified using the High Pure PCR Clean up Kit (Roche) and submitted for nucleotide sequencing.

2.3.6 Nucleotide Sequencing

Sequencing reactions were performed by using the DYEnamic ET Dye terminator cycle sequencing kit for MegaBACE (Molecular Dynamics) based on dideoxynucleotide chain termination chemistry (Sanger *et al.*, 1977). Reactions were performed according to manufactures' instructions and sequenced on a GeneAmp PCR system 9700 (Perkin Elmer, Applied Biosystems) using appropriate primers at concentrations of 5 μM and primer specific hybridization temperatures. Sequencing reaction products were analysed using an automated capillary DNA sequencing system, the MegaBASE 500 sequencer v2.4 (Molecular Dynamic, Amersham Pharmacia Biotech, Amersham Bioscience). Sequences were analysed using Lynnon Biosoft DNAMAN software v 4.13 and the NCBI BLAST program (Altschul *et al.*, 1997).

2.3.7 Southern Hybridization

Genomic DNA (20 μg) of both the *B. thetaiotaomicron* VPI-5482 and *B. thetaiotaomicron* Tn Met^R mutant, as well as 20 ng of the 13.1 kb transposon delivery vector, pYT646B, were each digested to completion with restriction enzyme *Pvu*II. Digested genomic (20 μg) and plasmid DNA (20 ng) were separated using 0.8 % agarose gel electrophoresis in TRIS-Acetate-EDTA buffer and transferred via the capillary alkali transfer procedure to a Hybond-N^+ nylon membrane (Sambrook *et al.*, 1989). The DNA probe used for hybridization was a 0.9 kb *Sac*I-*Eco*RV internal fragment of the pYT646B *tetQ* gene extracted from a 0.8 % agarose gel using the High Pure PCR Product Purification Kit (Roche). The purified fragment was non-radioactively labelled using the random primed Digoxygenin-11-dUTP (DIG) labelling and detection method (Roche). Hybridization and detection procedures were performed according to the manufacturers' instructions. Chemiluminscent signals were detected using CDP® Star (Roche).

2.4 RESULTS AND DISCUSSION

2.4.1 Growth of the *B. thetaiotaomicron* Tn Met^R mutant in complete medium

The growth of the *B. thetaiotaomicron* Tn Met^R mutant, relative to that of the parent strain was examined in BHIS broth (Fig. 2.4). *B. thetaiotaomicron* VPI-5482 grew well in comparison to the slower growing *B. thetaiotaomicron* Tn Met^R mutant. After 8 h of growth, *B. thetaiotaomicron* Tn Met^R had only reached an OD of ~0.7 whereas *B. thetaiotaomicron* VPI-5482 had reached an OD of ~1.6.

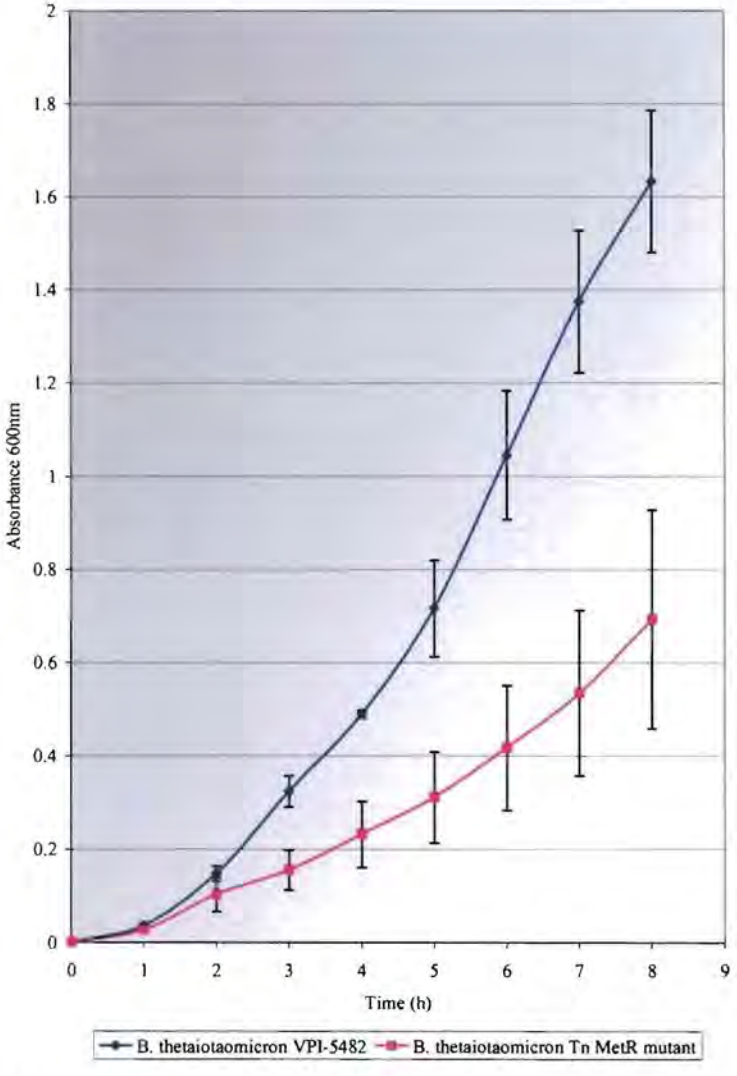


Figure 2.4: Growth of *B. thetaiotaomicron* VPI-5482 and *B. thetaiotaomicron* Tn Met^R in BHIS broth. Data points represent the mean values of 3 experiments. Standard error of the experiments is shown as error bars.

Slow growth of metronidazole resistant bacterial strains has also been observed in other published studies. For example, *Clostridium perfringens* metronidazole resistant mutant strains grew more slowly than the parent strain from which they were isolated (Sindar *et al.*, 1982). An explanation offered for slow growth in these mutant strains was that it might be due to improper catabolic reactions such as the phosphoroclastic reaction (Chapter 1), which would reduce energy production and slow down growth. Britz and Wilkinson (1979) also reported slow growing *B. fragilis* mutant strains displaying metronidazole resistance in complete medium, and also reported less cell mass in defined medium where glucose was the sole carbon source.

2.4.2 Metronidazole survival studies in complete medium

B. thetaiotaomicron Tn Met^R was originally isolated from BHIS agar containing 8 μg/ml of metronidazole. In order to verify that this mutant was resistant to metronidazole, a survival study was performed in which the actively growing mutant, capable of reducing metronidazole, was exposed to 20 μg/ml metronidazole and sampled at different time intervals to monitor viability as the log surviving fraction (Fig. 2.5).

Exposure of *B. thetaiotaomicron* Tn Met^R to metronidazole confirmed that this strain was more resistant to metronidazole than the *B. thetaiotaomicron* VPI-5482, parent strain. The log surviving fraction of *B. thetaiotaomicron* VPI-5482 decreased rapidly when the cells were exposed to 20 μg/ml of metronidazole. The ability of the *B. thetaiotaomicron* Tn Met^R mutant to survive may be due to the slower growth resulting in slower activation of metronidazole and hence a slower DNA damage process.

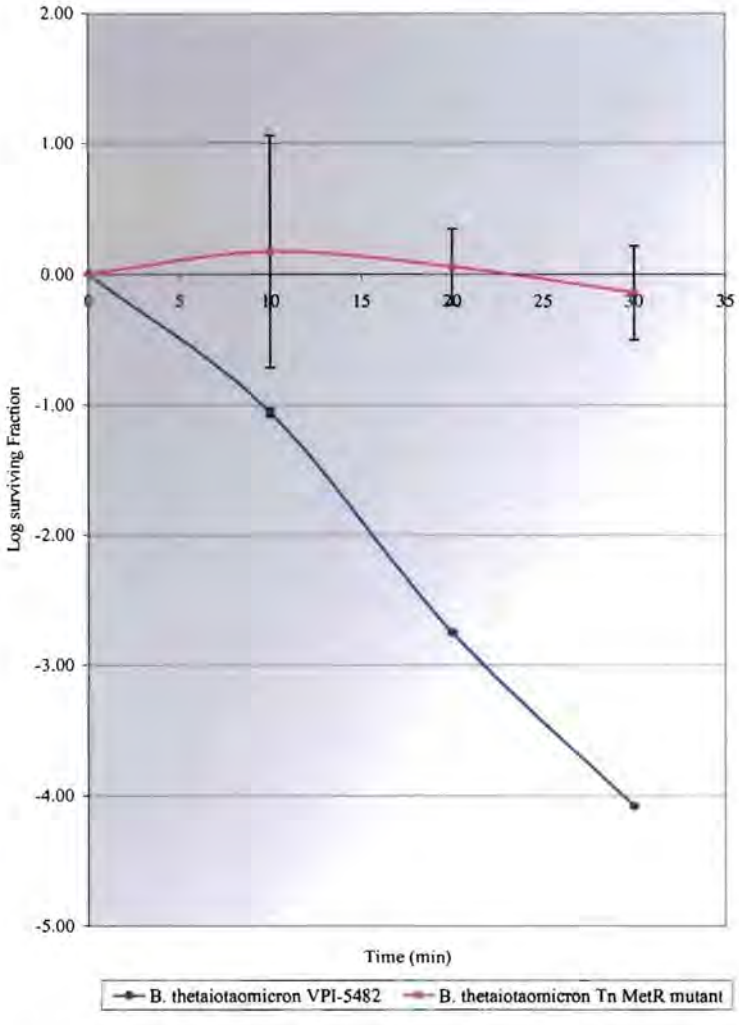


Figure 2.5: Survival studies of *B. thetaiotaomicron* VPI-5482 and *B. thetaiotaomicron* Tn Met^R mutant exposed to 20 µg/ml of metronidazole in complete medium. Error bars represent the standard deviation for three replicate experiments.

2.4.3 PCR Analysis of transposon insertion site

Plasmid rescue resulted in a single 4.5 kb plasmid being isolated. This, together with the fact that the mutant showed tetracycline resistance and erythromycin sensitivity, indicated that a single inverse insertion of *Tn4400* had occurred within the *B. thetaiotaomicron* Tn Met^R mutant strain, and that no co-integrate formation or direct transposition (discussed in section 2.2) had occurred. The nucleotide sequence of the junction region on the rescued plasmid, obtained using the L58 primer, was compared with the *B. thetaiotaomicron* VPI 5482 genome (ATCC 29148), using the NCBI BLAST program. Results showed sequence alignment to the

B. thetaiotaomicron VPI-5482 locus BT3767, indicating that this gene lay next to the transposon insertion site. In order to confirm that the rescued plasmid was a correct reflection of the insertion site in the genome, PCR of the *B. thetaiotaomicron* Tn Met^R mutant DNA region flanking the putative insertion sites was conducted using the genomic DNA and primers that were designed from within two locus tags, BT3767 (primer TnOF) and BT3768 (primer TnRR). Primer TnOF was used in combination with the L58 primer from within IS*4400*R to amplify one side of the junction region between the genomic DNA and transposon insertion. The downstream junction region was amplified using primer TnRR and a primer, named primer *c* within IS*4400*L (Fig 2.6).

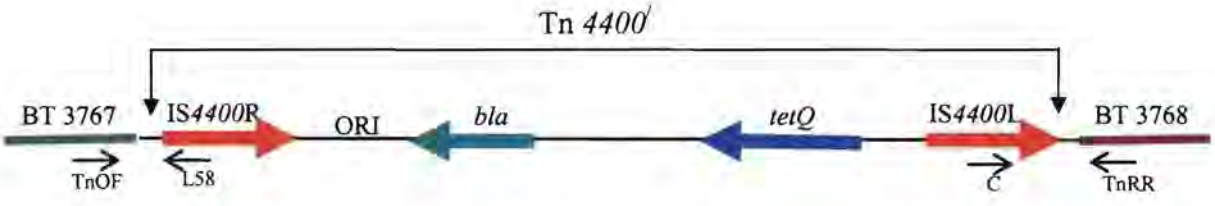


Figure 2.6: Schematic representation (not to scale) of the transposon insertion in an intergenic region between BT3767 and BT3768. Transposon (Tn*4400'*) inserted between *B. thetaiotaomicron* VPI-5482 locus tag BT3767 (green band) and BT3768 (purple band). Black arrows represent the primers used to amplify the junction regions.

Visualization of the PCR products on 0.8 % agarose gel resulted in a 400 bp and a 200 bp band for the upstream and downstream regions respectively (data not shown). Sequence analysis of the PCR products revealed that the transposon insertion had occurred within an intergenic region 12 bp downstream of BT3767 and 131 bp upstream of BT3786 (Fig 2.6).

2.4.4 Analysis of Southern Hybridization

The *B. thetaiotaomicron* Tn Met^R mutant was constructed using a transposon insertion system that was reported to cause predominantly single insertion events within the chromosome

although occasionally, multiple insertions could occur (Tang and Malamy, 2000). In order to verify the single insertion, Southern blot analysis was performed by digesting *B. thetaiotaomicron* VPI-5482, *B. thetaiotaomicron* Tn Met^R, as well as the positive control plasmid pYT646B with *Pvu*II and using the *Sac*I-*Eco*RV internal fragment of the *tetQ* gene as a probe. Restriction enzyme *Pvu*II was selected because pYT646B only has a single *Pvu*II site within this fragment of the *tetQ* gene, which would cause the plasmid to linearise upon digestion with this restriction enzyme. Insertion of the transposon into the genomic DNA would result in two hybridization bands per copy of the *tetQ* gene inserted.

As seen in Fig. 2.7, Lane 1 of the autoradiograph displayed a single band of the linearised plasmid. *B. thetaiotaomicron* VPI-5482 genomic DNA (Lane 2) showed no signal since there was no transposon to hybridize to the *tetQ* probe. *B. thetaiotaomicron* Tn Met^R genomic DNA (Lane 3) resulted in a doublet made up of two bands of very similar sizes, identifying a single inverse *Tn4400* insertion within the chromosome, with only a single copy of the *tetQ* gene being present.

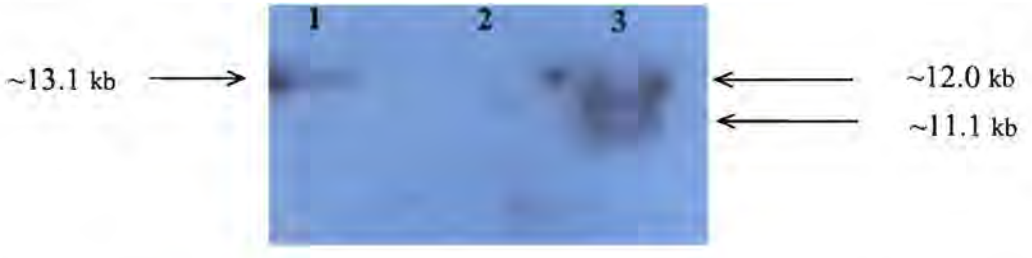


Figure 2.7: Autoradiograph of Southern hybridization to confirm a transposon insertion in *B. thetaiotaomicron* Tn Met^R mutant. Lane 1, pYT646B, Lane 2, *B. thetaiotaomicron* VPI-5482 genomic DNA, and Lane 3, *B. thetaiotaomicron* Tn Met^R genomic DNA. All DNA was digested with *Pvu*II and probed with a 0.9 kb *Sac*I-*Eco*RV internal fragment of the pYT646B *tetQ* gene.

In order to further explain the doublet observed in Lane 3, the published sequence of *B. thetaiotaomicron* was used to identify the *Pvu*II restriction sites in the regions flanking the

transposon insertion site (Fig. 2.8). *Tn4400* mutant containing a single insertion between BT3767 and BT3768 in the chromosome should result in two bands (12 kb and 11.1 kb) when digested with *Pvu*II, as was seen in the autoradiograph.

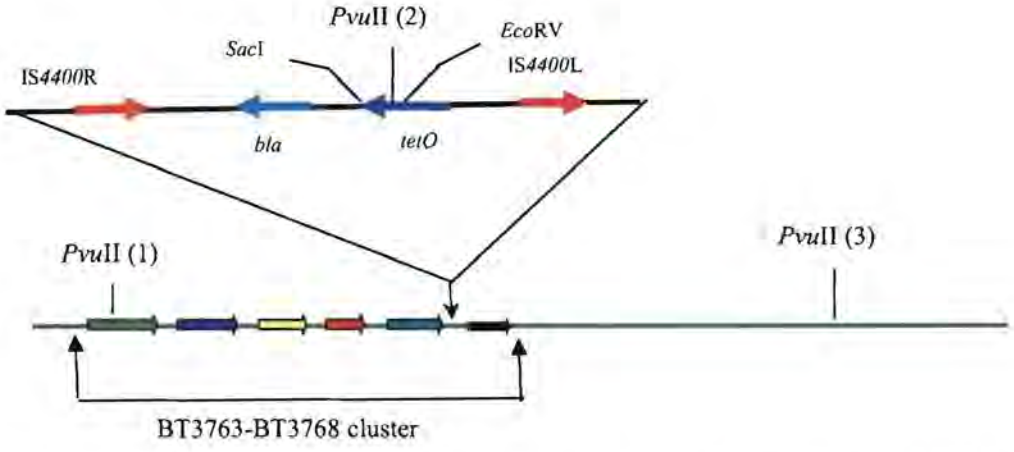


Figure 2.8: Schematic representation (not to scale) of the three *Pvu*II sites. *Pvu*II sites 1 and 2 and 3, located within BT3763 (green arrow), the *tetQ* gene (dark blue arrow) of the transposon, and within BT3774 (downstream on genomic DNA) of *B. thetaiotaomicron* VPI-5482, respectively. *Sac*I and *Eco*RV fragment of the *tetQ* gene was used as the DNA probe.

2.4.5 Bioinformatic analysis of the transposon insertion site and flanking regions

2.4.5.1 Analysis of the transposon insertion site

DNA sequence analysis of the transposon insertion site in the *B. thetaiotaomicron* VPI-5482 genome (ATCC 29148) showed that it had occurred in an intergenic region, 12 bp downstream of the BT3767 and 131 bps upstream of BT3768. These genes are annotated as forming part of a cluster having amino acid sequence similarity to the proteins of the *E. coli* rhamnose catabolic pathway (Table 2.1). BT3767 codes for a protein, predicted to have 384 amino acid residues, which is thought to play a role in converting L-lactaldehyde to L-1,2-propandiol, whereas BT3768 (299 amino acids) displays amino acid identity to a group of positive transcriptional regulators from the AraC/XylS family. It was, therefore, of interest to analyze whether these genes, and those surrounding them, formed part of a putative rhamnose catabolic gene cluster.

B. thetaiotamicron VPI-5482 Locus Tags	Deduced amino acid length	*B. thetaiotaomicron* gene name assigned in this study	Predicted protein function	% Identity to equivalent proteins in *E. coli* K12 & gene names of L-rhamnose pathway (Irino *et al.*, 1986) Accession N° AAA67618	
BT 3763	485	*rhaK*	rhamnulose/fuculose kinase	43%	*rhaB*
BT 3764	418	*rhaI*	L-rhamnose isomerase	54%	*rhaA*
BT 3765	339	*rhaP*	L-rhamnose/ H-symporter	32%	*rhaT*
BT 3766	269	*rhaA*	rhamnulose-1-phosphate aldolase	31%	*rhaD*
BT 3767	384	*fucO*	lactaldehyde reductase	44%	*fucO*
BT 3768	299	*rhaR*	transcriptional regulator	28%	*rhaR*

Table 2.1: Sequence identity of *B. thetaiotaomicron* VPI-5482 predicted gene products to proteins from *E. coli* K12.

2.4.5.2 Rhamnose catabolism

The putative rhamnose gene cluster in *B. thetaiotaomicron* VPI-5482 was found to consist of six genes, all transcribed in the same orientation (Fig 2.9A, Table 2.1). The gene nomenclature in this study for the *B. thetaiotaomicron* putative rhamnose pathway has been assigned according to the predicted function of each gene as is the convention in the *B. thetaiotaomicron* fucose gene cluster (Hooper *et al.*, 1999). For example, the gene coding for L-rhamnose isomerase in *B. thetaiotaomicron* has been assigned as *rhaI*. This differs from the nomenclatures of *E. coli* homologs (Table 2.1, Fig 2.9B).

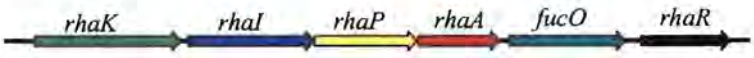
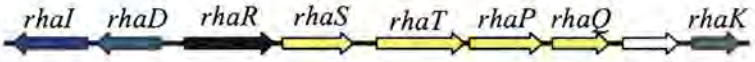
Bacterium	Gene arrangements of the Rhamnose pathway	Source
A) *B. thetaiotaomicron* VPI-5482.		This study
B) *E. coli* K-12		Power 1966
C) *S. Typhimurium* LT2		This study; Nishitani and Wilcox, 1991.
D) *Rhizobium leguminosarum*		Richardson *et al.*, 2004

Figure 2.9: Rhamnose gene cluster arrangements in *B. thetaiotaomicron* VPI-5482, *E. coli* K-12, *S. typhimurium* LT2 and *Rhizobium leguminosarum*. Red, dark blue, yellow, green, and light blue, arrows represent the aldolase, isomerase, permease/transport, kinase, and oxidoreductase genes, respectively. The black arrows represent regulators and the white arrows represent hypothetical proteins.

L-rhamnose is a methylpentose sugar that is metabolised and used as a carbon and energy source by organisms such as *E. coli*, *Salmonella* spp. and by the soil bacterium *R. leguminosarum* (Moralejo *et al.*, 1993; Chen *et al.*, 1987a; Richardson *et al.*, 2004). In *E. coli*, L-rhamnose is metabolised by an inducible gene cluster (Fig 2.9B) that is mediated by a kinase (*rhaB*), an isomerase (*rhaA*), a permease (*rhaT*), and an aldolase *(rhaD)* and the pathway consists of two diverging gene clusters *rhaBAD,* and *rhaSRT* (Moralejo *et al.*, 1993; Power, 1966). Similarly, in *S. typhimurium* (Fig 2.9C), the genes are arranged in the same orientation as the *E. coli* gene cluster. However, in both *B. thetaiotaomicron* (Fig 2.9A) and *R. leguminosarum* (Fig 2.9D), the genes of the rhamnose cluster are in a different orientation to that observed in *E. coli*. In *B. thetaiotaomicron*, the genes appear to be expressed in a single

orientation. However, in *R. leguminosarum*, the pathway consists of two diverging gene clusters, *rhaDI* and *rhaRSTPQUK*. There is no published information on rhamnose catabolism in *Bacteroides* spp. Therefore, other well-studied models, such as *E. coli* and *R. leguminosarum*, are reviewed here to better understand the events of rhamnose catabolism.

In *E. coli*, extensive studies have been carried out to elucidate the function of each of the genes in the rhamnose pathway as well as determine the catabolic end products (Fig 2.10). Here, the sugar is first brought into the cell by the permease (RhaT). It is then isomerized by L-rhamnose isomerase (RhaI), and subsequently phosphorylated by the kinase (RhaK). Finally the aldolase (RhaD) cleaves L-rhamnulose-1-phosphate into L-lactaldehyde and dihydroxyacetone phosphate (DHAP) as depicted in Fig 2.10 (Chen *et al.*, 1987a). The fate of L-lactaldehyde in *E. coli* depends on whether growth conditions are aerobic or anaerobic. Under anaerobic conditions, L-lactaldehyde, the common intermediate in both the rhamnose and fucose metabolism, gets reduced to L-1,2-propanediol via the NAD-linked propanediol oxidoreductase, FucO. This allows the regeneration of oxidized coenzyme, and allows the fermentation of rhamnose as well as fucose, a similar pentose sugar, to proceed (Boronat and Aguilar, 1981; Baldoma and Aguilar, 1988).

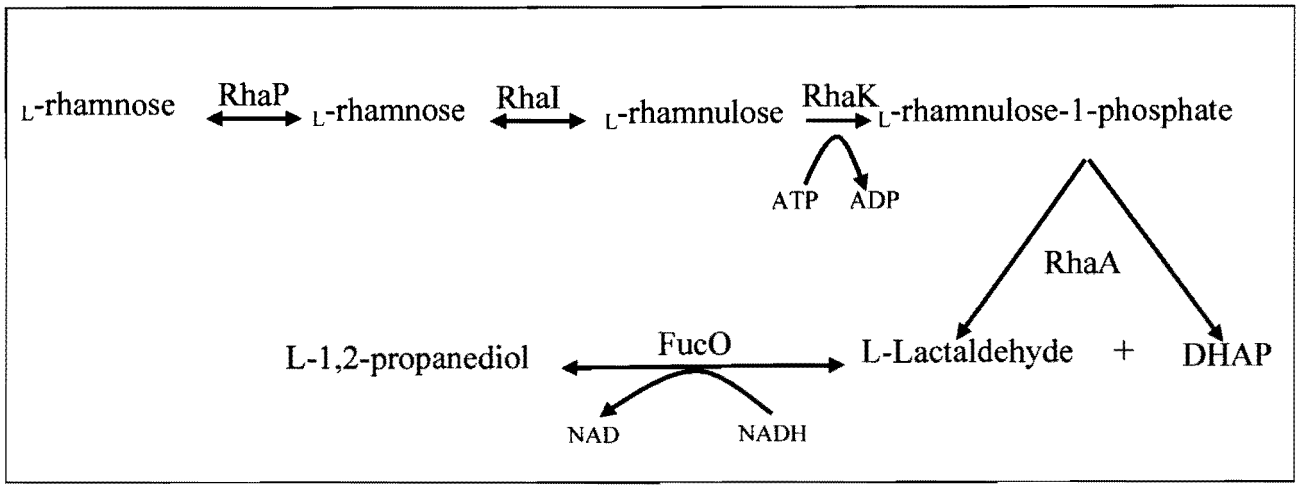


Figure 2.10: Schematic representation of L-rhamnose anaerobic metabolism in *E. coli* (Derived from Chen *et al.*, 1987a).

Under aerobic conditions, an inactive form of the propanediol oxidoreductase is produced. Instead, the L-lactaldehyde is oxidized to L-lactate that is, in turn, oxidized to pyruvate via a NAD-dependent lactaldehyde dehydrogenase and a flavin-linked lactate dehydrogenase. This allows all the carbons from rhamnose and fucose to enter the central metabolic pathways (Boronat and Aguilar, 1981; Baldoma and Aguilar, 1988).

The regulation of the rhamnose catabolic pathway in *R. leguminosarum* at the molecular level is currently under study (Richardson *et al.,* 2004). It has been reported that, unlike the situation in *E. coli*, this pathway contains the *rhaSTPQ* gene cluster and transports rhamnose via an ABC transporter. The periplasmic sugar binding protein is encoded by *rhaS*, *rhaT* encodes an ABC-type protein, where as *rhaP* and *rhaQ* encode a permease. The kinase, encoded by *rhaK* lies after a gene encoding the hypothetical protein, RhaU. All of these genes in this organism are under the regulation of a negative regulator, RhaR. The genes diverging from this cluster are the *rhaD*, coding for a putative oxidoreductase, and *rhaI*, an isomerase.

The regulation of the genes in the rhamnose utilization pathway in *B. thetaiotaomicron* VPI-5482 (Fig 2.8A) has not previously been studied, although those of the fucose catabolism have been elucidated (Hooper *et al.,* 1999; Chapter 1). In *E. coli*, *B. fragilis,* as well as *Salmonella* species, the *fucO* gene, encoding L-lactaldehyde oxidoreductase is located in the fucose gene clusters. However, in *B. thetaiotaomicron* VPI-5482 this gene is found in the putative rhamnose gene cluster. It is interesting to note, that in *R. leguminosarum*, *rhaD* codes for a putative oxidoreductase, however, its function is yet to be elucidated. FucO is a member of the group three iron-activated alcohol dehydrogenase that is NAD(P)-dependent (Obradros *et al.*, 1998; Reid and Fewson, 1994). Fe^{2+}-dependent alcohol dehydrogenases contain conserved histidine and cystine residues which assist in catalytic activity and protein stability respectively

(Montella *et al.*, 2005; Obradros *et al*, 1998). Members of this group of enzymes are induced in the presence of a methyl pentose, and remain fully active in the absence of oxygen. In the presence of oxygen, they get oxidatively inactivated by an iron catalyzed mechanism (Montella *et al.*, 2005; Obradros *et al.*, 1998).

Based on the genome sequence of *B. thetaiotaomicron* VPI-5482, the orientation and arrangement of the genes of the putative rhamnose cluster (Fig. 2.9A) it is possible that they may be expressed as an operon on a single transcript and induced by rhamnose. The final gene downstream of the cluster, the putative transcriptional regulator, might also be involved in regulation of the putative rhamnose cluster. These questions are addressed in Chapter 3.

2.5 CONCLUSIONS

To begin an evaluation of *B. thetaiotaomicron* Tn Met^R that displayed metronidazole resistance on plate MIC's, metronidazole survival studies in BHIS broth were conducted and the mutant was shown to display resistance to 20 μg/ml metronidazole. This resistance phenotype may partially be caused by the slow growth observed in the mutant in complete medium, which may be affecting the activation of drug (Chapter 1). Improper catabolic reactions in the mutant may be preventing the phosphoroclastic reaction (Pan and Imlay, 2001) and in turn preventing sufficient electrons to be generated to activate the drug to its toxic component (Chapter 1). Alternatively, the transposon insertion in an intergenic region of the putative rhamnose pathway may be exerting an as yet unknown effect which results in the metronidazole resistance phenotype. It would, therefore, be of interest to characterize this region further, as well as to investigate the effects of this insertion in the mutant.

Southern hybridization was conducted to confirm a single transposon insertion within the genomic DNA of the mutant. In addition, plasmid rescue and sequence analysis, confirmed the site of the insertion in an intergenic region between BT3767 and BT3768 (*fucO* and *rhaR* respectively). The transposon insertion may have affected either one or both the putative *fucO* or *rhaR* genes. There is a possibility that the transposon may have disrupted the formation of active FucO since the functional stop codon of this protein has not been confirmed. Alternatively, the transposon insertion may have affected the transcription of the downstream putative *rhaR* gene. If this gene, in fact, encodes a regulator of the rhamnose gene cluster, it may have other implications on the expression of these genes. Transposons are flanked with insertion sequence elements (Fig. 2.4) that can act as additional promoter sites (Podglajen *et al.*, 2001) and this may be affecting the *rhaR* transcription.

Based on this study, it would appear that the technique of using random transposon mutagenesis may not have been the ideal tool for examining metronidazole resistance, since their insertion into the genomic DNA can create mutants like *B. thetaiotaomicron* Tn Met^R where the transposon has integrated into an intergenic region. Mutants containing single gene disruptions can be complemented to confirm their function. However, a mutant created by an intergenic insertion needs to be first characterized to determine how the genes flanking the insertion site are affected and how an insertion in this region may be causing a metronidazole resistance phenotype. Random transposon mutagenesis is, however, an extremely useful tool for identifying genes or pathways involved in metronidazole resistance, which may not appear to be obvious candidates for study.

An alternative approach to addressing the question of what genes might control metronidazole resistance phenotype would be to create targeted gene disruptions using a suicide vector such as pGERM (Cheng *et al.*, 2000) whereby the process of homologous recombination ensures the targeting of specific candidate genes. A genetic tool such as this may be more effective and efficient, in comparison to random transposon mutagenesis, which brings with it the possibility of multiple transposon insertions, as well as that of IS elements integrating independently into other sites of the chromosome. This latter possibility will be dealt with in Chapter 4.

Based on the findings in this chapter, two areas of study will be addressed in this dissertation. Firstly, the genes of the putative rhamnose cluster will be studied to evaluate whether they are, in fact, involved in the catabolism of this substrate (Chapter 3). Secondly, a study will be conducted to address how the transposon insertion in the *B. thetaiotaomicron* Tn Met^R mutant, between the *fucO* gene and the putative transcriptional regulator, *rhaR*, might be causing a metronidazole resistance phenotype (Chapter 4). Further, to rule out the possibility of background mutations causing the resistance phenotypes, the mutant would be recreated genetically (Chapter 4).

CHAPTER 3

Genetic regulation of the rhamnose pathway in *B. thetaiotaomicron* VPI-5482

3.1 ABSTRACT

Bioinformatic analysis of ORFs BT3763 to BT3768 (Chapter 2) suggested that they may constitute a rhamnose catabolic gene cluster. This chapter analyzes the gene cluster of the putative rhamnose pathway in *B. thetaiotaomicron* VPI-5482 with respect to transcriptional regulation and function. Transcriptional hybridization studies showed that growth in the presence of the substrate, $_{L}$-rhamnose, induced expression of the putative rhamnose catabolic genes in *B. thetaiotaomicron* VPI-5482. ORFs BT3763 – BT3768 were shown by reverse transcriptase PCR to be expressed on a single transcript, *rhaKIPAO*, whereas BT3768, encoding the putative transcriptional regulator (RhaR) of the rhamnose gene cluster, was transcribed independently. Primer extension analysis identified the transcriptional start sites, both as cytosine bases, 41 bp upstream of the start codon for the putative catabolic genes (*rhaK-fucO*) and, 27 bp upstream of the start codon for the putative regulator (*rhaR*). The -7 promoter sequences of $P_{rhaKIPAO}$ and $P_{rhaR,}$ were identified and shown to resemble the *B. fragilis* promoter consensus motifs. Inactivation of the *rhaR* gene, in *B. thetaiotaomicron* VPI-5482, resulted in a mutant, *B. thetaiotaomicron rhaR*$^{-}$, which was unable to use $_{L}$-rhamnose, indicating that this gene may code for a positive transcriptional regulator of the rhamnose pathway. The complemented mutant, *B. thetaiotaomicron rhaR*$^{-}$ (pLYLrhaR) was shown to restore growth in defined medium supplemented with $_{L}$-rhamnose as the sole carbon source, confirming that the product of the *rhaR* gene is responsible for regulating the catabolic genes in the rhamnose gene cluster.

3.2 INTRODUCTION

As discussed in Chapter 2, the *B. thetaiotaomicron* VPI-5482 genome sequence provisionally annotates the presence of catabolic genes of the putative rhamnose gene cluster (*rha*) (Accession N° AAO76955). The gene nomenclature used in this study to name the genes of the putative rhamnose cluster has been described previously (Table 2.1). Having determined that the transposon insertion causing the metronidazole resistance phenotype occurred in an intergenic region between the *fucO* and *rhaR* genes of the putative rhamnose cluster, it was necessary to analyze this cluster in order to confirm its function and regulation in the parent strain, *B. thetaiotaomicron* VPI-5482. This would then serve as a basis, on which, to analyze the effect/s of the transposon insertion on the rhamnose catabolism in *B. thetaiotaomicron* Tn Met^R and determine how this might affect the metronidazole resistance phenotype in this strain. Therefore, this chapter deals with the characterization of the rhamnose gene cluster with respect to rhamnose utilization. Transcriptional hybridization studies carried out by Moralejo *et al.* (1993) showed that the *E. coli* rhamnose cluster contained the structural genes (*rhaBAD*). When RNA prepared from *E. coli* cells grown in glucose minimal medium was probed with the genes of interest, no transcription was seen. However, when RNA was prepared from cells grown in rhamnose minimal medium, the expected bands were visualized. Therefore, a similar approach was used for this study to determine if $_L$-rhamnose as the sole carbon source in the growth medium would induce the transcription of the *rhaK-rhaR* gene cluster in *B. thetaiotaomicron* VPI-5482. Bioinformatic analysis of the *rhaR* gene showed that the derived amino acid sequence shared similarity to a group of transcriptional regulators from the AraC/XylS family of regulators. It was of interest, therefore, to determine if this was the regulator of the rhamnose catabolic genes in *B. thetaiotaomicron*, as well as to establish whether it

functioned as a positive or negative regulator. A useful approach in this regard is the generation of a gene specific mutation using a suicide vector (Hooper *et al.*, 1999).

The bioinformatic analysis (Chapter 2) also showed that the genes of the putative rhamnose cluster may be transcribed on a single RNA transcript as an operon, from a single promoter upstream of *rhaK*. Methods such as 5′ RACE (Rapid Amplification of cDNA Ends, version 2; GibcoBRL-Life Technologies) have been used in mapping transcriptional start sites of the starch utilization genes and carbapenem resistance gene in *B. fragilis* (Spence *et al.*,2006; Podglajen *et al.*,2001, respectively). Alternatively, primer extension has also proven to be successful in identifying transcriptional start sites (Eikmanns *et al.*, 1994; Moon *et al.*, 2005). This latter method was selected for use in this study to identify the transcriptional start sites in the putative rhamnose gene cluster. Moon *et al.* (2005) used reverse transcriptase PCR (RT-PCR) to show that several of the ORFs were expressed as an operon in the regulation of the excision of the *Bacteroides* conjugative transposon, CTnDOT. For the purpose of this study, RT-PCR was used to determine if the *B. thetaiotaomicron rha* genes are expressed as an operon.

3.3 METHODS AND MATERIALS

3.3.1 Bacterial Strains, Plasmids and Growth Conditions

B. thetaiotaomicron VPI-5482 was grown anaerobically on BHIS as described in Section 2.3.1. *E. coli* DH5α and S17-1 strains have been described previously (Rasmussen *et al.*, 1994; Simon *et al.*, 1983) and were grown aerobically on Luria-Bertani (LB) broth or agar (1.5% w/v) at 37°C. The antibiotics gentamicin (200 μg/ml), ampicillin (100 μg/ml), tetracycline (2 μg/ml) and erythromycin (10 μg/ml) were added to BHIS and/or LB whenever necessary. Plasmids pGERM (Cheng *et al.*, 2000) and pLYL01 (Li *et al.*, 1995) have been described previously. For carbohydrate utilization studies, cells were grown in a defined medium (Van Tassel and Wilkinson, 1978) supplemented with either 0.1 % glucose (Merck), 0.1 % rhamnose (Sigma®) or 0.1% fucose (Sigma®) as the sole carbon source.

3.3.2 General Recombinant DNA procedures

B. thetaiotaomicron genomic DNA was prepared according to the method outlined by Wehnert *et al.* (1992). *E. coli* plasmid DNA was prepared using the QIAGEN® Plasmid Midi Kit (25) (QIAGEN). Restriction enzyme digestions were carried out according to the manufacturer's instructions.

3.3.3 RNA Extraction

B. thetaiotaomicron VPI-5482 was grown for 16 h in 50 ml defined medium supplemented with 0.1 % of either D-glucose (Merck) or L-rhamnose (Sigma®), and the total RNA was isolated according to method of Aiba *et al.* (1981). The quality of the RNA was confirmed by

electrophoresis in 1.5 % denaturing formaldehyde agarose gel (Fourney *et al.*, 1988) and it was quantified using the NanoDrop® ND-100 spectrophotometer (Nano Drop Technologies, Inc).

3.3.4 Construction of DNA probes for transcriptional hybridization studies

Internal regions of each of the putative rhamnose genes was PCR amplified in 50 µl reaction volumes. The master mix contained100 ng of *B. thetaiotaomicron* VPI-5482 genomic DNA, 0.5 µM of each primer (Table 3.1), 150 µM deoxynucleotide triphosphates, 1.5 mM of Mg^{2+}, and 2.5U *Taq* I polymerase (Roche). Samples were amplified for 30 cycles with appropriate annealing temperatures as shown in Table 3.1. The PCR products were purified using the High Pure PCR Purification Kit (Roche). The amplified products were DIG-labelled as described in Section 2.3.6.

Gene	Forward Primer	Reverse Primer	Temp °C	Band Size (bp)
16S rRNA	5'-AGA GTT TGA TCT TGG CTC AG-3'	5'-ACG GTT ACC TTG TTA CGA CTT-3'	55	500
rhaK	5'-CCG TTA TCT AAG CGG TGC C-3'	5'-CCG AAA TTC TCT TCT TGC AGT CC-3'	53.5	417
rhaI	5'-GCA ATA TTG ATG AAC TGC GTG CC-3'	5'-GAT TTC TGC AAT GAA ATC TTC GCC-3'	53	984
rhaP	5'-GAA AAT CGT CCG GCA CTT GCC-3'	5'-TCA TAC CAT CCG CTT CGA AGC C-3'	54.5	701
rhaA	5'-TAT AGC CAT CGG TAG CTT TTG CC-3'	5'-ATT ACT AAA GGT TAC GTT CAG CGC C-3'	53	855
fucO	5'-CTT ATT TCG GTG CAG GAT GCC-3'	5'-CGA ACA TAT CGC TCA TAG CCC-3'	52.8	507
rhaR	5'-GCT GAA GGA GCA TCC GTC-3'	5'-GTC TCA TCG CCA GTT CCT CC-3'	53.5	532

Table 3.1: Oligonucleotides used to amplify internal fragments of the putative rhamnose genes. List of primers internal to each of the genes in the putative rhamnose cluster, expected band sizes and annealing temperatures are shown. The 16S rRNA gene was used as an internal control.

3.3.5 RNA Transcriptional Hybridization Studies

RNA was extracted from *B. thetaiotaomicron* VPI-5482 cells grown in defined medium supplemented with 0.1 % of either $_{D}$-glucose (Merck) or $_{L}$-rhamnose (Sigma®) and equal amounts (1 µg), were spotted in equal volumes onto a nylon membrane (Roche). The membranes were hybridized with the DIG-labelled DNA probes. Hybridization and detection procedures were performed according to the manufacturers' instructions. Chemiluminescent signals were detected using CDP® Star (Roche).

3.3.6 Reverse Transcriptase PCR

RNA (1 µg) obtained from *B. thetaiotaomicron* VPI-5482, grown in defined medium supplemented with $_{L}$-rhamnose, was used for the synthesis of cDNA using the Omniscript® Reverse Transcription Kit (Qiagen), according to manufacturer's instructions. cDNA was synthesized from RNA using the reverse oligonucleotide (Table 3.2) and each of the cDNAs was amplified with the appropriate set of primers to obtain a fragment of the intergenic region (Table 3.2).

Intergenic Region	Forward Primers	Reverse Primers	Band size (bp)
rhaK-rhaI	5'-CCGTGGCGAATCTATTGCC-3'	5'-GAAGCATCGAAATAGTCGAGAACCG-3'	2100
rhaI-rhaP	5'-TGGCAGGCTGATGATGTAACGG-3'	5'-TGGCAGGCTGATGATGTAACGG-3'	1508
rhaP-rhaA	5'-TATCAAGGAAGCTGCTTTGGC-3'	5'-TATTTAGGAAGATTGAAAGCAACGG-3'	1250
rhaA-fucO	5'-GTAAACAAGGTAGCTGAAGTTGCC-3'	5'-CATTGAATGCAGCCACCGCC-3'	1115
fucO-rhaR	5'-CTTATTTCGGTGCAGGATGCC-3'	5'-GTCTCATCGCCAAGTTCCTC-3'	1140

Table 3.2: Oligonucleotides used to amplify intergenic regions of the putative rhamnose gene cluster. List of reverse primers used to synthesize cDNA, and used with the forward primers to amplify the cDNA are shown. Expected band sizes for each of the intergenic regions are indicated.

3.3.7 Primer Extension Studies

The transcriptional start sites of the catabolic genes (*rhaK-fucO*), and the putative transcriptional regulator, *rhaR* were identified using the non-radioactive method of Eikmanns *et al.* (1994). cDNA was synthesized from total RNA extracted from *B. thetaiotaomicron* VPI-5482, grown in defined medium supplemented with L-rhamnose (Sigma®) using the following Cy5 labelled primers: RhaKX (5'-CGA TTA ATC TCC TCC AGA TTT AAC CG- 3') corresponding to positions +98 to +71 bp downstream of the putative ATG start codon of *rhaK*, and primer RhaRX (5'-ACC GAA TTT GCG GTC TCC ATG -3') corresponding to positions +72 to +53 bp downstream of the putative ATG start codon of the putative transcriptional regulator.

3.3.8 Inactivation of the *rhaR* gene in *B. thetaiotaomicron* VPI-5482

An internal fragment of the *rhaR* gene was amplified from *B. thetaiotaomicron* VPI-5482 chromosomal DNA using PCR (forward primer, iRF 5'-GCT GAA GGA GCA TCC GTC-3' and reverse primer, iRR, 5'-GTC TCA TCG CCA GTT CCT CC-3'). The PCR product was blunt ended with T4 DNA polymerase, cloned into the *Sma*I site of the suicide vector pGERM (Cheng *et al.*, 2000), and transformed into *E. coli* DH5α. The resulting construct, pGERI, containing the internal fragment of the *rhaR* gene, was purified and retransformed into *E. coli* S17-1 according to standard methods (Sambrook *et al.*, 1989). Mobilization of pGERI from *E. coli* S-17 (pGERI) into *B. thetaiotaomicron* VPI-5482 was performed by an aerobic mating procedure as previously described (Li *et al.*, 1995). Briefly, *E. coli* S-17 (pGERI) was grown for 16 h in LB broth, containing ampicillin (100 μg/ml), while *B. thetaiotaomicron* VPI-5482 was grown anaerobically (16 h) in BHIS broth. Fresh LB or BHIS broth was inoculated with the relevant bacterial strain and grown until OD = 0.3 (1.44×10^8 cfu/ml of *E. coli* S-17 (pGERI), and 1.78×10^9 cfu/ml of

B. thetaiotaomicron). Twenty millilitres of *B. thetaiotaomicron* were mixed with 5 ml of *E. coli*, and the cells were pelleted by centrifugation (5,000 rpm for 10 min). The supernatant fraction was removed and the cells resuspended in 1 ml of BHIS broth. Aliquots (200 µl) were plated onto HAWP filters (0.45 µm, Millipore) placed on BHIS agar with no antibiotics, and incubated aerobically at 37°C for 16 h. Bacterial cells growing aerobically on the filters were washed off in BHIS broth and held anaerobically at 37°C for one hour. Aliquots (100 µl) were then spread-plated on BHIS agar containing gentamicin (100 µg/ml) and erythromycin (10 µg/ml) to select for the transconjugants (*B. thetaiotaomicron rhaR*⁻ mutants).

3.3.9 Confirmation of pGERI insertion in *B. thetaiotaomicron rhaR*⁻

Integration of pGERI within the *B. thetaiotaomicron rhaR*⁻ genomic DNA was confirmed using PCR. The junction between the *rhaR* gene in the genomic DNA and the plasmid, pGERI, was amplified using the following combinations of primers, RFF (5′-ATG ACT GAG GAT AAT AAT TTG GG-3′) with the M13 forward primer, and RFR (5′-TTA TTC TTC CCG ACC GAA G-3′) with the M13 reverse primer. The PCR products were purified and sequenced.

3.3.10 Complementation of *B. thetaiotaomicron rhaR*⁻ mutant with pLYLrhaR

A full length fragment of *rhaR*, including 239 bp upstream of the gene, was PCR amplified using the following primers; forward primer, cRF 5′-GTG AAG GAA GAG GAT TCT CCG G- 3′, and reverse primer, cRR, 5′-ACA GAT TAA AAC GGA AAG CTT AAA TCA CAT CG-3′. The PCR product was digested with *Hin*dIII and *Xmn*I, cloned into the *Hin*dIII and *Sma*I sites of the shuttle vector, pLYL01 (Li *et al.*, 1995) and transformed into *E. coli* DH5α (Rasmussen *et al.*, 1994). The

resultant plasmid, pLYLrhaR was extracted, purified and retransformed into *E. coli* S17-1 according to standard methods (Sambrook *et al.*, 1989). *E. coli* S-17 (pLYLrhaR) was conjugated with *B. thetaiotaomicron rhaR*$^-$ mutant, as described previously (Li *et al.*, 1995). The resulting complemented strain was grown on BHIS agar containing erythromycin (10 µg/ml), and tetracycline (2 µg/ml). The presence of pLYLrhaR in the *B. thetaiotaomicron rhaR*$^-$ mutant cytoplasm was confirmed by extracting plasmid from *B. thetaiotaomicron rhaR*$^-$ and retransforming it into *E. coli* DH5α which were then plated on LB agar with ampicillin (100 µg/ml). pLYLrhaR was re-extracted from *E. coli* DH5α and submitted for nucleotide sequencing to confirm the presence of the *rhaR* gene in the plasmid.

3.3.11 Growth of the *Bacteroides* strains in defined broth medium

B. thetaiotaomicron VPI-5482, *B. thetaiotaomicron rhaR*$^-$, as well as *B. thetaiotaomicron rhaR*$^-$ (pLYLrhaR) were grown separately under anaerobic conditions in 10 ml volume of BHIS broth for 16 h, at 37°C. Cultures were diluted (1:50) separately in 100 ml defined broth medium (Van Tassel and Wilkins, 1978) supplemented with 0.1 % of either $_D$-glucose (Merck), $_L$-rhamnose, or $_L$-fucose (Sigma®). Growth of these cultures was monitored at hourly intervals by recording the optical density (OD_{600}) using a Beckman DU®530 Lifescience UV/Vis Spectrophotometer.

3.4 RESULTS AND DISCUSSION

3.4.1 Transcriptional induction by L-rhamnose

In order to investigate if transcription of the genes of the putative rhamnose cluster was induced by L-rhamnose in the growth medium, transcriptional hybridization studies were performed using RNA extracted from cells grown in defined medium supplemented with L-rhamnose or D-glucose (Fig 3.1).

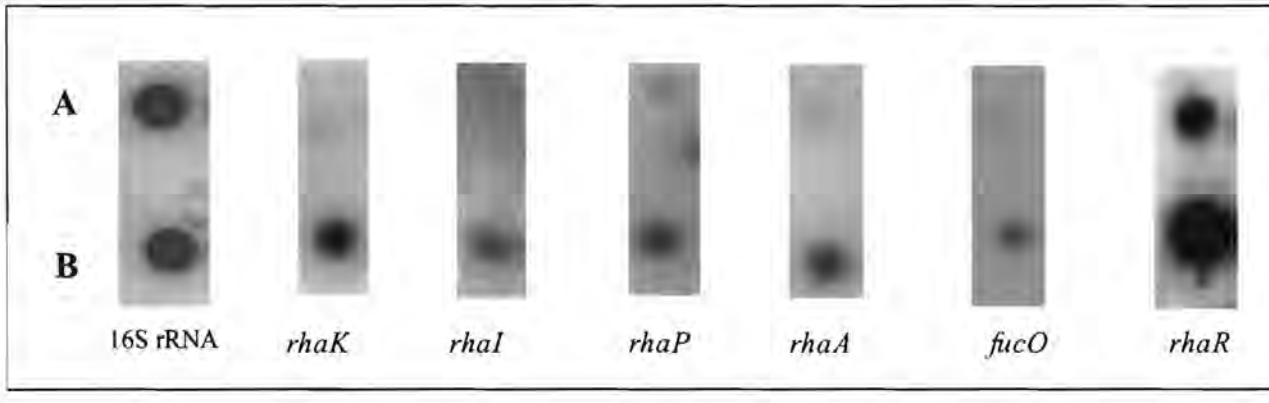


Figure 3.1: Transcription of the putative *rha* genes of *B. thetaiotaomicron* VPI-5482 during growth in the presence D-glucose or L-rhamnose as shown by dot blot analysis. Equal volumes (2 µl) and concentrations of total RNA (1 µg) extracted from *B. thetaiotaomicron* VPI-5482 grown in defined medium, supplemented with D-glucose (**A**), or L-rhamnose (**B**) were spotted onto nylon charged membranes. Membranes were probed with DNA probes specific for each of the *rha* genes as described in Section 3.3.4. The 16S rRNA gene (an internal control) was also used as a probe to show that equal amounts of RNA were loaded onto each membrane.

Results indicated that there was increased transcription of all of the rhamnose genes when cells were grown in L-rhamnose (**B**), as compared with growth in D-glucose (**A**). L-rhamnose, therefore, acts as an inducer of the transcription of these catabolic genes. In *E. coli*, it was shown that L-rhamnose was the direct inducer of the *rhaBAD* transcription since transcription of the gene

coding for rhamnulose kinase, was detected in strains carrying point mutations in each of the structural genes of the rhamnose operon (Moralejo *et al.*, 1993).

3.4.2 Transcription of the genes of rhamnose pathway as an operon

The *B. thetaiotaomicron* VPI-5482 rhamnose gene cluster is shown schematically in Fig 3.2. As can be seen, all the genes are transcribed in the same orientation and the intergenic regions (represented by the pink arrows) between ORFs *rhaK, rhaI, rhaP, rhaA, fucO* are quite short, leading to the hypothesis that the catabolic genes may be co-regulated as an operon, *rhaKIPAO*. The intergenic region between *fucO* and *rhaR*, the putative transcriptional regulator is, however, longer (142 bp), and may be transcribed independently, possibly from its own promoter. In order to determine whether the genes function as an operon, RT-PCR was performed.

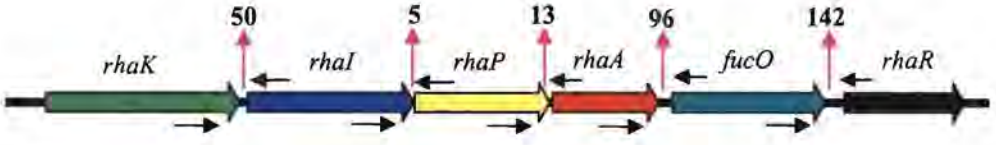
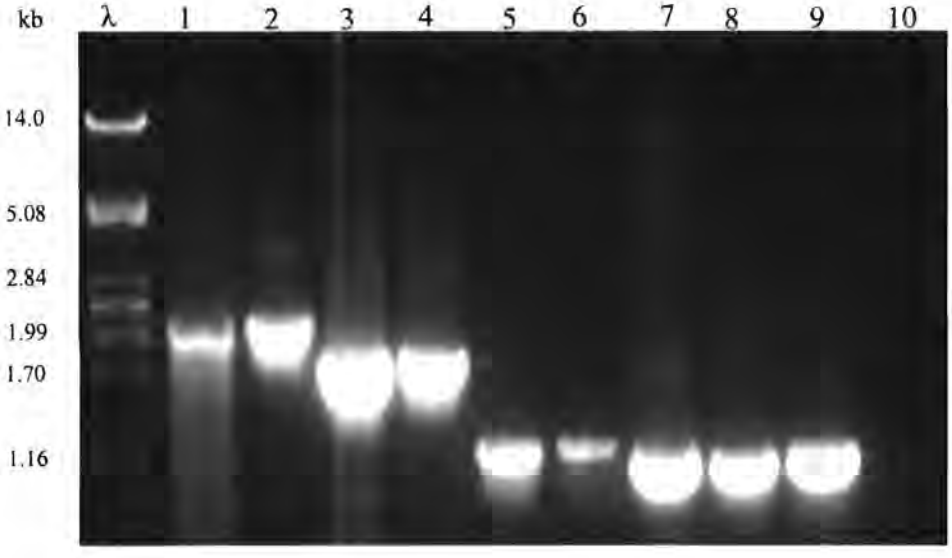


Figure 3.2: Schematic representation (not to scale) of the intergenic region between genes of the putative rhamnose pathway. The small black arrows represent the primers that were designed to amplify the intergenic regions (indicated by the pink arrows with their corresponding sizes in base pairs).

Primers were designed to amplify the mRNA of the intergenic regions between each of the genes (Table 3.2, Fig 3.2). Each set of primers was tested on genomic DNA as shown in Fig 3.3 (Lanes 1, 3, 5, 7 and 9) to determine the appropriate optimum conditions that would result in a single band of the predicted size (Table 3.2). The reverse primers were used to synthesize cDNA from *B. thetaiotaomicron* VPI-5482 RNA that was extracted from cells grown in the presence of

rhamnose (inducing conditions). The cDNA was amplified by PCR using the appropriate set of primers for each of the intergenic regions. The resulting PCR products were run on an agarose gel (Lanes 2, 4, 6, 8, 10) (Fig 3.3).



Lane	Intergenic region
1	Genomic *rhaK-rhaI*
2	cDNA *rhaK-rhaI*
3	Genomic *rhaI-rhaP*
4	cDNA *rhaI-rhaP*
5	Genomic *rhaP-rhaA*
6	cDNA *rhaP-rhaA*
7	Genomic *rhaA-fucO*
8	cDNA *rhaA-fucO*
9	Genomic *fucO-rhaR*
10	cDNA *fucO-rhaR*

Figure 3.3: RT-PCR analysis of the putative rhamnose gene cluster. PCR products from genomic DNA (odd numbers) and RT-PCR products from cDNA synthesis (even numbers). Key: Numbers on gel corresponding to PCR product loaded onto agarose gel. Molecular weight marker (kb) from lambda (λ) DNA digested with restriction enzyme, *Pst*I is also shown.

The results indicated that transcripts were obtained from the intergenic regions of *rhaK-rhaI, rhaI-rhaP, rhaP-rhaA,* and *rhaA-fucO* (Lanes 2, 4, 6, and 8 respectively) confirming that these five genes are expressed on a single mRNA transcript. However, no cDNA transcript was amplified for the *fucO-rhaR* region (Lane 10), despite visualizing the amplified PCR product on the agarose gel from genomic DNA (Lane 9). This suggests that *rhaR* may be expressed independently from the catabolic genes from its own promoter. The PCR products from cDNA synthesis were purified and sequenced, and it was confirmed that the expected intergenic regions had been amplified.

3.4.3 Identification of the transcriptional start sites using primer extension analysis

RT-PCR indicated that the catabolic genes were transcribed as an operon, *rhaKIPAO*, and that *rhaR* may be transcribed independently. Furthermore, genome analysis of the *B. thetaiotaomicron* VPI-5482 rhamnose gene cluster, revealed a 142 bp intergenic gap between *fucO* and *rhaR*. Primer extension analysis was, therefore, performed in order to identify the transcriptional start sites of the rhamnose catabolic gene cluster ($P_{rhaKIPAO}$), as well as that of the putative transcriptional regulator (P_{rhaR}). A single primer extension product was obtained for the catabolic genes, which identified the transcriptional start sites to be a cytosine, 41 bp upstream of the start codon for the putative kinase gene (*rhaK*). The transcriptional start site of the putative transcriptional regulator was also a cytosine, located 27 bp upstream of the start codon of the *rhaR* gene. (Fig 3.4 and Fig 3.5, respectively).

Studies carried out by Bayley *et al.* (2000) showed that *B. fragilis* had a typical promoter sequence with a -7 region containing the consensus sequence TAnnTTTG and a -33 consensus sequence of TTTG. This was confirmed by Podglajen *et al.* (2001) who used 5′ RACE to identify the transcriptional start sites of the *B. fragilis* carbapenemase gene, *cfiA*. Thus far, no typical promoter sequences for *B. thetaiotaomicron* have been reported. It is, therefore, interesting to point out that the -7 region identified in this study was typical of what was observed in *B. fragilis* (Fig 3.4C and 3.5C). However, the -33 regions of both promoters analyzed in this study showed a GCGT consensus sequence which is different from that reported in *B. fragilis*. Future studies will confirm whether this holds for all *B. thetaiotaomicron* promoter regions.

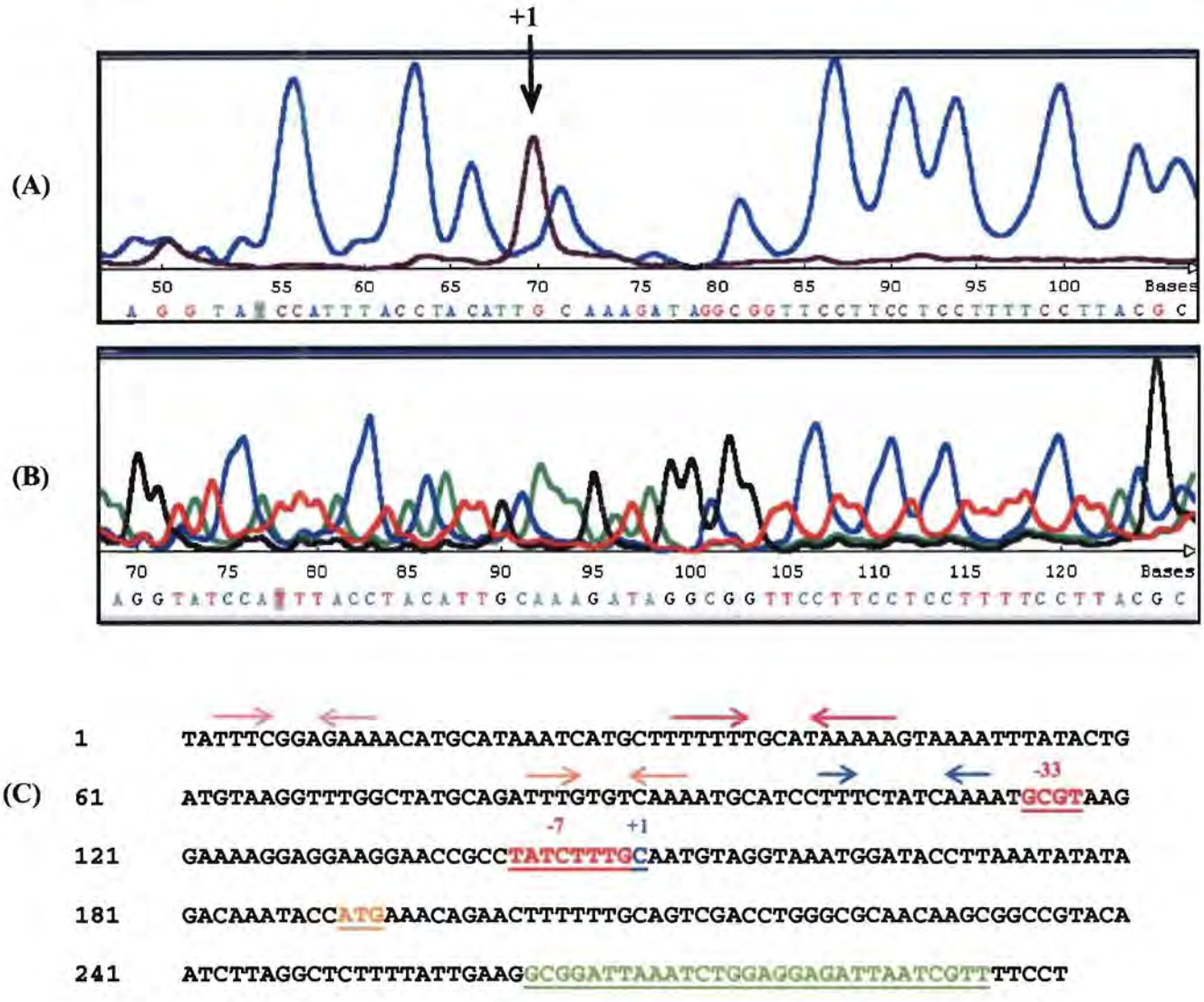


Figure 3.4: Identification of *B. thetaiotaomicron* putative rhamnulose kinase *(rhaK)* transcriptional start site by primer extension analysis. (A) Primer extension reaction product (black line) and A's in blue. Transcriptional start (+1) site is indicated by an arrow. (B) Chromatogram of the corresponding DNA sequencing reaction using the same primer RhaKX (underlined in green in C). (C) Nucleotide sequence of the putative rhamnulose kinase, *rhaK*: ATG start codon of *rhaK* (underlined in orange), transcriptional start site (blue, +1), putative -7 and -33 motifs (underlined in red). RhaKX Cy5 labelled reverse primer used to synthesis cDNA (underlined oligonucleotides in green). The 4 sets of inverted repeats are shown by the matching arrows.

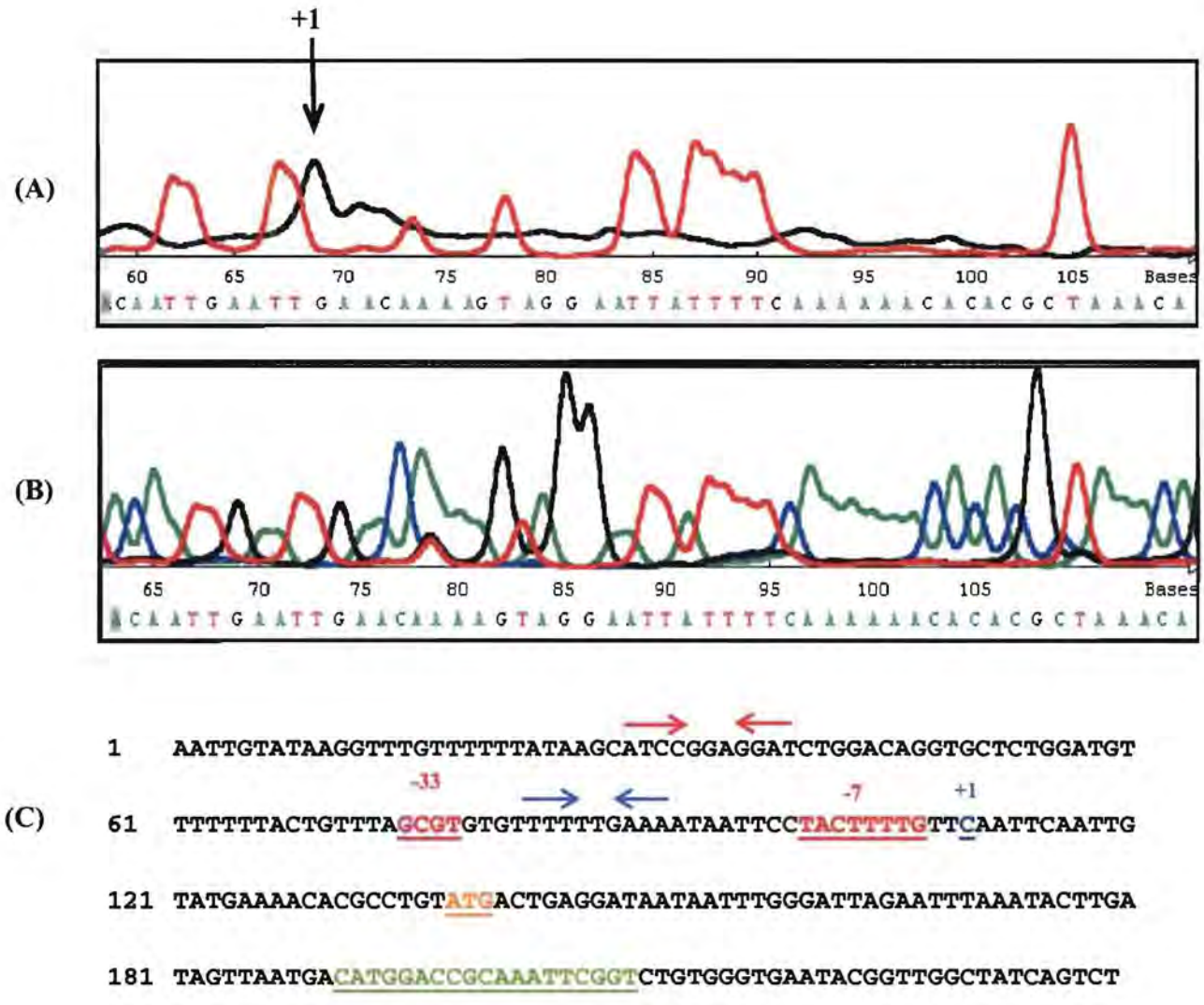


Figure 3.5: Identification of *B. thetaiotaomicron* putative transcriptional regulator *(rhaR)* transcriptional start site by primer extension analysis. (A) Primer extension reaction product (black line) and T's in red. Transcriptional start (+1) site is indicated by an arrow. (B) Chromatogram of the corresponding DNA sequencing reaction using the same primer RhaRX (underlined in green in C). (C) Nucleotide sequence of the putative transcriptional regulator, *rhaR*: ATG start codon of *rhaR* (underlined in orange), transcriptional start site (blue, +1), and putative -7 and -33 motifs (underlined in red). RhaRX Cy5 labelled reverse primer used to synthesis cDNA (underlined oligonucleotides in green). The 2 sets of inverted repeats are shown by the matching arrows.

Tobin and Schleif (1987), identified two inverted repeat (IR) sequences of the *E. coli rhaR* gene of the rhamnose pathway, located upstream of -35 region (5'TTTTT-----AAAAA-3') and (5'-AAAA------TTTT-3'). They also reported that these elements are often part of the binding site which are thought to be involved in regulating the protein binding. In this study, only one IR, 5'-TTTTT----AAAAA-3' (red arrows, Fig. 3.4C), was found upstream of the -33 region within the *B. thetaiotaomicron rhaK* promoter region. In addition, however, a further three sets of inverted repeats were identified. These are, 5'-TTTC---GAAA-3' where – is any base pair (pink arrows), 5'-TTTG---CAAA-3' (orange arrows) and 5'-TTT-----AAA-3' (blue arrows) (Fig. 3.4C). All of these IRs were found upstream of the -33 promoter sequence. In the *rhaR* promoter region, two sets of inverted repeats were identified, 5'-ATCC---GGAT-3' (red arrows) and 5'-TTT-AAA-3' (blue arrows) (Fig. 3.5C). The first one was found upstream of the -33 promoter sequence and the latter spanned the -33 and -7 promoter region.

3.4.4 Insertional inactivation and confirmation of *B. thetaiotaomicron rhaR*⁻ mutant

Gene specific disruption was performed to determine if the *rhaR* gene was responsible for regulating the rhamnose pathway. A schematic diagram of how the *rhaR* insertional inactivated mutant was created and genetically confirmed is shown in Fig. 3.6. Plasmid pGERI, containing an internal fragment of *rhaR* gene, was inserted into the genomic copy of the *B. thetaiotaomicron* VPI-5482 *rhaR* gene region via homologous recombination.

In order to confirm the single insertion of pGERI into the chromosome, PCR was used to amplify the junctions between the plasmid pGERI and the external fragments of *rhaR* gene from either end of the plasmid as shown by the blue bands (Fig 3.6D). M13 forward and reverse primers were

specific to the pUC based plasmid, pGERI, and these were matched with external primers to the *rhaR* gene (Fig 3.6C).

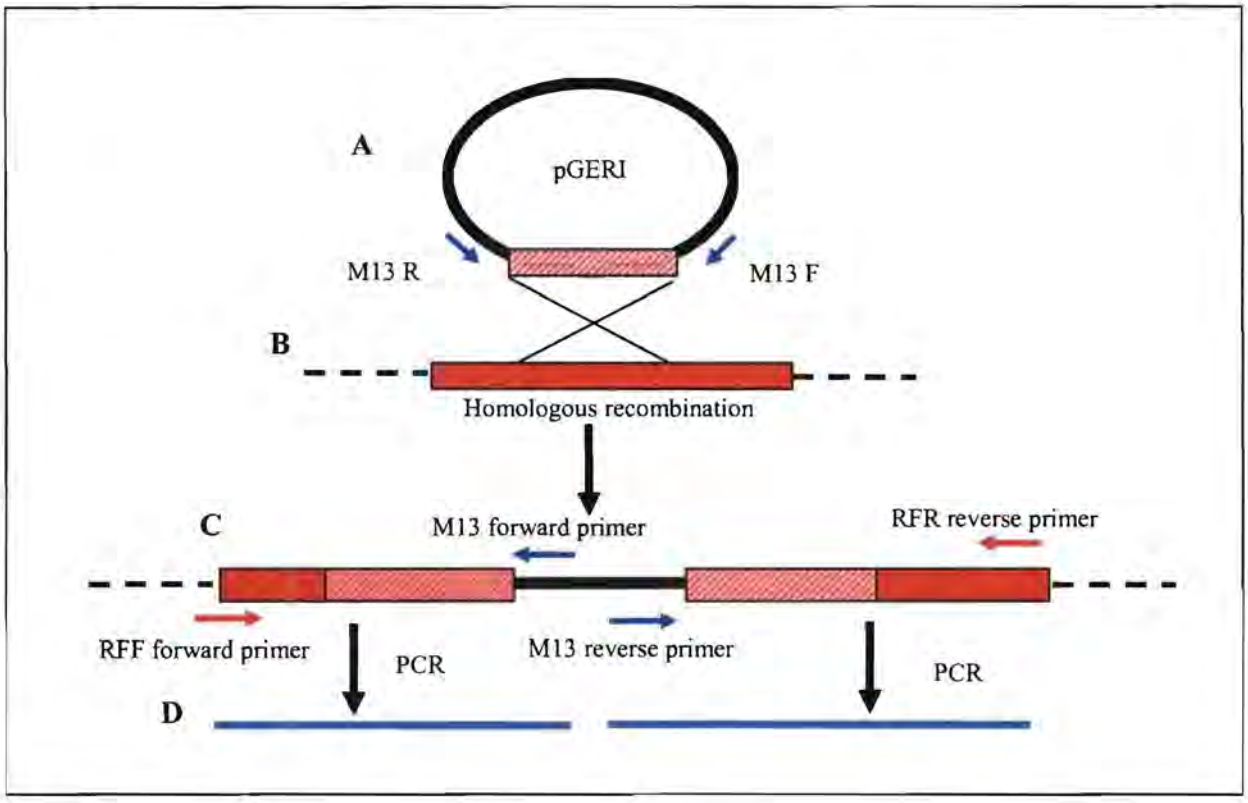


Figure 3.6: Schematic representation (not to scale) depicting the homologous recombination method used to create the insertionally inactivated *rhaR* mutant as well as confirmation of the mutation site. A) Plasmid pGERI (circular black band) with an internal fragment of the *rhaR* gene (red hatched band). B) *B. thetaiotaomicron* VPI-5482 genomic DNA (dashed line) flanking the full length *rhaR* gene (red band). C) The resulting mutant, *B. thetaiotaomicron rhaR'*, with an inactivated *rhaR* gene containing pGERI integrated into the genomic DNA. M13 primers are shown in blue arrows and *rhaR* primers shown in red arrows. D) PCR products (blue bands) amplified using M13 primers and gene specific primers (RFF and RFR) of the *rhaR* gene to genetically confirm the insertion of pGERI into the chromosome.

The amplified PCR products from either end were visualized on an agarose gel (Fig. 3.7 Lanes 1 and 4). No bands were visualized from the PCR samples loaded in lanes 2 and 3 given that the primer sets (RFF with M13 reverse, and RFR with M13 forward, respectively) used in these

reactions anneal to the DNA in the same direction (Fig 3.6C). Sequencing of the purified PCR products using the M13 primers showed the orientation of pGERI within the *B. thetaiotaomicron rhaR* gene, and confirmed the site of integration.

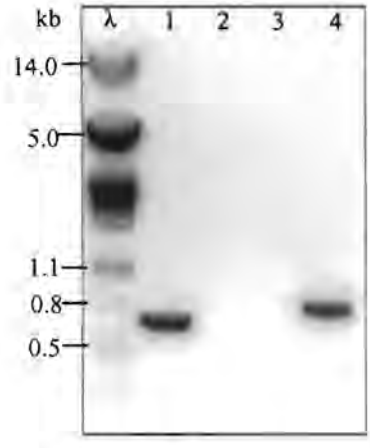


Figure 3.7: Agarose gel confirming pGERI insertion into *B. thetaiotaomicron rhaR*⁻ mutant genome using PCR. PCR products from RFF primer with M13 forward primer (Lane 1), RFF primer with M13 reverse primer (Lane 2), RFR primer with M13 forward primer (Lane 3), and RFR primer with M13 reverse primer (Lane 4). Molecular weight marker (kb) from lambda (λ) DNA digested with restriction enzyme, *Pst*I is also shown.

3.4.5 Role of RhaR in regulating the *B. thetaiotaomicron* VPI-5482 rhamnose utilizing pathway

The *B. thetaiotaomicron rhaR*⁻ mutant was created to determine if the product of this gene was responsible for regulating transcription of the rhamnose gene cluster. The role of RhaR in rhamnose utilization was further validated by transforming the plasmid pLYLrhaR into the *B. thetaiotaomicron rhaR*⁻ mutant to observe if this strain would restore growth in the rhamnose minimal medium. The presence of the cytoplasmic copy of pLYLrhaR was verified by extracting it from the complemented strain, *B. thetaiotaomicron rhaR*⁻ (pLYLrhaR). The yield of plasmid was too low to be used for restriction enzyme analysis, so it was transformed it into *E. coli* DH5α. The

plasmid was re-extracted from the resulting ampicillin resistant *E. coli* DH5α and the correct pLYLrhaR construct was verified by nucleotide sequencing.

The *B. thetaiotaomicron rhaR*⁻ mutant was unable to grow in defined medium supplemented with L-rhamnose as the sole carbon source. *B. thetaiotaomicron rhaR*⁻ (pLYLrhaR), however, grew in the minimal medium with L-rhamnose to the same extent as *B. thetaiotaomicron* VPI-5482 parent strain (Table 3.3). The results showed that RhaR is required to be active in order for it to positively regulate the uptake and catabolism of L-rhamnose.

Strains	**Absorbance (600nm) at 8 h**		
	L-rhamnose	**D-glucose**	**L-fucose**
B. thetaiotaomicron VPI-5482	0.633 (± 0.09)	1.74 (± 0.05)	0.76 (± 0.03)
B. thetaiotaomicron rhaR⁻ mutant	0.11 (± 0.00)	1.65 (± 0.08)	0.77 (± 0.11)
B. thetaiotaomicron rhaR⁻ (pLYLrhaR)	0.66 (± 0.05)	1.78 (± 0.02)	0.62 (± 0.02)

Table 3.3: Growth of the *Bacteroides* strains at 8 h in defined medium supplemented with L-rhamnose, D-glucose or L-fucose as the sole carbon source. The initial starting OD (600nm) of the cultures in the defined mediums was ~0.1 and grown for 8 h. Data points represent the mean values of three experiments, and the standard deviation is shown in brackets (±).

The *B. thetaiotaomicron rhaR*⁻ mutant was able to grow in medium supplemented with D-glucose or L-fucose (Table 3.3), demonstrating that RhaR plays an essential role in specifically regulating the utilization of L-rhamnose. In *E. coli*, it was reported that a point mutation in *rhaR* abolished transcription of *rhaBAD* (Moralejo *et al.*, 1993).

3.4.6 Bioinformatic analysis of the *B. thetaiotaomicron* positive transcriptional regulator, RhaR

Bioinformatic analysis of the *B. thetaiotaomicron* VPI-5482 *rhaR* gene reflected that it encoded a putative protein with an AraC binding region and a helix turn helix motif (Fig 3.8). It also shared (28 %) amino acid sequence identity with RhaR of *E. coli* which has been shown to act as a positive regulator and is a member of the AraC/XylS family of regulators (Gallegos *et al.*, 1997).

Figure 3.8: Schematic representation of *B. thetaiotaomicron* VPI-5482 RhaR amino acids showing conserved domains of the AraC_binding and the helix-turn-helix motifs. (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

The AraC/XylS family consists of positive transcriptional regulators that have been shown to regulate a diverse number of genes and their functions. Some of these control single operons or genes, and others control multiple genes forming a complex regulatory network (Tobes and Ramos, 2002; Gallegos *et al.*, 1997). Approximately 100 proteins are known to belong to the AraC/XylS family, and a few examples are given below. The proteins from this family can be divided into three main groups based on their regulatory functions, namely, the regulation of carbon metabolism, stress response and pathogenesis (Gallegos *et al.*, 1997). AraC/XylS family of proteins are found in many organisms, such as *E. coli, S. typhimurium, Citrobacter freundii* and *Pseudomonas putida*. Some of the proteins that are involved in the regulation of carbon metabolism include, AraC, RafR, RhaS/RhaR and XylS which are reported to regulate the degradation of arabinose, raffinose, rhamnose and xylose, respectively. Stress response regulation in *E. coli* and *S. typhimurium* involves the SoxS positive regulator protein. An example of a positive regulatory protein involved in pathogenesis is HrpB from *Burkholderia solanacearum*.

The conserved features of AraC/XylS positive regulators are the size of the proteins and the location of the conserved domains. Members of this family of regulators are 200-300 residues long, with a few exceptions like HrpB from *B. solanacearum*, the Ada protein from *Mycobacterium tuberculosis*, and Ybbb from *Bacillus subtilis*, all of which are 500 residues long. All members of this family of regulators contain a 17 residue conserved consensus domain (Gallegos *et al.*, 1997).

In *E. coli*, the rhamnose pathway is regulated by RhaSR (Chapter 2, Fig 2.9B) where RhaS is 30 % identical to RhaR at the amino acid level (Wickstrum *et al.*, 2005). In *E. coli*, RhaR is the transcriptional activator of the *rhaRS* operon, whereas RhaS is the transcriptional activator of the genes required for rhamnose catabolism (*rhaBAD*), as well as the rhamnose transporter gene, *rhaT* (Wickstrum and Egan, 2004; Gallegos *et al.*, 1997). In *S. typhimurium* (Chapter 2, Fig 2.9C) the RhaR and RhaS are also part of the AraC/XylS family of regulators and act in a similar manner to the *E. coli* orthologues (Nishitani and Wilcox, 1991).

Based on the analysis of AraC/XylS family of regulators conducted by Gallegos *et al.* (1997), the AraC regulator was found to contain a conserved consensus domain of 17 residues that is part of the C-terminus helix turn helix motif. However, sequence conservation at the N-terminus helix turn helix motif was low. It was also reported that while AraC might contact DNA sequences via two helix turn helix motifs, this may not be a general rule for members of this family. In order to analyze the putative conserved domains of RhaR in *B. thetaiotaomicron*, the RhaR amino acid sequence from the *E. coli*, *S. typhimurium* and *B. thetaiotaomicron* were aligned using Lynnon Biosoft DNAMAN software v 4.13 and the NCBI BLAST program (Altschul *et al.*, 1997), to observe if there were any conserved sequences within RhaR (Fig 3.9).

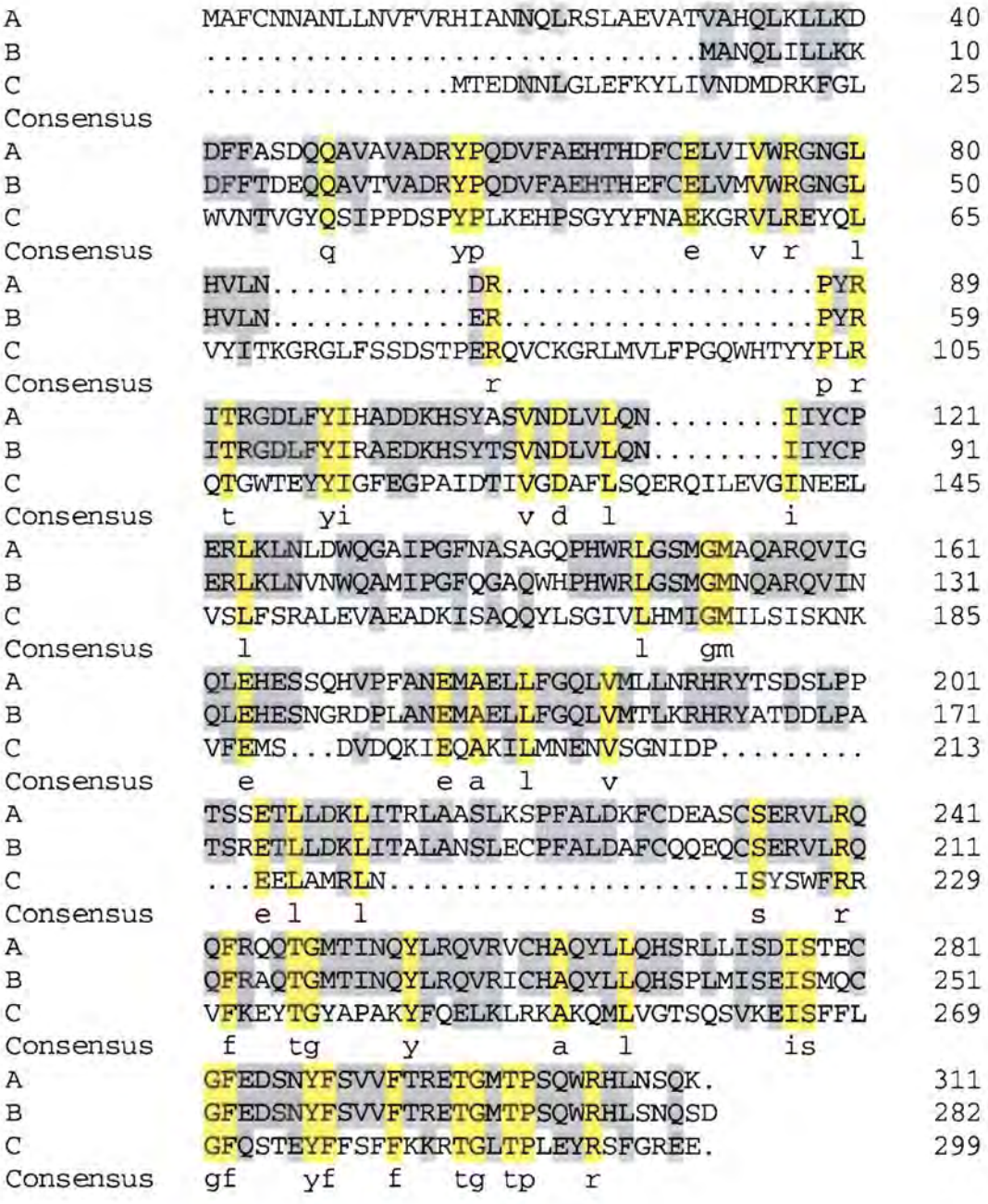


Figure 3.9: Multiple sequence alignment of RhaR. Amino acid sequences are shown for RhaR from (A) *E. coli* K12, (B) *S. Typhimurium* LT2 and (C) *B. thetaiotaomicron* VPI-5482. Amino acid residues that are identical in all sequences (100 %) are indicated in yellow shading and are displayed in the consensus lane. Residues that are 50 % conserved are indicated in grey shading.

Sequence alignment showed that 13 of the 17 highly conserved residues that constitute the C-terminus helix turn helix reported by Gallegos *et al.* (1997), have been identified in the RhaR amino acids of *B. thetaiotaomicron* VPI-5482 (Table 3.4).

AraC Family consensus[a]	A-----S---L---F----G----------R---A---L--------I/V-- I/V----GF-----F---FK/R---G--P---R
***B. thetaiotaomicron* VPI-5482 *rhaR* conserved residues**[b]	-------S--------F----G----------A---L---------I----------GF-----F---F--------G--P---R

Table 3.4: Comparison of the amino acid sequence for the AraC family of positive transcriptional regulators and *B. thetaiotaomicron* VPI-5482 RhaR.

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

b *B. thetaiotaomicron* VPI-5482 RhaR conserved amino acid residues from positions 223 to 292 in Fig 3.9.

Taken together, the functional, genetic and bioinformatic data suggests that the *B. thetaiotaomicron* VPI-5482 RhaR is the positive regulator of the rhamnose operon, since the presence of the active protein is a requirement for the growth on rhamnose as the sole carbon source.

3.5 CONCLUSIONS

In order to understand how the transposon insertion in an intergenic region of the putative rhamnose pathway was causing the metronidazole resistance phenotype, studies needed to be conducted to understand and confirm if the genes in this gene cluster were, in fact, involved in rhamnose utilization in *B. thetaiotaomicron* VPI-5482.

The results of this study verify that the genes in this cluster are involved in rhamnose catabolism, as they were shown to be induced by the presence of this substrate in the growth medium. It is possible that other metabolites of $_{L}$-rhamnose may also induce the rhamnose gene cluster, and future studies should be conducted to create point mutations in each of structural genes and determine if transcriptional activity is observed from these mutants grown in the presence of $_{L}$-rhamnose (Moralejo *et al.*, 1993).

The rhamnose genes in *B. thetaiotaomicron* VPI-5482 were also shown to be transcribed as a single operon (*rhaKIPAO*). Inactivation of the *rhaR* gene in the parent strain resulted in a mutant that was unable to utilize $_{L}$-rhamnose as the sole carbon source. However, it was able to utilize $_{D}$-glucose and $_{L}$-fucose confirming that RhaR plays a critical role as a positive regulator of the rhamnose pathway in *B. thetaiotaomicron* VPI-5482.

Future studies should involve two areas of analysis. Firstly, the function of each of the catabolic genes in this pathway should be identified. In order to do this, gene specific mutations in each of the catabolic genes could be created and their functional roles characterized (Hooper *et al.*, 1999; Richardson *et al.*, 2004). In order to identify the biochemical properties of the RhaR and the

rhamnose catabolic enzymes in *B. thetaiotaomicron* VPI-5482, purified proteins can be produced as described in Tobin and Schlief (1990).

Secondly, in order to confirm that RhaR is in fact the positive regulator, the DNA binding site for RhaR protein should be determined. This can be done using deletion mapping of the promoter regions and DNA mobility shift assays (Egan and Schleif, 1993). In studies conducted in *E. coli*, purification of the AraC DNA binding domain permitted measurements of the protein's specific activity, as well as of its DNA binding affinity (Timmes *et al.*, 2004). In this study on the *B. thetaiotaomicron* promoters several inverted repeats were identified and their possible role in RhaR binding should be evaluated. This can be done using DNase I footprinting and mutational analysis. These techniques were successful in showing that in *E. coli* RhaS binds to an inverted repeat region of the DNA in the promoter P_{rhaBAD} (Tobin and Schleif, 1987).

The findings reported in this chapter represent an overview and a basic understanding of the regulation of the rhamnose gene cluster in *B. thetaiotaomicron* VPI-5482. In the context of this thesis as a whole, these findings formed the foundation for asking further questions, namely, how does the transposon insertion in an intergenic region of the rhamnose pathway affect the utilization of rhamnose in the *B. thetaiotaomicron* Tn Met^R mutant, and more importantly, how might this be causing the observed metronidazole resistance phenotype (section 2.4.2)? These questions are addressed in the following chapter.

CHAPTER 4

Effects of the transposon insertion in the *B. thetaiotaomicron* Tn Met^R mutant

4.1 ABSTRACT

The *B. thetaiotaomicron* Tn Met^R mutant was shown to utilize $_L$-rhamnose as the sole carbon source indicating that the catabolic and regulatory genes of the rhamnose cluster were still functional and had not been disrupted by the transposon. Furthermore, the *B. thetaiotaomicron* Tn Met^R mutant grew better in this defined medium than the parent strain. A metronidazole resistance phenotype was also observed in the minimal medium supplemented with $_L$-rhamnose as the sole carbon source, which was not attributed to slow growth as seen in complete medium (Chapter 2). Transcriptional hybridization studies on the *B. thetaiotaomicron* Tn Met^R mutant showed higher levels of transcription of all the *rha* genes in the operon as well as the *rhaR* gene, under both inducing and non-inducing conditions, in comparison to *B. thetaiotaomicron* VPI-5482.

In Chapter 3, BT3768 was shown to encode the positive transcriptional regulator, RhaR, of the rhamnose gene cluster. In order to confirm that the metronidazole resistance phenotype observed in *B. thetaiotaomicron* Tn Met^R was due to the up-regulation of the *rha* genes, caused by the transposon insertion upstream of *rhaR*, a *B. thetaiotaomicron* strain overexpressing the *rhaR* gene on a cytoplasmic element was constructed, *B. thetaiotaomicron* (pLYLrhaR). Physiological, genetic and biochemical analysis of this strain, grown in $_L$-rhamnose as the sole carbon source, showed that increased expression of RhaR caused a metronidazole resistance phenotype. At the molecular level, *B. thetaiotaomicron* (pLYLrhaR) showed increased transcription of all the genes in the *rha* operon in comparison to *B. thetaiotaomicron* VPI-5482. This was comparatively less then the effect observed in *B. thetaiotaomicron* Tn Met^R containing the transposon upstream of *rhaR*, and may be due the lower copy number of pLYLrhaR found in the cytoplasm of *B. thetaiotaomicron* (pLYLrhaR). The biochemical analysis of both *B. thetaiotaomicron* Tn Met^R as well as *B. thetaiotaomicron* (pLYLrhaR)

showed that in both these strains, the lactate dehydrogenase (LDH) levels were elevated, and pyruvate oxidoreductase levels were depleted, relative to the *B. thetaiotaomicron* VPI-5482 parent strain. Both of these are key enzymes that are implicated in metronidazole activation (Narikawa *et al.,* 1991; Sindar *et al.,* 1982; Britz and Wilkinson, 1979).

4.2 INTRODUCTION

The aim of this study was to determine how the transposon insertion was affecting the regulation of the rhamnose pathway in *B. thetaiotaomicron* Tn Met^R, and whether it played a role in the observed metronidazole resistance (Chapter 2).

In Chapter 2, it was reported that *B. thetaiotaomicron* Tn Met^R displayed a metronidazole resistance phenotype, as well as slower growth in complete medium when compared to the parent strain. It was possible that the observed resistance phenotype was caused by a slower rate of metronidazole activation due to the slower growth. Therefore, it was of interest to determine if this strain would display resistance to metronidazole if slow growth was not a variable. In addition, it was important to establish whether the transposon insertion in an intergenic region of the rhamnose gene cluster in *B. thetaiotaomicron* Tn Met^R had affected the functionality of this rhamnose pathway and whether the transposon was responsible for causing the metronidazole resistance phenotype by specifically affecting the expression of the rhamnose genes.

The transposon insertion in the *fucO-rhaR* intergenic region may have affected FucO, RhaR or possibly both. It may have disrupted *fucO*, preventing the active formation of FucO. Briefly, as mentioned in Chapter 2, the *fucO* gene codes for a putative lactaldehyde reductase also known as a L-1,2-propanediol oxidoreductase, which is responsible for reducing L-lactaldehyde to

L-1,2-propanediol, using NADH as a cofactor (Montella *et al.*, 2005). In this regard, a biochemical assay to test for the production L-1,2-propanediol would verify the functionality of FucO in *B. thetaiotaomicron* Tn Met^R. This biochemical assay would also provide insight into rhamnose catabolism in *B. thetaiotaomicron* VPI-5482, as there have been no published reports on propanediol production in this organism.

Alternatively, the transposon may have affected the expression of the positive regulator, RhaR. The transposon, Tn*4400*' is flanked by two insertion sequence (IS) elements, IS*4400*R and IS*4400*L. IS elements have been shown to activate genes in *B. fragilis* (Podglajen *et al.*, 1994; Podglajen *et al.*, 2001), in which case, increased transcription of a positive regulator may result in increased levels of RhaR and possibly in turn, the rest of the rhamnose catabolic genes. In *B. thetaiotaomicron* Tn Met^R, both the IS elements (IS*4400*R and IS*4400*L) are in the same orientation as the positive transcriptional regulator, RhaR (Fig. 4.1). Furthermore, IS*4400*L may be located just upstream of the promoter region of the transcriptional regulator.

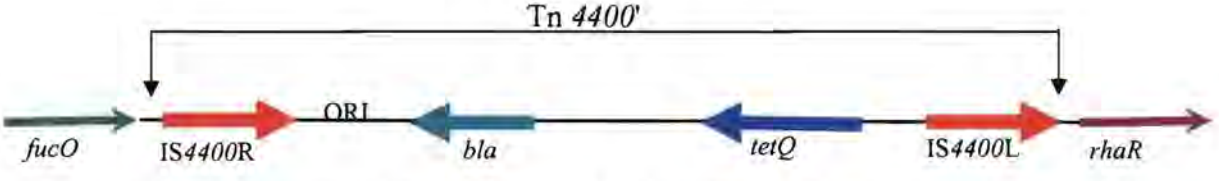


Figure 4.1: Schematic representation (not to scale) of the transposon insertion between *fucO* and *rhaR*. Arrows in red represent insertion sequence elements (IS*4400*L and IS*4400*R). Dark blue arrow represents, *tetQ* gene expressing tetracycline resistance in *Bacteroides* species, and the light blue arrow represents the *bla* gene. Green and purple bands represent the *fucO* and *rhaR* chromosomal genes, respectively.

In order to investigate the effects of the transposon insertion on the expression of the *rha* genes in the *B. thetaiotaomicron* Tn Met^R mutant, transcriptional hybridization studies were used as described by Kullin *et al.* (2006). In addition, the insertion site was analyzed at the DNA level relative to the *rhaR* promoter, P_{rhaR}. If the transposon insertion has affected the *rha* genes, and shown to be responsible for the metronidazole resistance phenotype observed in this mutant,

then a similar mutant would need to be recreated to verify that no other silent mutations exist within *B. thetaiotaomicron* Tn Met^R.

As discussed in Chapter 1, previous studies have reported in the literature that metronidazole resistant mutants of several different organisms displayed higher levels of lactate dehydrogenase (LDH) activity and depleted pyruvate ferredoxin oxidoreductase activity (Sindar *et al.,* 1982; Britz and Wilkinson, 1979; Narikawa *et al.,* 1991). Sindar *et al.* (1982) found that *C. perfringens* mutants displayed this phenotype. Similarly, Diniz *et al.* (2004) did not detect any pyruvate oxidoreductase activity in *B. fragilis* metronidazole resistant mutants, but higher levels of LDH activity were measured. Determining the levels of LDH and pyruvate oxidoreductase activity in the *Bacteroides* strains of this study, may provide insight into the metronidazole resistance phenotype being observed (Chapter 2), given that pyruvate oxidoreductase is a key enzyme in the activation of metronidazole (Chapter 1).

4.3 MATERIALS AND METHODS

4.3.1 Bacterial Strains and growth conditions

All the *B. thetaiotaomicron* strains were grown anaerobically on BHIS as described in Section 2.3.1. *E. coli* DH5α and S17-1 strains have been described previously (Rasmussen *et al.,* 1994; Simon *et al.,* 1983, respectively) and were grown aerobically on Luria-Bertani (LB) broth or agar (1.5% w/v) at 37°C. The antibiotics gentamicin (200 μg/ml), ampicillin (100 μg/ml), and tetracycline (2 μg/ml) were added to BHIS and/or LB whenever necessary. Plasmid pLYL01 has been described previously (Li *et al.,* 1995).

4.3.2 General Recombinant DNA procedures

B. thetaiotaomicron genomic DNA was prepared according to the method outlined by Wehnert *et al.* (1992). *E. coli* plasmid DNA was prepared by using the QIAGEN® Plasmid Midi Kit (25) (QIAGEN). Restriction enzyme digestions were carried out according to manufacturer's instructions.

4.3.3 Growth of *B. thetaiotaomicron* VPI-5482 and *B. thetaiotaomicron* Tn Met^R mutant in defined medium supplemented with $_L$-rhamnose

B. thetaiotaomicron VPI-5482 and *B. thetaiotaomicron* Tn Met^R were grown under anaerobic conditions at 37°C in defined medium supplemented with 0.1 % $_L$-rhamnose as described by Van Tassel and Wilkinson (1978). Cultures were grown separately in 10 ml volume of BHIS broth for 16 h. Cultures were then diluted (1:50) in 10 ml of defined broth medium and growth of these cultures was monitored at hourly intervals by recording optical density (OD_{600}) using a Beckman DU®530 Lifescience UV/Vis Spectrophotometer.

4.3.4 RNA extractions

The *B. thetaiotaomicron* strains were grown for 16 h in 50 ml defined medium supplemented with 0.1 % of either $_D$-glucose (Merck) or $_L$-rhamnose (Sigma®), and the total RNA was isolated according to method of Aiba *et al.* (1981). The quality of the RNA was confirmed by electrophoresis in 1.5 % denaturing formaldehyde agarose gel (Fourney *et al.*, 1988) and it was quantified using the NanoDrop® ND-100 spectrophotometer (Nano Drop Technologies, Inc).

4.3.5 Construction of DNA probes for transcriptional hybridization studies

Internal fragments of the genes *rhaK, rhaI, rhaP, rhaA, fucO* and *rhaR* were used to make DNA probes. This method has been described previously in Section 3.3.4.

4.3.6 RNA Transcriptional hybridization studies

RNA was extracted from *B. thetaiotaomicron* strains grown in defined medium supplemented with 0.1 % of either $_{D}$-glucose (Merck) or $_{L}$-rhamnose (Sigma®) and equal amounts (1 µg), were spotted in equal volumes onto a nylon membrane (Roche). The membranes were hybridized with the DIG-labelled DNA probes. Hybridization and detection procedures were performed according to the manufacturers' instructions. Chemiluminescent signals were detected using CDP® Star (Roche).

4.3.7 L-1, 2-propanediol assays

All the *B. thetaiotaomicron* cultures were grown separately in defined broth media supplemented with $_{L}$-rhamnose under anaerobic conditions at 37°C. After 16 h growth, cultures were centrifuged, and the concentration of L-1, 2- propanediol in the culture supernatant was measured in Molar (M) units by the method of Jones and Riddick (1957) using 1,2- propanediol (Merck) as a standard. The experiment was performed in triplicate, and a total of three readings were taken for each assay.

4.3.8 Construction of *B. thetaiotaomicron* VPI-5482 *rhaR* overexpresser

The plasmid, pLYLrhaR, containing the full length fragment of the *rhaR* gene and 239 bp upstream of the gene, was constructed using pLYL01 as described in Section 3.3.10 (Li *et al.*, 1995). The plasmid was extracted from transformed *E. coli* DH5α, purified and was retransformed into *E. coli* S17-1 according to standard methods (Sambrook *et al.*, 1989). *E. coli* S-17 (pLYLrhaR) was conjugated with *B. thetaiotaomicron* VPI-5482, as described previously. The resulting *B. thetaiotaomicron* (pLYLrhaR) was grown on BHIS agar with tetracycline (2 µg/ml). Confirmation of the presence of pLYLrhaR in the cytoplasm of *B. thetaiotaomicron* VPI-5482 was carried out according to Section 3.3.10.

4.3.9 Metronidazole survival studies in defined medium

Mid-logarithmic phase cultures of the *B. thetaiotaomicron* cultures were exposed separately to 15 μg/ml of metronidazole in defined medium supplemented with L-rhamnose. Cultures were incubated anaerobically at 37°C. Samples were removed at different time intervals, diluted appropriately and plated onto BHIS agar to determine the surviving fraction from viable counts.

4.3.10 Preparation of cell free extracts (CFE)

Cell free extracts (CFE) of the *B. thetaiotaomicron* cultures were prepared anaerobically. Cells were harvested by centrifugation (8K, 4°C, 10 min) after 16 h growth in defined media supplemented with L-rhamnose. Pellets were washed three times under anaerobic conditions in 10 mM Tris-HCL, pH 8.0 (for the lactate dehydrogenase assay), or 10 mM phosphate buffer with 250 μM β-mercaptoethanol, pH 7.0 (for the pyruvate oxidoreductase assay), and resuspended in 5 ml of the same buffers, respectively. Cells were ruptured by sonication using an MSE sonicator at amplitude of 18-24 μM for periods of 30 s/ml of cell suspension in tubes chilled at 0°C. Cell debris was removed by centrifugation (8K, 4°C, 20 min), the supernatant was collected (CFE), and protein concentrations were determined by the method of Bradford (1976) using BioRad reagents according to the manufacturer's instructions (Bio Rad Laboratories).

4.3.11 Lactate dehydrogenase assays

Enzyme assays were carried out by using the method outlined in Abbe *et al.* (1982). Lactate dehydrogenase (LDH) activity was measured anaerobically at 37°C by the rate of decrease in extinction coefficient of NADH at an absorbance of 340 nm using a Beckman DU®530 Lifescience UV/Vis Spectrophotometer. The assay mixture consisted of 0.24 mM NADH, 0.50 mM fructose-1,6-bisphosphate and 0.40 mM sodium pyruvate in a final volume of 1 ml, and

1 mg of protein was used. The reaction was started by adding the pyruvate substrate. A standard curve was set up using various concentrations of NADH prior to the assay and enzymatic activity was recorded as μM NADH/mg protein/min at 340nm. This experiment was performed in triplicate, and a total of five readings were taken for each assay.

4.3.12 Pyruvate oxidoreductase assay

Enzyme assays were carried out by using the method outlined in Lindmark and Müller (1973). Pyruvate oxidoreductase activity was measured spectrophotometrically at an absorbance of 600nm using a Beckman DU®530 Lifescience UV/Vis Spectrophotometer, with methyl viologen as the electron acceptor at 37°C under anaerobic conditions. The assay mixture consisted of 10 mM potassium phosphate buffer (pH 7.0), 2.5 mM potassium pyruvate, 250 mM β-mercaptoethanol, 0.25 mM CoA in a final volume of 1 ml, and 1 mg of protein was used. The reaction was started by adding the pyruvate, and the change in absorbance at 600nm was recorded before and after the addition of pyruvate. The difference in the measure of the slope was taken as the measure of enzyme activity and was recorded as μM of methyl viologen/mg protein/min at 600nm. This experiment was performed in triplicate, and a total of five readings were taken for each assay.

4.4 RESULTS AND DISCUSSION

4.4.1 Growth of *B. thetaiotaomicron* VPI-5482 and *B. thetaiotaomicron* Tn Met^R in defined medium containing $_L$-rhamnose

Growth studies of *B. thetaiotaomicron* VPI-5482 in comparison with the *B. thetaiotaomicron* Tn Met^R mutant were initially conducted in complete medium (Chapter 2), and the results showed that the mutant grew more slowly than the parent strain. Since the transposon insertion had been found to occur within the rhamnose gene cluster, it was necessary to determine if it

had affected the ability of the *B. thetaiotaomicron* Tn Met^R mutant to utilize $_L$-rhamnose as a sole carbon source. Therefore, growth studies were also performed in defined broth medium supplemented with $_L$-rhamnose (Fig 4.2).

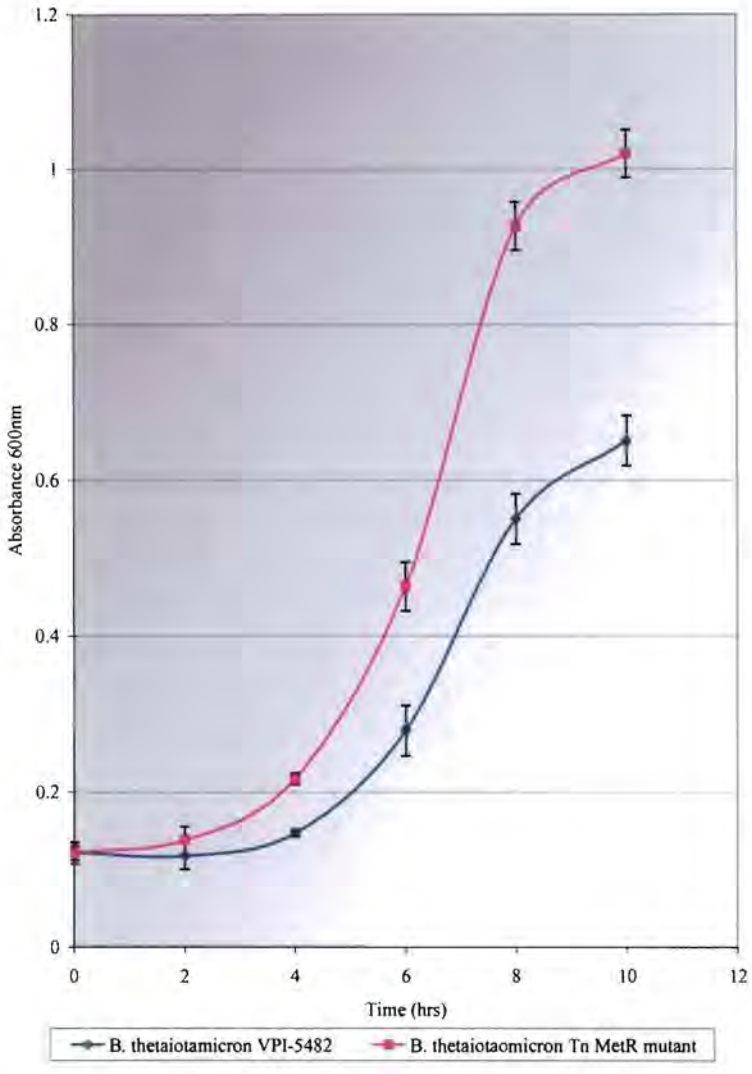


Figure 4.2: Growth pattern of *B. thetaiotaomicron* VPI-5482 and *B. thetaiotaomicron* Tn Met^R in defined medium supplemented with $_L$- rhamnose as sole carbon source. Data points represent the mean values of 3 experiments. Standard error of the experiments is shown as error bars.

Growth in the broth medium supplemented with $_L$-rhamnose as the sole carbon source (Fig 4.2), showed that *B. thetaiotaomicron* Tn Met^R grew better than the parent strain, indicating that the transposon insertion had not inhibited the utilization of $_L$-rhamnose in this mutant. The wild type strain, never reached the same O.D as the *B. thetaiotaomicron* Tn Met^R mutant even after

24 hr (data not shown). These findings indicated that the transposon insertion in the *fucO-rhaR* intergenic region had not disrupted the functioning of any of the enzymes expressed by the genes of the rhamnose operon, but may even have enhanced their expression resulting in better growth in the presence of rhamnose. RNA transcriptional hybridization studies were, therefore, undertaken to determine if this was so.

4.4.2 Transcriptional hybridization analysis of *B. thetaiotaomicron* VPI-5482 and *B. thetaiotaomicron* Tn Met^R under inducing and non-inducing conditions

In section 4.2, it was proposed that, since the transposon is flanked with IS elements, and is integrated between the *fucO-rhaR* genes, it may be possible that it was affecting the expression of the *rhaR* encoding the regulator of the rhamnose operon. In addition, the *B. thetaiotaomicron* Tn Met^R mutant grew better than the parent strain in the presence of rhamnose. Transcriptional analysis of the RNA was, therefore, conducted after growth in the presence of rhamnose (inducing conditions) or glucose (non-inducing conditions) (Fig 4.3A and Fig 4.3B) to determine if there were changes in the expression of the *rha* genes and if this may be linked to the metronidazole resistance phenotype.

During growth in the presence of rhamnose (Fig 3.4A), *B. thetaiotaomicron* Tn Met^R (**M**) showed an increase in the transcription of each of the rhamnose genes in comparison to *B. thetaiotaomicron* VPI-5482 (**WT**). During growth in the presence of glucose (Fig 3.4B), transcription of the genes of the *rha* cluster in the parent strain (**WT**) was barely detectable with the exception of the *rhaR* gene, coding for the positive regulator, which may be constitutively expressed under these conditions. In contrast, however, *B. thetaiotaomicron* Tn Met^R showed increased transcription even in the absence of rhamnose.

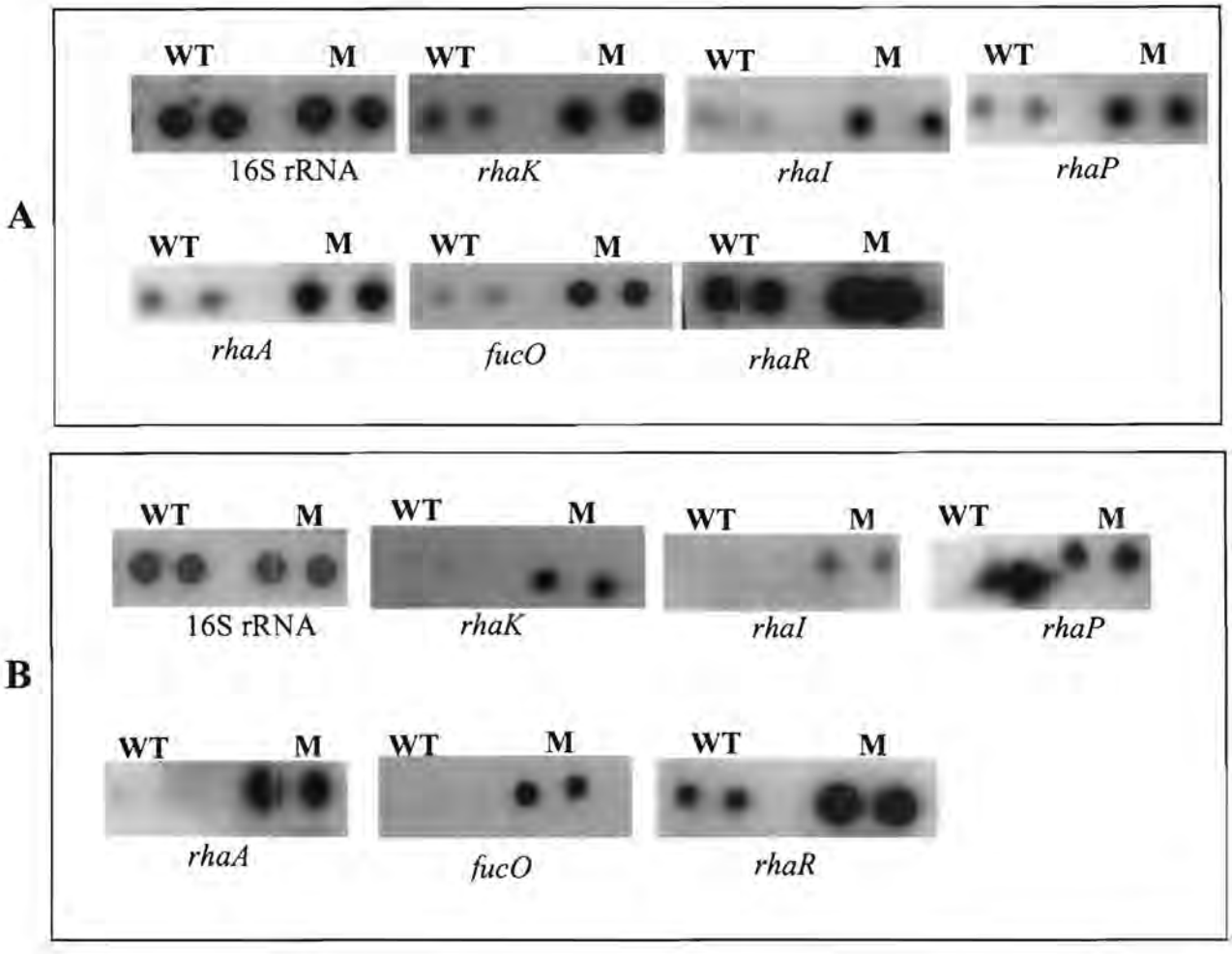


Figure 4.3: Transcription of the *rha* genes in *B. thetaiotaomicron* VPI-5482 (**WT**) and *B. thetaiotaomicron* Tn Met^R (**M**) as shown by dot blots analysis under inducing **(A)** and non-inducing **(B)** conditions. Cultures were grown anaerobically in $_L$-rhamnose **(A)** or $_D$-glucose **(B)** medium. An equal (2 µl) volume and concentration of total RNA (1 µg) were spotted in duplicate onto a nylon charged membrane. Membranes were probed with DNA probes specific for each of the *rha* genes as described in Section 3.3.4. The 16S rRNA gene (an internal control) was also used as a probe to show that an equal amount of RNA was loaded onto each membrane.

This data, suggested that the transposon insertion in *B. thetaiotaomicron* Tn Met^R was increasing transcription of the *rhaR* gene which, in turn, could up-regulate the expression of the whole operon, *rhaKIPAO*. Furthermore, transcription of the rhamnose genes in the mutant did not require rhamnose as an inducer since the increased transcription was seen in RNA extracted from *B. thetaiotaomicron* Tn Met^R grown in the presence of glucose as the sole carbon source.

Menon and Lee (1990) conducted studies on the transcriptional activation of the *E. coli* arabinose (*araBAD*) operon. Briefly, AraC, regulates the transcription of the arabinose operon in *E. coli,* and is a member of the AraC/XylS family of positive transcriptional regulators (Gallegos *et al.,* 1997). The protein binds $_{L}$-arabinose, the inducer, to activate transcription of the *ara* operon (Menon and Lee, 1990). It has been reported that AraC is biologically active in ligand-free and ligand-bound forms; therefore, in the absence of $_{L}$-arabinose, AraC activates the *araBAD* operon promoter, P_{BAD}, to 1 % in comparison to the induced level (Menon and Lee, 1990). In the *B. thetaiotaomicron* Tn Met^{R} mutant, it is evident that an inducer is not required to constitutively transcribe the rhamnose genes. Growth in the presence of rhamnose, however, further induces the transcription of the RhaR and hence the rhamnose operon, *rhaKIPAO.*

4.4.3 Analysis of IS*4400*L in relation to the RhaR promoter region in the *B. thetaiotaomicron* Tn Met^{R} mutant

A bioinformatic approach was used to determine how the transposon may have been responsible for the overexpression of *rhaR.* Based on the location of the transposon, and the orientation of IS*4400*L relative to the transcriptional direction of *rhaR* (Fig 4.1), it was possible that the IS element may be acting as an additional promoter (Fig 4.4).

Figure 4.4 illustrates the DNA sequence obtained from sequence analysis of the transposon insertion site relative to the promoter region and coding sequence of *rhaR.* Three possible typical *Bacteroides* putative -7 hexamers were identified within the IS*4400*L sequence, as highlighted in yellow.

Figure 4.4: Position of IS*4400*L in relation to transcriptional start site of *rhaR*. Nucleotide sequence of IS*4400*L (purple letters), the flanking *B. thetaiotaomicron* nucleotide sequence (black letters), resident -7 and -33 hexamers and transcriptional start site (red and blue letter/s underlined, respectively), and ATG start codon of *rhaR* (orange letters underlined). The putative -7 hexamers within the IS element are highlighted in yellow. (Mahillon and Chandler, 1998).

It not unusual for IS elements to act as strong promoters and activate genes (Mahillon *et al.*, 1999; Schneider and Lenski, 2004). IS elements are less than 2.5 kb in size, contain inverted repeats (IR) and are genetically compact DNA sequences encoding functions involved in translocation within and between bacterial genomes (Mahillon *et al.*, 1999). Nearly the entire IS element is consumed by 1 or 2 ORFs that encode the Tpase enzyme, and the IR found in the IS elements assist in Tpase binding (Mahillon and Chandler, 1998). There are over 600 IS elements identified, and approximately 20 distinct groups (Mahillon and Chandler, 1998; Mahillon *et al.*, 1999).

Many studies have shown that IS elements, or in some cases, compound transposons that are flanked by IS elements, can activate genes. For example, Ciampi *et al.* (1982) characterized Tn*10* transposon in *S. typhimurium* mutants and showed that when Tn*10* was inserted in the ribosomal RNA region of the histidine (*his)* operon there was weak expression of the *his* genes downstream that were not detected in the parent strain. In other studies, IS*1* and IS*5* elements

have been shown to activate the *bgl* operon in *E. coli*, irrespective of the elements being inserted upstream or downstream of the promoter region (Reynolds *et al.*, 1981).Yet again, the integration of IS*1* and IS*5* elements in the flagellar regulon (*fhlD*) region of *E. coli* mutants resulted in increased motility compared to the parent strain (Barker *et al.*, 2004). IS*1*, IS*2* and IS*5* have been shown to have outwardly directed -35 promoter hexamers that are located in the terminal IR of the IS elements (Mahillon and Chandler, 1998). During transposition events, if these elements are integrated at the appropriate distance from the host's -10 hexamer, then new promoters are created, expressing the genes downstream.

In *Bacteroides* spp., approximately 9 IS elements have been found, belonging to a range of IS families, viz: 1 from the IS*30* family, 2 from IS*4* and IS*21* and 4 from IS*5* family (Mahillon and Chandler, 1998). IS*4400*, IS*4351*L and IS*4351*R have been categorized under the IS*30* family having one and the same name, IS*4351* that was originally identified in *B. fragilis* V479-1 (Accession N^{o} M17124). Soki *et al.* (2006) reported that nine *nimA* genes were activated by IS*1168* and three of the *nimC* genes were activated by IS*1170*. Podglajen *et al.* (1994) reported that IS*1186* activated the expression of the carbapenem gene, *cfiA* in *B. fragilis* causing resistance. They later identified the typical *Bacteroides* consensus promoter sequences within two IS elements, IS*1187* and IS*1188* that were found upstream of *cfiA* (Podglajen *et al.*, 2001). Based on the analysis of the IS*4400*L in relation to RhaR, there is a strong possibility that the IS element may be activating the *rhaR* gene, possibly in the absence and presence of the substrate, $_{L}$-rhamnose, as observed in the transcriptional studies.

4.4.4 Enzymatic assays of L-1,2-propanediol

Hybridization analysis (Section 4.4.2), showed that there was transcription of the *fucO* gene during growth in the presence of the substrate $_{L}$-rhamnose. However, the functionality of the

FucO protein was still in question since the transposon insertion may have disrupted the end of the *fucO* gene. It was, therefore, of interest to determine the functionality of FucO in *B. thetaiotaomicron* Tn Met^R in relation to its parent strain. This was done using a biochemical assay to test for the production of L-1,2-propanediol during growth in rhamnose (Table 4.1). As mentioned earlier (Section 4.2, Chapter 1) the *fucO* gene codes for an oxidoreductase which, in *E. coli*, has been found oxidize L-lactaldehyde to L-1,2-propanediol under anaerobic conditions (Montella *et al.*, 2005).

Strains	L-1,2-propanediol (M)
B. thetaiotaomicron VPI-5482	0.166 (± 0.123)
B. thetaiotaomicron Tn Met^R	0.366 (± 0.259)

Table 4.1: L-1,2-propanediol activity measured in supernatant of *B. thetaiotaomicron* VPI-5482 and *B. thetaiotaomicron* Tn Met^R. Data represents the mean value of three experiments, and the standard deviation for the experiments is shown in brackets (±).

In this study, the results of the assay indicated that levels of L-1,2-propanediol produced by *B. thetaiotaomicron* Tn Met^R were not significantly different from those produced by *B. thetaiotaomicron* VPI-5482. The detection of L-1,2-propanediol in *B. thetaiotaomicron* VPI-5482 culture supernatant is the first report in this organism of anaerobic $_L$-rhamnose catabolism leading to the production of L-1,2-propanediol from L-lactaldehyde.

The results of this study confirmed that the transposon insertion had not disrupted the *fucO* gene and suggested that the effects of the transposon insertion in the mutant may solely be due to up-regulation the gene coding for a positive transcriptional regulator, *rhaR*, and hence increased transcription of the rhamnose gene cluster.

4.4.5 Construction of *B. thetaiotaomicron* overexpressing the positive regulator, RhaR

In Section 4.4.3 it was shown that *B. thetaiotaomicron* Tn Met^R containing a transposon flanked with an IS element upstream of the promoter region of the *rhaR* gene encoding the positive regulator, may be able to activate this gene. This together with the hybridization studies (Section 4.4.2) showed that the transposon insertion in *B. thetaiotaomicron* Tn Met^R caused an overexpression of the regulator and, in turn, the rest of the rhamnose catabolic genes. In order to re-create the transposon mutant, and determine whether the metronidazole resistance phenotype was caused by the up-regulation of RhaR, an additional copy of *rhaR*, together with its own promoter region, was introduced into *B. thetaiotaomicron* VPI-5482 on a multicopy plasmid. Plasmid pLYLrhaR was conjugated into *B. thetaiotaomicron* VPI-5482, to create *B. thetaiotaomicron* (pLYLrhaR), and its presence in the cytoplasm was verified as described in Section 3.3.10 by tetracycline resistance, plasmid extraction and sequencing.

4.4.5.1 Transcriptional hybridization analysis of *B. thetaiotaomicron* VPI-5482 and *B. thetaiotaomicron* (pLYLrhaR)

In order to determine that the rhamnose genes were being overexpressed in a similar manner to that observed in *B. thetaiotaomicron* Tn Met^R, transcriptional hybridization studies were performed on RNA extracted from *B. thetaiotaomicron* (pLYLrhaR) and *B. thetaiotaomicron* VPI-5482 grown in the presence of $_L$-rhamnose (Fig 4.5).

The results showed an increased amount of transcript from all the *rha* genes from *B. thetaiotaomicron* (pLYLrhaR) in comparison to the parent strain. The difference in transcriptional activity was, however, not as pronounced as seen in *B. thetaiotaomicron* Tn Met^R compared to the parent strain. This may be due to a lower level of RhaR, expressed from the pLYLrhaR plasmid in *B. thetaiotaomicron* (pLYLrhaR) as compared to the levels in the

B. thetaiotaomicron Tn Met^R mutant. pLYL01 from which pLYLrhaR was constructed is a low copy number plasmid and therefore lower expression levels were observed.

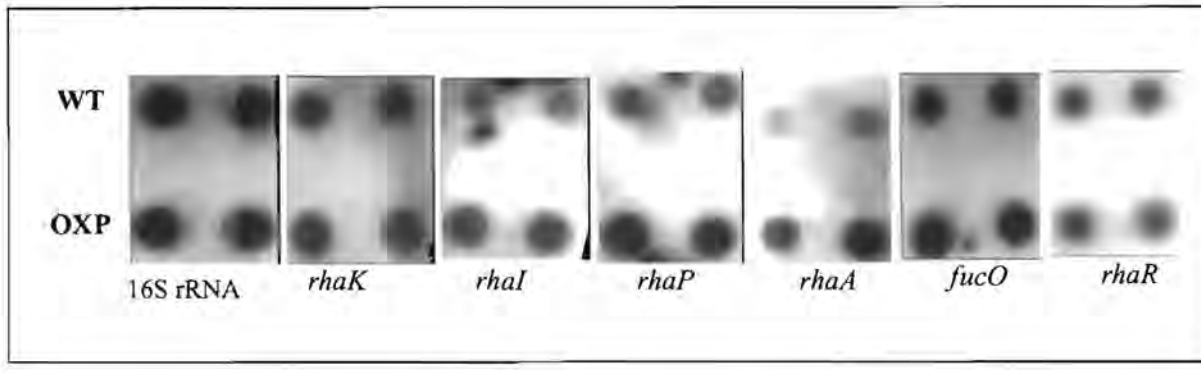


Figure 4.5: Transcription of the *rha* genes of *B. thetaiotaomicron* VPI-5482 (**WT**) and *B. thetaiotaomicron* (pLYLrhaR) (**OXP**) as shown by dot blots analysis under inducing conditions. Cultures were grown anaerobically in L-rhamnose medium. An equal (2 µl) volume and concentration of total RNA (1 µg) were spotted in duplicate onto a nylon charged membrane. Membranes were probed with DNA probes specific for each of the *rha* genes as described in Section 3.3.4. The 16S rRNA gene (an internal control) was also used as a probe to show that an equal amount of RNA was loaded onto each membrane.

4.4.6 Metronidazole survival studies in defined medium supplemented with L-rhamnose

Survival studies of *B. thetaiotaomicron* Tn Met^R in complete medium had shown slower growth and metronidazole resistance relative to the parent strain (Chapter 2). It may be possible that the metronidazole resistance phenotype in the complete medium may have been due to the slow growth. Therefore, in order to determine if *B. thetaiotaomicron* Tn Met^R would still display a metronidazole resistance phenotype, it was exposed to metronidazole in a defined medium supplemented with L-rhamnose as the sole carbon source, where the mutant grew better than the parents strain (Fig 4.2). It was also of interest to determine if overexpression of the rhamnose genes was linked to metronidazole resistance. *B. thetaiotaomicron* (pLYLrhaR) was, therefore, also exposed to metronidazole under the same growth conditions as *B. thetaiotaomicron* Tn Met^R. The cultures growing in the minimal medium supplemented with L-rhamnose were

exposed to 15 μg/ml of metronidazole and sampled at different time intervals to monitor viability as the log surviving fraction (Table 4.2).

Strains	Log surviving fraction after 40 min in L-rhamnose
B. thetaiotaomicron VPI-5482	- 2.78 (± 0.001)
B. thetaiotaomicron Tn Met^R	- 1.65 (± 0.007)
B. thetaiotaomicron (pLYLrhaR)	- 1.88 (± 0.010)

Table 4.2: Log surviving fraction of the *Bacteroides* strains exposed to 15 μg/ml of metronidazole in defined broth medium supplemented with L-rhamnose as sole carbon source after 40 min. Data represents the mean value of three experiments, and the standard deviation for the experiments is shown in brackets (±).

Exposure of *B. thetaiotaomicron* Tn Met^R and *B. thetaiotaomicron* (pLYLrhaR) to metronidazole in this defined medium showed that they were more resistant to metronidazole than *B. thetaiotaomicron* VPI-5482. This study proved that under these growth conditions, a slower growth rate does not contribute to the resistance phenotype in *B. thetaiotaomicron* Tn Met^R but was due to increased expression of *rhaR*. The *B. thetaiotaomicron rhaR*$^-$ mutant did not grow in defined medium supplemented with L-rhamnose, therefore, sensitivity to metronidazole could not be carried out.

The metronidazole resistance in *B. thetaiotaomicron* (pLYLrhaR) was only slightly less than in the *B. thetaiotaomicron* Tn Met^R mutant, which may be attributed to the difference in the amount of RhaR being expressed by the plasmid. These results showed that overexpressing the positive transcriptional regulator, RhaR, of the rhamnose gene cluster in *B. thetaiotaomicron* VPI-5482 contributed to a metronidazole resistance phenotype. This is the first study to report that altering a carbohydrate pathway could result in metronidazole resistance.

4.4.7 Analysis of lactate dehydrogenase and pyruvate oxidoreductase activity in the parent and metronidazole resistant strains

B. thetaiotaomicron Tn Met^R and *B. thetaiotaomicron* (pLYLrhaR) had been shown to display a metronidazole resistance phenotype during growth in the presence rhamnose. In order to determine if increased expression of RhaR had affected the enzymes responsible for metronidazole activation, biochemical studies were undertaken to determine their activity (Table 4.3). Elevated levels of lactate dehydrogenase (LDH) and depleted or undetectable levels of pyruvate oxidoreductase have been implicated in metronidazole resistance (Discussed in Chapter 1).

Strains	**Lactate dehydrogenase** μM NADH/mg protein/min	**Pyruvate oxidoreductase** μM methyl viologen/mg protein/min
B. thetaiotaomicron VPI-5482	3.24 (± 3.27)	0.230 (± 0.06)
B. thetaiotaomicron Tn Met^R	16.23 (± 6.04)	0.035 (± 0.05)
B. thetaiotaomicron (pLYLrhaR)	12.08 (± 3.50)	0.022 (± 0.02)

Table 4.3: Enzymatic activity of lactate dehydrogenase and pyruvate oxidoreductase measured from cell free extracts grown anaerobically in the presence of $_L$-rhamnose from *B. thetaiotaomicron* VPI-5482, *B. thetaiotaomicron* Tn Met^R mutant and *B. thetaiotaomicron* (pLYLrhaR). Data represents the mean value of three experiments, and the standard deviation for the experiments is shown in brackets (±).

The results showed that the lactate dehydrogenase activity of *B. thetaiotaomicron* Tn Met^R and *B. thetaiotaomicron* (pLYLrhaR), during growth in the presence of $_L$-rhamnose, was higher than *B. thetaiotaomicron* VPI-5482, whereas pyruvate oxidoreductase activity in these resistant strains was very low in comparison to the parent strain (Table 4.3). These results strongly suggest that the lower pyruvate oxidoreductase activity observed in the resistant strains may be contributing to the observed metronidazole resistance phenotype since it is a key enzyme involved in metronidazole activation.

The mechanism of metronidazole resistance is not well understood. However, *in vitro* studies have shown that resistant strains displayed higher levels of lactate or lactate dehydrogenase and, on occasion, no detectable levels of pyruvate oxidoreductase, a key enzyme that is thought to activate metronidazole (Edwards, 1993). In summary, Kulda (1999) reported that *T. vaginalis* metronidazole resistant strains had no detectable pyruvate oxidoreductase activity, and a higher lactate dehydrogenase activity in comparison to the parent strain. Also reported, is that the resistant strains became homolactic fermenters, converting 92 % of glucose into lactate (Kulda, 1999). Metronidazole resistant strains of *Giardia duodenalis* and *T. vaginalis* were reported to down-regulate pyruvate oxidoreductase and ferredoxin or have no detectable pyruvate oxidoreductase activity, respectively (Upcroft *et al.,* 2006). Kaihovaara *et al.* (1998) showed that a metronidazole resistant *H. pylori* strain had lower pyruvate oxidoreductase, and reported that metronidazole susceptible strains had lower lactate dehydrogenase activity. No pyruvate oxidoreductase activity was detected in *C. perfringens* metronidazole resistant strains (Sindar *et al.,* 1982).

There have been no reports on lactate dehydrogenase or pyruvate oxidoreductase activity in any *B. thetaiotaomicron* metronidazole resistant strains. However, there have been studies carried out on the more pathogenic organism, *B. fragilis*. Narikawa *et al.* (1991) reported very high levels of lactate dehydrogenase in a *B. fragilis* metronidazole resistant strain. In a more recent study, Diniz *et al.* (2004) created *B. fragilis* metronidazole resistant strains and reported that lactate dehydrogenase was up-regulated, but flavodoxin was down-regulated and there was no detectable pyruvate oxidoreductase activity in the resistant strain. The metronidazole resistance in the *B. thetaiotaomicron* Tn Met^R mutant and *B. thetaiotaomicron* (pLYLrhaR) may be attributed to the lack of metronidazole activation and or an altered electron flux.

4.5 CONCLUSIONS

In order to evaluate how the transposon insertion in the *fucO-rhaR* intergenic had affected the *B. thetaiotaomicron* Tn Met^R mutant, as well as to determine if the transposon insertion was responsible for causing the observed metronidazole resistance phenotype, several physiological, genetic and biochemical studies were conducted. The physiological evaluation aimed to determine whether the transposon had affected the mutant's ability to utilize $_L$-rhamnose as the sole carbon source. It was also used to determine if the metronidazole resistance in this strain was related to the slow growth rate observed in complete medium (BHIS) as reported in Chapter 2. Growth studies were performed in the defined medium and the results indicated that the *B. thetaiotaomicron* Tn Met^R mutant could utilize $_L$-rhamnose, and did so better than the parent strain. Resistance to metronidazole was tested under these growth conditions, and the results demonstrated that *B. thetaiotaomicron* Tn Met^R was resistant to 15 μg/ml of metronidazole regardless of the growth rate.

The increase in growth rate under the inducing conditions was examined at the genetic level by determining the transcriptional activity of the genes of the rhamnose operon. In addition, the transcription of the genes was also examined under non-inducing conditions revealing that the transposon in *B. thetaiotaomicron* Tn Met^R was overexpressing the genes of the rhamnose operon in comparison to the parent strain. This was also evident under the inducing conditions.

In the *B. thetaiotaomicron* Tn Met^R mutant, the transposon is flanked with IS elements. An analysis of this strongly suggested that the IS*4400*L may have formed a strong constitutive promoter functioning in conjunction with the existing inducible *rhaR* promoter of *B. thetaiotaomicron* VPI-5482 as identified in Chapter 3. Increased levels of the positive transcriptional regulator, RhaR, in turn, caused increased transcription of the rest of the *rha*

genes. Future studies can be conducted to identify the exact promoter region within the IS element by using primer extension or mapping with 5′RACE as conducted by Podglagen *et al.* (2001). Since the *B. thetaiotaomicron rhaR*⁻ strain (Chapter 3) did not utilize L-rhamnose, dot blots to show altered transcriptional levels in the defined medium could not be conducted.

The functionality of the *fucO* gene product, lactaldehyde reductase, was then examined at the biochemical level, where *B. thetaiotaomicron* Tn Met^R was shown to produce L-1,2-propanediol in equal amounts to the parent strain. These results showed that the *fucO* gene had not been disrupted by the transposon insertion, further confirming that the main effect of the transposon insertion was in causing overexpression of the *rha* genes. Additional biochemical studies were conducted to determine the levels of lactate dehydrogenase (LDH) and pyruvate oxidoreductase enzymes in the various strains, since both these enzymes are thought to be involved in metronidazole resistance. Pyruvate oxidoreductase, in particular, has been reported to be involved in metronidazole activation (Narikawa *et al.*, 1991; Upcroft *et al.*, 2006; Diniz *et al.*, 2004). *B. thetaiotaomicron* Tn Met^R was shown to have elevated levels of lactate dehydrogenase and depleted levels of pyruvate oxidoreductase in comparison to the parent strain under inducing conditions. These finding were consistent with other metronidazole resistant strains reported in literature.

One of the common factors observed in the *B. thetaiotaomicron* Tn Met^R mutant under the inducing and non-inducing conditions was the increased transcription of the *rha* genes. Therefore, in order to prove that overexpression of *rhaR* alone can cause the metronidazole resistance phenotype, and to show that no other silent mutations exist in the *B. thetaiotaomicron* Tn Met^R mutant, the *rhaR* gene, together with its own promoter, was introduced into the cytoplasm of the parent strain. The resulting RhaR overexpresser, *B. thetaiotaomicron*

(pLYLrhaR), was also physiologically, genetically and biochemically characterized under inducing conditions. *B. thetaiotaomicron* (pLYLrhaR) was shown to have elevated expression of the *rha* genes in comparison to the parent strain, and displayed resistance to 15 µg/ml of metronidazole. The biochemical analysis of this strain showed that LDH activity was high and pyruvate oxidoreducatase activities were similar to those observed in *B. thetaiotaomicron* Tn Met^R. Despite the similar enzymatic activities, the physiological resistance phenotype as well as the hybridization activity was not quite as pronounced as that observed in the *B. thetaiotaomicron* Tn Met^R mutant, which may have been due to the copy number of pLYLrhaR plasmid, resulting in less RhaR being produced than in the *B. thetaiotaomicron* Tn Met^R mutant. To alternatively demonstrate and verify the transcriptional activity observed between the overexpresser and the parent strain, a qualitative analysis using Real Time PCR can be preformed in the future. Future studies can focus on two-dimensional electrophoresis as conducted by Diniz *et al.* (2004) to identify differentially expressed proteins between *B. thetaiotaomicron* Tn Met^R mutant, *B. thetaiotaomicron* (pLYLrhaR) and the parent strain under inducing and non-inducing conditions.

A recent study conducted by Manjunatha *et al.*, (2006) showed that certain *Mycobacterium tuberculosis* mutants were resistant to a group of nitroimidazoles including PA-824, a nitroimidazole prodrug compound used in the treatment of tuberculosis. The mechanism of resistance in these mutants involved the loss of a specific glucose-6-phosphate dehydrogenase or its cofactor F_{420}, deazaflavin, since the two enzymes provide electrons to reduce the drug, demonstrating the role of a carbohydrate intermediate in metronidazole resistance.

The results of this study conclude that overexpression of the rhamnose operon can cause a metronidazole resistance phenotype. This is the first study to report a link between metabolism of rhamnose and metronidazole resistance.

CHAPTER 5

General Conclusions

5.1 General Conclusions

Metronidazole is a valuable drug for the treatment of *Bacteroides* infections. However, resistance to the drug in clinical isolates of *B. fragilis* has been reported in several countries including South Africa, UK, France, Morocco, India, and Hungry (Lubbe *et al.*, 1999; Gal and Brazier, 2004; Breuill *et al.*, 1989; Haggoud *et al.*, 2001; Chaudry *et al.*, 2001). Clinical isolates of *H. pylori* and *T. vaginalis* have also been reported to show resistance to metronidazole (Kaihovaara *et al.*, 1998; Upcroft *et al.*, 2006), and these and other organisms are under constant study to understand the mechanisms of metronidazole resistance (Upcroft *et al.*, 2006; Diniz *et al.*, 2004; Narikawa *et al.*, 1991; Kaihovaara *et al.*, 1998; Sindar *et al.*, 1982; Britz and Wilkinson, 1979).

The work presented in the current study was, therefore, designed to focus on the mechanism/s of metronidazole resistance in a *B. thetaiotaomicron* transposon-generated metronidazole resistant mutant (Casanueva, 2004), with a view to possibly extending the understanding of the resistance mechanism in the more pathogenic organism, *B. fragilis*. The major findings of this study led to the conclusion that, overexpression of the rhamnose carbohydrate utilizing pathway, via the positive transcriptional regulator, RhaR, in *B. thetaiotaomicron* VPI-5482 caused a metronidazole resistance phenotype. Although *B. fragilis* does not have a rhamnose utilizing pathway, it does have a fucose utilizing pathway that is similar to the rhamnose pathway and results in the formation of a common metabolic intermediate, L-lactaldehyde. This is metabolized to L-1,2-propanediol via the product of the *fucO* gene which is located within this putative fucose gene cluster in *B. fragilis*. It is noteworthy that the increasing availability of different genome sequence databases establishes the foundation on which comparative molecular genetic studies can be conducted.

In this study, the transposon generated metronidazole resistant mutant, *B. thetaiotaomicron* Tn Met^R was characterized using a range of experimental approaches. Tang and Malamy (2000) reported the possibility of multiple transposon integrations within the chromosome during transposon mutagenesis. This was not the case in *B. thetaiotaomicron* Tn Met^R since only a single copy of the *tetQ* gene was found in this strain conferring only tetracycline resistance which is the case with an inverse transposition event. No co-integrate or direct transposition events had occurred since this strain was not resistant to either tetracycline and erythromycin, or erythromycin alone, respectively. The location of the transposon insertion was identified in an A-T rich intergenic region between two locus tags, BT3767 and BT3768 of the putative rhamnose pathway. Based on the predicted function and amino acid sequence similarity to the rhamnose pathway in *E. coli*, the genes were named *fucO* and *rhaR* respectively.

Initially, it was observed that *B. thetaiotaomicron* Tn Met^R was resistant to metronidazole in complete medium. It also displayed slower growth than the parent, a phenotype resembling other reported metronidazole resistant strains (Gal and Brazier, 2004; Sindar *et al.*, 1982; Britz and Wilkinson, 1979). It was later shown that the slow growth did not contribute to the metronidazole resistance phenotype when the cells were grown in minimal medium supplemented with $_L$-rhamnose as the sole carbon source. Here the growth rate of the mutant exceeded that of the parent, yet a metronidazole resistance phenotype was still observed.

Since the transposon had integrated into an intergenic region and no genes were directly disrupted, it was important to identify which gene/s were responsible for the metronidazole resistance phenotype. Therefore, the putative rhamnose cluster in *B. thetaiotaomicron* VPI-5482, that had never previously been characterized in this organism, was used to understand

how the transposon had affected this carbohydrate pathway and how it was causing a metronidazole resistance phenotype.

Other well-documented studies on rhamnose carbohydrate utilization systems in organisms such as *E. coli* and *Rhizobium leguminosarum* were used as a platform to address the key molecular questions (Moralejo *et al.,* 1993; Powers, 1966; Richardson *et al.,* 2004). Evidence presented in this study indicated that the *rha* gene cluster encodes the rhamnose utilization system in *B. thetaiotaomicron* VPI-5482. Support for this is bioinformatic analysis of the genes. In addition, transcriptional analysis of the putative rhamnose cluster confirmed that the six ORFs, named *rhaK, rhaI, rhaP, rhaA, fucO* and *rhaR* were induced by the substrate $_L$-rhamnose. Of these, five genes (*rhaK – fucO*) were expressed on a single operon, *rhaKIPAO*. RT-PCR of the 142 bp intergenic region between *fucO – rhaR*, did not result in a product, suggesting that *rhaR* may be expressed from its own promoter. Bayley *et al.* (2000) and Podglajen *et al.* (2001) both identified typical *B. fragilis* promoters containing a -7 and -33 promoter consensus sequence. Two promoter sites, $P_{rhaKIPAO}$ and P_{rhaR} were identified in this study and contained the *B. fragilis* -7 promoter consensus sequence. However, the -33 promoter consensus sequence for both $P_{rhaKIPAO}$ and P_{rhaR} were not those typical of *B. fragilis*. Instead, the -33 regions of both $P_{rhaKIPAO}$ and P_{rhaR} showed a GCGT consensus sequence in *B. thetaiotaomicron*. It is possible that *B. thetaiotaomicron* may have its own unique -33 consensus sequences and this warrants further study.

In this study, it was shown that expression of the *rha* operon was mediated at the level of transcription by RhaR, which was shown to be responsive to $_L$-rhamnose. RhaR functioned as a positive transcriptional regulator with similarities to the family of AraC/XylS type regulators. A *rhaR* mutant, *B. thetaiotaomicron rhaR*$^-$, was unable to grow in minimal medium supplemented

with $_{L}$-rhamnose as the sole carbon source, although it was able to utilize other carbohydrates such as $_{D}$-glucose and $_{L}$-fucose. Complementing the *B. thetaiotaomicron rhaR*$^{-}$ mutant with RhaR on a plasmid restored growth in the defined medium. The focus of the future work should consist of identifying the binding site for RhaR upstream of RhaK using deletion mapping of the promoter region and DNA mobility shift assays (Egan and Schleif, 1993). Functionality of each of the rhamnose genes should also be confirmed by constructing and analyzing mutants with single rhamnose catabolic gene disruptions (Hooper *et al.*, 1999).

As mentioned earlier, (Section 2.4.2.5, Fig. 2.9) the *E. coli* rhamnose pathway lacks the *fucO* gene, however, it is found in the fucose pathway. Chen *et al.* (1987b) reported that anaerobic growth of *E. coli* on rhamnose induced the fucose regulon, including the *fucO* gene. Single mutations in the genes coding for the isomerase, kinase, aldolase and transcriptional regulators of both the rhamnose and fucose regulons, prevented rhamnose from inducing the fucose regulon. It is possible that a similar process might take place in *B. thetaiotaomicron*, since this organism has both regulons, a difference between this and *E. coli* being that the *fucO* gene in *B. thetaiotaomicron* lies within the rhamnose pathway. It would be of interest to determine if there is a cross induction of the rhamnose pathway by $_{L}$-fucose in *B. thetaiotaomicron.*

The transposon insertion in an intergenic region in *B. thetaiotaomicron* Tn Met^{R} did not disrupt the rhamnose pathway since the mutant was able to grow in minimal medium with $_{L}$-rhamnose as the sole carbon source. The strain displayed better growth in this medium compared to the parent, and also displayed a metronidazole resistance phenotype. The effects of the transposon insertion were further analyzed to determine whether the *fucO* gene, upstream of the transposon, or the *rhaR* gene downstream of the transposon had been affected.

A biochemical study was conducted to determine if *fucO* had been affected, and the results revealed some significant findings. Firstly, the parent strain was shown to produce L-1,2-propanediol under anaerobic conditions from cultures grown in $_{L}$-rhamnose. This suggests that rhamnose catabolism in *B. thetaiotaomicron* VPI-5482 may be similar to *E. coli*, where FucO is responsible for converting L-lactaldehyde to L-1,2-propanediol anaerobically (Boronat and Aguilar, 1981). Secondly, the ability of *B. thetaiotaomicron* Tn Met^{R} to produce L-1,2-propanediol at the same levels as the parent strain confirmed that the *fucO* gene was unaffected by the transposon and that there was active formation of FucO protein in order for the reaction to proceed.

Transcriptional hybridization studies showed that the transposon insertion in the mutant had increased the expression of the *rhaR* gene coding for the positive transcriptional regulator, and subsequently the rest of the rhamnose pathway. While there is no conclusive evidence as to why increased transcription of *rhaR* was observed in *B. thetaiotaomicron* Tn Met^{R}, it was proposed that the insertion sequence element on the transposon was acting as an addition constitutive promoter activating the genes (Schneider and Lenski 2004; Barker *et al.*, 2004; Soki *et al.*, 2004; Podglajen *et al.*, 2001; Kallastu *et al.*, 1998; Mahillon *et al.*, 1999). In support of this, nucleotide sequencing of IS*4400*L, which lies upstream of *rhaR*, identified three putative promoter sequences. Furthermore, *B. thetaiotaomicron* Tn Met^{R} was shown to express the rhamnose catabolic genes under non-inducing conditions in comparison to the parent strain. Together, this evidence strongly suggested that IS*4400*L could be transcribing the positive transcriptional regulator, RhaR, even in the absence of the substrate, and thus causing the expression of the rhamnose genes. Future studies should involve identifying these possible promoters within the IS elements.

A unique observation investigated in this study, was that the increased expression of the rhamnose genes resulted in a metronidazole resistance phenotype in *B. thetaiotaomicron* Tn Met^R. This was confirmed to be the cause of the metronidazole resistance phenotype, since the RhaR overexpressor, *B. thetaiotaomicron* (pLYLrhaR), containing an additional copy of *rhaR* on a plasmid, also displayed metronidazole resistance in minimal medium supplemented with $_L$-rhamnose as the sole carbon source. This result confirmed that no other undetected mutations conferring metronidazole resistance existed within *B. thetaiotaomicron* Tn Met^R.

Inefficient activation of metronidazole within the bacterial cells has been shown to be a mechanism of metronidazole resistance. A key enzyme thought to be involved in this mechanism of activation is pyruvate oxidoreductase, which is coupled with either ferredoxins or flavodoxin. The decarboxylation of pyruvate via the pyruvate oxidoreductase generates electrons that activate the drug. However, in certain metronidazole resistant strains, levels of lactate dehydrogenase are higher and levels of pyruvate oxidoreductase are lower in comparison to the parent strains (Diniz *et al.*, 2004; Upcroft *et al.*, 2006; Narikawa *et al.*, 1991). In this study, increased enzymatic activity of lactate dehydrogenase and decreased pyruvate oxidoreductase in the resistant strains, *B. thetaiotaomicron* Tn Met^R and *B. thetaiotaomicron* (pLYLrhaR), supported the findings in literature.

Based on this information and the overall findings of this study, two possible models will now be proposed to illustrate how increased rhamnose catabolism may be linked to metronidazole resistance.

5.1.1 Model 1: Decreased activation of metronidazole through reduced pyruvate oxidoreductase activity

If rhamnose catabolism in *B. thetaiotaomicron* yields L-lactaldehyde and dihydroxyacetone phosphate (DHAP), then DHAP would be isomerized to glyceraldehyde phosphate (GAP) (Prestwich, 2004) and in turn participate in pyruvate metabolism. Decarboxylation of pyruvate (Chapter 1) would then lead to the formation of Acetyl CoA and the electrons generated would be used to activate metronidazole.

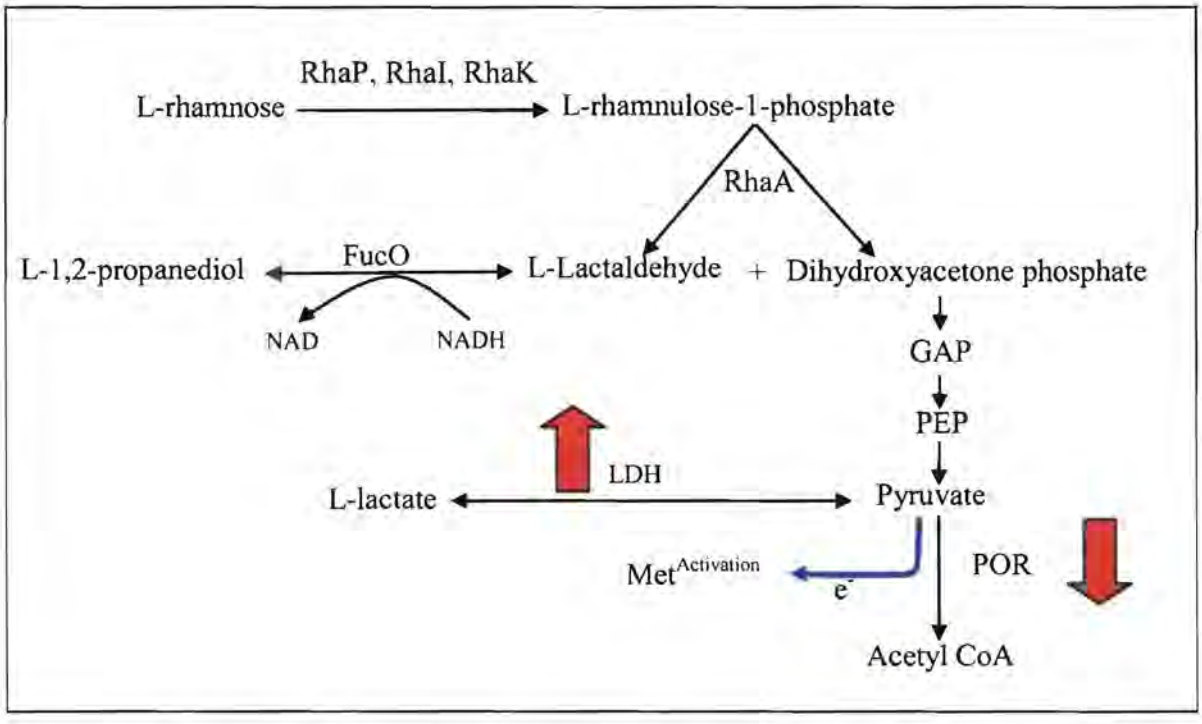


Figure 5.1: Proposed model for the metronidazole resistance phenotype in *B. thetaiotaomicron* Tn Met^R and the *B. thetaiotaomicron* (pLYLrhaR) overexpressor. RhaP, RhaI, RhaK, RhaA, FucO represent, rhamnose symporter, rhamnose isomerase, rhamnulose kinase, rhamnulose-1-phosphate aldolase, and lactaldehyde reductase, respectively. GAP, PEP, LDH and POR represents glyceraldehyde phosphate, phosphoenol pyruvate, lactate dehydrogenase and pyruvate (ferredoxins/flavodoxin) oxidoreductase, respectively. e^- donation to activate metronidazole is represented with a blue arrow.

However, if increased levels of rhamnose catabolism cause a shift in the metabolic pathway, then pyruvate could get oxidized to L-lactate by the reversible reaction using the a flavin-linked

lactate dehydrogenase (LDH) as reported in *E. coli* (Boronat and Aguilar, 1981). The concurrent reduction in pyruvate oxidoreductase activity would then lead to a decrease in metronidazole activation and account for the increased resistance observed in this study. Furthermore, since the resistance to metronidazole in *B. thetaiotaomicron* Tn Met^R mutant was observed in complete medium, the resistance may not be solely linked to rhamnose utilization. However, this strain did also grow slower in the complete medium which may explain the why the resistance was observed in the complete medium.

5.1.2 Model 2: Reduction of metronidazole to an inactive compound

Overexpression of one or more of the rhamnose catabolic genes may have directly affected the amount of activated metronidazole formed through reducing it to an inactive compound. For example, organisms containing nitroreductases, such as *Enterococcus casseliflavus* and *Enterococcus galliniarum* were shown to be resistant to metronidazole (Rafii *et al.*, 2003). It was reported that the nitroreductase identified in these strains should have reduced the drug, but the active form was not detected. It has also been reported that, like the *nim* genes, these nitroreductases may not metabolize metronidazole into toxic compounds since metronidazole susceptible *Clostridium perfringens* were reported to grow well in spent cultures of the *Enterococcus* spp. that were incubated with metronidazole (Rafii *et al.*, 2003).

In *B. thetaiotaomicron* Tn Met^R, it may be possible that overexpression of lactaldehyde oxidoreductase, which is an NAD-dependent dehydrogenase, and like the pyruvate oxidoreductase contains an iron cluster, may also reduce intracellular metronidazole, but not result in an activated compound. There are no reports of L-1,2-propanediol oxidoreductase having any flavodoxins or ferredoxins dependent mechanisms in *B. thetaiotaomicron*. However, in *S. typhimurium*, it is reported that propanediol metabolism results in excretion of

propanol into the medium and this provides the cells with an electron sink to help balance redox reactions (Walter *et al.,* 1997). It would, therefore, be of interest to investigate, *in vitro*, if metronidazole can be reduced by the L-1,2-propanediol oxidoreductase, in the presence of ferredoxins or flavodoxins, and whether the product would be in the toxic or non-toxic form.

It is also a possibility that any of the genes in the rhamnose operon may be responsible for causing a metronidazole resistance phenotype. Future studies may including making single point mutations in each of the gene and determine the mutant's sensitivity to metronidazole. Alternatively, knocking out the first gene, i.e. *rhaK*, and introducing an additional copy of the regulator in this mutant on a plasmid would determine is rhamnose is solely responsible for metronidazole sensitivity. Finally, it is also unknown if RhaR may be controlling the expression of some other genes out side of the rhamnose gene cluster.

The linking of metronidazole resistance to carbohydrate metabolism is potentially of clinical importance and warrants further study in the presence of carbon sources other than rhamnose. Future studies should also address the possible role of the NAD(P)-linked dehydrogenase, FucO, in metronidazole reduction, since the *fucO* gene is also found in the fucose pathway of the more pathogenic organism, *B. fragilis*. This may provide further insight into the various mechanisms of metronidazole resistance in this organism.

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