Metronidazole resistance in clinical *Bacteroides fragilis* isolates from Groote Schuur Hospital, Cape Town, South Africa

Rosemary L. Meggersee

Thesis Presented for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Molecular and Cell Biology

UNIVERSITY OF CAPE TOWN

February 2014

Acknowledgements

I would like to thank my supervisor Associate Professor Valerie Abratt for her never ending support and guidance throughout my thesis. Thank you for always having encouraging words when things were tough and for pushing me when I needed it. Without your support and gentle pushing I don't think that I would ever have managed to complete this thesis and while I am loathe to admit it that "one more experiment and last push" was all worth it.

A special thank you to my fellow anaerobes past and present, without you I would not have survived long enough to finish my thesis. A special mention to, Dr Clinton Moodley, Dr Bruna Galvao, 'Dr' Ailsa Davidson and Dr Samantha Nicholson for all your support, friendship, numerous hours of Sprocle and lots of laughter. I would also like to thank all of my other friends within the Molecular and Cell Biology Department who during many tea sessions provided advice, fun and the knowledge that I wasn't alone. To my friends outside of MCB, Dr Semira Irusen, Grant Godsmark, Kevin O'Connell, Bridget Price, Greg Bowden and many others words cannot express how grateful I am for your support and friendship during a long and not always easy journey. Thank you for the board games, braais, nights out and time away from work, without you this thesis would have ended with a mental breakdown. I would especially like to thank Byron Reeve for undertaking this journey with me from our early days of undergrad, through honours and finally the last days of our PhDs. I don't think this journey would have been quite the same without you there beside me. It was wonderful to have you around to lend a helping hand or provide some humour during difficult times and especially to have someone around who understood what I was going through. Thank you for everything and I hope I was as beneficial to you during your PhD.

My deepest and most heartfelt appreciation must go to, in particular, my parents as well as the rest of my family who have managed to show enthusiasm when I did not, belief when I had none and who have never wavered in their support or commitment to my dreams and aspirations. Thank you for always being there to provide whatever I needed, whether that was money when I could not afford to study or just a hug and gentle encouragement. Your belief in me and my abilities gave me strength I didn't think I had and means more to me then you could ever know.

Finally, I would like to thank my most amazing boyfriend, Troy Smuts. Without your continual, unwavering support and constant faith in me I don't think this thesis would have been completed. I don't think that I can ever truly convey how much you mean to me. You were always there to give me a hug or take my mind off work. You never complained about staying late with me in the lab or over the weekend so that I wouldn't be alone. You are my rock and were my anchor when times were tough and I didn't think I could manage. Your daily emails and words of encouragement helped me through each day and I will never forget that. Thank you for always being there and never letting me walk this path alone.

I would also like to acknowledge the financial support I received during the course of this thesis and that includes the DAAD/ National Research Foundation, the Ernst and Ethel Erikson Trust and the University of Cape Town Research Committee. Without the trust placed in me by these bodies this work would not have been possible.

Contents

Abstract

Bacteroides fragilis, an anaerobic gut commensal and opportunistic pathogen, is a leading cause of anaerobic abscesses and bacteraemias. Treatment of infections is complicated by the emergence of resistance to several of the antibiotics, specifically metronidazole, used in the clinical setting. The aim of this thesis was to examine the levels of antibiotic resistance of 23 *B. fragilis* strains isolated at Groote Schuur Hospital, Cape Town, and determine the metronidazole (Mtz) resistance mechanisms present in order to evaluate the clinical risk of the spread of drug resistance. It also reports the identification and functional characterisation of a putative, novel *nim* gene in the *B. fragilis* 638R genome.

Measurement of the minimum inhibitory concentration of the strains to antibiotics showed that 8% were highly resistant to imipenem and cefoxitin and 65% to tetracycline. All strains were sensitive to clindamycin. Two strains, *B. fragilis* GSH8 and GSH15 were Mtz resistant and strain GSH15 showed multidrug resistance to metronidazole, imipenem, cefoxitin and tetracycline. The genetic basis of the resistance to the various antibiotics was examined and could largely be attributed to the presence of previously published resistance genes. There were several exceptions to this which were investigated further at the genetic level with particular focus on imipenem and Mtz. In the cases of Mtz resistance, PCR screening did not detect any of the known *nim* genes but both strains showed increased lactate dehydrogenase (LDH) activity suggesting the involvement of the LDH/ pyruvate: ferredoxin oxidoreductase (PFOR) pathway. However, there was no reciprocal decrease in PFOR activity so the LDH/ PFOR pathway was not involved in the observed Mtz resistance. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) revealed that induction by exposure to sub-lethal doses of Mtz caused a slight increase in transcription of the efflux gene *bmeB5*. However, this was not statistically significant and still did not account for the observed Mtz resistance. A putative

nim-like gene was identified on the *B. fragilis* 638R genome and was present in most of the strains examined. Phylogenetic and three dimensional analysis of the derived amino acid sequence revealed that the 638Rnim was more closely related to NimA from *Deinococcus radiodurans* (drNimA) than to any of the other *nim* genes. Heterologous overexpression of *638Rnim* in an *Escherichia coli* Mtz sensitive mutant resulted in a 3-fold increase in Mtz resistance as compared to the control (6 vs 2 mg/L respectively). However, its possible role in Mtz resistance in *B. fragilis* could not be confirmed by overexpression or interruption of the gene in the homologous host. qRT-PCR showed that increased transcription of the gene approached statistically significant levels upon Mtz induction. This *nim*-like gene, therefore, warrants further investigation under a range of different induction conditions.

The findings of this research provide useful information on the state of *B. fragilis* antibiotic resistance present in Groote Schuur Hospital and the incidence of the associated resistance genes. It also identifies and presents a preliminary characterisation of a putative novel *nim* gene as a basis for further functional studies on Mtz resistance mechanisms.

Abbreviations

°C degree(s) Celsius

A adenosine

aa amino acid(s)

Amp ampicillin

bp base pair(s)

BHISA brain heart infusion supplemented agar

BHISB brain heart infusion supplemented broth

C- carboxy-(terminal)

C cytosine

cDNA complementary DNA

Cef cefoxitin

CFE cell free extract

Cln clindamycin

DNA deoxyribonucleic acid

dNTP deoxynucleotide triphosphate

Erm erythromycin

et al. *et alia*

FMN flavin mononucleotide

g acceleration due to gravity

g gram(s)

G guanine

gDNA genomic DNA

GSH Groote Schuur Hospital

h hour(s)

IPTG isopropyl β-D-1-thiogalactopyranoside

Imp imipenem

IS insertion sequence

kb kilobase pairs

kDa kilodalton(s)

L litre

LB Luria-Bertani medium

LDH lactate dehydrogenase

M molar

mg milligram

ml millilitre(s)

mM millimolar

MIC minimum inhibitory concentration

Min minute(s)

MSA multiple sequence alignment

Mtz metronidazole

MW molecular weight

N- amino-(terminal)

nm nanometre(s)

nM nanomolar

NCBI National Centre for Biotechnology Information

n number of samples

ODx optical density at x nanometres

ori origin of replication

P p-value; indicating probability

PAGE polyacrylamide gel electrophoresis

PCR polymerase chain reaction

PDB predicted data bank

PFOR pyruvate: ferredoxin oxidoreductase

qRT-PCR quantitative RT-PCR

RNA ribonucleic acid

rRNA ribosomal RNA

rpm revolutions per minute

RT room temperature

RT-PCR real time PCR

SDS sodium dodecyl sulphate

Sec second(s)

spp. species

T thymine

Tet tetracycline

Tig Tigecycline

U units

µg microgram(s)

µM micromolar

µl microlitre(s)

V volts

w/ v weight per volume

W watts

xR resistance to x

xS sensitive to x

Chapter 1

Literature review

1.1 General introduction

The human gastrointestinal tract contains the largest assortment of microbes in the human body. Approximately 500-1000 bacterial species are carried in the gut with the majority of these being anaerobes. The *Bacteroides* genus accounts for almost 25% of all bacteria cells present in the human intestines. *Bacteroides fragilis* is a non-spore forming, rod shaped, gram negative bacterium (Wexler, 2007, Xu *et al*., 2004). Although it accounts for only around 0.5% of the *Bacteroides* present in the gut it is a virulent opportunistic pathogen and is the leading cause of anaerobic infections. Normally it exists in harmony with its host, however, it may become pathogenic if the gut epithelium is injured and the bacterium enters sterile tissue (Wexler, 2007, Xu and Gordon, 2003). The widespread use of antibiotics to treat these infections has resulted in the emergence of resistance to many of the antibiotics used clinically (Snydman *et al*., 2010).

This chapter will review the literature on the mechanisms that cause an increase in antibiotic resistance in *Bacteroides* spp., with a particular focus on *B. fragilis*.

1.2 Commensal and opportunistic pathogen

Under normal circumstances, *B. fragilis* exists in a mutualistic relationship with its human host where it is able to utilize complex carbohydrates and break them down into usable fatty acids. These are then re-absorbed by the large intestine and provide a significant part of the daily energy requirement of the host (Wexler, 2007). *B. fragilis* is also critically important in the formation of the host immune system, where it has been linked to the development of gut associated lymphatic tissue (GALT) and the activation of $CD4^+$ T cells (Mazmanian and Kasper, 2006, Hooper *et al*., 2002). It has also been shown to play a pivotal role in preventing the intestinal colonization and movement through the gut of potential pathogens and has even been linked to preventing infection by *Clostridium difficile* (Hopkins and Macfarlane, 2003).

Although *B. fragilis* is usually a gut commensal and accounts for only 0.5% of *Bacteroides* present, it can cause disease within the gut, and is also the most commonly isolated anaerobic pathogen from anaerobic infections outside of the gut (Wexler, 2007). Within the gut, some strains of *B. fragilis*, known as enterotoxigenic *B. fragilis* (ETBF), can cause disease. These strains produce an enterotoxin called fragilysin, which can cause diarrhoea and extensive tissue damage within the gut and in rare cases, cause cancer (Sears, 2009). If the gut epithelium is breached, *B. fragilis* can escape from the gut and enter the sterile tissue and the bloodstream. ETBF strains are *B. fragilis* that possess one of three different *bft* gene alleles (*bft-1*, *bft-2* and *bft-3*), which are known to be carried on a *B. fragilis specific* pathogenicity island (BfPAI). This enables ETBF strains to pass the *bft* gene on to other *B. fragilis* isolates increasing the spread of the virulence factor (Sears, 2009). *B. fragilis* has a wide array of virulence factors which enable it to colonise the host outside the gut, evade the immune system and degrade the surrounding tissue (Wexler, 2007). *B. fragilis* possesses a number of adhesins which enable it to effectively colonise host tissues. These work in conjunction with a number of proteases such as hyaluronidase and chondroitin sulfatase, which degrade the host extracellular matrix. Some proteases, such as neuraminidases, are known to attack the immune system directly and can catalyse the removal of sialic acids from important immunoactive proteins such as IgG (Schauer, 2004). These virulence factors are aided by the remarkable ability of *B. fragilis* to avoid the host immune response and allow disease persistence (Patrick *et al*., 2009). The bacterial capsule in particular provides resistance to macrophages and prevents phagocytic uptake and killing, while a unique ability to modulate its cell surface polysaccharides helps evade the immune response (Patrick *et al*., 2009, Reid and Patrick, 1984). This ability to colonise, degrade and evade the host immune system makes *B. fragilis* a very efficient pathogen and very difficult to treat effectively.

1.3 Reservoir for resistance determinants

As described above, *B. fragilis* can function effectively as either a beneficial commensal or a harmful pathogen. However, some studies suggest that even as a gut commensal *B. fragilis* could be harming its host by acting as a reservoir of resistance determinants which can then be passed on to other species of the *B. fragilis* group as well as to much more virulent bacteria (Salyers *et al*., 2004). In addition, they may also be the recipients of resistance genes originating in other species. It is suggested that gut microbiota, such as *B. fragilis*, carry a variety of resistance genes which can be transferred within the population or to other related bacterial species (Shoemaker *et al*., 2001). Evidence of this can be seen in the frequency of *Bacteroides* species carrying the tetracycline and erythromycin resistance genes, *tetQ* and *ermF* respectively. A survey of 289 strains, during a 20 year period, found that in 1970 only 20-30% of isolates carried the *tetQ* gene, while few *ermF* genes were detected. However, in the 1990s this had risen to over 80% of strains carrying the *tetQ* gene and 15% containing the *ermF* gene (Shoemaker *et al*., 2001). It is of great concern that strains carrying both *tetQ* and *ermF* were found in people with no recent history of antibiotic use, which suggests that these two resistance genes are stably maintained (Salyers and Amabile-Cuevas, 1997). The study also observed that the increase in erythromycin (Erm) resistance genes was not just restricted to *ermF* as two other *erm* genes, *ermB* and *ermG*, were also identified. Both of these genes had previously not been observed in Gram-negative bacteria and analysis of the conjugative transposition that carried the two genes revealed that the DNA sequence was unrelated to any conjugative transposon carried by *Bacteroides* species. This indicated that *ermB* and *ermG* were transferred through conjugative transposons to *Bacteroides* species from Gram-positive bacteria (Gupta *et al*., 2003, Wang *et al*., 2003).

1.3.1 Transposons, conjugative transposons and plasmids

The most common method of resistance gene transfer is through the use of mobile genetic elements. *Bacteroides* spp. have a wide array of mobile elements which can participate in the process such as, mobilizable transposons, conjugative transposons and plasmids. These elements are relatively common and occur in nearly 80% of *Bacteroides* spp. (Shoemaker *et al*., 2001).

Mobilizable transposons are DNA elements which do not possess the required elements to enable them to self-transfer (Salyers *et al*., 1995). However, they are able to utilise the host's own machinery to transfer between species (Smith *et al*., 1998).

Conjugative transposons (CTn) are DNA elements which can form circular intermediates that integrate within the chromosome of the same cell (intracellular transposition). Alternatively, they can utilise conjugation to transfer the DNA into another bacteria, where they then integrate themselves into the new host genomes (intercellular transposition) (Cheng *et al*., 2000, Salyers *et al*., 1995). A bacterium can carry more than one CTn at a time and it is believed that more than one copy of a CTn in a strain can cause stimulation of transposition (Shoemaker *et al*., 2001). Thus, it is possible that several CTns with antibiotic resistance genes carried by a bacterium increase the chance of resistance genes being transferred to other bacteria. It is believed that CTns are responsible for over 75% of tetracycline (Tet) resistance in *Bacteroides* (Shoemaker *et al*., 2001).

The members of the CTnDOT family of conjugative transposons are the most well described and are known to carry both Tet (*tetQ*) and Erm resistance (*ermF*) genes (Wang *et al*., 2005, Cheng *et al*., 2000). A unique feature of CTnDOT is that the CTns excision and conjugative transfer is stimulated by exposure to low level Tet (Waters and Salyers, 2013). A study found

that cells exposed to Tet resulted in a 1000-10 000 fold increase in frequency of CTnDOT transfer (Whittle *et al*., 2001). Exposure to Tet caused a transcription cascade that resulted in the translation of the *tetQ-rteA-rteB* operon. This operon, in turn, activated the transcription of the excision genes which are needed to excise CTnDOT from the chromosome (Waters and Salyers, 2013).

In contrast to transposons and CTns, plasmids are generally able to replicate independently within in the cytoplasm of the host cell, although there are some plasmids that can also integrate into the chromosome (Smith *et al*., 1998). Plasmids are found in around 20 to 50% of *Bacteroides* strains. Many plasmids contain an origin of transfer and a mobilization gene, which allow them to be transferred by conjugation into another bacterium. Two different types of plasmids are carried by *B. fragilis*, namely, plasmids which confer antibiotic resistance, and cryptic plasmids which have no known function. Antibiotic resistance plasmids are able to confer resistance to many different classes of antibiotics and transfer these to other bacteria (Smith *et al*., 1998).

1.3.2 Insertion sequence (IS) elements

IS elements are double-stranded DNA sequences that contain a transposase gene, which is flanked by inverted repeat sequences. This allows the IS to duplicate short sections of nucleotides, which often cause mutations and rearrangements to the genome (Soki, 2013). However, they do not just provide new genetic information but are also capable of interacting with genetic material situated near to them (Soki, 2013). These interactions often take the form of providing a promoter, which is either coded for by the IS element itself, or is created as a result of the integration of the IS with the host DNA sequence upstream of the gene. Several bacterial genes can be activated in this way. Of greatest interest for the purposes of this review are the effects IS elements have on previously inactive antibiotic resistance genes (Soki, 2013).

The first discovery of IS elements associated with antibiotic resistance in *Bacteroides* was during identification of the Erm and clindamycin (Cln) resistance mechanism MLSB (macrolide, lincosamide and streptogramin B) (Soki, 2013). This mechanism is encoded by *ermF* and is able to confer resistance to three chemically different antibiotics (Smith *et al*., 1998). Analysis of the plasmid carrying this gene found that it was flanked on either side by inverted repeats of IS4351 (Shoemaker *et al*., 1985). It was later found that resistance to carbapenems in *B. fragilis* could occur through a single step mutation of the imipenem resistance gene, *cfiA*. Carbapenem sensitive isolates that carried the "silent" form of the *cfiA* gene could be made resistant through the insertions of IS1186 and IS942 upstream of the gene (Soki *et al*., 2004). Subsequently, many antibiotic resistance genes have been identified with an upstream IS element that, in most species, is specific to that exact resistance gene. In the case of the 5-nitroimidazole resistance genes (*nimA-E*) the activating IS elements were linked to each specific *nim* gene type (Soki, 2013). It appears that *Bacteroides* IS elements are ubiquitous and are capable of activating most antibiotic resistance genes (Soki, 2013). Thus, *nim* IS elements are interchangeable with those used to activate the *cfiA* resistance gene, while an IS element discovered for *cfiA* has been shown to activate the cefoxitin resistance gene, *cfxA* (Garcia *et al*., 2008, Podglajen *et al*., 1995). It is possible, therefore, that a single IS element could result in the activation of a whole suite of antibiotic resistance genes, which could then be spread to an entire population of bacteria increasing the spread of antibiotic resistance.

1.4 Antibiotic resistance

The *Bacteroides* species have the highest number of antibiotic resistance mechanisms and the greatest prevalence of antibiotic resistance of all anaerobic pathogens (Hecht, 2006). They are almost totally resistant to penicillins, cephalosporins and tetracycline. They show intermediate resistance to cefoxitin, clindamycin and most fluoroquinolones, while carbapenems, some β-lactamase combinations, tigecycline and metronidazole (Mtz) still remain mostly effective

(Nagy *et al*., 2010, Snydman *et al*., 2010). However, a number of *B. fragilis* isolates have been identified that are resistant to almost all antimicrobials used to treat it clinically (Hecht, 2006). Acquired resistance can be caused by a number of different mechanisms including: decreased permeability of the bacterial membrane, the presence of enzymes that inactivate the antimicrobial, alteration of the drug target binding site and antibiotic efflux systems (Wexler, 2007). The most relevant of these with respect to *B. fragilis* are reviewed below.

1.4.1 Tetracycline (Tet) resistance

The mechanism of Tet activity occurs through preventing the tRNA-amino acid from binding to the bacterial ribosome, inhibiting bacterial protein synthesis and ultimately causing cell death (Salyers *et al*., 1990). Tet was widely used throughout the 1950s and 1960s as the antibiotic of choice in treating anaerobic infections (Speer *et al*., 1992). However, nearly all isolates of *Bacteroides* are currently resistant to it through a combination of selective pressure and the acquisition of the resistance gene. Resistance occurs through the interaction of a protein (encoded by *tetQ*) with the ribosome that results in alteration of the antibiotic target site (Salyers *et al*., 1990). In 2004 a new Tet resistance gene, *tetX*, has been identified in *Bacteroides* and is believed to oxidise Tet and, therefore, inactive it (Yang *et al*., 2004). However, it is only active under aerobic conditions and has, therefore, not been shown to be active in *Bacteroides* spp. (Yang *et al*., 2004).

1.4.2 Erythromycin (Erm) resistance

Erythromycin binds to the 23S rRNA of the large 50S subunit of the bacterial ribosome. This prevents the tRNA-amino acid from binding, which inhibits the synthesis of novel proteins. It also prevents the assembly of new large ribosomal sub-units, leading to the functional ribosome sub-units being gradually exhausted in the cell (Pal, 2006). Resistance genes such as *ermF* are members of the macrolide-lincosaminde-streptogramin B (MLSB) family and code

for rRNA methylases that cause reduction in the affinity of the antibiotic for the 50s subunit (Pal, 2006). As mentioned earlier Erm and Tet resistance often occurs together due to the fact that their resistance genes can be carried on the same CTns.

1.4.3 β-lactam resistance

The most widely used antibiotics are the β-lactams, however, *B. fragilis* strains display high resistance to this class of drugs. Resistance to β-lactams can occur through the production of β-lactamases, changes in membrane permeability and alteration of the penicillin-binding proteins (PBPs) (Fang *et al*., 2002). β-lactamase production is very common in *B. fragilis* with almost 100% of isolates in the USA and 76% in the UK being found to produce them. The genes encoding them mostly occur on the chromosome and are often transcribed constitutively. Two types of β-lactamases exist, those that contain serine at their active sites and those that contain Zn^{2+}, known as metallo-β-lactamases (Jenkins, 2001). Normal β-lactamases (encoded by the *cepA* gene) are easily inhibited by β-lactam/ β-lactamase inhibitor antibiotic combinations such as sulbactam, clavulanic acid, and tazobactam (Jenkins, 2001). However, strains that produce metallo-β-lactamases (encoded by *cfiA*) are able to hydrolyse all β-lactam agents including carbapenems such as imipenem and meropenem (Podglajen *et al*., 2001).

Changes to the permeability of the outer membrane can also lead to increased β-lactam resistance (Fang *et al*., 2002). A decrease in the permeability of the outer membrane results in reduced antibiotic access to the cytoplasmic membrane (Rasmussen *et al*., 1994). Resistance to cefoxitin (Cef) has been linked to a decrease in outer membrane permeability and the loss of a 50 kDa outer membrane protein as well as the production of a cefoxitinase enzyme encoded by *cfxA* (Fang *et al*., 2002, Jenkins, 2001).

The PBPs are critical for bacterial growth as they are required for final stage cell wall synthesis (Jenkins, 2001). β-lactam antibiotics compete for the same active site as the one needed by the PBPs. This irreversible inhibition of the PBPs prevents the final crosslinking of the peptidoglycan layer and thus results in cell death (Jenkins, 2001). However, several resistant isolates have been identified that have an alteration to one or more PBP that results in reduced affinity of the β-lactam compound for the PBPs of that isolate resulting in resistance to that antibiotic (Wexler, 2007).

The multitude of resistance mechanisms present in *Bacteroides* render many of the antibiotics previously used to treat infections of current limited use. One of the few antibiotics still able to treat anaerobic infections successfully is metronidazole. However, like other antibiotics, resistance to this drug is also beginning to show an increase.

1.5 Metronidazole (Mtz)

Metronidazole is a 5-nitroimidazole antibiotic that is the drug of choice for treating anaerobic infections. It has been used clinically for over 45 years and was originally used to treat infections caused by *Trichomonas vaginalis* (Lofmark *et al*., 2010). The first recorded use of Mtz to treat anaerobic infections was in 1962 where it successfully treated a severe case of ulcerative gingivitis (Shinn, 1962). Ten years later it was shown to be effective in treating systemic anaerobic infections cause by *B. fragilis* (Tally *et al*., 1972). It was then introduced as an alternative to vancomycin therapy for the treatment of *Clostridium difficile* infections as well as for the clearance of *Helicobacter pylori* infections (Lofmark *et al*., 2010). Thus, Mtz has a very broad spectrum of antimicrobial activity against anaerobes and is highly effective against most anaerobic infections. This specificity for anaerobic organisms is due to its ability to utilise important biochemical processes only present in anaerobes for activation of the drug, while the broad spectrum of activity and low levels of resistance within this group is due to its

choice of drug target, namely, DNA. This makes it very difficult for resistance mechanisms to develop as altering the drug target is unlikely (Land and Johnson, 1997, Edwards, 1980).

1.5.1 Mode of action

Mtz structure consists of an imidazole ring with a nitro-group on the fifth position (Fig. 1.1). It is administered as an inactive pro-drug that upon entry into the cell, is reduced using the low redox potential of metabolic pathways found only in anaerobic bacteria (Edwards, 1980, Muller and Lindmark, 1976).

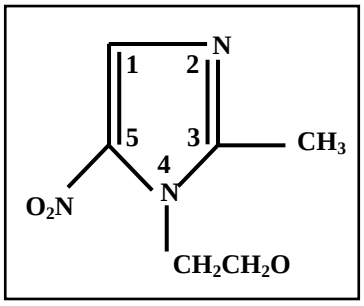


Figure 1.1: Structure of Metronidazole. The nitro-group is situated at position 5 (Adapted from Land and Johnson, 1997).

Activation of the drug is caused by reduction of the nitro group, in the fifth position of the imidazole ring, to produce three distinct species (Reysset, 1996). These include the nitro free radical (RNO_2^-), a nitroso group (RNO) and hydroxylamine (RNHOH) (Fig. 1.2). Of these, the short-lived nitro free radical, RNO_2^-, is known to cause damage. However, the role of the other two species is less clear but may also be important (Reysset, 1996).

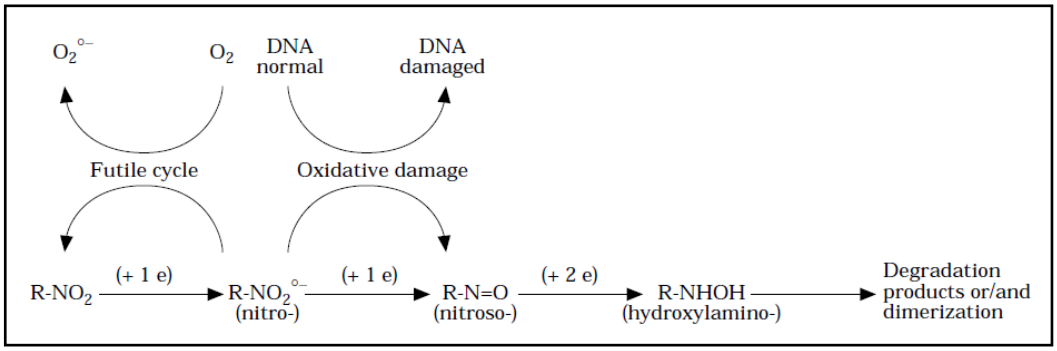


Figure 1.2: Suggested pathway of Mtz (RNO_2) reduction in anaerobic bacteria. The three products produced by Mtz reduction are shown. The process of futile cycling, occurring under aerobic conditions is also illustrated (Reysset, 1996).

It is believed that the nitro free radicals are able to oxidise macromolecules such as DNA. Oxidation of the DNA results in single and double strand breaks which cause cell death (Upcroft *et al*., 2006). The metabolic pathway believed to provide the electrons required to reduce Mtz to its active form is the pyruvate: ferredoxin oxidoreductase complex (Edwards, 1993). This pathway has a reduction potential more negative than that of Mtz and thus donates its electrons to the drug (Reysset, 1996). However, in the presence of oxygen the nitro radical anion can be reoxidised to its original, inactive form along with the production of a superoxide anion (O^-) (Fig. 1.2). This process is known as "futile cycling" and suggests why Mtz is only active against anaerobes (Reysset, 1996).

There are three main steps involved in Mtz activity: entry and uptake of the pro-drug into the cell, activation of the drug, and finally, DNA damage. These steps are reviewed in more detail below.

1.5.2 Entry and uptake

The exact mechanism of entry of Mtz into the bacterial cell is not fully understood. It is believed that Mtz enters the cell through passive diffusion due to its low molecular weight which aids its entry into most cell membranes (Muller, 1983). An investigation into the effect of oxygen on Mtz uptake provided some valuable information on Mtz activity (Muller and Lindmark, 1976). The study revealed that formation of the toxic form of the drug reduced the amount of pro-drug present in the cell and resulted in a concentration gradient that increased further Mtz uptake by the cell. The study further revealed that the rate of this process depended on competition for the free electrons required for drug activation (see next section), and that oxygen was a very effective competitor. It has been shown that oxygen reduced the rate of uptake to very low levels, or in some organisms, abolished it completely. This explains the lack of effective activity against aerobes but also suggests that Mtz may not be as effective against

anaerobes if the infection is present in highly oxygenated areas (Muller and Lindmark, 1976). No direct evidence exists for active transport of the drug into the cell, however, some studies suggests that Mtz uptake may be an energy dependent process. One study showed that the rate of Mtz uptake increased with a corresponding increase in the concentration of sucrose (Church and Laishley, 1995). It was also revealed that Mtz uptake was significantly inhibited by the addition of CCCP, a proton motive force inhibitor (Church and Laishley, 1995). This suggests that an active transport system may exist for Mtz uptake, however, this still needs to be conclusively proven.

1.5.3 Activation

Activation of Mtz relies upon the low redox potential of the drug to sequester electrons and reduce it to its nitro radical form. Reduction of Mtz is thought to occur through one of two ways: either through nitroreductase activity encoded by *rdxA*, a nonessential oxygen insensitive NADPH-dependent Mtz reductase (Sisson *et al*., 2000), or through the interaction with the pyruvate: ferredoxin oxidoreductase complex (Diniz *et al*., 2004).

Reduction of the 5-nitro group of Mtz by RdxA results in the formation of the mutagenic and DNA-damaging nitro radical product (Sisson *et al*., 2000). This was confirmed by the fact that nearly all Mtz resistant *H. pylori* clinical isolates were found to contain mutations in *rdxA* that resulted in loss of gene function (Goodwin *et al*., 1998). However, the *rdxA* gene product is not solely responsible for the activation of Mtz as treatment of *rdxA* mutant strains still resulted in cell death (Sisson *et al*., 2000). One of the enzymes that could also be responsible for Mtz activation is FrxA, a flavin nitroreductase. Studies revealed that inactivation of both *frxA* and *rdxA* resulted in increased Mtz resistance. In addition, inactivation of the *frxA* together with *fdxB*, a gene encoding a ferredoxin-linked protein, doubled the MIC of the strain tested (Sisson *et al*., 2000).

The more commonly accepted mechanism of Mtz activation is thought to be through its interaction with the pyruvate: ferredoxin oxidoreductase (PFOR) complex (Upcroft *et al*., 2006). The PFOR complex is formed through the metabolic reaction between pyruvate and the oxidoreductase enzyme to form acetyl CoA (Edwards, 1980). This results in the generation of electrons that have an efficiency of -430 mV which are sequestered by Mtz. Under normal conditions, pyruvate is oxidised to form acetyl CoA which is then metabolized to the end product, acetate (Fig. 1.3) (Edwards, 1980).

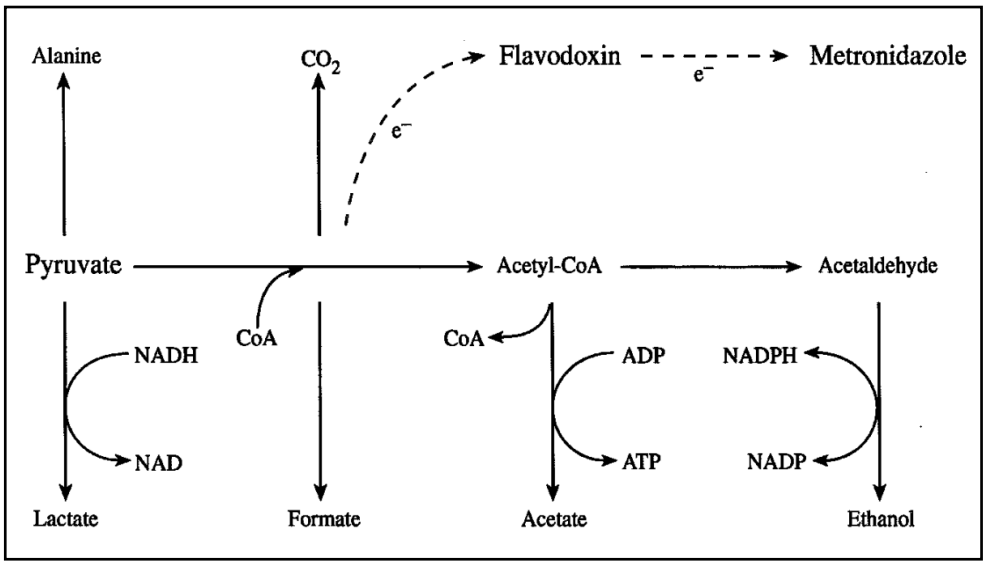


Figure 1.3: Schematic representation of the PFOR pathway in *H. pylori*. Pyruvate is converted to acetyl CoA, electrons are transferred to flavodoxin (or ferredoxin) and then Mtz to activate it (dashed line) (Kaihovaara *et al*., 1998).

As mentioned earlier the reduction potential of Mtz is less negative than that of the PFOR metabolic complex and thus acts as an electron sink and removes the electrons from the pathway. These electrons then reduce the nitro group of the drug and form the toxic nitro radicals. Numerous studies have identified Mtz resistant mutants that had decreased activity of the PFOR complex which implicated it in Mtz activation (Diniz *et al*., 2004, Sindar *et al*., 1982). The involvement of PFOR in Mtz activation was further confirmed by Kaihovaara *et al*. (1998) who showed that in *H. pylori*, the drug's activation was mediated through the PFOR complex, where flavodoxins are used instead of ferredoxins (Fig. 1.3). This was confirmed by

the fact that bacterial extracts depleted of flavodoxins lost their ability to reduce Mtz (Kaihovaara *et al*., 1998). Ferredoxins and flavodoxins are proteins which carry electrons in many bacterial species. They are characterised by their electron transport group, either an iron-sulphide or a flavin group respectively and are both present in most bacteria (Kaihovaara *et al*., 1998).

The only studies of this kind performed in *B. fragilis* have looked at Mtz resistant isolates. It was found that Mtz resistant mutants had decreased PFOR activity and metabolic end products that were different from Mtz sensitive parent strains (Britz and Wilkinson, 1979). Interestingly, no ferredoxins have been extracted from *B. fragilis*, and Diniz *et al*. (2004) instead reported the presence of a flavodoxin gene, *fldA* in *B. fragilis* 638R.

1.5.4 DNA damage

It is believed that the DNA helix is destabilised through its interaction with the nitro radical anions formed during Mtz activation, resulting in single and double strand breaks. This results in inhibition of DNA replication and synthesis (Sigeti *et al*., 1983). DNA breaks due to Mtz activity were observed in bacterial cells by Diniz *et al*. (2000). They reported that DNA breaks were detected in *Bacteroides distasonis* cultures 10 h after exposure to Mtz. This also coincided with the death of almost the entire cell culture. They were able to observe DNA breaks occurring even at time zero, suggesting that Mtz was acting even during the short time spent processing the samples (Diniz *et al*., 2000). Of most interest and possibly rather worrying is the observation that along with the strand breaks Mtz has been observed to cause DNA mutations in surviving cells (Sisson *et al*., 2000). In strains of *E.* coli treated with Mtz and grown aerobically (i.e. futile cycling conditions) it was found that there were high frequencies of CG-to-GC transversions and AT-to-GC transitions. It was also observed that Mtz was more mutagenic for Mtz resistant strains of *H. pylori* than Mtz sensitive ones (Sisson *et al*., 2000).

This suggests that Mtz resistant strains may possess resistance mechanisms which are aiding them in cell survival and allowing for DNA repair.

Mtz is highly effective against anaerobic bacteria and utilises an essential bacterial pathway to activate itself and cause DNA damage (Reysset, 1996). However, the mutagenic capacity of Mtz as well as its widespread use, have resulted in resistance. Resistance can occur at any of three possible levels, cell entry and uptake, activation of the drug, and repair of DNA damage (Reysset, 1996). This resistance is multifactorial and includes a number of different mechanisms which will be reviewed here.

1.6 Mechanisms of metronidazole resistance

Although Mtz has been in clinical use for more than 45 years, resistance to the antibiotic still remains relatively rare (1-5%) (Lofmark *et al*., 2010). However, the incidence and severity of resistance is continuing to rise (Hedberg and Nord, 2002). A number of Mtz resistance mechanisms have been identified in bacteria. Those present in *B. fragilis* involve the removal of Mtz through efflux pumps, inhibition of the electron transport chain and alteration of metabolic pathways, Mtz inactivation through 5-nitroimidazole reductases and DNA repair systems (Wexler, 2007).

1.6.1 Mtz export *via* efflux pumps

After an antimicrobial enters the cell, the first stage of resistance is active export of the drug from the cytoplasm (Ueda *et al*., 2005). This is accomplished through the use of efflux systems which are known to expel antimicrobials from the cell and into the surrounding environment and are not given the opportunity to cause any cell damage. Sixteen efflux pumps (bmeABC1-16) have been identified in *B. fragilis* (Ueda *et al*., 2005). These belong to the resistance-nodulation-division (RND) as well as the multidrug and toxic extrusion class (MATE). These

pumps are made up of three parts: the pump component, a membrane fusion protein and an outer membrane channel protein (Pumbwe *et al*., 2006a). It has been shown that in *B. fragilis* each of the 16 operons has all three components. The inner membrane pump is encoded by *bmeB*, while the membrane fusion protein is encoded by *bmeA* and *bmeC* codes for the outer membrane channel. It was revealed that transcripts of 15 of the 16 inner membrane pump genes were detectable in *B. fragilis* (Pumbwe *et al*., 2006a). Exposure to antibiotics revealed that the efflux pumps transported a variety of microbial agents including: antibiotics, detergents, dyes and biocides and that deleting three or more of the genes caused an increase in transcription of the other pump genes as well as an increase in antimicrobial MICs (Wexler, 2012). This increase in MIC was reversible upon the addition of CCCP. It is believed that each of the 16 Bme pumps has a specific function in the bacterium and that certain pumps (including *bme5*, *bme15*, possibly *bme7*) are normally transcribed at high levels. However, under antibiotic pressure, these genes are upregulated and may contribute to multidrug resistance (Wexler, 2012).

Another potential mechanism for Mtz resistance involves a point mutation in a regulatory region upstream of the gene coding for the efflux pump BmeABC5 (Pumbwe *et al*., 2007a). This mutation resulted in the inability of the transcriptional regulator repressor, TetR, to bind to this regulatory region. Further experiments showed that a null mutant of the putative repressor gene *bmeR5*, in the *B. fragilis* strain ADB77, caused the overexpression of the three efflux genes, *bmeB5*, *bmeA5* and *bmeC5* resulting in an increase in resistance to Mtz and other antibiotics (Pumbwe *et al*., 2007a).

1.6.2 Inhibition of metabolic activation of Mtz

Once Mtz has entered the cell it requires activation by the bacterial metabolic pathways. Prevention of activation will inhibit Mtz activity and result in Mtz resistance in the bacterial

cell. Inactivation of elements of the electron transport chain were identified when it was observed that metronidazole resistant *B. fragilis*, generated by passaging a sensitive strain across media containing a Mtz gradient, exhibited reduced activity of the enzymes PorA, Fld and Rdx which have been shown to be involved in the electron transport chain (Diniz *et al*., 2004). The reduction in activity of these enzymes is believed to result in fewer electrons being available to reduce Mtz to its active form. Thus, it is theorized that those strains with decreased or impaired enzyme function in the electron transport chain, will be more resistant to Mtz (Diniz *et al*., 2004). In a study performed on *Porphyromonas gingivalis* it was observed that a reduction in iron availability resulted in an increase in Mtz activity (Moon *et al*., 2011). It was believed that this was due to an increase in the reaction between Mtz and the PFOR complex. Thus, it is possible that an increase in the availability of iron may increase bacterial resistance to Mtz (Moon *et al*., 2011).

As described, above Mtz is activated by removing electrons which would normally be transferred to hydrogen ions along the PFOR complex (Upcroft *et al*., 2006). Alteration to elements of this metabolic pathway were identified as a possible resistance mechanism when *Clostridium perfringens* mutants were observed to have reduced levels of pyruvate dehydrogenase and high levels of lactic acid end products (Sindar *et al*., 1982). A study in a *B. fragilis* Mtz resistant isolate, investigated whether lactate dehydrogenase (LDH) levels were affected by decreased PFOR activity (Narikawa *et al*., 1991). It was revealed that detectable levels of LDH were present upon Mtz exposure. However, PFOR was completely undetectable after exposure to even low levels of Mtz. This suggested that LDH was compensating for reduced PFOR activity (Narikawa *et al*., 1991). Alteration to the metabolic pathway by increasing the activity of LDH means that rather than pyruvate being oxidised to acetyl CoA by

PFOR and thus generating electrons for the reduction of Mtz, it is instead converted to lactate (Fig. 1.4) (Diniz *et al*., 2004).

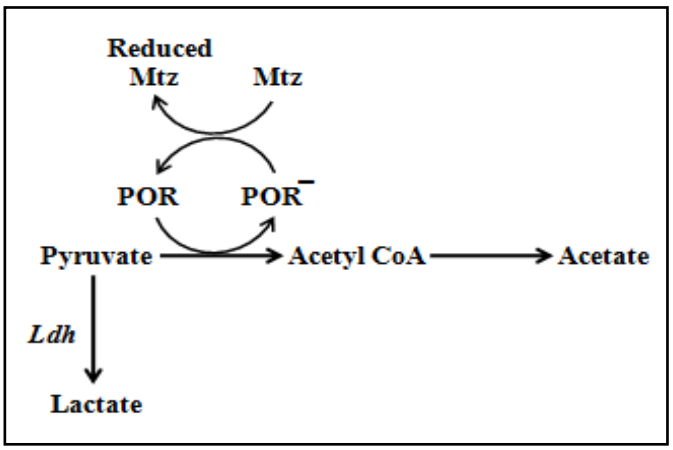


Figure 1.4: Pyruvate pathway showing increased lactate dehydrogenase activity (Diniz *et al*., 2004).

It has been hypothesized that this results in a decrease in the number of electrons available for the reduction of Mtz causing an increase in the resistance of the strain to the drug (Diniz *et al*., 2004). Analysis of a *B. fragilis* Mtz resistant strain revealed that it had a flavodoxin gene that was down regulated compared to a wild type strain. It was also revealed that no PFOR activity could be detected in any of the resistant strains and analysis of the end products showed high levels of lactate (Diniz *et al*., 2004). A single crossover insertion mutant was made in Mtz sensitive *B. fragilis* 638R by inactivating the *porA* gene which codes for PFOR. It was found that this mutant had significantly increased levels of lactate present in its end products and had increased Mtz resistance compared to the wild type strain (Diniz *et al*., 2004). Two other mutants, defective in *rdxA* (nitroreductase) and *fldA* (flavodoxin) respectively, were also found to have increased Mtz resistance. Double mutants defective in either *porA* and *rdxA*, or *porA* and *fldA* were found to be more Mtz resistant than any of the single mutants (Diniz *et al*., 2004). All of this data supports the hypothesis that electrons are being shunted down the LDH pathway instead of the PFOR route.

Interestingly, the *porA* deficient mutant was only able to achieve a Mtz MIC of 1 mg/L which was 2-fold higher than the wild type strain (Diniz *et al*., 2004). However, this MIC is well below the resistance levels observed in clinical isolates, which can often be as high as 256 mg/L (Galvao *et al*., 2011). Even the double mutants described above were only able to generate MICs of 3.5 and 6 mg/L respectively (Diniz *et al*., 2004), suggesting that alteration of a single gene or multiple genes does not confer clinical levels of resistance.

Recently, it has been demonstrated, in *Bacteroides thetaiotaomicron*, that there was a link between the rhamnose carbohydrate catabolism regulatory protein (RhaR) and Mtz resistance (Patel *et al*., 2009). It was revealed that overexpression of the regulator increased transcription of the rhamnose operon. It was also shown that overexpression of RhaR resulted in increased production of LDH and decreased production of PFOR, and an increase in Mtz resistance (Patel *et al*., 2009). Although *B. fragilis* has no equivalent rhamnose operon, the involvement of modifications in carbohydrate metabolism in Mtz resistance remains a possibility.

1.6.3 Mtz inactivation

Mtz can be inactivated by specific reductases that convert the drug to its inactive form. The most widely described mechanism of this type of resistance in *B. fragilis* involves the contribution of the nitroimidazole resistance (*nim*) genes. These genes are thought to encode various types of nitroimidazole reductases. Ten *nim* genes, namely *nimA-J*, which are believed to confer reduced susceptibility to Mtz, have been identified (Husain *et al*., 2013, Soki *et al*., 2006). These genes share approximately 70% sequence similarity with each other and may occur in all *Bacteroides* species. The first four genes, *nimA-D*, were identified on individual mobile genetic elements specific for each gene type and with activational insertion sequences (Soki *et al*., 2006). Three of the genes are carried on plasmids *nimA* (pIP417), *nimC* (pIP419) and *nimD* (pIP421) with *nimB* being found on the chromosome (Soki *et al*., 2006). Little

further information is available about the other four *nim* genes E – H, except that they can all be detected by PCR using "universal" primers. However, a new *nim* gene, *nimJ*, was recently identified in *B. fragilis* which was not amplified by the universal *nim* primers (Husain *et al*., 2013). This gene is located on the chromosome of *B. fragilis* strains HMW615 and HMW616. Overexpression of this gene resulted in a low level increase in Mtz resistance providing proof that *nimJ* was a novel nitroimidazole resistance gene (Husain *et al*., 2013).

The exact function of the *nim* genes is not known, however, it is speculated that they encode various homologous of a nitroimidazole reductase that converts 5–nitroimidazole to 5-aminoimidazole (Fig. 1.5), thus preventing the reduction of Mtz to its nitro radical anion form (Reysset, 1996, Edwards, 1980).

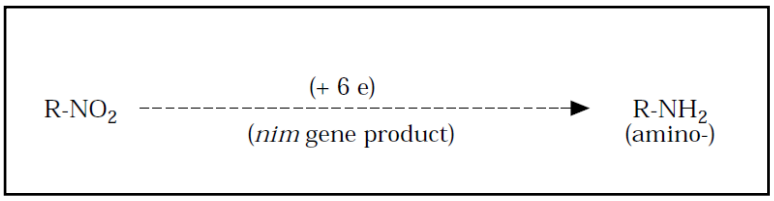


Figure 1.5: Proposed mechanism of resistance encoded by the *nim* genes (Reysset, 1996).

It is interesting to note that the presence of *nim* genes does not necessarily translate into Mtz resistance, as *nim* genes have been detected in *Bacteroides* species which have shown sensitivity much lower than that of the standard breakpoint of 32 mg/L. The converse has also been observed, whereby strains have shown extreme Mtz resistance in the absence of any known *nim* genes (Gal and Brazier, 2004). The possible reason for the lack of Mtz resistance, while harbouring a *nim* gene, may be due to the lack of an activating IS element. As has been discussed earlier, IS elements often cause the transcription or overexpression of a gene when it is inserted upstream of that gene (Soki, 2013).

Expression of *nimA*, *nimC* and *nimD* in Mtz sensitive strain, *B. fragilis* 638R, resulted in MICs of 24, 6 and 16 mg/L respectively (Lofmark *et al*., 2005). A similar situation is observed with overexpression of *nimJ* in *B. fragilis* 638R, however, this only produced a Mtz MIC of 0.19 mg/L (Husain *et al*., 2013). This, once again, is well below the resistance levels detected in clinical isolates and suggests that expression of *nim*, alone, may not be responsible for clinical resistance.

1.6.4 DNA damage repair

Once Mtz has been activated to its nitro radical form and caused numerous single and double stranded DNA breaks, resistance to the effects of the drug can still occur through efficient repair of DNA damage. DNA repair mechanisms play an important role in Mtz resistance as the ability to survive DNA damage dealt by the drug provides an advantage to a bacterium (Steffens *et al*., 2010). The mechanisms for DNA damage repair in *B. fragilis* are not well understood, although, some studies have investigated these systems. RecA is an important DNA repair protein which is involved in homologous recombination repair and expression of many other DNA repair proteins in certain bacterial species (Kuzminov, 1999). One of the first such studies investigated the role of the RecA protein in *B. fragilis* DNA repair. A putative *recA* gene was isolated from *B. fragilis* which complemented a RecA deficient *E. coli* strain (Goodman *et al*., 1987). In *H. pylori* it was demonstrated that mutations to the *recA* gene increased the strains sensitivity to Mtz by 10-fold compared to the parent strain (Thompson and Blaser, 1995). The effect of RecA on Mtz sensitivity in *B. fragilis* was identified by Steffens *et al*. (2010), who reported that a *recA* deficient mutant was highly metronidazole sensitive. It was revealed that overexpression of a *recA* gene in a Mtz sensitive *B. fragilis* wild type strain caused an increase in Mtz resistance (Steffens *et al*., 2010). Several other DNA repair systems exist in *B. fragilis* which may also be involved in repairing Mtz strand breaks. The AddAB helicase and nuclease complex was found to be important in *B. fragilis* survival

following DNA strand breaks (Reuter *et al*., 2010). An excision repair pathway was identified in *B. fragilis* in response to UV and mitomycin C damage (Abratt *et al*., 1986), while bioinformatic analysis revealed that putative recombination repair genes with homologues to RecF, RecO and RecR were present in *B. fragilis* 638R (Steffens *et al*., 2010). It was also revealed that inactivation of the genes encoding a putative RecQ helicase present in *B. fragilis* resulted in increased susceptibility to Mtz (Paul *et al*., 2011). This suggested that both *recA* and *recQ* were important specifically in Mtz resistance and that the DNA other repair mechanisms present in *B. fragilis* may also play a role.

Although, significant research has been conducted on identifying Mtz resistance mechanisms present in *B. fragilis*, no single mechanism has been revealed that can cause the level of resistance observed in clinical strains (Husain *et al*., 2013). This has led many researchers to hypothesise that Mtz resistance is a multifactorial system involving numerous different mechanisms, each playing a role that, together, enables the cell to tolerate high Mtz levels (Husain *et al*., 2013, Soki, 2013). Alternatively, there may be an as yet undiscovered resistance mechanism present in *B. fragilis* that is causing the high clinical levels of Mtz resistance.

1.7 Aims and objectives

In comparison with the rest of the world, very few *B. fragilis* antibiotic surveys have been performed in South Africa. Little information is, therefore, available about the levels of antibiotic resistance, the presence of resistance genes or the spread of multidrug resistance. This dissertation aimed to investigate the occurrence and genetic basis for antibiotic resistance present in specific *B. fragilis* strains isolated at Groote Schuur Hospital, Cape Town, South Africa, with particular focus on Mtz resistance and possible novel resistance mechanisms.

The specific objectives of the study were as follows:

Objective 1: To perform an antibiotic resistance survey of 23 isolates of *B. fragilis* isolated from Groote Schuur Hospital, as well as determining whether known resistance determinants and mechanisms are responsible for this observed resistance to a range of antibiotics in particular, metronidazole.

Objective 2: To identify and characterise a putative novel *nim* gene present in the *B. fragilis* 638R genome and investigate its possible role in Mtz^R from a genetic and functional perspective using a variety of molecular and physiological techniques.

Chapter 2

Antibiotic susceptibility and resistance gene survey

2.1 Introduction

Bacteroides fragilis is one of the leading causes of intra-abdominal infections and bacteraemias, which, without antibiotic treatment, can prove fatal (Wexler, 2007). Treatment regimens are often determined from surveillance reports of the susceptibility patterns of *B. fragilis* (Nagy *et al*., 2010) since recent surveillance data has revealed that the incidence of *B. fragilis* antibiotic resistance is increasing worldwide. This includes resistance to antibiotics previously thought to be universally active such as metronidazole and carbapenems (Nagy *et al*., 2010, Snydman *et al*., 2010). The reason for this widespread resistance is the ability of *B. fragilis* to efficiently and effectively transfer antibiotic resistance determinants. These determinants carry antibiotic resistance genes and include conjugative transposons, integrated genetic elements and conjugative and mobilizable plasmids (Eitel *et al*., 2013, Nagy *et al*., 2010).

Clindamycin (Cln) is one of the most commonly used antibiotics for the treatment of *B. fragilis* infections (Eitel *et al*., 2013). Clindamycin resistance is mainly attributed to the presence of resistance genes (*ermB, ermF* and *ermG*) which interfere with the antibiotic's ability to attach to the ribosomal binding site of bacteria (Pal, 2006). Many *B. fragilis* isolates show reduced susceptibility if not total resistance to Cln, and surveys performed in Europe found that between 24.2 and 28.5% of isolates were Cln resistant (Eitel *et al*., 2013, Nagy *et al*., 2010). A similar survey completed across Japan observed slightly higher resistance with 33% of all *B. fragilis* isolates showing reduced susceptibility (Tran *et al*., 2013). An American survey reported worrying indications of increasing Cln resistance when they observed only 6% resistance in 1980 but 35% in 2007 (Snydman *et al*., 2010).

Although *B. fragilis* is nearly completely resistant to most common β-lactam antibiotics, a few of the cephalosporins and carbapenems remain effective. The best examples of these classes

are cefoxitin (Cef) and imipenem (Imp) respectively (Eitel *et al*., 2013, Soki *et al*., 2011). Cefoxitin resistance has been linked to the presence of the *cfxA* gene which encodes a class A β-lactamase (Fang *et al*., 2002). Carbapenem resistance has been shown to be due to the presence of the *cfiA* gene which is believed to encode a metallo-β-lactamase. The *cfiA* gene may be expressed to various degrees or may remain completely silent resulting in various levels of resistance (Soki *et al*., 2011). Although mechanisms of resistance exist for both Cef and Imp, they still remain quite effective. No Imp resistant *B. fragilis* were detected in Japan (Tran *et al*., 2013) and both the USA (Snydman *et al*., 2010) and Europe (Nagy *et al*., 2010) reported less than 1% resistance. Resistance levels were slightly higher for Cef in Europe (13.7% resistance) and in the USA (9%). However, this is still an increase from 1980 when only 4% of *B. fragilis* strains showed resistance (Nagy *et al*., 2010, Snydman *et al*., 2010).

Tetracycline (Tet) is one of the least effective antibiotics for treating *B. fragilis* infections as most strains show partial if not complete resistance (Bartha *et al*., 2011). The *tetQ* gene is responsible for most of the Tet resistance observed in *B. fragilis* and encodes a ribosomal protection protein (Rasmussen *et al*., 1993). Tetracycline resistance in Japan was reported a 86% of isolates (Tran *et al*., 2013), while in Brazil it was found that 68% of the strains were resistant (Boente *et al*., 2010). The related compound tigecycline (Tig) is the first of a novel family of antimicrobial agents, the glycylcyclines. These are derived from a tetracycline nucleus, but are not affected by the existing tetracycline-resistance mechanisms. The only currently known mechanism of Tig resistance is through the products of the *tetX* and *tetX1* genes which encode a FAD-dependent monooxygenase, which destroys Tet (Bartha *et al*., 2011). In contrast to Tet resistance levels, Tig resistance remains very low, with two separate European studies reporting levels of only 1 and 1.7% resistance respectively (Hawser *et al*., 2010, Nagy *et al*., 2010).

One of the most effective antibiotics used for treating *B. fragilis* infections is metronidazole (Mtz) (Lofmark *et al*., 2010). Mtz is a 5-nitroimidazole agent which is often used as the last line of defence when other antibiotics have failed to properly treat an infection. Resistance to Mtz is most often attributed to the presence of the nitroimidazole resistance genes (*nimA-J*), although other resistance mechanisms such as drug efflux, prevention of drug activation and enhanced DNA repair have also been shown to play a role (Chapter 1) (Lofmark *et al*., 2010, Steffens *et al*., 2010, Wexler, 2007). Worldwide, the incidence of resistance has been reported to be between 1 and 5% (Hedberg and Nord, 2002). Both the European study and the survey performed in the USA observed less than 1% resistance, while the Japanese were unable to detect any Mtz resistance in their *B. fragilis* isolates (Tran *et al*., 2013, Nagy *et al*., 2010, Snydman *et al*., 2010).

In comparison to the rest of the world very few antibiotic resistance surveys are performed in South Africa, and those that are performed do not include resistance gene screening. The aims of this chapter were, therefore, to determine the antibiotic resistance profiles of pilot group of *B. fragilis* clinical isolates from patients admitted to GSH Cape Town, South Africa and screen these strains for the presence of resistance genes. It was hoped that this would provide information with regards to the susceptibility patterns and genetic makeup of the bacterial species in a South African context and form a basis for future larger surveillance studies.

2.2 Materials and Methods

2.2.1 Bacterial strains and plasmids, media and growth conditions

Twenty-five *B. fragilis* clinical isolates were sourced from the Groote Schuur Service Laboratory at the Groote Schuur Hospital in Cape Town, Western Cape, South Africa (Galvao *et al*., 2011). The strains and plasmids used are described in Table 2.1.

All *Bacteroides* strains were grown in brain heart infusion supplemented with hemin (5 mg/L), menadione (0.5 mg/L) and cysteine (0.5 mg/L) broth (BHISB) or agar (BHISA) at 37°C under anaerobic conditions (Holdeman and Moore, 1972).

2.2.2 Polymerase chain reaction (PCR) techniques

All PCR reactions used Kapa Ready Mix (Inqaba Biotech) with 2.5 mM $MgCl_2$ as per manufacturer's instructions, and the thermal cycling was performed using a GeneAmp® PCR system 9700 (Applied Biosystems). The standard PCR parameters used consisted of an initial 5 min denaturation step at 95°C, followed by primer pair specific number of cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at primer pair specific temperatures, and elongation at 72°C for 1 min. The thermal cycling was then concluded with a 7 min elongation step at 72°C. All PCR primers and primer specific cycling parameters used are described in Table 2.2.

2.2.3 16S rRNA gene sequencing for identification of Groote Schuur Hospital (GSH) clinical isolates

All GSH isolates were identified to the genus and species level by sequencing of the 16s rRNA gene which was amplified using the universal primers F27 and R5 (Table 2.2). DNA sequencing was performed by Macrogen Inc., Seoul, Korea. The sequences obtained were analysed using BLAST 2.2.17 (Altschul *et al*., 1997).

Table 2.1: Strains and plasmids used in this study

Strain/Plasmid	Genotype/Phenotype	Source/Reference
Plasmids		
pLYL01	Mob^{+}, $Tet^{R}Amp^{R}$, *tetQ*	(Li *et al*., 1995)
pYT646	Mob^{+}, $Tet^{R}Amp^{R}Erm^{R}$, *tetX1*, *ermF*	(Tang and Malamy, 2000)
pMCL140:616*nimJ*	Mob^{+}, Amp^{R}, *nimJ*	(Husain *et al*., 2013)
Strains		
Bacteroides vulgatus		
CLA341	Clinical Strain, Tet^{R} Cfx^{R}, *tetQ cfxA*	(Soki *et al*., 2011)
Bacteroides fragilis		
638R	Clinical Strain, Rif^{R} $Gent^{R}$	(Privitera *et al*., 1979)
Gbr7	Clinical Strain, Mtz^{R}, *nimA*	(Lofmark *et al*., 2005)
GSH2	Clinical Isolate	This study
GSH3	Clinical Isolate	This study
GSH5	Clinical Isolate	This study
GSH6	Clinical Isolate	This study
GSH7	Clinical Isolate	This study
GSH8	Clinical Isolate	This study
GSH9	Clinical Isolate	This study
GSH10	Clinical Isolate	This study
GSH12	Clinical Isolate	This study
GSH13	Clinical Isolate	This study
GSH14	Clinical Isolate	This study
GSH15	Clinical Isolate	This study
GSH16	Clinical Isolate	This study
GSH17	Clinical Isolate	This study
GSH18	Clinical Isolate	This study
GSH19	Clinical Isolate	This study
GSH20	Clinical Isolate	This study
GSH21	Clinical Isolate	This study
GSH22	Clinical Isolate	This study
GSH23	Clinical Isolate	This study
GSH24	Clincal Isolate	This study
GSH25	Clinical Isolate	This study
GSH26	Clinical Isolate	This study
GSH28	Clinical Isolate	This study
GSH30	Clinical Isolate	This study

Table 2.2: Primers and PCR parameters used in this study

Primers	PCR annealing temperatures and cycles and gene target	Source/Reference
F27 5'-AGAGTTTGATCITGGCTCAG-3'	55°C and 25 cycles 16S rRNA gene	(Cheneby *et al*., 2000)
R5 5'-ACGGITACCTTGTTACGACTT-3'		
nim3 5'-ATGTTCAGAGAAATGCGGCGTAAGCG-3'	57°C and 25 cycles *nimA-I* gene	(Trinh and Reysset, 1995)
nim5 5'-GCTTCCTTGCCTGTCATGTGCTC-3'		
615-616nimJ-qRT-F 5'- TGACAAGGCTTCGTTCTGTG-3'	55°C and 31 cycles *nimJ* gene	(Husain *et al*., 2013)
615-616nimJ-qRT-R 5'- GTCGAAACGAATCATCAGCA-3'		
tetQ1 5'-GGCTTCTACGACATCTATTA-3'	50°C and 30 cycles *tetQ* gene	(Shoemaker *et al*., 2001)
tetQ2 5'-CATCAACATTTATCTCTCTG-3'		
cfiA1 5'-CCATGCTTTTCCCTGTCGCAG-3'	50°C and 35 cycles *cfiA* gene	(Podglajen *et al*., 1992)
cfiA2 5'-GGGCTATGGCTTTGAAGT-3'		
cfxA1 5'-ATCGTAGTTTTGAGTATAGCT-3'	57°C and 30 cycles *cfxA* gene	(Garcia *et al*., 2008)
cfxA2 5'-TAAAAGCACTCCGATAACGAT-3'		
ermF1 5'-CCTTATGGCATTACTTCCGA-3'	55°C and 30 cycles *ermF* gene	(Pumbwe *et al*., 2007b)
ermF2 5'-GGACCTACCTCATAGACAAG-3'		
IS942B 5'-AGAAAAGCATGGTCTTTAACCAAAGTC-3'	50°C and 35 cycles Various IS elements	(Soki *et al*., 2004)
IS1186A 5'-GAGAATCAAGCTTCTCGCC-3'		
IS4351C 5'-AACCGAGGATCCAAGGTATGCAATTTCT-3'		
IS1169/1 5'-TGAGTCAGAGAATCGTG-3'		
IS1170/1 5'-CTTCTGTGTGTCATGAG-3'		
Up2 5'-TACGCTTTTCTGTGCCATAACTGC-3'	52°C and 35 cycles Upstream region of *cfiA* gene	(Soki *et al*., 2004)
G 5'-CGCCAAGCTTTGCCTGCCATTA-3'		

2.2.4 Antibiotic susceptibility testing of the GSH isolates

The susceptibility of the strains to metronidazole (Mtz), tetracycline (Tet), cefoxitin (Cfx), imipenem (Imp), erythromycin (Erm), Tigecycline (Tig) and Clindamycin (Cln) was determined by measuring the minimum inhibitory concentration (MIC) on BHISA plates using E-test strips according to the manufacturer's instructions (AB Biodisk).

2.2.5 Screening of GSH isolates for antibiotic resistance genes

Known antibiotic resistance genes were amplified using published primers (Table 2.2). The following genes were screened for: *nimA*-*J* genes (Mtz), *tetQ* gene (Tet), *cfxA* (Cfx), *cfiA* (Imp), *ermF* (Erm/ Cln). The following bacterial strains and plasmids were used as positive controls: *B. fragilis* Gbr7 (*nimA*), *Bacteroides vulgatus* CLA341 (*cfx*A), pMCL140:616*nimJ* (*nimJ*), pLYL01 (*tetQ*) and pYT646 (*ermF*).

2.2.6 Identification of insertion sequences (IS) upstream of *cfiA*

The PCR strategy of Soki *et al*., 2004 was followed. Briefly, to determine whether the IS element was upstream of the *cfiA*, PCR was performed with a forward primer specific to the IS (IS942B, IS1186A, IS4351C, IS1169/1 or IS1170/1) and a reverse primer to either the 5' (primer Up2) or 3' (primer cfiA2) end of the *cfiA* (Table 2.2). To identify novel IS elements, PCR was done using a forward primer annealing to the upstream region of *cfiA* gene (primer G) and a reverse primer recognising the 5' region of *cfiA* (primer Up2). The strategy described above is depicted in Fig. 2.1A. The PCR amplification protocol for the IS elements included 5 min at 94°C followed by 35 cycles of 30 sec at 94°C, 1 min at annealing temperature (Table 2.2), 3 min at 72°C and 10 min at 72°C. PCR products were sequenced (Macrogen Inc., Seoul, Korea) and bioinformatics analysis was performed using the National Centre for Biotechnology Information (www.ncbi.nih.gov). Multiple sequence alignments were carried out with DNAMAN version 4.13 (Lynnon BioSoft).

2.2.7 Determination of stability of Mtz resistance of subpopulation colonies

The stability of the resistance was evaluated by determining the MIC of Mtz after three consecutive subcultures onto antibiotic free BHISA. Resistance was regarded as stable when the MIC of Mtz for the mutants was maintained after the passages (Pelaez *et al*., 2008).

2.2.8 Total DNA extraction and detection of the presence of plasmids

Total *B. fragilis* DNA was extracted using the high-salt buffer total DNA extraction method (Campbell and Yasbin, 1984). The DNA was subjected to gel electrophoresis on a 1% agarose gel at 60 V for 2 h. Southern blotting was done according to the method described in the DIG application manual for filter hybridization (Roche) on the total DNA of *B. fragilis* strains showing the presence of plasmids. A DNA *tetQ* gene probe was made from primer tetQ1. Labelling was by DIG High Prime DNA Labelling and Detection II kit (Roche) according to manufactures instructions.

2.3 Results and Discussion

2.3.1 Identification of GSH clinical isolates

The 16S rRNA genes of all 25 clinical isolates from Groote Schuur Hospital were sequenced in order to confirm their identities as *B. fragilis*. The sequencing results and identity of the isolates is shown in Table 2.3. All strains were identified as *B. fragilis* except for GSH24 and GSH26 which were identified as *Bacteroides thetaiotaomicron* and were thus excluded from this study.

Table 2.3: Identification of clinical isolates from Groote Schuur Hospital

Strain	Identification	Accession Number	Max. Identity
GSH2	*B. fragilis* 12491	X83940.1	99%
GSH3	*B. fragilis* 12491	X83940.1	99%
GSH5	*B. fragilis* YCH46	AP006841.1	99%
GSH6	*B. fragilis* 638R	EU728706.1	99%
GSH7	*B. fragilis* B119	X83942.1	99%
GSH8	*B. fragilis* 2991	X83938.1	99%
GSH9	*B. fragilis* TAL3636	X83941.1	99%
GSH10	*B. fragilis* JCM 11017	AB542764.1	99%
GSH12	*B. fragilis* 638R	EU728706.1	99%
GSH13	*B. fragilis* YCH46	AP006841.1	99%
GSH14	*B. fragilis* 2991	X83938.1	99%
GSH15	*B. fragilis* Bfr81	X83943.1	99%
GSH16	*B. fragilis* 638R	EU728706.1	99%
GSH17	*B. fragilis* 638R	EU728706.1	100%
GSH18	*B. fragilis* YCH46	AP006841.1	99%
GSH19	*B. fragilis* YCH46	AP006841.1	99%
GSH20	*B. fragilis* 638R	EU728706.1	100%
GSH21	*B. fragilis* 638R	EU728706.1	99%
GSH22	*B. fragilis* JCM 11019	AB510701.1	99%
GSH23	*B. fragilis* 638R	FQ312004.1	99%
GSH24	*B. thetaiotaomicron* JCM 5827	AB510710.1	99%
GSH25	*B. fragilis* 638R	EU728706.1	99%
GSH26	*B. thetaiotaomicron* 17.4	AY319392.1	99%
GSH28	*B. fragilis* JCM 11017	AB542764.1	99%
GSH30	*B. fragilis* JCM 11019	AB510701.1	99%

2.3.2 Determination of the minimum inhibitory concentrations (MIC) of GSH isolates

The susceptibility of the GSH strains to a variety of antibiotics was tested using the E-test method of MIC determination. It was found that no strains were Cln, Erm or Tig resistant, however, 4 of the strains showed greater than sensitive levels of resistance to Erm (Table 2.4). Mtz and Imp resistance was revealed to be 8%, while only one strain was Cef resistant (4%). A second strain was revealed to have Cef resistance levels well above those considered to be intermediate. The greatest number of strains were found to be resistant to Tet, with nearly 65% of isolates showing reduced susceptibility. In addition to this high percentage of resistance 3 of the strains show Tet resistance above intermediate level. This is important to note since, if those strains increased their resistance levels slightly, the occurrence of Tet resistance in the study group would increase to nearly 83%.

Table 2.4: Minimum inhibitory concentrations of antibiotics and resistance genes present in GSH isolates

Strains	MTZ (mg/L)	IMP (mg/L)	CEF (mg/L)	CLN (mg/L)	ERM (mg/L)	TET (mg/L)	TIG (mg/L)	*nimA-J*	*ermF*	*tetQ*	*cfiA*	*cfxA*
Gbr7	≥256	1.5	6	0.032	1	0.094	0.064	*nimA*	-	-	-	-
638R	0.125	0.094	0.5	0.094	0.125	0.047	0.023	-	-	-	-	-
GSH 2	0.094	0.094	4	0.047	0.25	**24**	0.032	-	-	*tetQ*	-	-
GSH 3	0.19	0.064	2	<0.016	0.25	12	<0.016	-	-	*tetQ*	-	-
GSH 5	0.19	0.125	3	0.094	2	**16**	0.094	-	-	*tetQ*	-	-
GSH 6	0.19	0.094	3	0.25	1.25	0.125	0.032	-	-	-	-	-
GSH 7	0.038	0.19	12	0.125	0.75	0.094	<0.016	-	-	-	*cfiA*	-
GSH 8	**≥256**	0.125	3	0.023	1	0.032	0.032	-	-	-	-	-
GSH 9	0.064	**≥32**	**64**	0.75	5.5	**24**	0.047	-	-	*tetQ*	*cfiA*	-
GSH 10	0.125	0.125	4	0.094	0.75	0.094	0.032	-	-	-	-	-
GSH 12	0.125	0.125	4	0.064	1	**16**	<0.016	-	-	*tetQ*	-	-
GSH 13	0.064	0.38	2	0.032	1	**24**	0.125	-	-	*tetQ*	-	-
GSH 14	0.047	0.094	2	0.032	0.75	**16**	0.047	-	-	-	-	-
GSH 15	**≥256**	**16**	48	0.50	0.75	**48**	0.75	-	-	*tetQ*	*cfiA*	-
GSH 16	0.19	0.094	6	<0.016	5	12	0.047	-	-	*tetQ*	-	-
GSH 17	0.125	0.125	3	0.125	5.5	**24**	0.19	-	-	*tetQ*	-	-
GSH 18	0.064	0.19	2	0.016	1.5	**32**	0.38	-	-	*tetQ*	-	-
GSH 19	0.25	0.094	8	0.016	0.38	**48**	0.094	-	-	*tetQ*	-	-
GSH 20	0.125	0.125	6	0.094	0.75	**16**	0.5	-	-	*tetQ*	-	-
GSH 21	0.19	0.064	1	0.064	0.5	**32**	0.38	-	-	*tetQ*	-	-
GSH 22	0.19	0.094	2	0.50	0.5	12	0.094	-	-	*tetQ*	-	-
GSH 23	0.25	0.094	6	0.064	0.75	**16**	0.047	-	-	*tetQ*	-	-
GSH 25	0.19	0.064	4	0.19	2	**16**	0.25	-	-	*tetQ*	-	-
GSH 28	0.25	0.047	3	0.047	6	**16**	0.38	-	-	*tetQ*	-	-
GSH 30	0.25	0.047	1.5	<0.016	1	0.032	0.25	-	-	-	-	-

Values shown in bold indicate resistance, all units are in mg/L. MIC breakpoints of resistance for antibiotics according to the National Committee for Clinical Laboratory Standards (2011) and are as follows: S = sensitive, I = intermediate and R = resistant, Mtz S≤8, I=16, R≥32; Imp S≤4, I=8, R≥16; Cef S≤16, I=32, R≥64; Cln S≤2, I=4, R≥8; Erm S≤4, I=8, R≥16; Tet S≤4, I=8, R≥16; Tig S≤4, I=8, R≥16.

Interestingly, it was observed that Tig was able to overcome the Tet resistance carried by these isolates. This is probably due to its ability to evade the Tet(A-E) efflux pumps and to bind to bacterial ribosomes even if they have been modified by ribosomal protection proteins which account for most of the observed Tet resistance (Livermore, 2005).

The pattern of resistance observed for Imp, Cef and Tig is fairly consistent with reported worldwide trends. However, the sensitivity to Cln and the high incidence of Mtz resistance differs quite substantially from published international data. The worldwide incidence of Cln resistance is closer to 30% and Mtz resistance is only approximately 1-5% (Nagy *et al*., 2010, Snydman *et al*., 2010, Hedberg and Nord, 2002). The results reported in the current study differ from the trends obtained from the same sampling site in 1998, where it was observed that no strains were Mtz resistant and only 5% of isolates were resistant to Cef and Cln (Koch *et al*., 1998). This shows an increase in resistance to Mtz and Cef and a decrease in Cln resistance since 1998 although the statistical significance of this could not be determined because of the small sample size. In one of the few other surveys performed in South Africa, it was determined that 39% of *Bacteroides* spp were resistant to Cln and 22% were resistant to Mtz (Meyer *et al*., 2006). This is different from the incidence of resistance reported in this study and may indicate the difference in preferred antibiotic treatments used at different hospitals. It has been suggested that changes in treatment regimens can result in a change in the observed patterns of clinical resistance and that this could possibly account for the reduction in resistance to some antibiotics and the increase in resistance to others (Chan *et al*., 2010). However, a study correlating treatment protocols with bacterial resistance has yet to be done to prove this.

Interestingly two of the isolates, GSH9 and GSH15, appeared to be multidrug resistant. They both showed resistance to at least three antibiotics with GSH15 being resistant to four of those tested. As most other surveillance studies only ever look at resistance as a group rather than on a strain by strain basis, it is not known exactly how these results compare to those obtained internationally.

2.3.3 Presence of known resistance genes and *cfiA* insertion sequences in isolates

To determine whether the GSH strains possessed known resistance genes, PCR analysis was performed using published primers (Table 2.4) and the plasmids and strains indicated in the methods as positive controls. It was observed that none of the experimental strains carried the *nimA-J*, *cfxA* or *ermF* genes which confer Mtz, Cef and Erm/ Cln resistance respectively. However, all of the positive controls for these genes generated the correct PCR products and the *cfiA* positive samples were sequenced to confirm their identity. This means that the clinical resistance to Mtz and Cef observed in GSH8, GSH9 and GSH15 is due to mechanisms other than the presence of the tested genes. With regard to Mtz, there are a number of metabolic and physiological mechanisms that can result in resistance. These include alteration of the LDH/ PFOR pathway which reduces the presence of free electrons and prevents Mtz activation (Carlier *et al*., 1997) and the presence of efflux systems that actively remove Mtz from the cell (Wexler, 2012). The other known Cef resistance mechanism, which is not dependent on the *cfxA* gene product, is the production of penicillin-binding proteins which bind to the β–lactam ring of the antibiotic and prevent it acting on the cell (Soki *et al*., 2011).

In contrast, 17 of the GSH isolates contained the *tetQ* gene which accounted for almost all of the strains which were found to be Tet resistant. None of the strains that were identified as sensitive harboured the gene. However, there was a discrepancy with one of the resistant isolates, GSH14, which did not possess the *tetQ* gene at all. The resistance shown by this strain

may be linked to Tet efflux pumps that are known to cause reduced susceptibility (Livermore, 2005). It could also be that there is a mutation to the primer binding site of the *tetQ* primers which is preventing amplification but leaves the gene fully functional. It is interesting to note that no *ermF* resistance genes were identified. This suggests that conjugative transposons are not present in the isolates studied here, as the *tetQ* and *ermF* genes are often carried together in *B. fragilis* on the same mobilizable element (e.g. CTnDOT) (Whittle *et al*., 2001).

Screening for the *cfiA* gene revealed that it was only carried in three of the isolates (GSH7, GSH9 and GSH15), and of those, only two of the strains (GSH9 and GSH15) were Imp resistant. Imipenem sensitive strains that carry a 'silent' version of the *cfiA* gene can be converted to their resistant form through a single step mutation (Soki *et al*., 2004). This involves the integration of an IS element into the upstream region of the *cfiA* gene (Podglajen *et al*., 1994). It was, therefore, possible that the difference between the Imp^R strains (GSH9 and GSH15) carrying the *cfiA* gene and the Imp^S (GSH7) strain which also contained the gene (by PCR detection) is the presence of an IS element. The three *cfiA* positive strains were, therefore, screened for the presence of IS elements upstream of *cfiA* using various PCR strategies. It was found that PCR using primers designed specifically to IS elements: IS942, IS1186, IS4351, IS1169 and IS1170 did not detect their presence in any of the three strains (data not shown). A more general approach using primers binding to the adjacent upstream and internal *B. fragilis* sequence was, therefore, used (Fig. 2.1A).

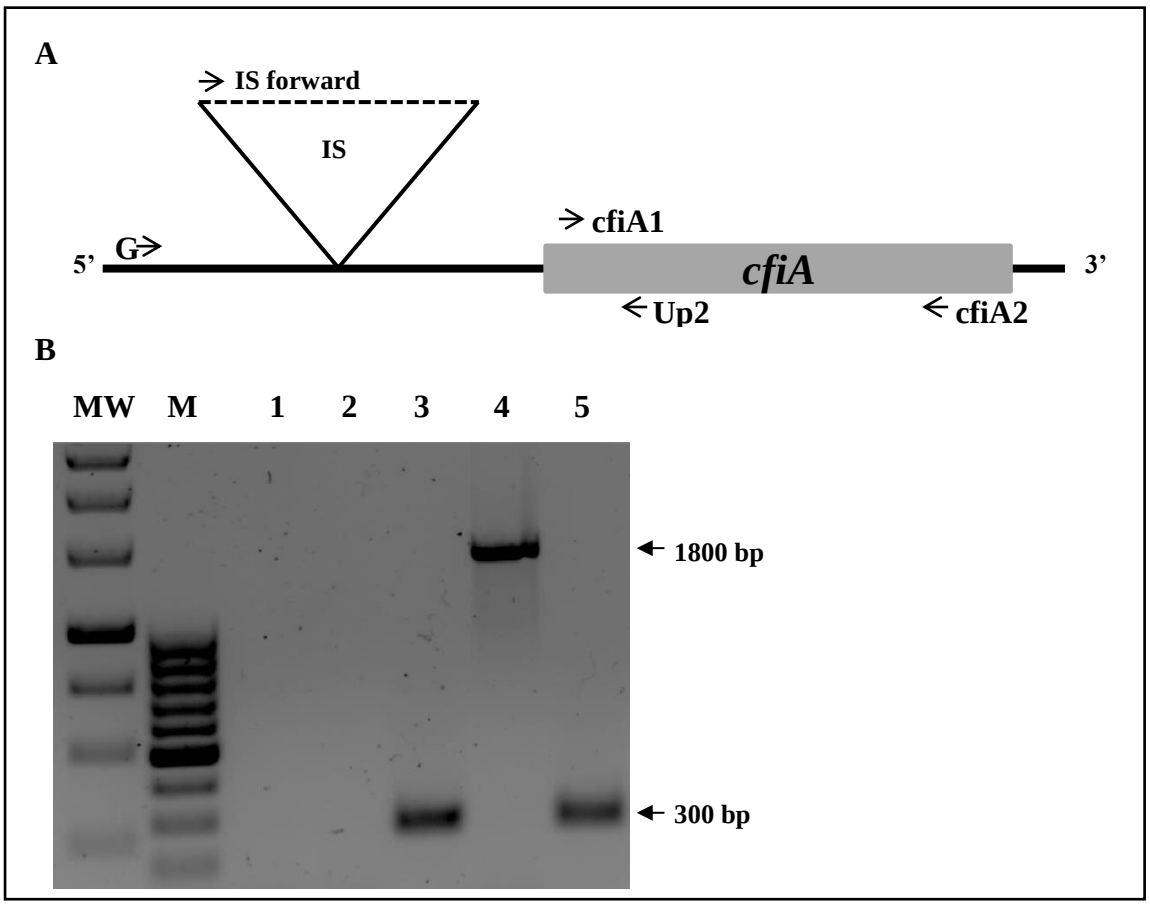


Figure 2.1: *cfiA* IS detection. **(A)** Schematic diagram of the PCR strategy. The *cfiA* gene is represented by the bar and the 5' and 3' flanking regions are indicated by the lines. The estimated position of a possible IS insertion is depicted by the dotted line and the position and direction of the primers are indicated by the arrows (Adapted from Soki *et al*., 2004). **(B)** PCR products generated from the upstream region of the *cfiA* gene using primers G and Up2. **Lanes: (MW)** molecular weight marker O'GeneRuler 1 kb DNA Ladder (Fermentas), **(M)** molecular weight marker O'GeneRuler 100 bp DNA Ladder (Fermentas #SM1143). **(1)** No template control, **(2)** *B. fragilis* 638R negative control, **(3)** GSH7, **(4)** GSH9, **(5)** GSH15. Sizes of the PCR products indicated in base pairs (bp) with arrows.

PCR using primers G and Up2 which bind upstream and internal to the *cfiA* gene respectively (Fig. 2.1A), revealed that GSH9, the *cfiA* positive strain, contained an IS element (Fig. 2.1B, Lane 4). Sequence analysis of the PCR product identified this IS element present as IS4351, which is usually carried on transposon Tn4351. In contrast, the same PCR strategy revealed that neither the Imp^S strain, GSH7, nor the Imp^R, GSH15, contained an IS element. These findings explain why GSH9 showed resistance to Imp and why GSH7, which has the *cfiA* gene, is still sensitive. They do not, however, explain the phenotype of GSH15 which has the *cfiA* gene and is Imp^R but does not contain an IS element. A similar situation was observed by Sóki *et al*. (2004), where the authors found that five of their isolates that were Imp^R and carried the

cfiA gene did not, however, contain an IS element. Analysis of the upstream regions of the *cfiA* genes of these five resistant strains revealed that they all had various nucleotide changes compared to *B. fragilis* Bfr81 (a susceptible strain). Two of them had identical upstream regions with a particular C → T transition, which was speculated to result in a more active promoter (Soki *et al*., 2004). Sequence analysis of the upstream regions of GSH7 and GSH15 in this study revealed that they too had nucleotide changes when compared to Bfr81 (Fig. 2.2). Neither of the strains possessed the specific C → T transition in the promoter region believed to account for the increased *cfiA* resistance (Soki *et al*., 2004). However, both of the strains had the same A → T substitution and adenine deletion observed by Sóki *et al*. Interestingly, only the *cfiA* resistant strain (GSH15) had the G → A transition noted by Sóki *et al*. in their resistant strains. It is, thus, possible that this change may be contributing to the *cfiA* resistance shown by these strains and may indicate that a novel mechanism of *cfiA* activation could be occurring.

			RBS	***cfiA***	**Strain**	**Imp**
-40		-20		0		
cggaaaaacaaccataagatatagatgttagtttgaatacagcaaataaa			**aagaa**	taaa**atg**aaa	Bfr81	S
cggaaaaacaac**T**ataa**A**atata**A**atgttagtttgaatacagca**T**at-aa			**aagaa**	taaa**atg**aaa	16997/ 21216	R
cggaaaaacaaccataagatatagatgttagtttgaatacagca**T**ata-a			**aagaa**	taaa**atg**aaa	GSH7	S
cggaaaaacaaccataagatata**A**atgttagtttgaatacagca**T**ata-a			**aagaa**	taaa**atg**aaa	GSH15	R

Figure 2.2: Comparison of the nucleotide sequences of the upstream region of the *cfiA* gene lacking an IS. The *B. fragilis* strains shown from Sóki *et al*. are Bfr81 (*cfiA* positive, Imp^S) and consensus sequence of 16997 and 21216 (*cfiA* positive, Imp^R). The *B. fragilis* strains shown from this study are GSH7 (*cfiA* positive, Imp^S) and GSH15 (*cfiA* positive, Imp^R). The hypothetical promoter regions are indicated above the sequences and the ribosome binding site (RBS) and the *cfiA* start site are in bold. Changes in the sequences compared to Bfr81 are shown in bold while nucleotide deletions are represented by a dash (Adapted from Soki *et al*., 2004).

2.3.4 Determination of stability of Mtz resistant subpopulations

During the course of the antimicrobial screening it was noted that several Mtz^R breakthrough colonies were growing within the zones of inhibition on the E-test plates of *B. fragilis* 638R. This was surprising as 638R is overall Mtz sensitive and this phenomenon was not observed for any other strains (Fig. 2.3). Three of these colonies, designated R1, R2 and R3, were isolated and later confirmed to be *B. fragilis* 638R by 16S rRNA gene sequencing and resistance to rifampicin.

Figure 2.3: Metronidazole E-test plate of strain *B. fragilis* 638R showing the resistant subpopulation of cells.

The MICs of these colonies were retested, after subculture in the absence of Mtz, in order to determine whether their MICs were different from that of their parent. The results revealed that the breakthrough isolates grew at higher concentrations of Mtz (Table 2.5) as compared to their parent strain. Interestingly, no further sub populations were observed when the E-tests were repeated for these isolates. The mutants were tested two further times after serial passages on BHISA without antibiotic selective pressure (Pelaez *et al*., 2008). After the first passage the Mtz, MICs were 0.75 mg/L for all three mutants. This was an increase in resistance for both R2 and R3, but a decrease in resistance for R1. The second passage resulted in a further decrease in resistance for R1 (0.5 mg/L) and maintenance of resistance for R2 and R3 (0.75 mg/L). This indicates that R1 may have inducible resistance as the antibiotic susceptibility decreased after each successive passage in the absence of Mtz selective pressure, while R2 and R3 have

permanent mutations due to the fact that they maintained their resistance even during growth in the absence of selective pressure.

Table 2.5: MICs of *B. fragilis* 638R and breakthrough colonies after each passage

Strain	Initial MIC (mg/L)	Passage 1 (mg/L)	Passage 2 (mg/L)
638R (Parent)	0.125	0.125	0.125
R1	1	0.75	0.5
R2	0.5	0.75	0.75
R3	0.5	0.75	0.75

This phenomenon has previously been observed in a *Prevotella baroniae* study (Alauzet *et al*., 2010), where it was shown that 30 of the isolates tested produced Mtz^R sub-populations within the inhibition zone of the E-test. These isolates had MICs ranging from 8 to 32 mg/L and in most cases showed an 8 fold increase in resistance from their parent strains (Alauzet *et al*., 2010). The development of resistant cells within a Mtz^S population may be due to the fact that Mtz targets DNA and causes extensive double stranded breaks. It is, therefore not surprising that DNA mutations leading to increased Mtz^R could occur following exposure to this known mutagen (Edwards, 1980). Although the levels of resistance reported here are well below clinically resistant levels (≥32 mg/L) it is still important to monitor them. Since resistance to Mtz is a multifactorial process and may include a number of different mechanisms each providing a small increase in Mtz resistance (Leiros *et al*., 2004). When these small increases are combined they can, together, result in clinically resistant bacteria. It would be interesting to do a comparative proteomic study comparing the parent strain to the subpopulations and determine whether there is a change in protein expression levels.

2.3.5 Plasmid analysis of the GSH isolates

In order to determine whether the observed antibiotic resistance was chromosomally or plasmid located, total DNA for each of the GSH isolates was extracted and electrophoresed on a 1%

agarose gel (Fig. 2.4). It was observed that 7 of the 23 (30%) GSH isolates contained at least one plasmid, although the exact number of plasmids could not be determined using this technique as the various circular plasmid configurations could account for multiple bands seen. This result is similar to the reported observation that between 20 to 50% of clinical and faecal isolates of *Bacteroides* species carry plasmids (Smith *et al*., 1998).

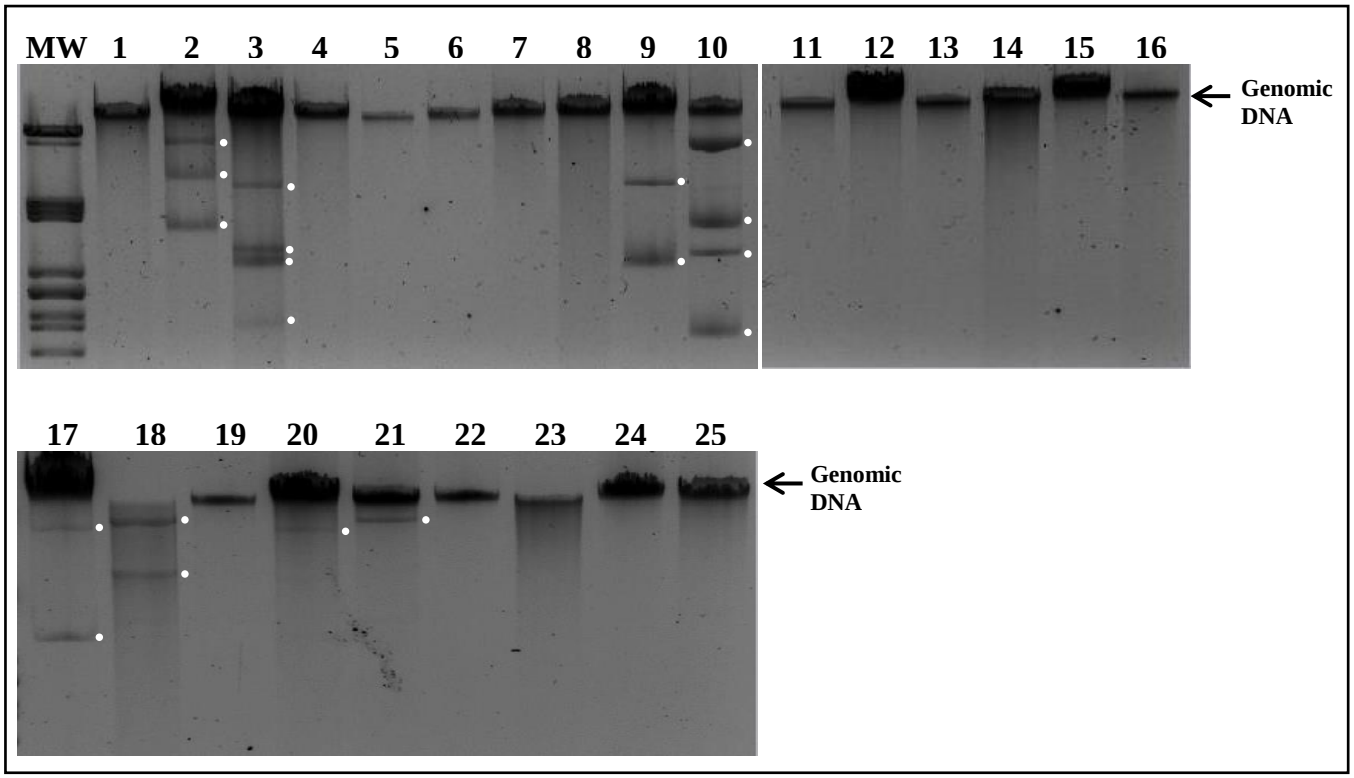


Figure 2.4: Total DNA extractions of *B. fragilis* GSH strains, *B. fragilis* 638R and *B. fragilis* Gbr7 showing the distribution of plasmids throughout the isolates. White dots indicate plasmids. **Lanes: (MW)** molecular weight marker of λ-phage DNA digested with *Pst*I. **(1)** 638R (negative control), **(2)** Gbr7 (positive control), **(3)** GSH2, **(4)** GSH3, **(5)** GSH5, **(6)** GSH6, **(7)** GSH7, **(8)** GSH8, **(9)** GSH9, **(10)** GSH10, **(11)** GSH13, **(12)** GSH14, **(13)** GSH15, **(14)** GSH16, **(15)** GSH17, **(16)** GSH18, **(17)** GSH12, **(18)** GSH19, **(19)** GSH20, **(20)** GSH21, **(21)** GSH22, **(22)** GSH23, **(23)** GSH25, **(24)** GSH28, **(25)** GSH30.

B. fragilis can carry two different types of plasmids: plasmids which carry antibiotic resistance genes, and cryptic plasmids that do not confer any known phenotype (Smith *et al*., 1998). The resistance gene most often associated with the presence of plasmids is the *tetQ* gene. This gene is often carried on transposons such as the CTnDOT conjugative transposon (Whittle *et al*., 2001). Thus, it is possible that the *tetQ* gene identified in this study by PCR in 16 of the

isolates may be carried on the plasmids present in those strains. Of the strains that carried the *tetQ gene*, 10 of these did not possess plasmids, and no strains carried an *ermF* gene which is often associated with this type of transposon (Bartha *et al*., 2011). Interestingly, of the two multidrug resistant strains (GSH9 and GSH15) only GSH9 possessed plasmid(s).

In order to determine whether the *tetQ* gene was being carried on the chromosome or plasmids, Southern blot hybridisation was performed using the *tetQ* gene as a probe on all of the plasmid carrying strains (personal communication from N. Hlatshwayo in this laboratory). It was found that the probe only hybridized to a single band equivalent to the position of the chromosomal DNA and no *tetQ* signal was observed on any of the plasmids (data not shown). It is important to note that plasmids can be as large as 80 kb and thus could be running along with the chromosomal DNA. However, it does appear that the plasmids harboured by the strains do not carry the *tetQ* gene conferring resistance to Tet.

Bacteroides species are known to carry another type of plasmid known as a cryptic plasmid. These are plasmids with no known or as yet undetectable phenotype and are often relatively small, <8 kb (Smith *et al*., 1998). They can be divided into three classes based on their sequence similarity and sizes: class I – 2.7 kb, class II – 4.2 kb and class III – 5.6 kb (Soki *et al*., 1999, Nagy *et al*., 1990). It is estimated that approximately 30% of intestinal *Bacteroides* and 50% of clinical isolates harbour these cryptic plasmids (Shkoporov *et al*., 2013). Thus, it is possible that the plasmids carried by the GSH isolates may be cryptic plasmids and not directly linked to the observed antibiotic resistance.

2.4 Conclusion

The aim of this chapter was to characterise the GSH *B. fragilis* clinical isolates based on their antibiotic resistance profiles and resistance genes. It was hoped that this study would provide

useful data about the state of drug resistance in the South African setting as not much information is available due to the lack of a regular surveillance program. The E-test method of MIC determination was used to identify the levels of antibiotic resistance present in the strains. Cln, Erm and Tig were revealed to be the most effective antibiotics, while Tet was identified as the least effective. Mtz, Imp and Cef were reasonably active with only low levels of resistance present in the group investigated. Although still relatively low, this observation marks an increase in resistance when compared to our previous study performed in 1998. This is an important finding as it suggests that antibiotic resistance in South Africa is increasing and indicates the need for further more regular antibiotic surveys. It is important to note, however, that the work reported here should be considered a pilot study since the sample size was small in comparison to other surveillance studies. Future work would include testing these strains against other antibiotics used to treat *B. fragilis* infections and to do more regular and larger surveys of antibiotic resistance at GSH and other sites across South Africa.

PCR using published primers was performed on the isolates to determine whether known resistance genes were present. It was determined that most of the antibiotic resistance observed could be related to the presence of known resistance genes as many of the strains possessed them. Plasmid analysis and Southern hybridisation of the *tetQ* gene, on those strains that did harbour plasmids, revealed that the gene appeared to be chromosomally and not plasmid located. Although most of the resistance was related to previously described genes, a few of the strains which showed reduced susceptibility did not carry any of the known resistance genes. This suggests that resistance mechanisms other than those already described are involved.

Analysis of the upstream region of the *cfiA* gene of the *cfiA* positive, IS negative strains revealed that both the Imp^S and Imp^R strains had various nucleotide changes. Most of the

changes within the promoter region of the gene were the same for both strains. However, the Imp^R strain (GSH15) had a particular G to A substitution that the Imp^S strain did not. It is possible that this change could alter the promoter of the *cfiA* gene in some way resulting in expression of Imp resistance. Future studies should involve identifying all of the antibiotic resistance mechanisms present in the resistant strains which do not carry resistance genes. This is particularly interesting in the case of the Mtz^R strains (GSH8 and GSH15) which contained none of the *nim* genes which are often identified as the leading cause of Mtz resistance. This dissertation therefore, focused on the alternative mechanisms of Mtz resistance potentially present in these two strains. This is investigated further in Chapter 3.

Chapter 3

Mtz resistance mechanisms present in *B. fragilis* GSH8 and GSH15

3.1 Introduction

Two highly metronidazole (Mtz) resistant *B. fragilis* clinical isolates (GSH8 and GSH15) were identified as described in Chapter 2. This Mtz resistance could not be attributed to the presence of nitroimidazole (*nimA-I*) resistance genes as the universal *nim* primers were unable to detect their presence in either of these two strains. The *nimJ* gene could also not be detected using the *nimJ* specific primers. It was, therefore, concluded that the high level of resistance was being caused by an alternative resistance mechanism and that this required further investigation in this chapter.

As described in Chapter 1, the pyruvate: ferredoxin oxidoreductase (PFOR) enzyme is crucial for Mtz activation (Edwards, 1980). The metabolic reaction catalysed by the PFOR enzyme provides free electrons which are thought to cause the activation of Mtz to its toxic nitro radical form (Upcroft *et al*., 2006). Mtz resistant bacteria, therefore, often show reduced PFOR enzyme activity and, instead, the pathway shifts towards an increase in activity of the lactate dehydrogenase (LDH) enzyme, which removes the free electrons that Mtz usually scavenges (Diniz *et al*., 2004). Several Mtz resistant bacteria have been identified as possessing this resistance mechanism (reviewed in Chapter 1). For example Sindar *et al*. (1982) found that Mtz resistant strains of *C. perfringens* had no detectable levels of PFOR and had increased levels of lactate production. In *B. fragilis*, 2D gel electrophoresis revealed that there was an upregulation of LDH in Mtz resistant strains, and that mutants lacking the PFOR gene were less susceptible to Mtz (Diniz *et al*., 2004). Thus, the levels of PFOR and LDH should be investigated in this current study as this pathway may be responsible for the Mtz resistance observed in the two clinical isolates.

Another possible Mtz resistance mechanism is via the activity of drug efflux pumps, which are known to cause resistance to Mtz as well as multidrug resistance (Alekshun and Levy, 2007).

Since efflux pumps either import or export substances into or out of the bacterial cell, inactivating an import pump could prevent antimicrobials from entering the cell while overexpressing an export system could expel any antibiotics present before they can cause cell damage. Many different types of efflux systems exist in bacteria and these have been widely investigated (Alekshun and Levy, 2007). In *B. fragilis*, 16 resistance-nodulation-division (RND) efflux pumps BmeABC(1–16) have been identified. These efflux systems consist of three separate parts: the pump component (BmeB), a membrane fusion protein (BmeA), and an outer membrane channel protein (BmeC) (Ueda *et al*., 2005).

Reverse transcriptase PCR (RT-PCR) of the pump components (*bmeB1-16*) of each of the complexes was performed on a Mtz sensitive strain (Pumbwe *et al*., 2006a). It was determined that all of the *bmeB* genes, except *bmeB9*, were expressed at detectable levels. This suggests that the efflux system is being transcribed constitutively without the presence of antibiotics (Pumbwe *et al*., 2006a). Quantitative RT-PCR (qRT-PCR) of the BmeABC5 complex in a *nim* negative Mtz resistant strain revealed significantly increased transcription of each of the components (Pumbwe *et al*., 2007a). This suggests that the BmeABC5 efflux pump may be contributing to the observed Mtz resistance (Pumbwe *et al*., 2007a).

The aim of this Chapter was to identify and characterise the Mtz resistance mechanism(s) present in the Mtz resistant clinical isolates identified in Chapter 2, using both a biochemical and genetic approach.

3.2 Materials and Methods

3.2.1 Bacterial strains and plasmids, media and growth conditions

All *Bacteroides* strains were grown in supplemented brain heart infusion broth (BHISB) or on plates (BHISA) at 37°C under anaerobic conditions as described in Chapter 2. The strains used are described in Table 3.1.

Table 3.1: Strains and primers used in this study

Strain	**Genotype/Phenotype**	**Source/Reference**
638R	*B. fragilis* Clinical strain $Rif^R Gent^R$	(Privitera *et al*., 1979)
GSH8	*B. fragilis* Clinical strain described in Chapter 2	This study
GSH15	*B. fragilis* Clinical strain described in Chapter 2	This study
Primers	**Sequence**	**Source/Reference**
PorA F	5'-CACTAACACCGGTTCATGTTAATCC-3'	This study
PorA R	5'-ACACCAGCGAGGCTGACC-3'	This study
PorAint F	5'-ACCGATATTAATGGTAACAAAGCC-3'	This study
PorAint R	5'-GGAGTCATGTGTTCAAGAGC-3'	This study
F27	5'-AGAGTTTGATCITGGCTCAG -3'	(Cheneby *et al*., 2000)
R5	5'-ACGGITACCTTGTTACGACTT -3'	(Cheneby *et al*., 2000)
16S F	5'-ACGTTGTTGGTGAGGTAACG-3'	This study
16S R	5'-CCAATATTCCTCACTGCTGC-3'	This study
bmeB5 Wex F	5'-CACATCGCGCAGATAGACAT-3'	(Pumbwe *et al*., 2006a)
bmeB5 F	5'-GGGCCACATCGCGCA-3'	This study
bmeB5 R	5'-TTCGATTATCGGAGCCGAGA-3'	(Pumbwe *et al*., 2006a)
bmefull F	5'-ATGAGTTTATACGAAGGAGCGG-3'	This study
bmefull R	5'-CACCACCATACCGATGGC-3'	This study

3.2.2 Mtz induction studies

Two different methods were used to determine whether exposure of *B. fragilis* to incrementally increased doses of Mtz could induce drug resistance. These were a gradient plate passage method, and a doubling dilution in broth method.

3.2.2.1 Gradient plate passage

B. fragilis strains 638R, GSH8 and GSH15 were inoculated onto Mtz gradient plates with a maximum Mtz concentration of 1 mg/L. Resistant colonies are then re-inoculated onto a gradient plate with increased Mtz concentration and this process is repeated until reaching a

final concentration of 6 mg/L. Resistant colonies are then grown for 16 h in BHISB without Mtz and the MIC was determined using E-tests (AB Biodisk).

3.2.2.2 Doubling dilutions of Mtz in broth

B. fragilis strains 638R, GSH8 and GSH15 were grown for 16 h in BHISB they were then diluted to an OD_{600} = 0.1. Aliquots (0.1 ml) were used to inoculate sets of doubling dilutions of various Mtz ranges (Table 3.2) and were incubated for 24 h. The tube with the highest Mtz concentration that produced growth was used to inoculate subsequent sets of doubling dilutions. The sets used are shown in Table 3.2. The highest resistant tube of the final set was used to inoculate fresh BHISB and grown for 16 h without Mtz. The MIC was determined using E-tests (AB Biodisk).

Table 3.2: Sets of doubling dilution broths showing the range of Mtz concentrations.

Set	Range
1	5 mg/L - 0.3215 mg/L
2	10 mg/L - 0.625 mg/L
3	20 mg/L - 1.25 mg/L
4	40 mg/L - 2.5 mg/L
5	60 mg/L - 3.75 mg/L
6	80 mg/L - 5 mg/L

3.2.2.3 Stability of Mtz resistance in induced strains

The stability of the induced Mtz resistance observed in *B. fragilis* strains 638R, GSH8 and GSH15 from both the induction methods was evaluated by several passages in BHISB without Mtz as described for *B. fragilis* 638R subpopulation Mtz resistant strains (Chapter 2).

3.2.3 Genomic DNA (gDNA) extractions

Genomic DNA (gDNA) was extracted using the Genomic DNA Purification Kit (Fermentas) as per manufacturer's instructions, and quantitated using a NanoDrop spectrophotometer (NanoDrop ND-1000). Analysis of the quality of the gDNA extracted was done by

electrophoresis on 0.8% (w/v) agarose gels in Tris-acetate buffer with ethidium bromide (Sambrook *et al*., 2001).

3.2.4 Preparation of cell free extracts (CFE)

Cell free extracts (CFE) were prepared anaerobically from *B. fragilis* 16 h cultures. Culture volumes of 20 ml were harvested anaerobically using the Spectrafuge 6C centrifuge (6000 rpm or 3000 g, 10 min). Pellets were washed twice in half culture volumes of 10 mM Tris-HCl, pH 8.0, and then resuspended in 2 ml of the same buffer. Cells were ruptured by sonication on ice using a Misonic sonicator 3000 at a power output of 3 W for 5 rounds of 30 s. The cell debris was removed by centrifugation under anaerobic conditions in an Eppendorf 5415C bench top centrifuge (14 000 rpm or 16 000 g, 4°C, 10 min). The supernatant was collected (CFE) and protein concentrations were determined using Bio-Rad Protein Assay Dye Reagent Concentrate according to the manufacturer's instructions (Bio-Rad Laboratories, cat # 1500 - 0006). CFE was stored anaerobically before use in subsequent assays.

3.2.5 Lactate dehydrogenase (LDH) assay

The LDH assay was performed anaerobically, according to the manufacturer's instructions, using the LDH based *In vitro* Toxicology Assay Kit (Tox7) from Sigma-Aldrich. The assay was measured at OD = 450 nm using the Bio-Tek powerwaveX5 MQX200R spectrophotometer. Enzymatic activity was recorded as µM NADH/ mg protein/ min and was performed in biological triplicate and technical duplicate.

3.2.6 Pyruvate oxidoreductase assay

The PFOR assay was performed using the method described in Lindmark and Müller (1973). Pyruvate activity was measured spectrophotometrically at an absorbance of 600 nm using an Ultrospec 10 cell density meter, 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 using 0.5 mg of protein. The change in absorbance at 600 nm was recorded before and after the addition of pyruvate. The difference in the measure of the slope was taken as the enzyme activity and was reported as µM methyl viologen/ mg protein/ min, calculated using the extinction coefficient of methyl viologen (6300 $M^{-1}cm^{-1}$). The experiment was performed in biological triplicate and technical duplicate.

3.2.7 Pyruvate oxidoreductase (PFOR) gene sequencing and MSA

The *porA* genes of *B. fragilis* GSH8 and GSH15 were amplified using the primers PorAF and PorAR (Table 3.1). The PCR was performed using Kapa Ready Mix (Inqaba Biotech) and the GeneAmp® PCR system 9700 as described in Chapter 2. The PCR conditions consisted of an initial 5 min denaturation step at 95°C, followed by 25 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 52°C and elongation at 72°C for 2 min. The thermal cycling was then concluded with a 7 min elongation step at 72°C. DNA sequencing was performed by Macrogen Inc., Seoul, Korea using primers PorAF and PorAR as well as PorAintF and PorAintR. The *porA* nucleotide and amino acid sequences for *B. fragilis* strains 638R, NCTC9343 and YCH46 were obtained from the National Centre for Biotechnology Information (www.ncbi.nih.gov). The sequences were aligned using DNAman software 4.13 (Lynnon BioSoft).

3.2.8 Sequencing and MSA of the *bmeB5* efflux gene

The *bmeB5* gene of *B. fragilis* GSH15 was amplified using the primers bmefull F and bmefull R (Table 3.1). The PCR was performed using Kapa Ready Mix (Inqaba Biotech) and the GeneAmp® PCR system 9700 as described in Chapter 2. The PCR conditions consisted of an initial 5 min denaturation step at 95°C, followed by 25 cycles of denaturation for 30 sec at

95°C, annealing for 30 sec at 50°C and elongation at 72°C for 30 sec. The thermal cycling was then concluded with a 7 min elongation step at 72°C. DNA sequencing was performed by Macrogen Inc., Seoul, Korea. The sequences were aligned using DNAman software 4.13 (Lynnon BioSoft).

3.2.9 RNA extraction and integrity studies

RNA was extracted using the RNeasy Mini Kit with RNA protect (Qiagen) according to manufacturer's instructions. RNA was treated with DNaseI (Fermentas) to completely remove any DNA contamination. DNA contamination was evaluated by PCR of the 16S rRNA gene using the universal F27/ R5 primer pair and the RNA preparation as template (Table 3.1). The products were electrophoresed on a 1% agarose gel. RNA quantity and quality were determined using the NanoDrop spectrophotometer (NanoDrop Technologies) and denaturing agarose gel electrophoresis (2% Formaldehyde, MOPS running buffer), respectively.

3.2.10 Quantitative RT-PCR (qRT-PCR) of the *bmeB5* during Mtz induction

3.2.10.1 Sample preparation, storage and Primer design

B. fragilis 638R and GSH15 were grown to mid-log phase OD_{600}=0.6 and then half of the culture was exposed to 1 mg/L Mtz for 60 min. The other half of the culture was used as the uninduced control. Three biological repeats were performed. RNA was extracted from all samples as described in 3.2.9 and assessed for integrity and DNA contamination as described above. All RNA samples were then frozen and stored at -70°C. RNA (500 ng) was converted to cDNA using the Tetro cDNA synthesis kit (Bioline) according to the manufacturer's instructions. Random primers were used for first strand cDNA amplification of RNA transcripts. Evaluation of the conversion of RNA to cDNA was performed using the 16S rRNA universal primers mentioned above (Table 3.1). The cDNA (2 µl) was then used as template for the amplification of the qRT-PCR target gene primers. The cDNA was stored at -20°C before

use in any experiment. The amplicon of each primer set was within 30 bp in size from the other. This was done in order to compensate for any error associated with SYBR green intercalation of products with varying lengths (Fig. 3.1). The primers were synthesised and purified using HPLC methods (University of Cape Town Oligo Synthesis Service) and their site specificity was tested using BLAST (Altschul *et al*., 1997) as well as by standard PCR methods.

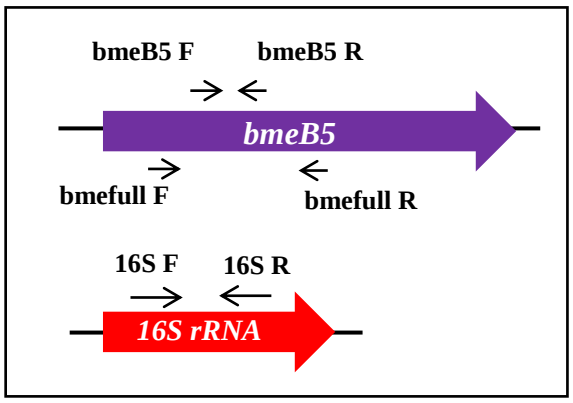


Figure 3.1: Schematic representation of the position of the qRT-PCR primers. A representation of the regions of the *bmeB5* and *16S rRNA* genes is shown. The positions of the primers used in the qRT-PCR experiment as well as the primers used in the gene sequencing are indicated. Primers are reported in Table 3.1.

3.2.10.2 qRT-PCR reaction conditions, controls and optimisation

The design and implementation of the quantitative RT-PCR experiment was done according to the relevant MIQE guidelines (Bustin *et al*., 2009) using the Rotogene 6000 (Corbet). All runs were set up using the QIAgility pipetting robot (Qiagen) and done using the 100 tube rotor. All qPCR tips and tubes belonged to the QIAgility Consumables range (Qiagen). Optimisation was first undertaken for the PCR reaction mixture using gDNA extracted as previously described (3.2.2), a known concentration for each primer pair, and using Kapa Ready Mix (Lasec). The reaction was optimised for the final PCR reactions using Sensimix (Celtic Diagnostics). The $MgCl_2$ concentration, annealing temperature, annealing time, elongation time and cycle number for each primer pair were tested and optimised. Once the optimal conditions for the primers had been established, the optimum amount of cDNA added in the reaction mix was tested. The final cycle conditions were as follows: 95°C for 10 min, 95°C for 15 sec, 50°C for 20 sec, and 72°C for 15 sec for 45 cycles. The final reaction mix was as follows: 15 µl

Sensimix (Celtic Diagnostics), 2 µl cDNA template, 0.25 µl forward primer, 0.25 µl reverse primer and 7.5 µl MilliQ water for a final reaction volume of 25 µl. Three biological samples of each strain were tested, with each biological sample being analysed in technical triplicate. The standard curves were created by mixing cDNA for each technical repeat under each condition (induced and uninduced) and then a dilution series from $10^0 – 10^{-3}$ and 1:5 – 1:500 was made with sterile MilliQ water in technical duplicate for each primer pair. Each run had its own standard curve for each primer pair including the reference gene to allow for differences in cDNA and PCR efficiency. All R^2 values of 0.99 and all efficiency values between 0.6 and 0.9 for each primer pair were used in the data analysis and the validation of the qRT-PCR experiment (Bustin *et al*., 2009). The controls for each run included a no template control (to determine the baseline of SYBR green fluorescence), an RNA template control (to confirm no DNA contamination), and a gDNA control (to ensure that the PCR was successful).

3.2.10.3 Data Analysis

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

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

3.3 Results and Discussion

3.3.1 Mechanism of Mtz resistance of *B. fragilis* GSH8 and GSH15

In the studies reported in Chapter 2, it was found that two *B. fragilis* clinical isolates (GSH8 and GSH15) were extremely Mtz resistant. However, preliminary MIC evaluation before performance of the LDH and PFOR assays described in this chapter, found that the MIC for *B. fragilis* GSH8 had dropped from 256 mg/L (Chapter 2) to 0.19 mg/L, and *B. fragilis* GSH15 was reduced from 256 mg/L (Chapter 2) to an MIC of 0.5 mg/L (Table 3.3). The time interval between the two evaluations was approximately 6 months and the strains had been subcultured on a routine basis in the absence of Mtz selective pressure while the genetic investigations reported in Chapter 2 were carried out. 16S rRNA sequencing and rescreening of the antibiotic resistance profiles of the strains revealed that both were the same strains that had been previously described and had maintained all other previously reported characteristics (Chapter 2). It was hypothesized that the continuous sub-culture of the strains in the absence of selective pressure may have resulted in the loss of resistance. Thus, the original culture stocks (stored in BHISB/ glycerol at -80°C) were revived and the Mtz MICs retested. Unfortunately, these cultures were also no longer highly Mtz resistant and showed the same Mtz resistance levels as the subcultured strains.

The phenomenon of a decrease in Mtz resistance on storage or subculture of bacteria has previously been observed in *Clostridium difficile*, where loss of Mtz resistance was detected

after passages on drug-free media (Pelaez *et al*., 2008). The authors also found that after thawing stored samples, initially resistant strains showed either reduced resistance or complete susceptibility (Pelaez *et al*., 2008). Thus, it appears that without the constant selective pressure of Mtz, certain strains may lose their resistance. It was, therefore, decided that pre-exposure to Mtz induction would be attempted in order to determine whether the resistance observed previously in the *B. fragilis* isolates could be induced back to the recorded levels.

3.3.2 Induction of Mtz resistance

Mtz induction was performed using two different methods, namely, Mtz gradient plate serial passage and broth doubling dilutions. Mtz induction using gradient plate serial passage revealed that Mtz sensitive *B. fragilis* 638R and the previously Mtz resistant GSH8 and GSH15 strains could all be induced to grow on higher Mtz concentrations after passaging them on increasing concentrations of 1 mg/L to 6 mg/L of Mtz (Table 3.3). All showed a reproducible increase in MIC when the cultures were tested directly post induction using E-test strips under standard conditions.

Table 3.3: Mtz resistance levels of *B. fragilis* strains after gradient plate serial passages.

***B. fragilis* strains**	**Original MIC* (mg/L)**	**Basal MIC Chapter 3 (mg/L)**	**Post induction MIC (mg/L)**	**Fold increase**
638R	0.125	0.125	3	24
GSH8	256	0.19	0.38	2
GSH15	256	0.5	0.75	1.5

MIC determined using E-test method
*As determined in Chapter 2 upon original isolation

It is interesting to note that Mtz sensitive *B. fragilis* 638R was induced to the highest MIC (3 mg/L) and also had the greatest fold increase. This ability to show increased Mtz resistance is possibly due to the production of the resistant sub-populations of cells observed in Chapter 2. Paláez *et al.* (2008) found that many of their *C. difficile* cultures also had this ability which

they called heteroresistance. The mutagenic capacity of Mtz as a DNA damaging agent may also be playing a role.

The doubling dilution method was able to induce both *B. fragilis* 638R and GSH15 to much higher levels of Mtz resistance (Table 3.4). *B. fragilis* 638R was once again highly Mtz inducible, however, this time GSH15 had the highest MIC after induction (16 mg/L). In contrast, although GSH8 was able to grow on a higher Mtz concentration during the doubling dilution experiment, it was unable to maintain any increase MIC in the absence of selective pressure and reverted to a basal MIC of 0.19 mg/L after a single subsequent passage in the absence of the drug.

Table 3.4: Mtz resistance levels of *B. fragilis* strains after doubling dilution induction and serial passages on BHISA without antibiotic.

***B. fragilis* strains**	**Basal MIC (mg/L)**	**Passage 1 (mg/L)**	**Passage 2 (mg/L)**	**Passage 3 (mg/L)**	**Post passage 1 Fold increase**
638R	0.125	8	8	4	64
GSH8	0.19	0.19	0.19	0.19	0
GSH15	0.5	16	8	6	8

MIC determined using E-test method

As *B. fragilis* 638R and GSH15 had slightly elevated Mtz MICs, it was decided to determine how stable this resistance was. Each strain was exposed to several passages in BHISB without Mtz and after each passage the MIC was retested (Table 3.4). Once again neither *B. fragilis* 638R nor GSH15 were able to maintain their Mtz resistance, and after two passages both strains had at least halved their post induction MICs. Therefore, viewed using 2 different methods, all three strains, were to some extent, able to have resistance induced by Mtz even though none of them could maintain it in the absence of selective pressure. The induction phenomenon could, therefore, be a strain specific regulated response.

3.3.3 LDH activity of *B. fragilis* 638R, GSH8 and GSH15

In view of the fact that some residual Mtz resistance was still being observed in GSH15, it was decided that it should be examined further with respect to other known Mtz resistance mechanisms as compared to GSH8 and 638R.

As the *nim* genes are not responsible for the Mtz resistance observed in the resistant strains (Chapter 2) it was decided to investigate, whether any of the other known, major Mtz resistance mechanisms could be contributing to the residual increase Mtz resistance seen in GSH15. One of the metabolic mechanisms associated with Mtz resistance is the LDH/ PFOR pathway (Diniz *et al*., 2004). When associated with Mtz resistance, this pathway results in increased LDH levels and reduced or undetectable levels of PFOR (described in Chapter 1).

An LDH assay was, therefore, performed to determine the enzyme levels in GSH15 as compared to GSH8. *B. fragilis* 638R was used as a control. The LDH assay showed that both GSH8 and GSH15 had significantly increased LDH activity compared to 638R (Table 3.4).

Table 3.5: Enzyme activity of lactate dehydrogenase present in *B. fragilis* strains 638R, GSH8 and GSH15 after loss of Mtz resistance.

***B. fragilis* strains**	**Lactate Dehydrogenase** μM NADH.mg protein^{-1}.min^{1}	**Standard Error**
638R	0.58	±0.063
GSH8	1.56*	±0.095
GSH15	1.14*	±0.088

Data represents the mean value of at least 3 experiments and the standard error of the mean (±).* Results statistically different from 638R using a student's t-test ($P < 0.05$).

However, there was no difference in the levels of GSH15 relative to GSH8 indicating that LDH levels were not linked to the slightly increased Mtz resistance phenotype of GSH15 and that they are probably due to biological and strain variation. In order to complete the analysis of whether there were changes in the LDH/ PFOR pathway in GSH15, PFOR enzyme analysis was performed.

3.3.4 Pyruvate oxidoreductase (PFOR) analysis

3.3.4.1 PorA amino acid MSA

In order to determine whether the strains with increased LDH activity showed a reciprocal decrease in PFOR activity, genetic and biochemical analysis of the PFOR gene and its encoded protein were done. The *porA* gene of the three strains (*B. fragilis* 638R, GSH8 and GSH15) was sequenced and analysed (Figure 3.2). The MSA analysis revealed that both *B. fragilis* 638R and GSH8 had 100% identical PorA amino acid sequences. In contrast, *B. fragilis* GSH15 had numerous amino acid changes, some of which were conserved, while others resulted in completely different amino acids.

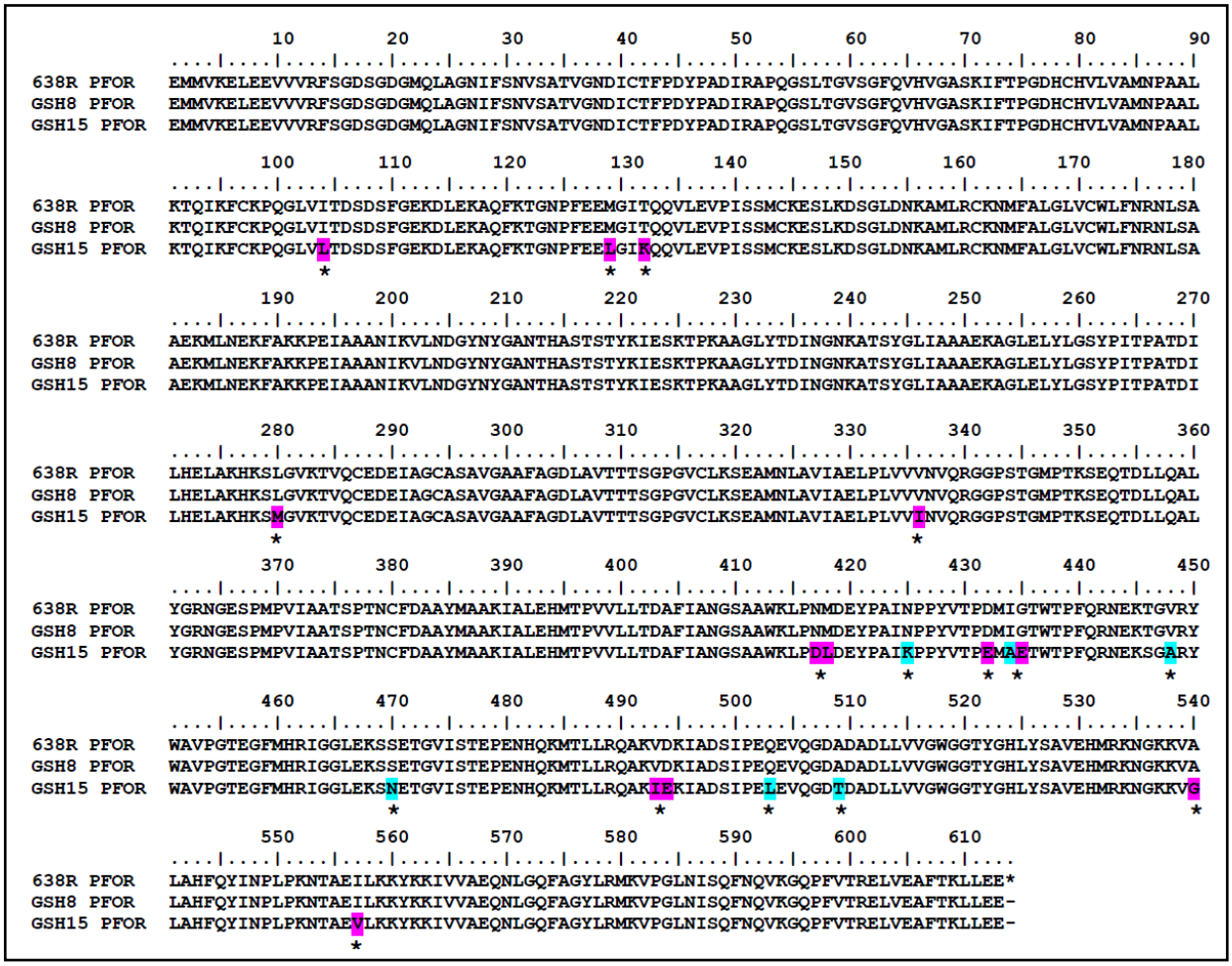


Figure 3.2: Multiple sequence alignment of the amino acid sequence of the *porA* gene of *B. fragilis* 638R, GSH8 and GSH15. Identical sequences are shown in white. Asterisks indicate amino acid changes: pink for a conserved change and blue for a completely different amino acid substitution.

This could indicate that GSH15 may have a functional change in the PorA enzyme activity. As there is no evidence in the literature indicating whether these amino acid changes would affect the activity, a PFOR biochemical assay was performed on *B. fragilis* GSH15 in comparison with 638R and GSH8.

3.3.4.2 Determination of the PFOR activity in the strains

The PFOR activity of the three *B. fragilis* strains was determined anaerobically using a previously described PFOR assay described (Lindmark and Muller, 1973). The assay revealed that both GSH8 and GSH15 had PFOR activities which were not statistically different from the activity recorded for 638R (Table 3.6).

Table 3.6: Enzyme activity of pyruvate oxidoreductase present in *B. fragilis* strains 638R, GSH8 and GSH15.

***B. fragilis* strains**	**Pyruvate oxidoreductase** µM methyl viologen/ mg protein/ min	**Standard Error**
638R	0.055	±0.0036
GSH8	0.063	±0.0010
GSH15	0.064	±0.013

This suggests that the increased LDH activity (Table 3.5) observed previously in the two clinical isolates (GSH8 and GSH15) is probably not involved in Mtz resistance as there is no reciprocal decrease in PFOR activity. Thus, the increased LDH activity and altered PFOR sequence is probably related more to strain variation than to Mtz resistance. It is possible that the amino acid changes present in GSH15 may be affecting the interaction of Mtz with the metabolic pathway even though the enzyme itself is still completely functional. It may be that this subtle effect on Mtz is contributing the basal levels of Mtz resistance observed in GSH15. However, it is more likely that the LDH/ PFOR pathway is not responsible for the slight Mtz resistance observed in *B. fragilis* GSH15 and probably was not the cause of the original resistance.

Although, the LDH/ PFOR pathway has been correlated with Mtz resistance in anaerobic bacteria, it is not always the cause of the resistance. Some studies have found that Mtz resistant strains have fully functional PFOR activity (Leitsch *et al*., 2011). In a study performed on several lines of Mtz resistant *Giardia lamblia* it was found that all of them had completely functional PFOR activity and ultimately the Mtz resistance mechanism could not be identified (Leitsch *et al*., 2011).

3.3.5 qRT-PCR analysis of the Mtz efflux gene *bmeB5*

3.3.5.1 *bmeB5* selection for qRT-PCR and primer choice

Due to the inducible nature of the Mtz resistance observed in the three *B. fragilis* strains (638R, GSH8 and GSH15) it was hypothesized that efflux systems might be responsible. As discussed earlier, RND type efflux pumps are considered to be largely responsible for the steady increase in multi-drug resistance (Alekshun and Levy, 2007). This is mainly due to their ability to export numerous classes of antimicrobial agents out of the cell as well as their ability to increase transcription upon antibiotic induction (Wexler, 2012, Alekshun and Levy, 2007). All of these characteristics make efflux systems possible candidates for contributing to the Mtz resistance initially observed in the isolates as well as for its inducible nature in the strains tested. Sixteen RND-family efflux pump gene clusters (*bmeABC1-16*) have been identified in *B. fragilis* (Ueda *et al*., 2005). Of these *bmeB5*, in particular, showed an increase in transcription levels in Mtz resistant mutants. When it was deleted, it caused a 2-fold reduction in Mtz resistance (Wexler, 2012). It was, therefore, chosen as an initial candidate in this study for further investigation.

qRT-PCR was used in order to determine whether *bmeB5* was responsible for the slightly elevated Mtz resistance observed in GSH15 and whether Mtz induction could increase *bmeB5* transcription. Published qRT-PCR *bmeB5* primers (bmeB5 Wex F and bmeB5 R) were initially

used. However, during primer optimisation, no PCR product was produced for *B. fragilis* GSH15. A new set of primers (bmefull F and bmefull R) were designed to a slightly larger region of the *bmeB5* gene, which included the binding region of the published forward primer (Fig. 3.3). The sequence analysis revealed that while the published reverse primer (bmeB5 R) bound almost perfectly, the published forward primer (bmeB5 Wex F) had four significant base pair changes (Fig. 3.3).

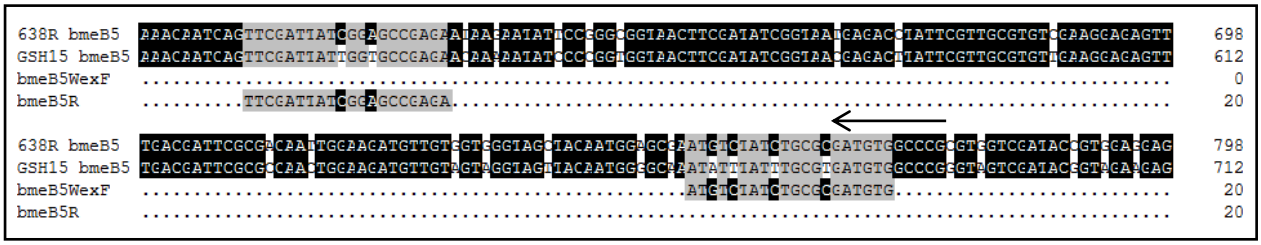


Figure 3.3: Region of the *bmeB5* gene in *B. fragilis* 638R and GSH15 showing published primer binding sites. Sequence changes are represented as white blocks. Identical sequences are shown in black blocks. Primer sequences and binding sites are shown in grey blocks. The arrow indicates the new primer position (bmeB5 F).

All four of the changes were from either a G or C to an A or T. This means that the overall annealing temperature of that primer would be reduced by almost 8°C and, therefore, amplification of the product was highly unlikely to occur. With this in mind a new forward primer (bmeB5 F) was designed and utilised for all subsequent experiments (Fig. 3.3, arrow).

3.3.5.2 qRT-PCR analysis of *bmeB5* in *B. fragilis* 638R and GSH15

qRT-PCR analysis was performed on cDNA created by the reverse transcription of RNA from Mtz induced and uninduced cell cultures from *B. fragilis* 638R (Mtz sensitive) and GSH15 (low level Mtz resistant) as described previously. An example of a melt curve and standard curve for each primer set can be seen in the appendix (Fig. S1 and S2).

Analysis using the Pfaffl criteria (2001) revealed that both *B. fragilis* 638R and GSH15 showed an increase in transcription of *bmeB5* after Mtz induction (Table 3.7), but this did not reach significance levels (greater than 2 fold according to Pfaffl, 2001).

Table 3.7: CT values, relative fold increase in transcript and standard errors for *B. fragilis* 638R and GSH15.

Strain	CT Value	Fold Change	Standard Error
638R	22.5	1.03	±0.15
GSH15	21.7	1.52	±0.28

This suggests that the efflux gene *bmeB5* is not responsible for the Mtz resistance observed in *B. fragilis* GSH15 during induction. However, it is possible that the lack of a significant increase in transcription was due to the specific design of the induction experiment which may not reflect the optimal time of transcript response to Mtz induction. In this study, cultures were induced with Mtz for 60 min before the RNA was harvested. It was decided not to use the induction conditions from the doubling dilution experiment as those cultures were of stationary phase cells and there would not be sufficient mRNA transcript present for qRT-PCR analysis. The qRT induction conditions were specifically chosen as they had been shown, in a previous study, to cause increased gene transcription (of *recA*) in *B. fragilis* 638R in response to Mtz (Nicholson, 2012). However, it is possible that the mRNA transcripts of *bmeB5* had already been expressed and degraded by this point. In a study performed in *E. coli*, which looked at 1036 open reading frames and 329 operons, it was determined that most mRNA has a half-life of less than 10 min (Selinger *et al*., 2003). However, no information is available about the mRNA half-life in *B. fragilis*. Thus, a more extensive qRT-PCR analysis needs to be performed. Sampling should be done at various time intervals after induction with sub-lethal concentrations of Mtz in order to optimise this study.

As there are 15 other RND-type efflux gene clusters it is possible that one of these is involved in the Mtz resistance or that a combination of the efflux systems is being employed. Thus, future work should include a full scale qRT-PCR induction and investigation of the other 15 efflux gene systems. Another possible target for investigation would be the *bmeB5* regulator, *bmeR5*. BmeR5 functions as a local TetR-family repressor of the expression of *bmeABC5* efflux genes (Pumbwe *et al*., 2007a). It has been shown that mutants deficient in this regulator have significant increases in transcription levels of the *bmeABC5* efflux genes as well as showing a two-fold increase in minimum inhibitory concentrations of various antibiotics including Mtz (Pumbwe *et al*., 2007a). Therefore, future work should also include investigating whether this regulator has been inactivated in *B. fragilis* GSH15.

3.4 Conclusion

The observed loss of the high Mtz resistance phenotype in two *nim* negative clinical strains GSH8 and GSH15 was a very unexpected finding of this study. Only GSH15 retained some low level residual resistance. Two Mtz induction methods (doubling dilution and gradient plate passage) were attempted in order to induce the previously reported levels of resistance. The doubling dilution method was the most effective and managed to induce resistance. However, MIC determination after several passages on BHISA without Mtz revealed that none of the strains maintained their resistance in the absence of selective pressure.

The loss of clinical resistance in these strains was a major set-back to this study, as there was no longer a high Mtz resistant candidate to investigate. However, since one of the strains, GSH15, retained a low level of resistance relative to the full sensitive 638R strain. LDH assays were performed on it in order to determine if there was any indication of changes to the levels of enzymes in LDH/PFOR pathway. The assay revealed that both previously resistant *B. fragilis* GSH15 and GSH8 had significantly increased LDH activity relative to 638R. The

subsequent PFOR assay, however, did not show any significant difference between the activity obtained for both GSH8 and GSH15 and that observed for 638R. This confirmed that the LDH/ PFOR pathway was not responsible for the residual slight Mtz resistance observed in the GSH15 clinical isolate. Thus, an alternative mechanism might be responsible. Future work should include a full comparative analysis of all of the GSH strains, which would reveal more about the strain specific variation within the LDH/ PFOR pathway and provide a full picture of possible cumulative effects if they operated in the case of the resistant strains.

Due to the observed inducible nature of the Mtz resistance detected in this chapter and the fact that *B. fragilis* GSH15 displayed multidrug resistance (Chapter 2), one of the components of an efflux pump which was reported in the literature as being transcribed at a higher level in a Mtz resistance clinical strain (Wexler, 2012), was investigated. qRT-PCR analysis of the *bmeB5* gene suggested that there was an increase in gene transcription in response to Mtz induction, however this was not significant. Future work should attempt to investigate the possibility that the recently discovered efflux pump regulator, *bmeR5*, might be responsible for the Mtz resistance (Pumbwe *et al*., 2007a). It is also possible that one of the other 16 *bmeABC* genes is responsible. Thus, future work should investigate the involvement of the other 15 gene clusters including their regulators. The induction conditions could also be varied to take into account the possible duration of the mRNA transcript half-life. Alternate Mtz resistance mechanisms should also be investigated as potential contributors to the Mtz resistance. These include the DNA repair systems such as *recA* and *recQ* which have both been shown to be involved in Mtz resistance (Paul *et al*., 2011, Steffens *et al*., 2010), although, neither one has shown the high levels of Mtz resistance initially observed in both clinical isolates.

In this study, the loss of the high basal levels of Mtz resistance resulted in the 2 clinical strains being very poor candidates for further analysis. In addition, no mechanism could be conclusively determined to be the cause of the residual levels of Mtz observed in GSH15. The inducibility of Mtz resistance observed in all the *B. fragilis* strains, including 638R led to the hypothesis that the genome may contain a novel Mtz resistance gene that may be silent under normal conditions but active upon Mtz induction. The emphasis of the research was then turned to investigating this possibility.

Chapter 4

Bioinformatic analysis of a putative *nim* gene present in *B. fragilis* 638R

4.1 Introduction

The analysis reported in Chapter 3 was unable to account for the Mtz resistance initially observed in the two clinical isolates, the low residual level observed in one of the strains, or the inducible nature of the resistance, on the basis of known resistance mechanisms. It was, therefore, hypothesized that the *B. fragilis* genome contained a putative, novel, inducible gene which was responsible for the observed inducible resistance.

The most widely described mechanism of Mtz resistance in *B. fragilis* involves the nitroimidazole resistance (*nim*) genes (Gal and Brazier, 2004). Homologs of these genes are found in a wide range of organisms including both Gram positive and negative bacteria. They can be carried either on the chromosome or on plasmids (Carlier *et al*., 1997). Screening and detection of *nim* genes has been routinely carried out using "universal" *nim* primers which are designed to a conserved region of the gene. It was originally believed that these primers were able to detect and amplify this region in all of the *nim* gene types (Trinh and Reysset, 1995) and this technique has been used in almost all studies to determine whether or not *B. fragilis* isolates are *nim* negative or positive (Lofmark *et al*., 2010). Further identification of the specific *nim* type (A-F) has been accomplished by restriction enzyme digestion of the PCR product using Hsp9211 which produced unique banding patterns for *nimA-F* (Lofmark *et al*., 2005).

More recently, it has been found that *nim* genes may exist which cannot be detected using the universal *nim* primers. To date, 10 *nim* genes have been identified (*nimA-J*), with 9 of them being described in *B. fragilis* (*nimI* was described in *Prevotella baroniae*). All but one of them, *nimJ*, were identified using the universal primers (Husain *et al*., 2013, Alauzet *et al*., 2010). Discovery of this novel *nimJ* gene brings into question the reliability of universal primers to identify novel *nims*. An alternative approach could be to analyse the annotated whole genomes

of bacteria in order to identify candidates which have the potential to be novel *nim* genes. This was done successfully in the case of *nimJ* where the gene was identified, by the RAST annotation server as coding for a pyridoxamine 5'-phosphate oxidase-related protein. This is the same family to which the known *nim* genes belong (Husain *et al*., 2013).

The *B. fragilis* 638R gene BF638R0416 was annotated in the complete genome sequencing project for the bacterium as a "putative nitroimidazole resistance-like protein" with an orthologue in the complete genome of NCTC9343 (Patrick *et al*., 2010, Cerdeno-Tarraga *et al*., 2005). There are a variety of computer programs available to help predict the tertiary structure of a protein following identification of a possible candidate from the genome. This is considered one of the best ways to determine putative function as the protein structure is often very well conserved (Leiros *et al*., 2004). These programs use the principles of homology modelling to generate a predicted tertiary structure based on the multiple sequence alignments with those of published structures (Kelley and Sternberg, 2009). The predicted protein structure can indicate whether the candidate has the characteristics of a nitroimidazole resistance protein.

This chapter describes the identification and bioinformatic analysis of a putative novel *nim* gene present in the *B. fragilis* 638R genome. This analysis acts as the first stage in determining whether the gene has the characteristics of possibly encoding a Nim-like protein. It also acts as a foundation for further functional characterisation, which may account for the induced Mtz resistance observed in the clinical isolates described in Chapter 3.

4.2 Materials and Methods

4.2.1 Bacterial strains and plasmids, media and growth conditions

All *Bacteroides* strains were grown in supplemented brain heart infusion broth (BHISB) or on plates (BHISA) at 37°C under anaerobic conditions as described in Chapter 2. The strains and plasmids used are described in Table 4.1.

Table 4.1: Strains and primers used in this study

Strain/Primer	Genotype/Phenotype/Use	Source/Reference
B. fragilis		
638R	Clinical strain Rif^R	(Privitera *et al*., 1979)
GSH2-GSH30	Clinical strains described in Chapter 2	This study
Primers		
F27 (16S) 5'-AGAGTTTGATCITGGCTCAG -3'	16S rRNA gene	(Cheneby *et al*., 2000)
R5 (16S) 5'-ACGGITACCTTGTTACGACTT -3'		
638RnimF (1) 5'-ACGCACTATCGGGTTGATATTCC -3'	Full length *B. fragilis 638Rnim* gene	This study
638RnimR (2) 5'-ATCGCAAGTACTCTCCACTTACG-3'		
IntF (3) 5'-TATATCAACGATTTAATCCGACGG-3'	*638Rnim* internal fragment	This study
IntR (4) 5'-GAGAATATTTATCAAGCAGAAGTTCC-3'		
NimUpF (5) 5'-CTACTGCGTGGAGCATATTG -3'	Region covering upstream gene and internal *638Rnim*	This study
NimUpR (6) 5'-CCGTCGGATTAAATCGTTGATATA -3'		
Up2F (7) 5'-GATTGTAGAGGTAGATAGACGC -3'	Region covering upstream gene	This study
Up2R (8) 5'-ACCCACTTCGAGGAATGGC -3'		

F = forward primer; R = reverse primer; Rif = rifampicin; Amp = ampicillin; ^{R} = resistance

4.2.2 Bioinformatics

All of the strains used for bioinformatic analysis are shown in Table 4.2. The amino acid and nucleotide sequences were obtained from the National Centre for Biotechnology Information (www.ncbi.nih.gov). Sequence alignments and phylogenetic analysis were conducted using the Mega5 program with MUSCLE alignment (Tamura *et al*., 2011). The predicted protein structure was determined by the Phyre Protein Fold recognition server (Kelley and Sternberg,

2009). The Predicted Data Bank (PDB) structure was submitted to the Dali server to determine the closest structural neighbours (Holm *et al*., 2006). The Dali Lite server was used to thread the PDB onto the crystal structure of *Deinococcus radiodurans*. The similarity of the structures was measured by Dali Z scores with significant similarities being assigned a Z score of above 2 (Holm *et al*., 2008).

Table 4.2: Strains used in bioinformatics analysis

Strain	Accession Number	Mtz Resistance Proteins	Reference
Deinococcus radiodurans	Q9RW27	NimA	(Leiros *et al*., 2008)
Bacteroides vulgatus	Q45801	NimA	(Haggoud *et al*., 1994)
Bacteroides fragilis	Q45146	NimB	(Haggoud *et al*., 1994)
Bacteroides thetaiotaomicron	Q45778	NimC	(Trinh *et al*., 1995)
Bacteroides fragilis	Q45150	NimD	(Trinh *et al*., 1995)
Bacteroides fragilis	Q9L4E6	NimE	(Stubbs *et al*., 2000)
Bacteroides vulgatus	Q8GJ54	NimF	(Lofmark *et al*., 2005)
Bacteroides fragilis	FJ969397	NimH	(Alauzet *et al*., 2010)
Prevotella baroniae	FJ940883	NimI	(Alauzet *et al*., 2010)
Bacteroides fragilis	K1G767	NimJ	(Husain *et al*., 2013)

4.2.3 PCR *638Rnim* gene screening of *B. fragilis* GSH isolates

All 23 GSH isolates were screened for the *638Rnim* gene using primers 638RnimF and 638RnimR (Table 4.1). Strains that did not amplify *638Rnim* were screened with a combination of primers (Fig. 4.8A and Table 4.1). All PCRs were done as described in Chapter 2 using the following PCR parameters: an initial 5 min denaturation step at 95°C, followed by 30 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 50°C, and elongation at 72°C for 1 min. The thermal cycling was then concluded with a 7 min elongation step at 72°C.

4.3 Results and Discussion

4.3.1 Identification and genomic context of a putative *nim* gene BF638R_0416

Analysis of the *B. fragilis* 638R genome, published on NCBI, revealed the presence of a gene annotated as a "putative nitroimidazole resistance-like protein" (Fig. 4.1). *B. fragilis* 638R is

not known to carry any of the known *nim* genes and is Mtz^S in the clinical treatment setting. The universal *nim* primers did not recognize this gene or detect any other *nim* genes present in *B. fragilis* 638R (Chapter 2).

The two other published *B. fragilis* genomes, NCTC9343 and YCH46, both confirmed the presence of the *638Rnim* gene. The genome context of the gene, in *B. fragilis* 638R, is shown in Fig. 4.1 (Patrick *et al*., 2010, Cerdeno-Tarraga *et al*., 2005, Kuwahara *et al*., 2004). These ORF's were chosen due to the fact that they lie on either side of the gene in question (BF638R_0416). It was decided not to proceed further downstream than BF638R_0414 as these genes are transcribed in the opposite direction to BF638R_0416.

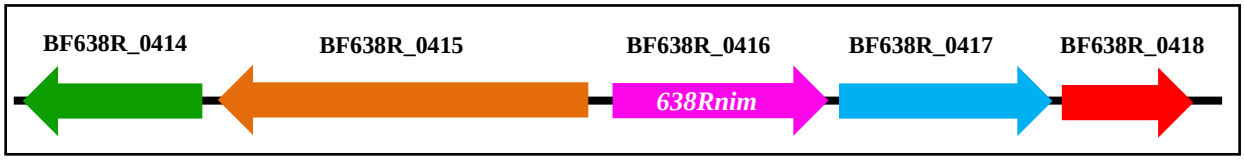


Figure 4.1: Genome structure of region 484488bp – 488087 bp in *B. fragilis* 638R genome. The *638Rnim* gene is shown as BF638R_0416.

This genome context was then compared between the 3 genomes. The ORFs within the nucleotide sequence of the 484488bp – 488087 bp region in 638R were shown to be identical in relation to each other within the 3 sequenced *B. fragilis* genomes. The five putative ORFs, including 638Rnim, present in this region and their annotated functions are shown in Table 4.3.

Table 4.3: Hypothetical functions of genes located within the region of interest of the *B. fragilis* 638R, NCTC9343 and YCH46 genomes.

638R	NCTC9343	YCH46	Annotated Function
BF638R_0414	BF0357	BF0416	Hypothetical function with signal polypeptide, no significant database matches.
BF638R_0415	BF0358	BF0417	Putative transmembrane protein.
BF638R_0416	BF0359	BF0418	Putative nitroimidazole resistance-like protein.
BF638R_0417	BF0360	BF0419	Putative napC/nirT cytochrome c-type protein.
BF638R_0418	BF0361	BF0420	Putative napC/nirT cytochrome c-type protein.

4.3.2 Multiple sequence alignment (MSA) of 638Rnim and known Nim proteins

A MSA and the resulting homology matrix between BF638R_0416 (638Rnim) and known Nim proteins from various other bacteria revealed that it showed very little amino acid conservation in relation to them (Fig. 4.1 and Fig. 4.2). While the known Nims all appear quite well conserved with respect to each other, with numerous shared amino acids and homology scores around 70%, 638Rnim appears to have only a few regions of commonality and very low percentage homology (approximately 25%). The Nim protein with the highest homology to 638Rnim is NimF from *B. vulgatus* with 27.6%. Interestingly, NimJ, which is also not amplified by the universal *nim* primers, has relatively good homology (approximately 50%) and very similar amino acid sequence to the known Nim proteins. This includes conservation of the site of primer binding used by the universal primers (FREMRRK), which suggests that a change in codon usage could be the reason for amplification failure (Husain *et al*., 2013).

	638Rnim	NimA	NimB	NimC	NimD	NimE	NimF	NimH	NimI	NimJ	D. radio NimA
638Rnim	100%										
NimA *B._vulgatus*	24.7%	100%									
NimB *B._fragilis*	24.1%	73.8%	100%								
NimC *B._theta*	22.8%	67.5%	71.8%	100%							
NimD *B._fragilis*	22.8%	75.6%	83.5%	73.0%	100%						
NimE *B._fragilis*	25.5%	75.2%	80.0%	70.3%	76.6%	100%					
NimF *B._vulgatus*	27.6%	74.3%	78.9%	71.1%	78.9%	71.5%	100%				
NimH *B._fragilis*	25.7%	78.3%	82.9%	74.3%	85.5%	77.8%	80.8%	100%			
NimI *P._baroniae*	25.0%	55.6%	58.9%	56.3%	57.6%	54.5%	58.7%	60.0%	100%		
NimJ *B._fragilis*	20.6%	53.1%	54.3%	51.2%	53.1%	53.8%	54.6%	57.2%	73.0%	100%	
NimA *D._radio*	7.2%	14.9%	12.2%	11.7%	12.8%	14.5%	12.5%	14.5%	12.5%	8.5%	100%

Figure 4.1: Homology matrix of amino acid sequences used to make multiple sequence alignment. Numbers are expressed as percentage homology. NimA: *B. vulgatus*, NimB: *B. fragilis*, NimC: *B. thetaiotaomicron*, NimD: *B. fragilis*, NimE: *B. fragilis*, NimF: *B. vulgatus*, NimH: *B. fragilis*, NimI: *P. baroniae*, NimJ: *B. fragilis*, NimA *D. radiodurans*.

In contrast, NimA from *D. radiodurans* (drNimA), which is also not identified by the universal primers, does not share much in common with the known Nim protein sequences (less than 15% homology) and like 638Rnim, does not have conservation in the universal primer binding region. Its closest match was to NimA from *B. vulgatus* with 14.2% homology. Interestingly,

drNimA and 638Rnim have the lowest sequence homology (7.2%) of any of the Nim proteins analysed. However, even with this low homology, all of the Nim proteins (including 638Rnim) share the His71 residue (Fig. 4.2), which was shown to be covalently linked to pyruvate by Leiros *et al* (2004). It is also believed to be the active-site residue of drNimA.

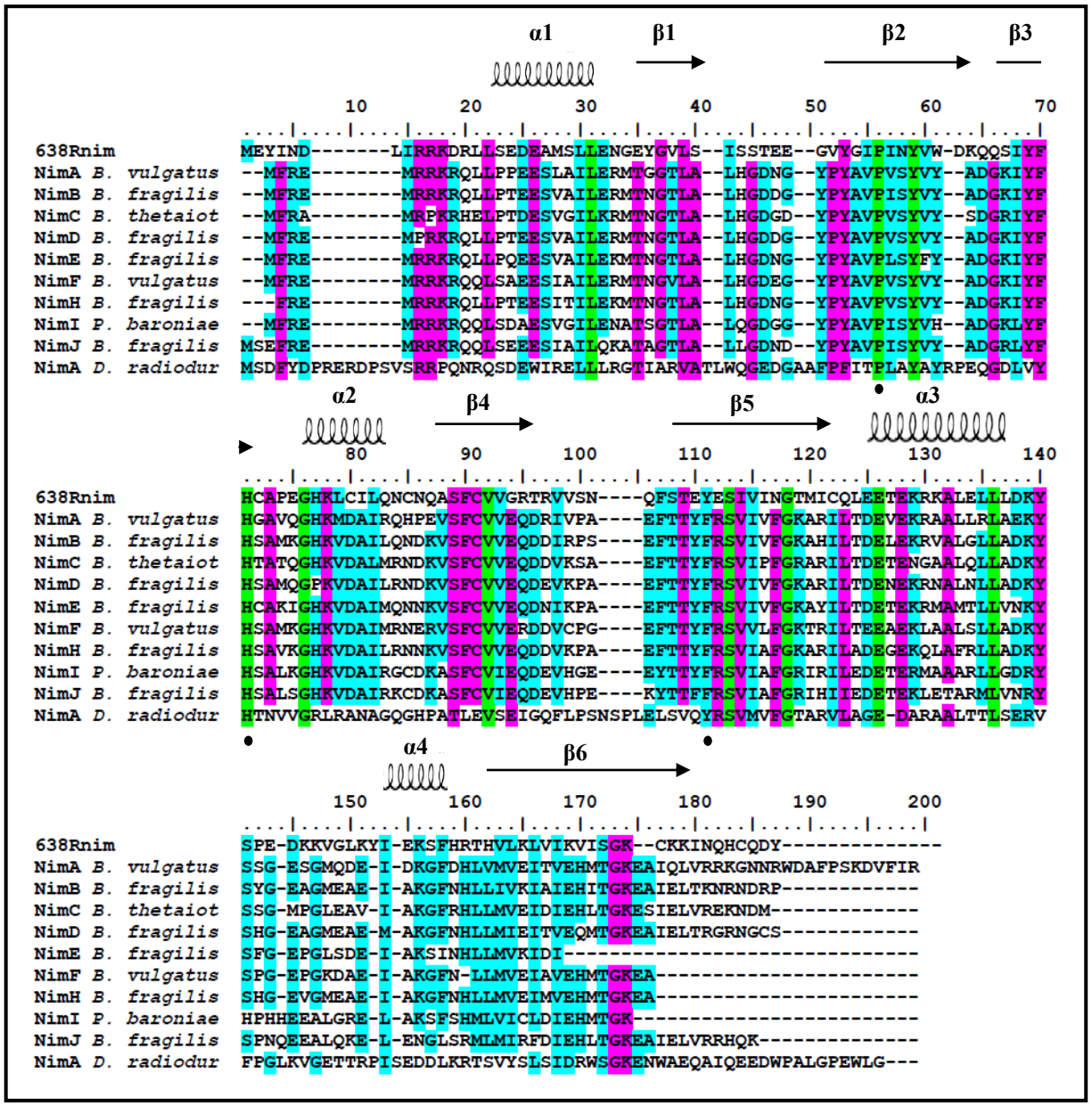


Figure 4.2: Amino acid multiple sequence alignment of *638Rnim* and known *nim* genes.
NimA: *B. vulgatus*, NimB: *B. fragilis*, NimC: *B. thetaiotaomicron*, NimD: *B. fragilis*, NimE: *B. fragilis*, NimF: *B. vulgatus*, NimH: *B. fragilis*, NimI: *P. baroniae*, NimJ: *B. fragilis*, NimA *D. radiodurans*. Shading of amino acids: green – identical; pink – highly similar; light blue – similar. The marked (•) residues indicate the reactive His71 as well as the Pro56 and Tyr111 present in the Mtz-binding site.

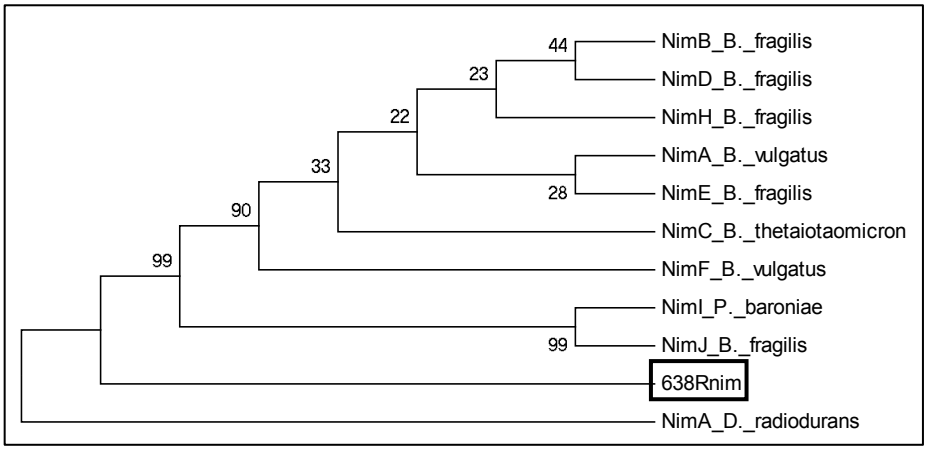
Two other important amino acids are shared by 638Rnim and the other Nim proteins. These are Pro56 and Tyr111. Both of these were shown to be part of the Mtz-binding site of the protein (Leiros *et al*., 2004). Crystallised drNimA protein soaked in Mtz revealed that the antibiotic was contained between these two amino acids suggesting their importance in Mtz inactivation (Leiros *et al*., 2004).

Interestingly, while the Pro56 is highly conserved among all the strains (Fig. 4.2) the Tyr111 is substituted with the similar amino acid, phenylalanine, in all of the sequences except 638Rnim and drNimA. This suggests that while an aromatic ring is important for interaction with the antibiotic it does not need to be identical (Leiros *et al*., 2004). However, it is noteworthy that both *D. radiodurans* and 638Rnim carry the Tyrosine at position 111 rather than the phenylalanine. This suggests that 638Rnim could quite possibly be a novel nitroreductase.

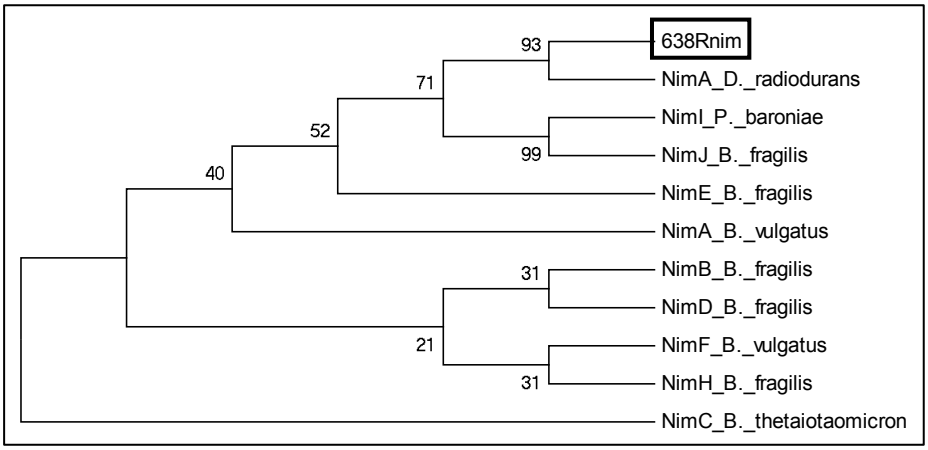
4.3.2 Phylogenetic analysis of *nim* genes in relation to *638Rnim*

In order to further determine the relationship between the protein encoded by *638Rnim* and the known Nim proteins, phylogenetic analysis was performed on the derived amino acid sequences. Three phylogenetic trees were constructed using different algorithms namely, neighbour-joining (Fig. 4.3A), maximum-parsimony (Fig. 4.3B) and maximum-likelihood (Fig. 4.3C). All three phylogenetic trees revealed that the 638Rnim protein was more closely related to the Nim proteins from *D. radiodurans* NimA and *B. fragilis* NimJ than to the other Nim proteins. However, in the neighbour-joining (Fig. 4.3A) and maximum–likelihood (Fig. 4.3C) trees 638Rnim and drNimA appear to be "rooting" both trees, suggesting that they are only quite distantly related to the other Nims, including NimJ.

A) Neighbour-joining



B) Maximum-parsimony



C) Maximum-likelihood

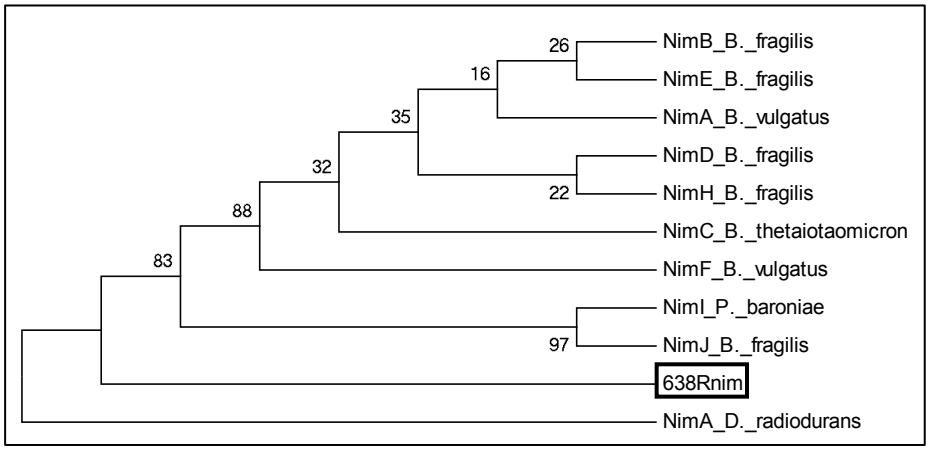


Figure 4.3: Phylogenetic analysis of *B. fragilis* 638Rnim represented by three phylogenetic trees. Phylogenetic analysis was performed with the MEGA 5 program using a MUSCLE alignment. The phylogenetic trees were reconstructed using the neighbour-joining **(A)**, maximum-parsimony **(B)** and maximum likelihood **(C)** methods. The reliability for internal branching was assessed using the bootstrapping method (1000 bootstrap replicates).

Interestingly, while all three trees grouped the known Nim protein sequences together none of them had the same overall tree structure. Even within each tree the bootstrap values of the various clades of the known Nims were not very strong (all below 50%). This could be due to the high conservation between the known Nim sequences. In comparison, all three trees grouped 638Rnim and drNimA together no matter where they were situated within in the tree itself. This suggests that either 638Rnim and drNimA are very similar evolutionarily, or that they are both so different to the known Nim proteins that they are being 'rooted' together. Further structural analysis may provide the answer and reveal how similar 638Rnim really is to other Nim proteins.

4.3.3 Predicted protein structure

In order to determine whether 638Rnim was structurally similar to nitroreductases and not just evolutionarily similar, the predicted protein sequence of 638Rnim was submitted to the Phyre Protein Fold recognition server (Kelley and Sternberg, 2009). Here, the amino acid sequence was used to construct a predicted protein structure which was then matched to template structures provided by known protein structures (usually provided from crystallised structures). The predicted protein structure of 638Rnim (Fig. 4.4A) revealed it to be part of the pyridoxine 5′-phosphate oxidase (PNP-ox) like family and the flavin mononucleotide (FMN) binding superfamily. Proteins that belong to the PNP-ox family and catalyse FMN reactions (including nitroreductases) have a characteristic two-fold dimer organisation and beta barrel folds important for electron transfer (Leiros *et al*., 2008). The closest structural match suggested by PHYRE (with 100% confidence) for 638Rnim was to model d2vpaa1 (Fig. 4.4B) which corresponds to the high resolution crystal structure of the antibiotic resistance protein NimA (drNimA) from *D. radiodurans*.

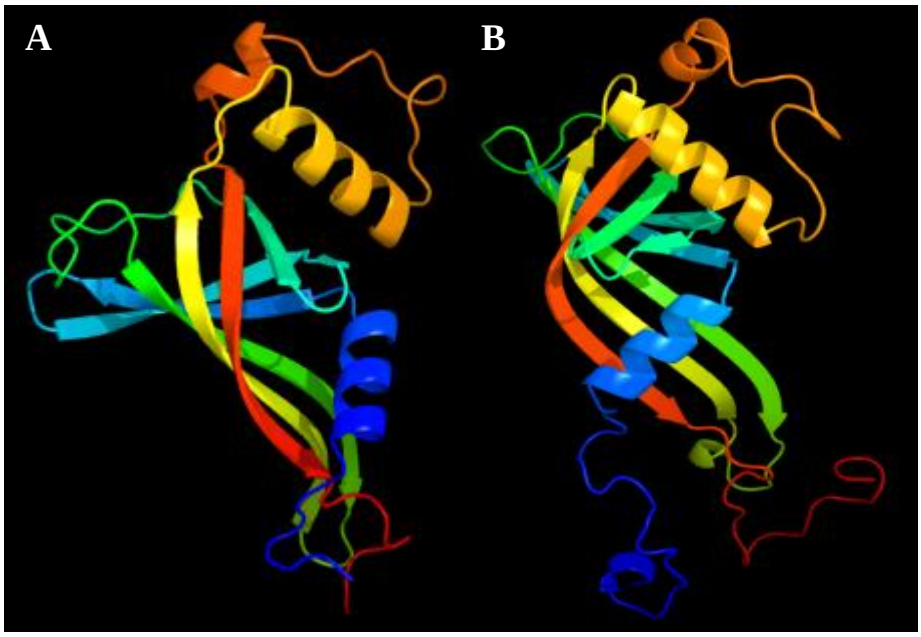


Figure 4.4: Predicted protein structures generated by PHYRE. Image coloured by rainbow (red to dark blue) N to C terminus. Arrows indicate beta sheets and barrels indicate alpha helices. **(A)** Three dimensional model of the predicted protein structure of 638Rnim. **(B)** Three dimensional model of the protein structure of drNimA.

The PDB generated by PHYRE for 638Rnim was compared to the database of known PDBs using the Dali server (Holm *et al*., 2006). The top eight matches to 638Rnim were all NimA related proteins with the best match (Z-score, 24.2) being to 2vpa-A. This protein is described as "the high resolution crystal structure of the antibiotic resistance protein NimA from *Deinococcus radiodurans*". Using the Dali server, it was possible to superimpose the structures of 638Rnim and drNimA onto one another. This enabled visual determination of how closely the protein structure under investigation matched that of a query structure, in this case drNimA. The predicted superimposition of the two proteins is depicted in Fig. 4.5.

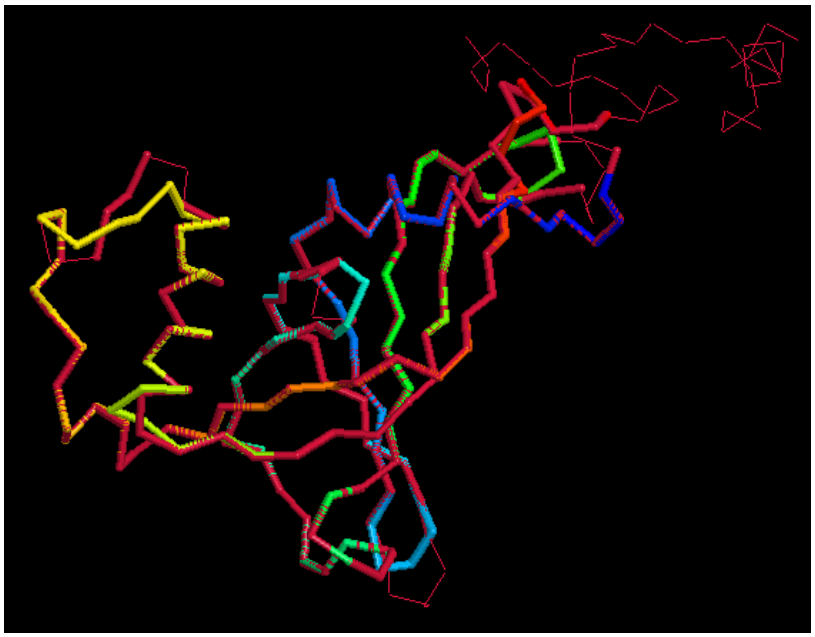


Figure 4.5: Superimposed protein structures of 638Rnim and closest match drNimA using the Dali Lite server. The rainbow coloured backbone (red to dark blue) N to C terminus represents 638Rnim. The red thread represents drNimA.

Overall, the overlap appears to be good with most of the two proteins superimposing on one another perfectly. The only real area of discord appears to be at the N terminus of drNimA which does not align with any part of 638Rnim. However, structurally this is still a very good match and clear indication that the predicted 638Rnim protein has many similarities to drNimA.

It was possible, using the Dali Lite server, to thread 638Rnim onto the crystal structure of drNimA soaked with Mtz, pyruvate and acetate (1W3R.pdb) (Holm *et al*., 2008). It was revealed that the structure of drNimA was altered slightly when bound with these substrates (Leiros *et al*., 2004). Thus it would be interesting to observe whether structurally 638Rnim will still be similar to drNimA when it is bound to Mtz (Fig. 4.6).

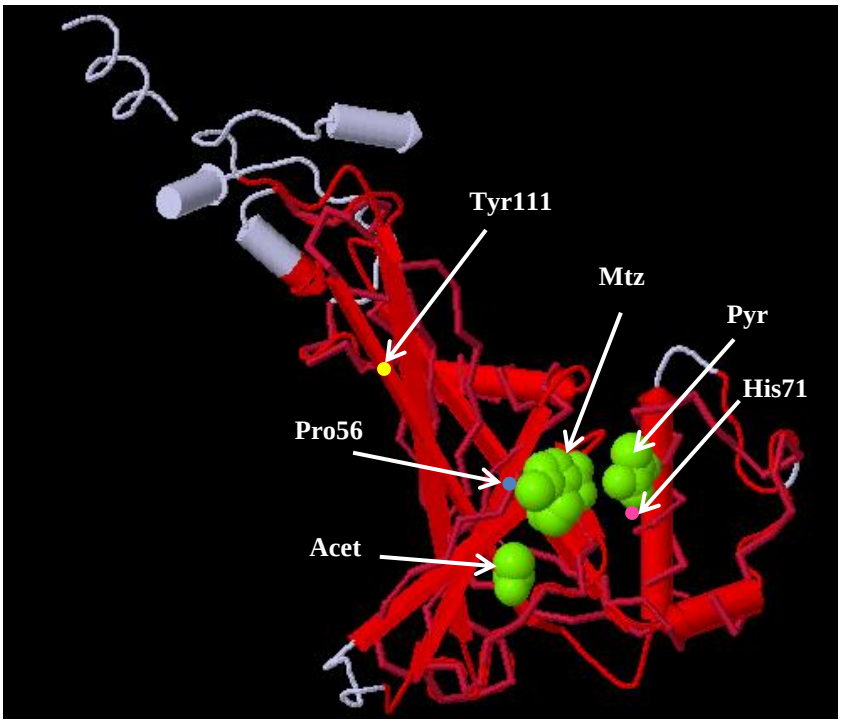


Figure 4.6: Superimposed structures of 638Rnim and Mtz soaked drNimA (1W3R.pdb) using the Dali Lite server. drNimA is in cartoon mode with 638Rnim threaded around it. Identical sequences are shown in red and dissimilar regions are grey. The positions of Mtz, pyruvate (Pyr) and acetate (Acet), are all indicated by green bubbles. The positions of the important amino acids His71 (pink dot), Pro56 (blue dot) and Tyr111 (yellow dot) are also represented.

As was observed in the previous superimposition, the ends of the proteins are not very similar (coloured in grey) while the main body of the structure appears to be very similar (coloured in red). This includes the potential active site (His71), the Mtz binding site and the conserved Pro56 and Tyr111. This was corroborated by a Dali Z-score of 22.8 which is well above the value of 2 that indicates significant similarities and folding. From this data it appears that 638Rnim may encode a 5-nitroimidazole reductase since it has all of the structural characteristics necessary for such an enzyme.

4.3.4 Prevalence of 638Rnim gene in *B. fragilis* GSH clinical isolates

As *638Rnim* has been identified in two other *B. fragilis* strains (YCH46 and NTCT9343) it was decided to screen for the gene's presence in other clinical isolates. This was done in order to determine whether the occurrence of the gene was a widespread phenomenon in the *B. fragilis* GSH strains under investigation in this study. Due to the fact that the universal *nim* primers did not detect the presence of *638Rnim* in *B. fragilis* 638R (Chapter 2), specific primers were designed to the two flanking regions on either side of *638Rnim* gene (primers 1 and 2, Fig. 4.8A). These were then used to screen the *nimA-J* negative GSH strains (described in Chapter 2) for the presence of *638Rnim*. PCR products were obtained for all except three of the 23 strains, namely GSH6, GSH7 and GSH8 (Fig. 4.8B). This suggested that these strains either did not carry the *638Rnim* gene, or that the base sequence or genomic context was altered such that the primers could not bind to the specific DNA regions.

In order to detect whether the *638Rnim* gene itself was present in the three GSH isolates (GSH6, 7 and 8) primers were designed internal to the *638Rnim* gene (primer positions 3 and 4 on Fig. 4.8A). PCR product was obtained from GSH7 and GSH8 revealing that both these strains carried the *638Rnim* gene (or at least the internal fragment of it) (Fig. 4.8C, lanes 15 and 21). However, GSH6 once again did not produce a PCR product which indicated that it either did not possess the *638Rnim* gene or that the primers could not bind to an altered internal sequence (Fig. 4.8C, lane 9). The possibility of poor quality DNA was ruled out due to strong product produced by the 16S rRNA primers using the same genomic DNA (Fig. 4.8C, lane 8). In order to determine why the full length 638Rnim primers (primer positions 1 and 2 on Fig. 4.8A) did not produce product for GSH6, GSH7 and GSH8, various primer combinations were used (primer pairs: 1 & 4 and 2 & 3, Fig. 4.8A).

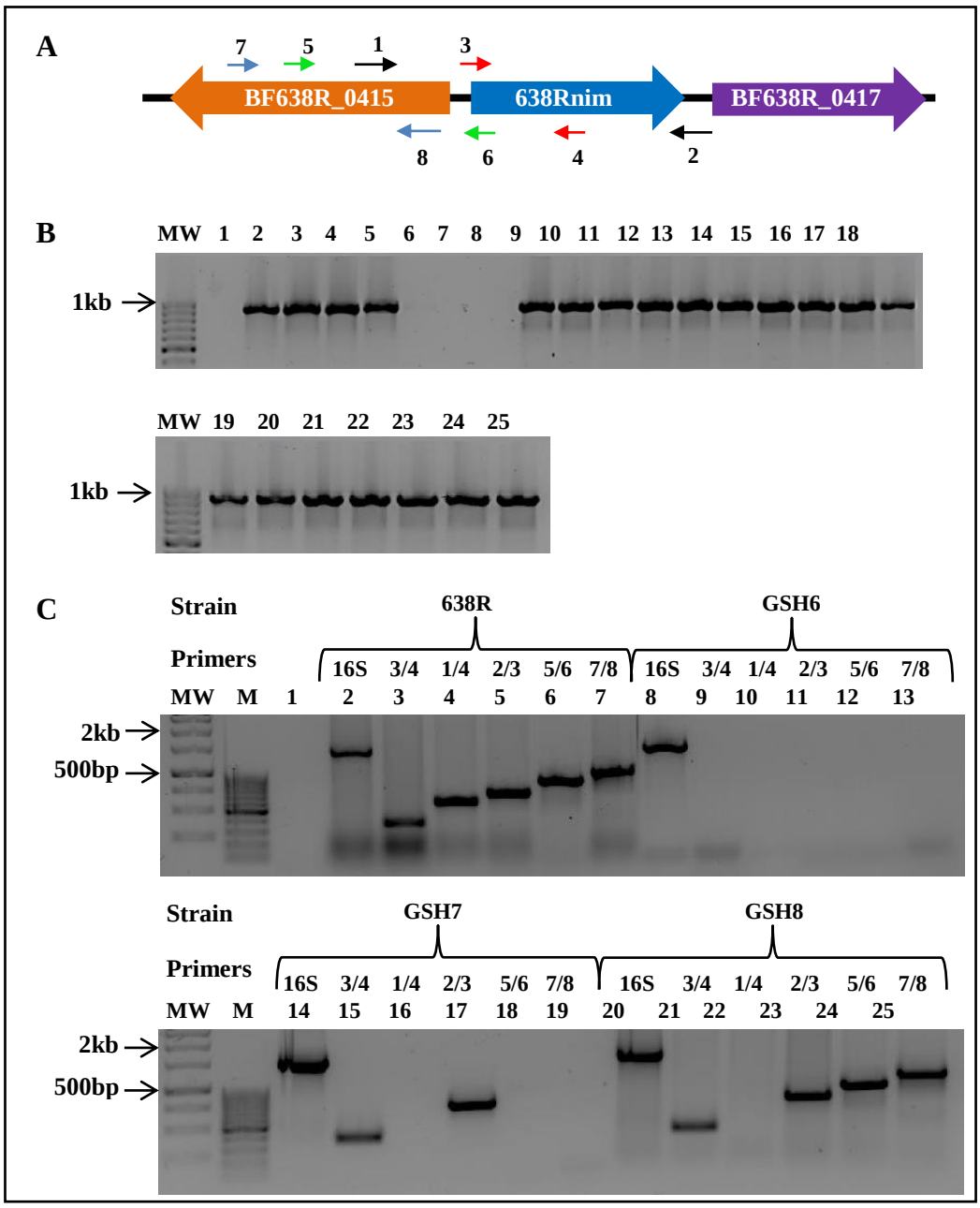


Figure 4.8: *638Rnim* PCR analysis. **(A)** Schematic representation of the *638Rnim* gene structure and the relative positions of the primer pairs used for the PCR analysis. Primers are reported in Table 4.1. **(B)** PCR products generated from the primer pair 638RnimF/R. **Lanes: (MW)** molecular weight marker O'GeneRuler 100 bp DNA Ladder (Fermentas), **(1)** No template control, **(2)** 638R positive control, **(3)** GSH2, **(4)** GSH3, **(5)** GSH5, **(6)** GSH6, **(7)** GSH7, **(8)** GSH8, **(9)** GSH9, **(10)** GSH10, **(11)** GSH12, **(12)** GSH13, **(13)** GSH14, **(14)** GSH15, **(15)** GSH16, **(16)** GSH17, **(17)** GSH18, **(18)** GSH19, **(19)** GSH20, **(20)** GSH21, **(21)** GSH22, **(22)** GSH23, **(23)** GSH25, **(24)** GSH28, **(25)** GSH30. **(C)** PCR products generated from the various primer pairs indicated above. **Lanes: (MW)** molecular weight marker O'GeneRuler 1 kb DNA ladder (Fermentas), **(M)** molecular weight marker O'GeneRuler 100 bp DNA ladder (Fermentas), **(1)** no template control. **Lanes 2–7**, 638R template (positive controls); **lanes 8-13**, GSH6 template; **lanes 14-19**, GSH7 template; **lanes 20-25**, GSH8 template. **Lanes 2, 8, 14 and 20**, 16S rRNA primers; **lanes 3, 9, 15 and 21**, primers 3 and 4; **lanes 4, 10, 16 and 22**, primers 1 and 4; **lanes 5, 11, 17 and 23**, primers 2 and 3; **lanes 6, 12, 18 and 24**, primers 5 and 6; **lanes 7, 13, 19 and 25**, primers 7 and 8.

These two combinations would enable detection of whether the reason for the lack of PCR product, when using primers 1 and 2, was due to changes in BF638R_0415 or BF_638R_0417 of the *638Rnim* gene region. PCR product was obtained from GSH7 and GSH8 using primers 2 and 3 but not 1 and 4, suggesting that there were changes in the BF638R_0415 primer 1 binding region (Fig. 4.8C, lanes 16, 17, 22 and 23). Alternatively, there may be an insertion in the intergenic region between BF_638R_0415 and *638Rnim* resulting in a DNA region that is too large to yield PCR product. In the case of GSH6, however, no such conclusions could be drawn as, once again, no PCR product was detected with either primer set (Fig. 4.8C, lanes 10 and 11).

It is known that *B. fragilis* carry numerous IS elements which often insert themselves in front of antibiotic resistance genes. These elements, when inserted before a gene or near to a promoter, can sometimes enhance transcription (Soki, 2013). Thus, it is possible that the lack of PCR product from the upstream region may be due to this kind of insertion. In order to detect whether there was an IS element present in GSH6, GSH7 and GSH8, primers were designed further into BF638R_0415 (Fig. 4.8C, primers 5 and 6). Primer 6 was chosen specifically because it was the reverse complement of primer 3, which from the internal PCR (primers 3 and 4) in GSH7 and GSH8 had proved to be functional. Thus, it could provide an anchor for this PCR as if no product was detected the problem could only be with the upstream primer (primer 5). For both GSH6 and GSH7 this combination once again failed to produce product indicating either that a large IS element has been inserted or that modifications to the sequence had occurred to the region preventing primer 5 binding. In the case of GSH6, it is also possible that the *638Rnim* gene is not present and primer 6 could not bind. In the case of GSH8, however, PCR product was produced using primers 5 and 6. This product was sequenced in order to determine why primer 1 was unable to bind (Fig. 4.9). It was revealed

that the sequence of GSH8 in this region was identical to that of *B. fragilis* 638R except for a region of 11 bp that has been deleted from GSH8. This region happens to be the exact binding area of primer 1. Thus, the reason for the lack of full length product for GSH8 is due to the deletion of 11 bp from the total 23 bp of primer 1.

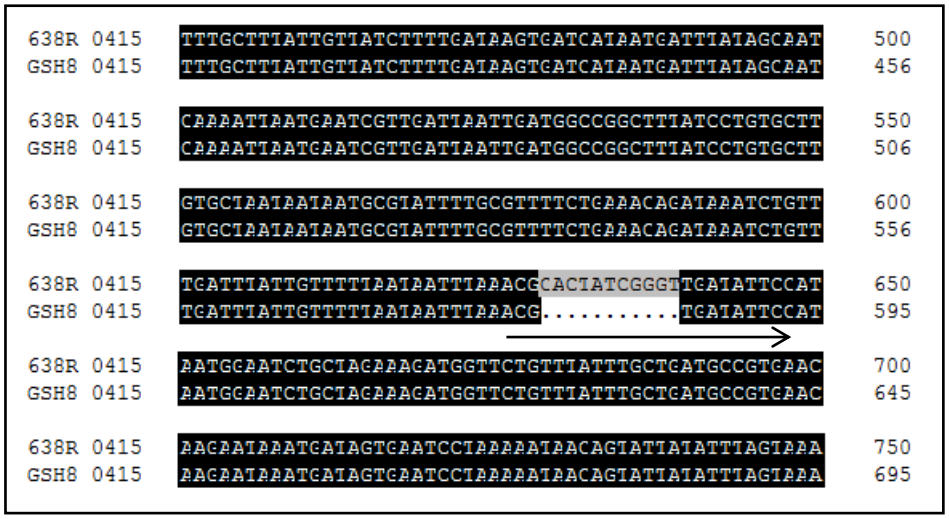


Figure 4.9: MSA of a region of BF638R_0415 from 638R and GSH8. Black = identical sequence, while grey indicates changes. The arrow indicates the position of primer 1.

Due to the fact that all primer combinations, so far, were unable to produce product for the region of BF638R_0415 in either GSH6 or GSH7 a final primer combination was attempted (Fig. 4.8C, primers 7 and 8). This combination was designed to cover approximately a third of the upstream gene in order to determine whether the upstream gene was still present at all. Interestingly, this PCR also failed to produce product in GSH6 and GSH7 which suggests that there has been some significant alterations to this region of BF638R_0415 in these strains. Analysis of the three published *B. fragilis* genomes (638R, YCH46 and NCTC9343) revealed that the upstream region was very well conserved across all three genomes.

4.4 Conclusion

A variety of bioinformatic tools were used to study the putative nitroimidazole resistance gene, *638Rnim*, identified in the *B. fragilis* 638R genome but not amplified by the universal *nim* primers. Amino acid MSA and the resulting homology matrix revealed that 638Rnim was not similar to the known Nim proteins with no protein having more than 28% sequence homology. However, it was shown that 638Rnim does share important amino acids identified in structural studies of NimA from *D. radiodurans* (drNimA). These include the possible active-site residue His71 and the Mtz-binding site of Pro56 and Tyr111 (Leiros *et al*., 2004). Phylogenetic analysis confirmed the MSA data by grouping 638Rnim separately from the known Nims. However, the analysis did suggest that 638Rnim was more closely related to drNimA than to any of the other 9 Nim proteins, including NimJ, which is also not amplified by the universal primers (Husain *et al*., 2013). This is interesting when compared to the percentage homology which indicates that 638Rnim and drNimA share less than 8% direct amino acid sequence homology.

However, the structure of a protein is often more conserved than the amino acid sequence and can provide valuable information on the potential function of that protein (Kelley and Sternberg, 2009, Holm *et al*., 2008, Holm *et al*., 2006). The PHYRE and Dali servers were used to determine the predicted protein structure of 638Rnim and identify possible structural matches. This analysis identified drNimA as the closest structural match with 100% confidence and a Z-score of 22.8. Threading the structures of 638Rnim and drNimA onto one another revealed that there was very good structural conservation between them, especially with the addition of drNimA's preferred ligands (Mtz, pyruvate and acetate). Taken together the above mentioned analysis suggests that 638Rnim protein may be a novel Nim and warrants further functional investigation.

PCR screening of the clinical strains was performed in order to determine how common *638Rnim* was throughout them, since analysis of the three other published *B. fragilis* genomes revealed it was present in all of them (Patrick *et al*., 2010, Cerdeno-Tarraga *et al*., 2005, Kuwahara *et al*., 2004). PCR analysis showed it could be detected in all of the clinical isolates except GSH6, while GSH7 and 8 contained at least an internal portion of it. Upstream analysis showed that GSH8 contained an 11 bp change at the binding site of the forward primer in the region of BF638R_0415, but otherwise possessed an unaltered gene when compared to sequence data from 638R. GSH7, however, revealed significant upstream alterations that resulted in failure to amplify any part of BF638R_0415. This failure to amplify the upstream portion of GSH7 and any section of GSH6 (including *638Rnim* itself) could well be due to the use of directed PCR. Directed PCR relies on finding a specific, conserved region of the gene of interest for the primer binding sites in order to amplify any product. Thus, failure to produce product may not be due to the gene not being present but instead due to the primers not being able to bind to the target area. Therefore, a better method would be complete genome sequencing of this region of GSH6 and GSH7 in order to determine whether any portion of *638Rnim* is still present in GSH6 and identify the changes to the upstream region of GSH7. This would provide a much more comprehensive analysis of the region of interest and resolve the issues associated with the direct PCR approach.

It is not clear at this early stage of the research whether the *638Rnim* gene is involved in Mtz resistance or not. However, it does not appear to have a significant effect on the basal Mtz resistance in the Mtz sensitive clinical isolates (Chapter 2). It is possible that this gene may be responsible for the low level intrinsic resistance observed in *B. fragilis* isolates or may require some type of IS element activation. It may be that the gene is present but inactive in most *B. fragilis* strains and requires an IS element to cause Mtz resistance as observed with other

antibiotic resistance genes including the universal *nim* genes (Soki, 2013). However, further analysis needs to be performed in order to determine if this is the case.

The bioinformatic analysis performed in this study strongly suggests that *638Rnim* could be a novel 5-nitroimidazole resistance gene. However, bioinformatic analysis can only provide an indication of a possible function of a putative protein. In order to ascribe a direct role for a gene, functional analysis *in vivo* is required. Functional characterisation of *638Rnim* was, therefore, carried out in order to determine the true properties of the gene and its encoded protein product. This work is described in Chapter 5 of this thesis.

Chapter 5

Functional characterisation of the *638Rnim* gene present in *B. fragilis* 638R

5.1 Introduction

A putative nitroreductase-like gene was identified in the *B. fragilis* 638R genome (Chapter 4). Bioinformatic analysis revealed that *638Rnim* was structurally similar to the *nimA* from *Deinococcus radiodurans* and this warrants functional analysis as it has the potential to be a novel *nim* gene with possible links to metronidazole (Mtz) resistance.

As described previously (Chapter 1) Mtz is a 5-nitroimidazole compound that enters the cell as an inactive pro-drug where it sequesters electrons, resulting in its activation to a toxic nitroso radical intermediate (Sisson *et al*., 2000). This intermediate then binds to the DNA, causing single- and double-stranded DNA breaks (Trinh and Reysset, 1998). The *nim* genes are believed to encode a nitroimidazole reductase which reduces the nitro group of Mtz to an amino group (Carlier *et al*., 1997). This results in the formation of the inactive compound, 5-aminoimidazole, instead of Mtz's toxic nitroso radicals that are essential for its activity. However, the exact method of action of enzymes encoded by the *nim* genes is not yet known (Gal and Brazier, 2004).

Of the 10 *nim* genes that have been identified only *nimJ* has had any functional studies performed on it (Husain *et al*., 2013, Alauzet *et al*., 2010), while the only crystal structure that exists of a Nim protein is that encoded by *nimA* from *Deinococcus radiodurans*. As described in Chapter 4, the drNimA protein was expressed and purified allowing for the crystal structure to be solved and function to be assigned to the gene (Leiros *et al*., 2004). The *nimJ* gene, like *638Rnim*, was identified using bioinformatic tools and provides the closest comparison to *638Rnim*. Overexpression of *nimJ* revealed that it increased Mtz resistance from 0.03 mg/L to 0.14 mg/L in a *B. fragilis* 638R background, which was a 4-fold increase in resistance (Husain *et al*., 2013). This study suggests a useful experimental approach for the identification of a potential *nim* gene as a functional resistance gene.

While the functional analysis performed on the *nimJ* only used a genetic approach, reductases linked to Mtz resistance in other organisms have been characterised using protein analysis and biochemical assays. In a study performed on RdxA, a nitroreductase present in the anaerobic bacterium *Helicobacter pylori*, the authors developed a protocol which measured the reduction of Mtz anaerobically (Olekhnovich *et al*., 2009). This assay confirmed that RdxA was 60 times more active than any other enzyme tested in its ability to reduce Mtz (Olekhnovich *et al*., 2009). A similar study performed in *Giardia lamblia* utilized both an aerobic and anaerobic nitroreductase assay in order to determine that GlTrxR, a thioredoxin reductase, was able to reduce 5-nitroimidazoles to non-radical anions (Leitsch *et al*., 2011).

This chapter aims to functionally characterise *638Rnim* and its product employing a variety of techniques. These include a genetic approach using mutation and heterologous overexpression and transcriptional analysis using qRT-PCR. A functional protein approach was also attempted with a view to demonstrating nitroreductase enzymatic activity.

5.2 Materials and Methods

5.2.1 Bacterial strains and plasmids, media and growth conditions

All *Bacteroides* strains were grown in supplemented brain heart infusion broth (BHISB) or on plates (BHISA) at 37°C under anaerobic conditions as described in Chapter 2. *B. fragilis* strains with pMCL140 or pGerm integrations were grown on BHISA with 10 mg/L Erm (Husain *et al*., 2013, Bonheyo *et al*., 2001), with the addition of 2 mg/L Tet for *B. fragilis* containing plasmid pLYL01. The strains and plasmids used are described in Table 5.1.

Table 5.1: Strains and plasmids used in this study

Strain/Plasmid	Genotype/Phenotype	Source/Reference
B. fragilis		
638R	Clinical strain $Rif^R Gent^R$	(Privitera *et al*., 1979)
638R *recA*	*recA*$^-$ $Rif^R Gent^R Erm^R$ Mtz^S strain	(Steffens *et al*., 2010)
638R(pLYL01)	$Rif^R Gent^R Tet^R$	(Steffens *et al*., 2010)
638R(pLYL638Rnim)	*638Rnim*$^+$ $Rif^R Gent^R Tet^R$ *638Rnim* gene overexpressor strain	This study
638R *recA*(pLYL01)	*recA*$^-$ $Rif^R Gent^R Erm^R Tet^R$ Mtz^S strain	(Steffens *et al*., 2010)
638R *recA*(pLYL638Rnim)	*638Rnim*$^+$ *recA*$^-$ $Rif^R Gent^R Erm^R Tet^R$ *638Rnim* gene overexpressor strain	This study
638R(pMCL140)	$Rif^R Gent^R Erm^R$	This study
638R(pMCL638Rnim)	*638Rnim*$^+$ $Erm^R Rif^R Gent^R$ *638Rnim* gene overexpressor strain	This study
638R *638Rnim*$^-$	*638Rnim*$^-$ $Rif^R Gent^R Erm^R$ 638R with pGerm-nim integrated into the *nim* gene	This study
GSH15	Clinical strain Mtz^R (Chapter 2)	This study
E. coli		
S17-1	RP4-2-Tc::Mu *aph*::Tn*7 recA* $Strep^R$	(Simon *et al*., 1983)
AB2463	*recA*$^-$ $Strep^R$ Mtz^S strain	(Howard-Flanders and Theriot, 1966)
AB2463(pTZ57Rcontrol)	Mob+, Amp^R containing a stuffer fragment	This study
AB2463(pTZ57R638Rnim)	Mob+, Amp^R *nim*$^+$ *638Rnim* gene overexpressor strain	This study
BL21DE3	*E. coli* B strain with DE3, a λ prophage carrying the T7 RNA polymerase gene and $lacI^q$	(Studier and Moffatt, 1986)
BL21DE3(pLysS)	*E. coli* BL21DE3 strain expressing T7 lysozyme	Novagen
BL21DE3(pET22b(+))	BL21DE3 with empty pET22b(+) vector	This study
BL21DE3(pET22b638Rnim pro)	BL21DE3 derivative with pET vector expressing full length *B. fragilis 638Rnim*	This study
Plasmids		
pLYL01	Mob+, $Tet^R Amp^R$	(Li *et al*., 1995)
pLYL638Rnim	pLYL01 containing *638Rnim*	This study
pMCL140	pFD340-based vector, Mob+, $Erm^R Amp^R$	(Chatzidaki-Livanis *et al*., 2010)
pMCL638Rnim	pMCL140 containing *638Rnim*	This study
pTZ57R	Mob+, Amp^R	Thermo Scientific
pTZ57R(control)	pTZ57R containing a stuffer fragment (Control)	Thermo Scientific
pTZ57R(638Rnim)	pTZ57R containing *638Rnim*	This study
pGerm	pUC19-based suicide vector, Erm^R	(Bonheyo *et al*., 2001)
pGerm638Rnim-int	pGerm with *638Rnim* insertion sequence	This study
pET22b(+)	*pelB* coding sequence, His-tag at C-terminal	Novagen
pET22b638Rnimpro	pET22b(+) plasmid with full length 638Rnim protein	This study

Escherichia coli BL21DE3 and BL21DE3(pLysS) were grown under aerobic conditions at 30°C on Luria Agar (LA) or in Luria Broth (LB) with no selection. Growth was under aerobic conditions at 30°C. *E. coli* AB2463 was grown on LA or in LB supplemented with 0.5% glucose and 0.2% sodium nitrate with no selection. Growth was under anaerobic conditions at

37°C. *E. coli* BL21DE3, BL21DE3(pLysS) and AB2463 strains expressing the pET22b638Rnimpro, pET22b(+), pTZ57R(control) or pTZ57R(638Rnim) plasmids were grown with ampicillin (Amp) (100 mg/L) selection.

5.2.2 DNA techniques

Genomic DNA (gDNA) was extracted using the Genomic DNA Purification Kit (Fermentas) and, plasmid DNA was extracted using the BioSpin Plasmid Extraction Kit (BioFlux). DNA was quantitated using a NanoDrop spectrophotometer (NanoDrop ND-1000). Analysis of gDNA, plasmid or PCR DNA products was routinely done by electrophoresis on 0.8% (w/v) agarose gels in Tris-acetate buffer with ethidium bromide. Plasmids were transformed into *E. coli* S17-1 $CaCl_2$ competent cells (Dagert and Ehrlich, 1979). Plasmids were transferred into *B. fragilis* strains from these *E. coli* transformants by conjugation (Bonheyo *et al*., 2001).

5.2.3 Metronidazole survival curves

Cultures of *B. fragilis* 638R(pLYL01), 638R*recA*(pLYL01) and 638R*recA*(pLYL638Rnim) were incubated for 16 h at 37°C in BHISB under strict anaerobic conditions. The 16 h culture was inoculated into fresh BHISB, grown to log phase (OD600=0.6), and exposed to 0.5 mg/L Mtz. Cell samples were collected at 0, 20 and 40 min intervals, dilution series made from 10^{-1} – 10^{-6} in anaerobically reduced water, and the cells plated on BHISA. The plates were then incubated anaerobically at 37°C for 3 days and the surviving fraction of cells was calculated for each time point. The experiment was done in biological triplicate and technical duplicate. Statistical significance was determined using the student t-test with a p-value for statistical significance at $p < 0.05$.

5.2.4 Insertional mutagenesis

An internal fragment of the *638Rnim* gene from *B. fragilis* 638R (BF638R_0416), of approximately 0.5 kb, was amplified by PCR with primers IntF and IntR (Table 5.2) at an annealing temperature of 55°C, for an elongation period of 30 sec.

Table 5.2: Primers and PCR parameters used in this study

Primers	Characteristic/ Use	Source/ Reference
F27 5'-AGAGTTTGATCITGGCTCAG-3'	16S rRNA published primers	(Cheneby *et al*., 2000)
R5 5'-ACGGITACCTTGTTACGACTT-3'		
M13F 5'-CGCCAGGGTTTTCCCAGTCACGAC-3'	Plasmid confirmation, published primers	(Yanisch-Perron *et al*., 1985)
M13R 5'-GAGCGGATAACAATTTCACACAGG-3'		
638RnimF 5'-ACGCACTATCGGGTTGATATTCC-3'	Full length *B. fragilis* 638R *638Rnim* gene	This study
638RnimR 5'-ATCGCAAGTACTCTCCACTTACG-3'		
IntF 5'-CCGACGGAAAGACCGCC-3'	Internal fragment of *B. fragilis* 638R *638Rnim* gene	This study
IntR 5'-CTAGACGTGGCTACGATGTTAAGA-3'		
16S F 5'-ACGTTGTTGGTGAGGTAACG-3'	qPCR 16S rRNA primers	(Pumbwe *et al*., 2006a)
16S R 5'-CCAATATTCCTCACTGCTGC-3'		
NimF 5'-GCATCGTTTTGCGTGGTAGG-3'	qPCR *B. fragilis* 638R *638Rnim* gene primers	This study
NimR 5'-AGAAGTTCCAATGCCTTTCGC-3'		
ProNimF 5'-ACCATGGAATATATCAACGATTTAATCC-3' *Nco*I	Primers for directional cloning of full length *B. fragilis* 638R *638Rnim*	This study
ProNimR 5'-GGTAGGCTCGAGTTTATAATCCTGAC-3' *Xho*I		

Translation stop codons were designed into these primers to ensure that no truncated version of the protein was produced. The resulting PCR products were cloned into the *Sma*I site of the suicide vector pGerm (Bonheyo *et al*., 2001) by blunt cloning using standard protocols (Maniatis *et al*., 1982). Confirmation of insertion of the DNA to form the plasmid pGermnim-int was done by PCR of erythromycin and gentamycin resistant *B. fragilis* transconjugant

colonies with primer pairs 638RnimF/ R and M13F or M13R (Table 5.2) followed by sequencing of the products as described previously (Chapter 4).

5.2.5 Metronidazole resistance

Resistance to Mtz was determined using the E-test (AB Biodisk) method as described in Chapter 2.

5.2.6 RNA extraction and integrity studies

RNA was extracted using the RNeasy Mini Kit with RNA protect (Qiagen). RNA was treated with DNaseI (Fermentas) to completely remove any DNA contamination. DNA contamination of the RNA was evaluated by PCR of the 16S rRNA gene using the universal F27/R5 primer pair combination and the RNA preparation as template (Table 5.2). The products were electrophoresed on a 1% agarose gel. RNA quantity and quality were determined using the NanoDrop spectrophotometer (NanoDrop Technologies) and denaturing agarose gel electrophoresis (2% Formaldehyde, MOPS running buffer), respectively.

5.2.7 Reverse transcriptase PCR (RT-PCR)

The synthesis of the cDNA molecules was carried out using the Tetro cDNA Synthesis Kit (Bioline) according to the manufacturer's instructions. Random primers were used for first strand cDNA amplification of RNA transcripts. Conversion of RNA to cDNA was evaluated using the 16S rRNA PCR primer pair (F27/R5). The cDNA (2 µl) was then used as template for the target genes using gene specific primers (NimF/ NimR). The GeneAmp PCR System 9700 was used for the cycling reactions (Perkin Elmer, Applied Biosystems). The PCR was done using Kapa Ready Mix (Lasec). The PCR parameters were: initial denaturation of 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, and

elongation at 72°C for 30 sec. A final elongation step was carried out at 72°C for 5 min. Primers and priming positions are shown in Fig. 5.1 and Table 5.2.

5.2.8 Quantitative RT-PCR (qRT-PCR)

Sample preparation, storage and Primer design

Mtz induction, sample preparation, primer design, reaction conditions and data analysis were all done as described in Chapter 3. The positions of the primers used in this experiment are shown in Fig. 5.1.

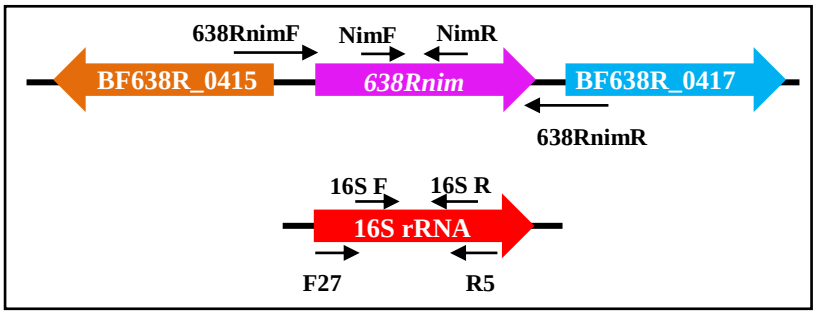


Figure 5.1: Schematic representation of the *B. fragilis* 638R *638Rnim* and 16S rRNA genes and the primer pairs used to amplify them. A region of the chromosome from nucleotide positions 484761bp – 487896 bp is shown with annotated ORFs BF638R_0415-BF638R_0417. Primers are reported in Table 5.2.

5.2.9 Protein expression and purification

Protein expression was undertaken using the pET expression system (Novagen) and plasmids expressed in *E. coli* BL21DE3 or BL21DE3pLysS. Full length sequence of the *B. fragilis* 638R *638Rnim* gene was obtained by PCR using ProNimF and ProNimR. The details of these primers can be seen in Table 5.2. The PCR was carried out using Kappa Ready Mix and the parameters were: initial denaturation of 95°C for 5 min, then 30 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, and elongation at 72°C for 3 min. A final elongation step was carried out at 72°C for 5 min. PCR products were purified using the BioSpin PCR purification kit (BioFlux) and cloned using the instaclone kit (Fermentas) as per the manufacturer's instructions. The recombinant plasmids were sequenced (Macrogen) and those

carrying the correct full length sequence of the *638Rnim* gene were digested using the *Nco*I and *Xho*I restriction enzymes. The full length gene was then directionally cloned into the pET22b (+) (Novagen) vector. The sequence was analysed to ensure that the reading frame was correct and that the 6xHis residue tag was present on the C-terminus of the protein. The construct was then transformed into *E.coli* BL21DE3 or BL21DE3pLysS and successful transformation was confirmed by PCR using ProNimF and ProNimR primers. The cells containing the correct plasmid constructs were then grown for 16 hours. During optimisation cells were grown to an OD600 of 0.4, after which various concentrations of IPTG were added and samples were taken at a range of time intervals. These cells were sonicated (Sonix 5000) and the insoluble and soluble fractions were run on a 15% SDS-PAGE gel. These gels were then visualised using Coomassie staining, and novel bands were assessed to determine optimum induction time. The protein fractions were also analysed by western blot using Anti-His antibodies (Sigma) and visualised using Pierce ECL (Thermo Scientific). The soluble cell free extract was passed through a Nickel-Affinity Chromatography Column (His-Select Nickel Affinity gel Sigma) which was prepared according to the manufacturer's instructions and stored at 4°C before use. The column with bound protein was sequentially washed with 30 ml of 10 mM imidazole wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole), and then 30 mM imidazole (50 mM NaH_2PO_4, 300 mM NaCl, 30 mM imidazole). The bound purified protein was eluted using 250 mM imidazole (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole) and the purity of the protein was assessed using both SDS-PAGE and anti-His-tag Western Blots.

5.3 Results and Discussion

5.3.1 Reverse transcription PCR (RT-PCR)

The bioinformatic analysis performed in Chapter 4 revealed that the *638Rnim* gene present in *B. fragilis* 638R has the potential to be a nitroreductase and PCR showed that it was present in almost all the clinical strains. The first stage in characterising this gene involved a

determination of whether it was being transcribed under standard *in vitro* growth conditions. This was achieved using RT-PCR (Fig. 5.2).

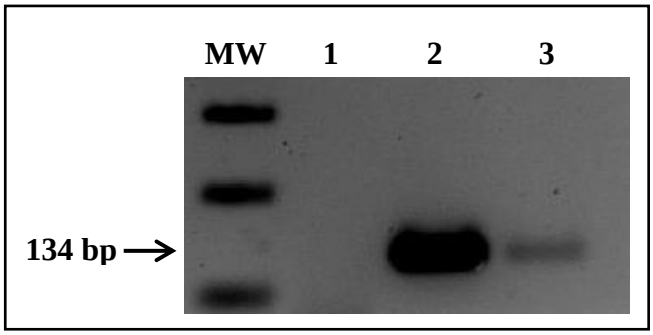


Figure 5.2: RT-PCR analysis of *B. fragilis* 638R *nim* gene. Agarose gel (2%). **Lanes: (MW)** molecular weight marker O'GeneRuler 100 bp DNA ladder (Fermentas), **(1)** 638R RNA template, **(2)** 638R genomic DNA template, **(3)** 638R cDNA template.

A cDNA conversion was performed on DNA-free RNA extracted from exponential phase *B. fragilis* 638R cultures, under normal growth conditions. The RT-PCR revealed that the *nim* gene present in *B. fragilis* 638R was being transcribed (Fig. 5.2).

5.3.2 638Rnim protein expression and purification

Transcription of the *638Rnim* gene in *B. fragilis* 638R suggested that a protein could possibly be produced from this ORF. In order to determine the functional identity of the 638Rnim protein, attempts were made to purify it from the cloned gene expressed in *E. coli* with a view to performing nitroreductase assays using Mtz as a substrate.

The *E. coli* protein expression strain BL21DE3 was used to express the *638Rnim* gene from the pET22b (+) vector. Very little protein expression was observed and all of the protein that was expressed was found in the insoluble fraction (Fig. 5.3A). This was confirmed by the anti-His-tag Western blot (Fig. 5.3B). All attempts to improve the expression and solubility of the protein by changing the temperature or IPTG concentration were unsuccessful (data not shown).

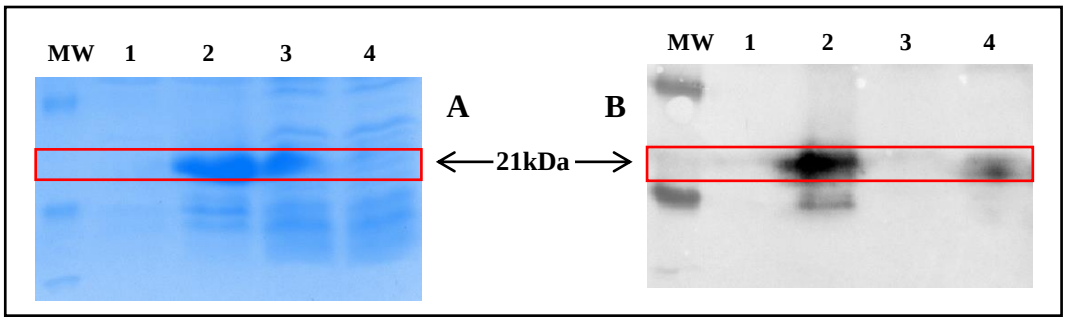


Figure 5.3: Expression of the *B. fragilis* 638Rnim encoded protein in *E. coli* BL21DE3. **(A)** 12% SDS-PAGE gel showing expression of pET22b638Rnimpro plasmid in BL21DE3. **Lanes: (MW)** Marker Protein Ladder (Fermentas), **(1)** pEt22b (+) insoluble fraction, **(2)** pET22b638Rnimpro insoluble fraction, **(3)** pEt22b (+) soluble fraction, **(4)** pET22b638Rnimpro soluble fraction. **(B)** His-Tag Western showing expression of pET22bnimpro plasmid in BL21DE3. **Lanes: (MW)** Marker Protein Ladder (Fermentas), **(1)** pEt22b (+) insoluble fraction, **(2)** pET22b638Rnimpro insoluble fraction, **(3)** pEt22b (+) soluble fraction, **(4)** pET22b638Rnimpro soluble fraction.

It was decided that a more stringent expression system may provide better soluble protein expression and thus, *E. coli* BL21DE3 pLysS was used which completely represses recombinant protein production until it is specifically induced under controlled conditions. Initially, no change in the amount of protein expressed in the soluble fraction was observed using the standard optimizing conditions mentioned in the methods (data not shown). However, after reducing the growth temperature to 30°C, the IPTG concentration to 0.25 mM and the induction time to 30 min a small amount of soluble protein was observed (Fig. 5.4A and B).

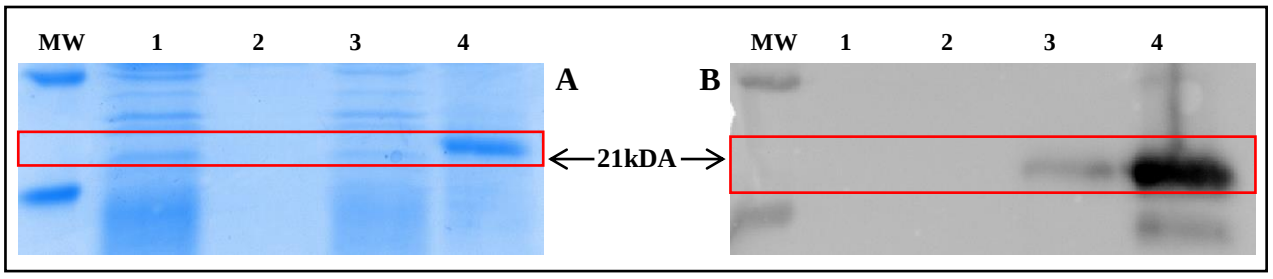


Figure 5.4: Expression of the *B. fragilis* 638Rnim encoded protein in *E. coli* BL21DE3 pLysS. **(A)** 12% SDS-PAGE gel showing expression of pET22b638Rnimpro plasmid in BL21DE3 pLysS. **Lanes: (MW)** Marker Protein Ladder (Fermentas), **(1)** pEt22b (+) soluble fraction, **(2)** pEt22b (+) insoluble fraction, **(3)** pET22b638Rnimpro soluble fraction **(4)** pET22b638Rnimpro insoluble fraction. **(B)** His-Tag Western showing expression of pET22b638Rnimpro plasmid in BL21DE3 pLysS. **Lanes: (MW)** Marker Protein Ladder (Fermentas), **(1)** pEt22b (+) soluble fraction, **(2)** pEt22b (+) insoluble fraction, **(3)** pET22b638Rnimpro soluble fraction **(4)** pET22b638Rnimpro insoluble fraction.

Attempts to improve the soluble protein yields using the addition of chloramphenicol to disrupt possible inclusion bodies (Carrio and Villaverde, 2001) were unsuccessful (data not shown). Thus, these protein induction conditions were used for Nickel-Affinity Chromatography in order to attempt to purify the Nim protein for use in a nitroreductase assay (Fig. 5.5A and B).

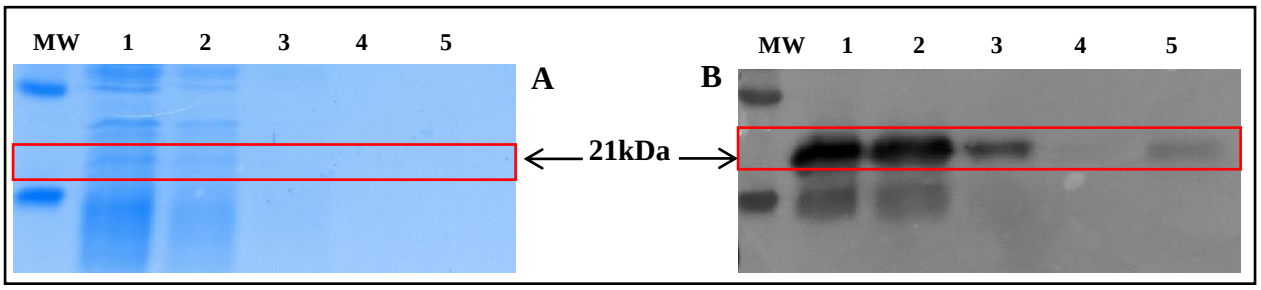


Figure 5.5: Purification of the *B. fragilis* 638Rnim expressed in *E. coli* BL21DE3 pLysS. **(A)** 12% SDS-PAGE gel stained with coomassie showing purification of *B. fragilis* 638Rnim **Lanes: (MW)** Marker Protein Ladder (Fermentas), **(1)** crude fraction, **(2)** cell free extract flow through, **(3)** 10 mM Imidazole wash, **(4)** 30 mM Imidazole wash, **(5)** purified 638Rnim. **(B)** His-Tag Western showing purification of *B. fragilis* 638Rnim. **Lanes: (MW)** Marker Protein Ladder (Fermentas), **(1)** crude fraction, **(2)** cell free extract flow through, **(3)** 10 mM Imidazole wash, **(4)** 30 mM Imidazole wash, **(5)** purified 638Rnim.

Unfortunately, the purification process never yielded enough protein to be visible on a coomassie gel even though anti-His-tag Westerns confirmed the presence of the protein (Fig. 5.5A and B). No increase in protein yield was observed even with alterations to the wash and elution buffers (data not shown).

Chemical denaturation and dissolution of inclusion bodies and the refolding of proteins as described by Middelberg (2002) were considered as possible methods to improve 638Rnim protein solubility. Since these may exert inhibitory effects on protein activity, they were not attempted in the current research. However, future work should include a detailed analysis of chemical denaturation and its effects on 638Rnim soluble protein production and enzyme activity. *E. coli* is well known for insoluble inclusion body formation for some gene sequences (Middelberg, 2002) and thus, future studies should attempt to use alternate methods and systems for protein expression in order to obtain soluble 638Rnim protein. This includes a

relatively new system developed for overexpression of a protein using a *B. fragilis* specific expression system which contains a C-terminal His-tag (Parker and Smith, 2012). This method was not available at the time the studies reported here were undertaken. It might, however, solve the problem of inclusion body formation associated with *E. coli* and prevent any post-translational modification which may be *B. fragilis* specific. Due to the lack of purified protein, no nitroreductase assay could be performed and thus functional characterisation utilizing the protein approach was not possible in this study. Therefore, a genetic approach to functional characterisation was attempted.

5.3.3 Determination of the effect of the *B. fragilis 638Rnim* gene on cellular Mtz resistance

5.3.3.1 Heterologous expression of the *638Rnim* gene in *E. coli* strain AB2463

E. coli is a facultative anaerobe that is not usually Mtz sensitive under aerobic conditions due to its high redox potential and futile cycling of the Mtz as opposed to activation of the pro-drug (Edwards, 1980). However, the cells do become sensitive when treated under anaerobic conditions, and this is enhanced further in a *recA* deficient *E. coli* mutant that is highly sensitive to Mtz when grown anaerobically (Howard-Flanders and Theriot, 1966). *E. coli recA* strain was, therefore, used in an experiment to determine the effect of *nim* gene overexpression on Mtz resistance in a Mtz sensitive heterologous bacterium. The plasmids pTZ57R (control) and pTZ57R (*638Rnim*) were transformed into *E. coli* AB2463 (*recA*$^-$). Under anaerobic conditions, the Mtz resistance level of *E. coli* AB2463 pTZ57R (control) was 2 mg/L. In contrast *E. coli* AB2463 carrying pTZ57R (*638Rnim*) was found to be 6 mg/L (Table 5.3).

Table 5.3: Mtz resistance levels determined by the E-test method. Each E-test was performed in duplicate.

Strain (Plasmid system)	Effect on *638Rnim*	Control	Experimental
E. coli AB2463 (pTZ57R)	Overexpression	2 mg/L	6 mg/L
B. fragilis 638R (pLYL01)	Overexpression	0.125 mg/L	0.125 mg/L
B. fragilis 638R (pMCL140)	Overexpression	0.125 mg/L	0.125 mg/L
B. fragilis 638R (pGERM)	Interruption	0.125 mg/L	0.125 mg/L

Control = Empty vector or vector with a stuffer fragment; Experimental = Vector with the *638Rnim* gene

This 3-fold increase in the level of Mtz resistance suggested that overexpression of *638Rnim* in *E. coli* did result in increased Mtz resistance and that it may also serve this function in *B. fragilis*. Thus, investigation into the effects of the *638Rnim* gene on the response to Mtz in *B. fragilis* was undertaken.

5.3.3.2 Expression of the *B. fragilis* 638R *638Rnim* gene in *B. fragilis*

The impact of the presence of *638Rnim* on *B. fragilis* Mtz sensitivity was determined using both a gene overexpression and a gene interruption approach. The overexpressor strains were constructed by transformation of the *B. fragilis* 638R wild type strain with one of the following plasmids: pLYL01, pLYL638Rnim, pMCL140 or pMCL638Rnim. The *638Rnim* interruption strain was constructed using the pGerm interruption method (Bonheyo *et al*., 2001). The pGerm construct containing the internal portion of the *638Rnim* gene was created and conjugated into *B. fragilis* 638R. To confirm the insertion of the suicide vector into the gene of interest, PCR of the putative transconjugants was performed using the full length gene primers, in combination with M13 primers which bind to the pGerm vector sequence (Fig. 5.6).

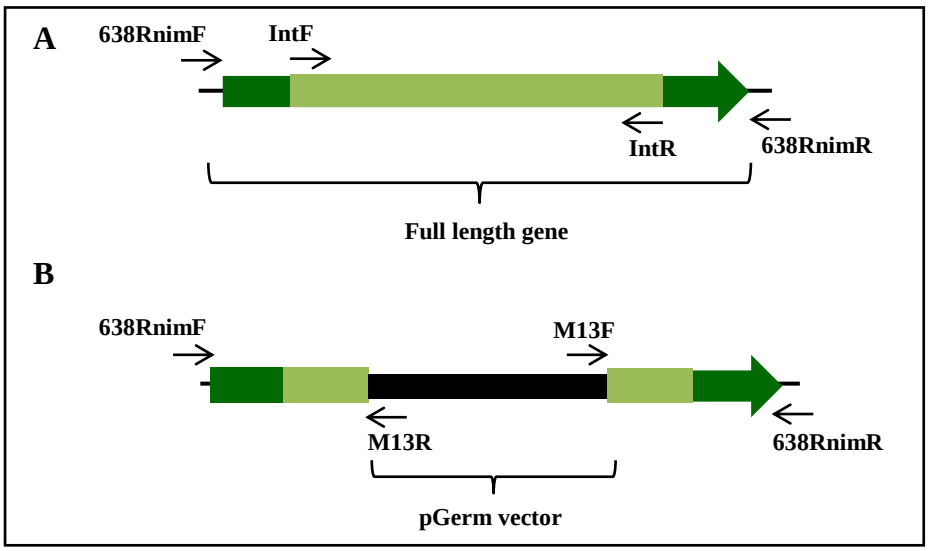


Figure 5.6: Schematic diagram of the pGerm interruption of the *638Rnim* gene. **(A)** Wild-type *B. fragilis* 638R *638Rnim*. **(B)** *B. fragilis* 638R *638Rnim*- mutant. The light green area indicates the internal fragment of the *638Rnim* gene used for the homologous recombination. The dark green area is the rest of the *638Rnim* gene. The black block represents the pGerm plasmid DNA and the arrows indicate the primers used and their annealing sites.

The production of PCR products with primer pairs 638RnimF/ M13R and 638RnimR/ M13F confirmed that the *638Rnim* gene was interrupted (Fig. 5.7, lanes 7 and 9 respectively). These fragments were not produced when wild-type colonies were used (lanes 6 and 8). PCR with 638RnimF and 638RnimR amplified the full length *638Rnim* gene from wild-type *B. fragilis* 638R (lane 2) and not from the mutant (lane 3). The mutant PCR products were sequenced (Fig. 5.7, Lanes 7 and 9) with primers M13F and M13R and confirmed the junction between the pGerm inserted vector and the interrupted *638Rnim* gene.

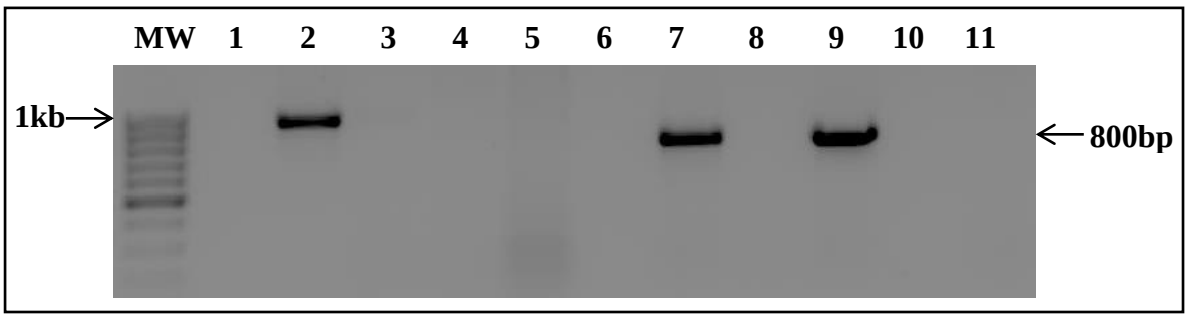


Figure 5.7: PCR of wild-type *B. fragilis* 638R and *B. fragilis* 638R *638Rnim*- mutant for confirmation of insertion. **Lanes: (MW)** O'GeneRuler 100 bp DNA ladder (Fermentas), Primers 638RnimF and 638RnimR with **(1)** no DNA template, **(2)** wild-type DNA, **(3)** mutant DNA, Primers 638RnimF and M13F with **(4)** wild-type DNA, **(5)** mutant DNA, Primers 638RnimF and M13R with **(6)** wild-type DNA, **(7)** mutant DNA, Primers 638RnimR and M13F with **(8)** wild-type DNA, **(9)** mutant DNA, Primers 638RnimR and M13R with **(10)** wild-type DNA, **(11)** mutant DNA.

Overexpression of *638Rnim* using the pLYL01 vector revealed that there was no difference in Mtz resistance compared to the control (Table 5.3). The resistance levels observed for both the experiment (pLYL638Rnim) and the control (pLYL01), were 0.125 mg/L, which is comparable to those seen for wild type *B. fragilis* 638R (Chapter 2). This would suggest that overexpression of *638Rnim* under these *in vitro* conditions does not increase Mtz resistance. However, it may be possible that the *638Rnim* gene expression in a wild type genetic background may not be sufficient to be detected physiologically due to the presence of the efficient endogenous *B. fragilis* DNA repair systems, such as RecA, which normally recognise Mtz damage. In order to determine if this is the case overexpression of the gene was attempted in a highly Mtz sensitive *B. fragilis recA*$^{-}$ DNA repair mutant analogous to the *E. coli* mutant described previously.

This RecA deficient *B. fragilis* mutant was created by Steffens *et al*. (2010) and lacks an essential DNA repair system required to repair damage caused by Mtz. The strain has a reported Mtz MIC of below 0.016 mg/L which is the lowest detectable level on an E-test strip (Steffens *et al*., 2010). Since these values could not be measured accurately in the current study by E-test, the survival of the cells following Mtz treatment was measured in broth over time. This approach was used in a study by Paul *et al*. (2011) where subtle but significant changes in Mtz resistance were measured. In the current study reported here, the Mtz survival curve revealed that even in a highly Mtz sensitive *B. fragilis* strain, overexpression of *638Rnim* does not significantly increase Mtz resistance (Fig. 5.8).

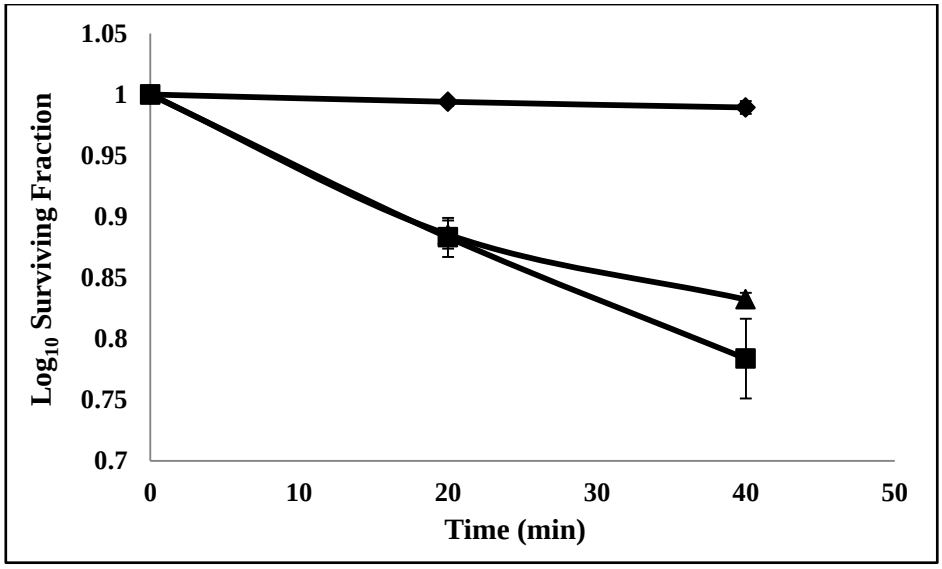


Figure 5.8: Survival curve of *B. fragilis* strains in response to Mtz (0.5 mg/L). **Diamonds**, *B. fragilis* 638R(pLYL01); **Squares**, *B. fragilis* 638R *recA*(pLYL01); **Triangles**, *B. fragilis* 638R *recA*(pLYL638Rnim). The errors bars represent the standard error calculated from triplicate repeats of data.

There did appear to be a slight increase in cell survival with *638Rnim* (triangles) compared to the control (squares) between 20 and 40 min but this was not significant. It, therefore, appears that overexpression of the *638Rnim* gene in *B. fragilis* does not affect Mtz resistance. It should be noted that the cloning strategy described here used the *nim* gene together with 120 bases upstream, assuming that this would include a full putative promoter and all regulatory recognition sequences necessary for transcription. Since the exact functional promoter region of this gene has not been established it is possible this may not have been included and that overexpression of *nim* may not be occurring.

In order to ensure *638Rnim* expression, the gene was cloned into pMCL140, which is a *B. fragilis* vector with an experimentally confirmed functional promoter (Chatzidaki-Livanis *et al.*, 2010). Overexpression of *638Rnim* using this vector, however, also did not result in a change in Mtz resistance (Table 5.3), as both the control (pMCL140) and the experimental (pMCL638Rnim) strains had the same level of resistance (0.125 mg/L). Thus, it appears that

overexpression of *638Rnim* using both its own putative promoter and an experimentally confirmed functional promoter does not affect Mtz resistance.

In order to confirm these findings, the converse experiment was performed where the chromosomal copy of the *638Rnim* gene in *B. fragilis* 638R was inactivated to determine whether the mutant cells became more sensitive to Mtz. It was observed that interruption of the gene did not result in a loss of Mtz resistance as the MIC levels were the same (0.125 mg/L) for both the wild type parent and the mutant (Table 5.3).

At the current stage of this investigation, it is still not clear whether there is genuinely no functional link between the *638Rnim* gene and Mtz resistance, or whether the experimental conditions used were simply not able to demonstrate its function. Although no visible effect on Mtz was seen when *638Rnim* was either overexpressed or interrupted it is still possible that the gene might be involved in the intrinsic resistance observed in *B. fragilis* 638R (Chapter 2) and that the 638Rnim protein requires other ancillary cofactors or proteins for it to have appropriate activity against Mtz. Alternatively, it may be that the gene requires Mtz to be present initially at lower levels before actual challenge with the drug in order to induce a response. Since an induced Mtz resistance response was previously reported in the studies described in Chapter 3, it is possible that induced *638Rnim* transcription may play a role in this phenomenon. The transcriptional response of the *638Rnim* gene to cell exposure to Mtz was, therefore, investigated.

5.3.4 Quantitative real time reverse transcription PCR (qRT-PCR)

qRT-PCR was performed in order to determine if Mtz induction would cause an increase in transcription of the *638Rnim* gene, and if so, whether this was higher in a strain with slightly elevated Mtz resistant (GSH15) as compared to a sensitive strain (638R). The investigation

was done on cDNA created by the reverse transcription of RNA from Mtz induced and uninduced cell cultures from *B. fragilis* 638R (Mtz sensitive) and GSH15 (Mtz resistant) as described in Chapter 3. Optimisation for qRT-PCR and analysis of the data was performed as described in Chapter 3. An example of a melt curve and standard curve for each primer set can be seen in the appendix (Fig. S1 and S2).

Analysis using the Pfaffl criteria (2001) revealed that both *B. fragilis* 638R and GSH15 showed some upregulation of *638Rnim* after Mtz induction (Table 5.4) but neither strain reached the level of significance which is set at greater than 2-fold difference according to Pfaffl (2001).

Table 5.4: CT values, relative fold increase in transcript and standard errors for *B. fragilis* 638R and GSH15.

Strain	CT Value	Fold Change	Standard Error
638R	21.1	0.84	±0.13
GSH15	21.7	1.26	±0.13

Although both values were below significance levels, they did confirm that *638Rnim* is being transcribed in both a sensitive (638R) and resistant strain (GSH15). It is possible that the lack of observation of a significant increase in *638Rnim* transcription was due to the specific design of the induction experiment which may not reflect the optimal time of transcript response to Mtz induction. In this study cultures were induced with Mtz for 60 min before the RNA was harvested. It is possible that the mRNA transcripts had already been expressed and degraded by this point, as described in Chapter 3. However, no information is available about the mRNA half-life in *B. fragilis*. Thus, in order to determine unequivocally whether *638Rnim* transcription is induced by exposure to Mtz or not, a more comprehensive qRT-PCR analysis needs to be performed. Sampling should be done at various time intervals after induction with sub-lethal concentrations of Mtz in order to optimise this study.

Transcription of *638Rnim* genes has been investigated by Husain *et al*. (2013) in a study where endogenous *nimJ* transcription was investigated in two Mtz resistant strains, *B. fragilis* HMW615 and HMW616. They did not investigate transcription of *nimJ* under Mtz inducing conditions. The average fold increase in *nimJ* transcription in HMW615 compared to *B. fragilis* 638R carrying *nimJ* on a multicopy plasmid was found to be 4.26. In comparison the level of transcription observed for *nimJ* in *B. fragilis* HMW616 compared to *B. fragilis* 638R carrying *nimJ* on a multicopy plasmid (also Mtz resistant) was only 1.76. This value is close to the fold change of 1.26 observed in the current study for *B. fragilis* GSH15, the partially Mtz resistant clinical strain.

5.4 Conclusions

A variety of molecular techniques were employed in order to determine whether the *638Rnim* gene affects Mtz resistance in *B. fragilis*. The overall outcome of the investigation was that it could not establish an outright link between the presence and transcription of the *638Rnim* gene and a Mtz resistance phenotype. This does suggest that while *638Rnim* may have predicted structural similarity to a Nim-like nitroreductase, it might not utilise Mtz as its substrate and, therefore, may not be a novel *638Rnim* gene. However, the data does show some interesting positive trends which warrant further future investigation.

RT-PCR revealed that the *nim* gene was being transcribed at basal levels in the absence of Mtz in a sensitive strain. Overexpression of the gene in a heterologous, Mtz sensitive *E. coli recA* mutant strain resulted in a 3-fold increase in Mtz resistance, indicating a functional link for the gene product in resistance to the drug. However, overexpression of *638Rnim* in wild type *B. fragilis* or a *B. fragilis recA*$^-$ strain using the *638Rnim* gene's own promoter and a known *B. fragilis* promoter did not reproduce this result or reveal any change in Mtz resistance. Interruption of the *638Rnim* gene also did not alter the level of Mtz resistance observed

compared to the wild type control strains. The results of these experiments have, therefore, not, as yet, demonstrated any direct link between the *638Rnim* gene and the metronidazole phenotype of the cells. Future work should consider the possibility that the gene product could be working in concert with other genetic elements, such as co-factors, or that insertion sequences are required for full expression of the gene. It is possible that 2-D gel electrophoresis could be used to determine whether any other novel proteins are working in complex with 638Rnim in response to Mtz. Complete deletion of the *638Rnim* gene and its promoter, instead of simply mutating it by insertional inactivation, may also remove the possibility that the truncated protein formed has any residual function. A deletion system has been described, by Patrick *et al*. (2009) for *B. fragilis* and could be used for this purpose.

The evidence from the bioinformatics and structural analyses reported in Chapter 4 strongly suggested that the 638Rnim protein was a nitroreductase. The protein expression and purification protocol attempted in this study did not, however, yield sufficient purified, soluble protein for use in a nitroreductase assay using Mtz as the substrate. Future work should focus on attempting to purify the protein from a *B. fragilis* overexpression plasmid (Parker and Smith, 2012) with the aim of obtaining sufficient product for both biochemical and protein structural analyses.

The findings reported in Chapter 3 suggested that metronidazole resistance was inducible in *B. fragilis*. The possibility that transcription of the *638Rnim* gene was enhanced by exposure of Mtz was, therefore, investigated by qRT-PCR analysis of a sensitive (638R) and resistant (GSH15) *B. fragilis* strain under inducing conditions. The analysis revealed that there was not a significant upregulation of the *638Rnim* gene in both the sensitive and resistant strains under the experimental conditions used. The slight increase observed may suggest that Mtz could

elicit a response from the gene, but that the *in vitro* induction conditions used in this study may not have been ideal for viewing its optimal effect. Thus, future work should include a more comprehensive qRT-PCR analysis that encompasses a variety of sub-lethal Mtz conditions and a number of different induction time points in both a Mtz sensitive and resistant strain.

6.1 General conclusions

There were two main aims of this thesis. The first aim was to perform an antibiotic resistance survey of *B. fragilis* isolates from Groote Schuur Hospital and determine whether known resistance determinants and mechanisms were responsible for this observed resistance with particular focus on Mtz. The second aim was to identify and characterise a putative novel *nim* gene present in *B. fragilis* 638R from a genetic and functional perspective.

Very few antibiotic resistance surveys have been performed in South Africa particularly on *B. fragilis*, which means that very little information is available about the levels of antibiotic resistance present in our hospitals. This thesis found that of the 23 clinical isolates of *B. fragilis* from Groote Schuur Hospital, 8% of them were highly resistant to Imp and Cef, while 65% were resistant to Tet. This pattern of resistance is consistent with trends reported worldwide (Nagy *et al*., 2010, Snydman *et al*., 2010). Two strains, *B. fragilis* GSH8 and GSH15 were identified as Mtz resistant and strain GSH15 showed multidrug resistance to Mtz, Imp, Cef and Tet. However, as this analysis was performed using a small sample size, future work should include larger surveys encompassing more strains and more than one hospital. It should also be performed more regularly as these samples only represent sampling over a single short period of time and, therefore, do not give a complete view of the temporal increase in antibiotic resistance.

In order to determine the mechanisms behind the antibiotic resistance identified in the isolates, the genetic basis of the resistance was examined. It was found that most of the resistance phenotype could be attributed to the presence of known resistance genes. However, certain strains showed a deviation from this trend and were, therefore, of particular interest. These were two highly Mtz^R strains, which were found to be *nim* negative, as well as the presence and structure of the *cfiA* genes found in three isolates. Two of these three isolates carrying the

cfiA gene were identified as Imp^R, while one was found to be Imp^S even though it carried the resistance gene. Analysis of the upstream regions of the *cfiA* genes in each strain revealed that none of the strains had the C to T transition in the promoter region believed to switch on a silent gene and account for the increased *cfiA* resistance (Soki *et al*., 2004). However, the Imp^R strain did have a specific G to A substitution that the Imp^S strain did not. This specific substitution was also observed by Soki *et al*. (2004) in one of their Imp^R strains. It is, thus, possible that this change may be contributing to the *cfiA* resistance shown by these strains. Future studies could investigate the mechanisms behind the resistance present in the other isolates which were found not to carry any of the resistance genes screened for in this study. These were GSH14 which was Tet resistant but did not carry the *tetQ* gene, GSH9 which was Cef resistant but did not possess the *cfxA* gene as well as, GSH8 and GSH15 which were both Mtz initially resistant without the presence of the *nim* genes. This could be accomplished through whole genome sequencing and genome analysis software. This was done in a published study on two *nim*-negative, Mtz and multidrug resistant *B. fragilis* strains where the RAST annotation software was used. This software identified a gene coding for a pyridoxamine 5'-phosphate oxidase-related protein which was later identified as *nimJ* (Husain *et al*., 2013).

The loss of high levels of resistance in both Mtz^R strains in the course of this study caused difficulties in this research which initially aimed to investigate their molecular mechanisms. An investigation into why the phenotype was lost was, therefore, attempted. Both strains were shown to have inducible resistance to Mtz, however, this resistance was not stably maintained. Experiments were then performed to determine whether the residual resistance in GSH15 could be linked to the levels of enzymes thought to be involved in Mtz resistance. Both previously resistant isolates showed significantly higher LDH activity compared to *B. fragilis* 638R,

suggesting the involvement of the LDH/ PFOR pathway. However, there was no corresponding, detectable decrease in PFOR activity. Interestingly, GSH15, which still retained some residual Mtz resistance, had numerous alterations to its PFOR gene sequence even though there was no difference in PFOR activity compared to *B. fragilis* 638R. It is possible that these amino acid changes are affecting the interaction of Mtz with the metabolic pathway even though the enzyme itself is still completely functional. It may be that this subtle effect on Mtz is contributing the basal levels of Mtz resistance observed in GSH15.

B. fragilis GSH15 displayed multidrug resistance and residual low level Mtz resistance. It was, therefore, hypothesised that an efflux pump might be involved. Such a pump was reported in the literature as being transcribed at a higher level in a Mtz resistance clinical strain (Wexler, 2012), and this system was, therefore, investigated. qRT-PCR revealed that Mtz induction caused a slight increase in transcription of the efflux gene *bmeB5*. However, this was not statistically significant. In comparison, Pumbwe *et al*. (2006a) reported a 2.7-fold increase in *bmeB5* transcription in a Mtz^R strain. However, this Mtz^R strain did have a Mtz MIC of 64 mg/L which is much higher than the basal resistance observed in GSH15. It is possible that the exact conditions needed to increase gene transcription were not used in this study. Future work should investigate the possibility of using different induction concentrations and times. In order to confirm if these efflux pumps are causing the multidrug resistance observed in GSH15, future studies could also investigate whether induction with other antibiotics increases gene transcription since exposure to some antibiotics has been reported in the literature as causing the overexpression of multiple efflux genes (Pumbwe *et al*., 2006b). Another experiment that could be attempted would be to add efflux pump inhibitors and determine whether the MIC decreases for the other antibiotics tested here. It has been demonstrated that

resistance to numerous antimicrobial agents can be significantly reduced by the addition of efflux pump inhibitors (Pumbwe *et al*., 2006a).

The inducible nature of the Mtz resistance observed in *B. fragilis* 638R led to the hypothesis that the genome may contain a novel as yet, undescribed Mtz resistance gene. Analysis of the published genome identified the presence of a putative *nim*-like gene. Bioinformatic analysis of the derived amino acid sequence revealed that 638Rnim was structurally more closely related to NimA from *D. radiodurans* (Leiros *et al*., 2004) than to any of the other known Nims. Both 638Rnim and NimA are not amplified by the universal *nim* gene primers. The only other *nim* gene not to be amplified by these primers is *nimJ*, which has been shown to increase Mtz resistance (Husain *et al*., 2013). This suggests that the universal primers may not be identifying all nitroimidazole resistance genes present in all strains and, therefore, surveillance programs may be under reporting the presence of resistance genes present in isolates.

PCR screening of the clinical strains revealed that the gene encoding the 638Rnim protein was present in all of the clinical isolates investigated in this study except *B. fragilis* GSH6, while GSH7 and 8 contained at least an internal portion of it. Analysis of the gene upstream of *638Rnim* in these three strains revealed that GSH8 contained an 11 bp change in the forward primer binding site but otherwise contained an unaltered *638Rnim* gene homologue when compared to sequence data from 638R. In contrast, GSH6 appeared to have alterations to the gene that resulted in failure to amplify any part of it. GSH7, meanwhile, contained the internal portion of the gene but alterations to the upstream region of *638Rnim* resulted in failure to amplify product. It would be interesting to fully sequence this region of GSH6 and GSH7 in order to identify the changes that have occurred in it. In addition, studies could be performed to determine whether insertion of a promoter could cause increased transcription of the *638Rnim*

gene and whether this results in increased Mtz resistance. This phenomenon has been observed with the *cfiA* genes (Soki *et al*., 2004).

Heterologous expression of *638Rnim* in an *E. coli* expression system was used in an attempt to obtain soluble protein to use for a nitroreductase assay to prove that 638Rnim is a functional nitroreductase that uses Mtz as its substrate. However, after much optimization not enough soluble protein was ever produced in order to perform the assay. This could be due to protein expression in *E. coli* which is known to produce inclusion bodies and often fold proteins incorrectly (Middelberg, 2002). In the future, attempts should be made to express the protein using the *B. fragilis* IPTG-inducible his-tagged plasmid construct developed by Parker *et al*. (2012). Expression of the protein in its own host may result in correct folding and processing of the protein, producing enough soluble protein for use in the nitroreductase assay.

Heterologous overexpression of *638Rnim* in an *E. coli* Mtz^S mutant resulted in a 3-fold increase in Mtz resistance as compared to the control, giving strong evidence that the protein might function in this way. However, its possible role in Mtz resistance in *B. fragilis* could not be confirmed by overexpression or interruption of the gene as all experiments revealed no change in Mtz resistance compared to control strains. It may be that the gene is working in concert with other genes such as co-factors and thus overexpression of the gene alone may not reveal its true effect unless these were overexpressed as well. It is possible that the gene interruption mutant created in this study did not completely inactivate *638Rnim*, therefore, targeted deletion of the gene should be attempted using the protocol described by Patrick *et al*. (2009).

The possibility that transcription of *638Rnim* was enhanced by exposure to Mtz was investigated using qRT-PCR analysis of a sensitive (638R) and resistant (GSH15) *B. fragilis* strain under inducing conditions. qRT-PCR revealed that increased transcription of the gene approached statistically significant levels upon Mtz induction. However, as suggested earlier it is possible that the exact induction conditions required to optimally increase gene transcription were not met. This *nim*-like gene, therefore, warrants further investigation under a range of different induction conditions as well as a full comparative study comparing the transcription levels in a Mtz sensitive and fully clinically resistant strain. In contrast, it is also entirely possible that this *nim*-like gene may not be able to utilise Mtz as a substrate and instead may be a completely unrelated nitroreductase. It is possible that the *nim*-like gene may have a protective role in shielding sensitive molecules from low doses of Mtz by sequestering activated Mtz (Leitsch *et al*., 2014). However, this result would provide a completely different avenue for investigation as determining the reason behind the structural similarity of this alternative nitroreductase and the true *nims* would be interesting.

In February 2014, a study was published investigating the correlation between Nim protein levels and Mtz resistance (Leitsch *et al*., 2014). The authors determined that there was no link between Nim protein levels and exposure to increasing doses of Mtz and most interestingly they could not demonstrate nitroreductase activity of recombinantly expressed NimA *in vitro* (Leitsch *et al*., 2014). This suggests that the products of the *nim* genes may not be acting directly on Mtz as previously thought.

This thesis has provided valuable information about the antibiotic resistance profile of *B. fragilis* strains present in patients admitted to Groote Schuur Hospital. It also provides a basis for future work to be performed in order to provide more information about the state of

drug resistance in South Africa as a whole. Additionally this thesis has also identified a potential, novel *nim* gene encoding a protein that is structurally very similar to the NimA identified in *D. radiodurans*. Although, this could not be confirmed functionally, the studies reported in the thesis have provided a platform for more research to be conducted on this gene in order to elucidate its true function either as being involved in Mtz resistance or acting in an alternative metabolic capacity.

Appendix

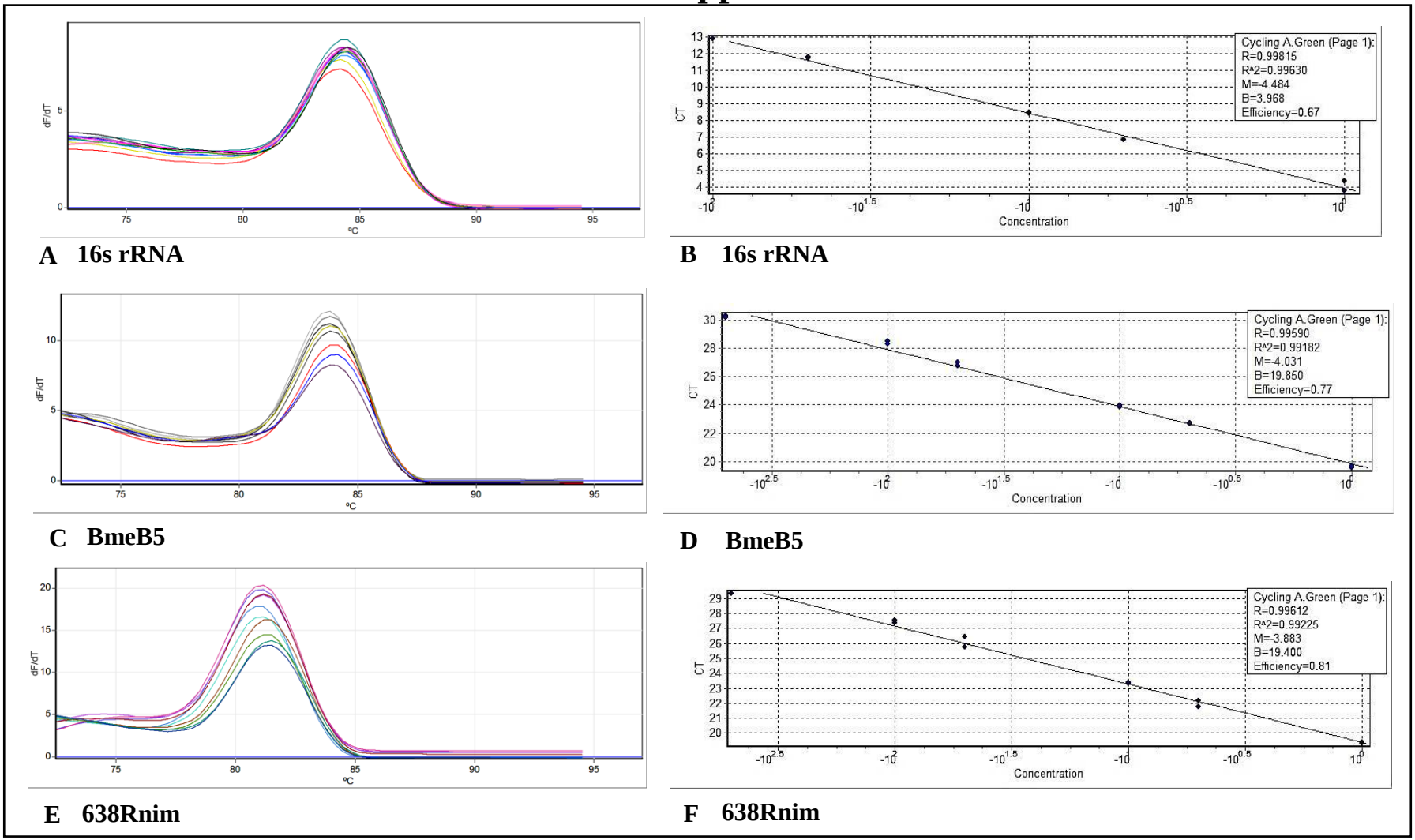


Figure S1: Representative melt and standard curves for *B. fragilis* 638R for each primer pair for one biological. Melt curves for A: 16S rRNA gene primers; C: *BmeB5* gene primers; E: *638Rnim* gene primers. Standard curves showing R^2 and efficiency values for B: 16S rRNA gene primers; D: *BmeB5* gene primers; F: *638Rnim* gene primers.

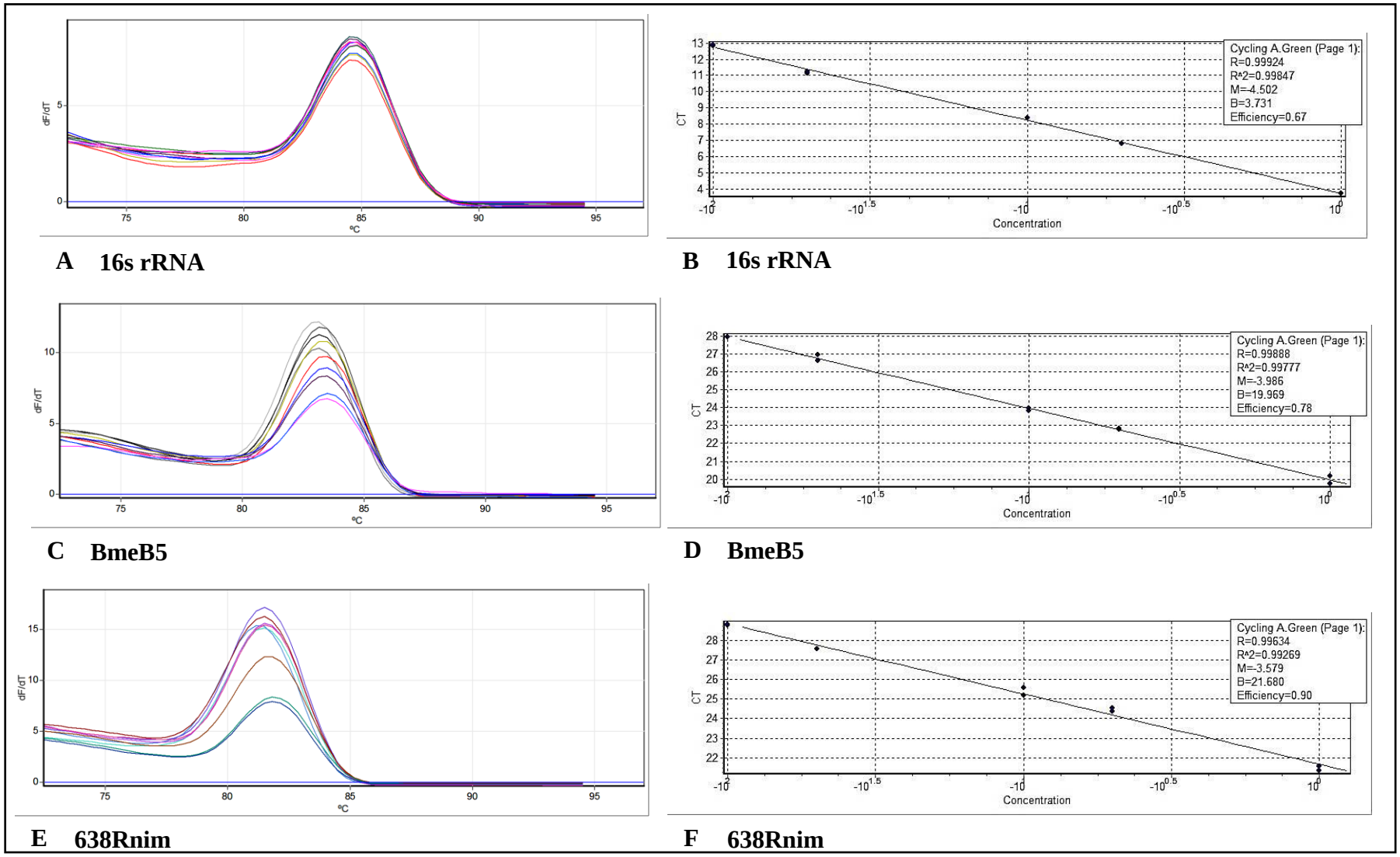


Figure S2: Representative melt and standard curves for *B. fragilis* GSH15 for each primer pair for one biological. Melt curves for A: 16S rRNA gene primers; C: *BmeB5* gene primers; E: *638Rnim* gene primers. Standard curves showing R^2 and efficiency values for B: 16S rRNA gene primers; D: *BmeB5* gene primers; F: *638Rnim* gene primers.

Reference List

ABRATT, V., LINDSAY, G. & WOODS, D. 1986. Pyrimidine dimer excision repair of DNA in *Bacteroides fragilis* wild-type and mitomycin C-sensitive/UV-sensitive mutants. *J Gen Microbiol,* 132**,** 2577-81.

ALAUZET, C., MORY, F., TEYSSIER, C., HALLAGE, H., CARLIER, J., GROLLIER, G. & LOZNIEWSKI, A. 2010. Metronidazole resistance in *Prevotella* spp. and description of a new *nim* gene in *Prevotella baroniae*. *Antimicrob Agents Chemother,* 54**,** 60-64.

ALEKSHUN, M. & LEVY, S. 2007. Molecular mechanisms of antibacterial multidrug resistance. *Cell,* 128**,** 1037-50.

ALTSCHUL, S., MADDEN, T., SCHAFFER, A., ZHANG, J., ZHANG, Z., MILLER, W. & LIPMAN, D. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res,* 25**,** 3389-402.

BARTHA, N., SOKI, J., URBAN, E. & NAGY, E. 2011. Investigation of the prevalence of *tetQ*, *tetX* and *tetX1* genes in *Bacteroides* strains with elevated tigecycline minimum inhibitory concentrations. *Int J Antimicrob Agents,* 38**,** 522-525.

BOENTE, R., FERREIRA, L., FALCAO, L., MIRANDA, K., GUIMARAES, P., SANTOS-FILHO, J., VIEIRA, J., BARROSO, D., EMOND, J., FERREIRA, E., PAULA, G. & DOMINGUES, R. 2010. Detection of resistance genes and susceptibility patterns in *Bacteroides* and *Parabacteroides* strains. *Anaerobe,* 16**,** 190-194.

BONHEYO, G., HUND, B. D., SHOEMAKER, N. & SALYERS, A. 2001. Transfer region of a *Bacteroides* conjugative transposon contains regulatory as well as structural genes. *Plasmid,* 46**,** 202-9.

BRITZ, M. & WILKINSON, R. 1979. Isolation and properties of metronidazole-resistant mutants of *Bacteroides fragilis*. *Antimicrob Agents Chemother,* 16**,** 19-27.

BUSTIN, S. A., BENES, V., GARSON, J. A., HELLEMANS, J., HUGGETT, J., KUBISTA, M., MUELLER, R., NOLAN, T., PFAFFL, M. W., SHIPLEY, G. L., VANDESOMPELE, J. & WITTWER, C. T. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem,* 55**,** 611-22.

CAMPBELL, L. & YASBIN, R. 1984. Mutagenesis of *Neisseria gonorrhoeae*: abscence of error-prone repair. *J Bacteriol,* 160**,** 288-93.

CARLIER, J., SELLIER, N., RAGER, M. & REYSSET, G. 1997. Metabolism of a 5-nitroimidazole in susceptible and resistant isogenic strains of *Bacteroides fragilis*. *Antimicrob Agents Chemother,* 41**,** 1495-1499.

CARRIO, M. & VILLAVERDE, A. 2001. Protein aggregation as bacterial inclusion bodies is reversible. *FEMS Microbiol Lett,* 489**,** 29-33.

CERDENO-TARRAGA, A., PATRICK, S., CROSSMAN, L., BLAKELY, G., ABRATT, V., LENNARD, N., POXTON, I., DUERDEN, B., HARRIS, B., QUAIL, M., BARRON, A., CLARK, L., CORTON, C., DOGGETT, J., HOLDEN, M., LARKE, N., LINE, A., LORD, A., NORBERTCZAK, H., ORMOND, D., PRICE, C., RABBINOWITSCH, E., WOODWARD, J., BARRELL, B. & PARKHILL, J. 2005. Extensive DNA inversions in the *B. fragilis* genome control variable gene expression. *Science,* 307**,** 1463-1465.

CHAN, K., LEE, K., MOU, J., CHEUNG, S., SIHOE, J. & TAM, Y. 2010. Evidence-based adjustment of antibiotic in pediatric complicated appendicitis in the era of antibiotic resistance. *Pediatr Surg Int,* 26**,** 157-160.

CHATZIDAKI-LIVANIS, M., WEINACHT, K. & COMSTOCK, L. 2010. Trans locus inhibitors limit concomitant polysaccharide synthesis in the human gut symbiont *Bacteroides fragilis*. *Proc Natl Acad Sci USA,* 107**,** 11976-80.

CHENEBY, D., PHILIPPOT, L., HARTMANN, A., HENAULT, C. & GERMON, J. 2000. 16S rDNA analysis for characterization of denitrifying bacteria isolated from three agricultural soils. *FEMS Microbiol Ecol,* 34**,** 121-8.

CHENG, Q., PASZKIET, B., SHOEMAKER, N., GARDNER, J. & SALYERS, A. 2000. Integration and excision of a *Bacteroides* conjugative transposon, CTnDOT. *J Bacteriol,* 182**,** 4035-43.

CHURCH, D. & LAISHLEY, E. 1995. Reduction of metronidazole by hydrogenase from *Clostridia*. *Anaerobe,* 1**,** 81-92.

DAGERT, M. & EHRLICH, S. 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene,* 6**,** 23-8.

DINIZ, C., FARIAS, L., CARVALHO, M., ROCHA, E. & SMITH, C. 2004. Differential gene expression in a *Bacteroides fragilis* metronidazole-resistant mutant. *J Antimicrob Chemother,* 54**,** 100-108.

DINIZ, C., SANTOS, S., PESTANA, A., FARIAS, L. & CARVALHO, M. 2000. Chromosomal breakage in the *B. fragilis* group induced by metronidazole treatment. *Anaerobe,* 6**,** 149-53.

EDWARDS, D. 1980. Mechanisms of selective toxicity of metronidazole and other nitroimidazole drugs. *Br J Vener Dis,* 56**,** 285-290.
EDWARDS, D. 1993. Nitroimidazole drugs-action and resistance mechanisms. I. Mechanisms of action. *J Antimicrob Chemother,* 31**,** 9-20.
EITEL, Z., SOKI, J., URBAN, E. & NAGY, E. 2013. The prevalence of antibiotic resistance genes in *Bacteroides fragilis* group strains isolated in different European countries. *Anaerobe,* 21**,** 43-49.
FANG, H., EDLUND, C., HEDBERG, M. & NORD, C. 2002. New findings in beta-lactam and metronidazole resistant *Bacteroides fragilis* group. *J Antimicrob Chemother,* 19**,** 361-370.
GAL, M. & BRAZIER, J. 2004. Metronidazole resistance in *Bacteroides* spp. carrying *nim* genes and the selection of slow-growing metronidazole-resistant mutants. *J Antimicrob Chemother,* 54**,** 109-116.
GALVAO, B., MEGGERSEE, R. & ABRATT, V. 2011. Antibiotic resistance and adhesion potential of *Bacteroides fragilis* clinical isolates from Cape Town, South Africa. *Anaerobe,* 17**,** 142-146.
GARCIA, N., GUTIERREZ, G., LORENZO, M., GARCIA, J., PIRIZ, S. & QUESADA, A. 2008. Genetic determinants for *cfxA* expression in *Bacteroides* strains isolated from human infections. *J Antimicrob Chemother,* 62**,** 942-947.
GOODMAN, H., PARKER, J., SOUTHERN, J. & WOODS, D. 1987. Cloning and expression in *Escherichia coli* of a *recA*-like gene from *Bacteroides fragilis*. *Gene,* 58**,** 265-71.
GOODWIN, A., KERSULYTE, D., SISSON, G., VAN ZANTEN, S., BERG, D. & HOFFMAN, P. 1998. Metronidazole resistance in *Helicobacter pylori* is due to null mutations in a gene (*rdxA*) that encodes an oxygen-insensitive NADPH nitroreductase. *Mol Microbiol,* 28**,** 383-93.
GUPTA, A., VLAMAKIS, H., SHOEMAKER, N. & SALYERS, A. 2003. A new *Bacteroides* conjugative transposon that carries an *ermB* gene. *Appl Environ Microbiol,* 69**,** 6455-63.
HAGGOUD, A., REYSSET, G., AZEDDOUG, H. & SEBALD, M. 1994. Nucleotide sequence analysis of two 5-nitroimidazole resistance determinants from *Bacteroides* strains and of a new insertion sequence upstream of the two genes. *Antimicrob Agents Chemother,* 38**,** 1047-1051.
HAWSER, S., HACKEL, M. & HOBAN, D. 2010. Antibiotic susceptibility profiles of European *Bacteroides fragilis* with reduced carbapenem susceptibility. *J Antimicrob Chemother,* 65**,** 803-804.
HECHT, D. 2006. Anaerobes: antibiotic resistance, clinical significance, and the role of susceptibility testing. *Anaerobe,* 12.
HEDBERG, M. & NORD, C. 2002. Antimicrobial susceptibility of *Bacteroides fragilis* group isolates in Europe. *Clin Infect Dis,* 9**,** 475-488.
HOLDEMAN, L. & MOORE, W. 1972. Anaerobe laboratory manual. 4th Ed. Virginia polytechnic institute and state university anaerobe laboratory, Blacksburg, VA.
HOLM, L., KAARIAINEN, S., ROSENSTROM, P. & SCHENKEL, A. 2008. Searching protein structure databases with DaliLite v. 3. *Bioinformatics,* 24**,** 2780-1.
HOLM, L., KAARIAINEN, S., WILTON, C. & PLEWCZYNSKI, D. 2006. Using Dali for structural comparison of proteins. *Curr Protoc Bioinformatics,* Chapter 5**,** Unit 5.5.
HOOPER, L., MIDTVEDT, T. & GORDON, J. 2002. How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu Rev Nutr,* 22**,** 283-307.
HOPKINS, M. & MACFARLANE, G. 2003. Nondigestible oligosaccharides enhance bacterial colonization resistance against *Clostridium difficile in vitro*. *Appl Environ Microbiol,* 69**,** 1920-7.
HOWARD-FLANDERS, P. & THERIOT, L. 1966. Mutants of *Escherichia coli* K-12 defective in DNA repair and in genetic recombination. *Genetics,* 53**,** 1137-50.
HUSAIN, F., VEERANAGOUDA, Y., HSI, J., MEGGERSEE, R., ABRATT, V. & WEXLER, H. 2013. Two multidrug-resistant clinical isolates of *Bacteroides fragilis* carry a novel metronidazole resistance *nim* gene (*nimJ*). *Antimicrob Agents Chemother,* 57**,** 3767-74.
JENKINS, S. 2001. Infections due to anaerobic bacteria and the role of antimicrobial susceptibility testing of anaerobes. *Rev Med Microbiol,* 12**,** 1-12.
KAIHOVAARA, P., HOOK-NIKANNE, J., UUSI-OUKARI, M., KOSUNEN, T. & SALASPURO, M. 1998. Flavodoxin-dependent pyruvate oxidation, acetate production and metronidazole reduction by *Helicobacter pylori*. *J Antimicrob Chemother,* 41**,** 171-7.
KELLEY, L. & STERNBERG, M. 2009. Protein structure prediction on the Web: a case study using the Phyre server. *Nat Protoc,* 4**,** 363-71.
KOCH, C., DERBY, P. & ABRATT, V. 1998. In-vitro antibiotic susceptibility and molecular analysis of anaerobic bacteria isolated in Cape Town, South Africa. *J Antimicrob Chemother,* 42**,** 245-248.
KUWAHARA, T., YAMASHITA, A., HIRAKAWA, H., NAKAYAMA, H., TOH, H., OKADA, N., KUHARA, S., HATTORI, M., HAYASHI, T. & OHNISHI, Y. 2004. Genomic analysis of *Bacteroides fragilis* reveals extensive DNA inversions regulating cell surface adaptation. *Proc Natl Acad Sci U S A,* 101**,** 14919-14924.

KUZMINOV, A. 1999. Recombinational repair of DNA damage in *Escherichia coli* and bacteriophage lambda. *Microbiol Mol Biol Rev,* 63**,** 751-813.
LAND, K. & JOHNSON, P. 1997. Molecular mechanisms underlying metronidazole resistance in Trichomonads. *Exp Parasitol,* 87**,** 305-308.
LEIROS, H., KOZIELSKI-STUHRMANN, S., KAPP, U., TERRADOT, L., LEONARD, G. & MCSWEENEY, S. 2004. Structural basis of 5-nitroimidazole antibiotic resistance: the crystal structure of *NimA* from *Deinococcus radiodurans*. *J Biol Chem,* 279**,** 55840-55849.
LEIROS, H., TEDESCO, C. & MCSWEENEY, S. 2008. High-resolution structure of the antibiotic resistance protein *NimA* from *Deinococcus radiodurans*. *Acta Crystallogr Sect F Struct Biol Cryst Commun,* 64**,** 442-447.
LEITSCH, D., BURGESS, A., DUNN, L. A., KRAUER, K., TAN, K., DUCHENE, M., UPCROFT, P., ECKMANN, L. & UPCROFT, J. 2011. Pyruvate:ferredoxin oxidoreductase and thioredoxin reductase are involved in 5-nitroimidazole activation while flavin metabolism is linked to 5-nitroimidazole resistance in *Giardia lamblia*. *J Antimicrob Chemother,* 66**,** 1756-65.
LEITSCH, D., SOKI, J., KOLARICH, D., URBAN, E. & NAGY, E. 2014. A study on Nim expression in *Bacteroides fragilis*. *Microbiology,* (In press)**,** http://dx.doi.org/10.1099/mic.0.074807-0.
LI, L., SHOEMAKER, N. & SALYERS, A. 1995. Location and characteristics of the transfer region of a *Bacteroides* Conjugative transposon and regulation of transfer genes. *J Bacteriol,* 177**,** 4992-4999.
LINDMARK, D. & MULLER, M. 1973. Hydrogenosome, a cytoplasmic organelle of the anaerobic flagellate *Tritrichomonas foetus,* and its role in pyruvate metabolism. *J Biol Chem,* 248**,** 7726-7728.
LIVERMORE, D. 2005. Tigecycline: what is it, and where should it be used? *J Antimicrob Chemother,* 56**,** 611-614.
LOFMARK, S., EDLUND, C. & NORD, C. 2010. Metronidazole is still the drug of choice for treatment of anaerobic infections. *Clin Infect Dis,* 50 Suppl 1**,** 16-23.
LOFMARK, S., FANG, H., HEDBERG, M. & EDLUND, C. 2005. Inducible metronidazole resistance and *nim* genes in clinical *Bacteroides fragilis* group isolates. *Antimicrob Agents Chemother,* 49**,** 1253-1256.
MANIATIS, T., FRITSCH, E. & SAMBROOK, J. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbour, N.Y.
MAZMANIAN, S. & KASPER, D. 2006. The love-hate relationship between bacterial polysaccharides and the host immune system. *Nat Rev Immunol,* 6**,** 849-58.
MEYER, L., BRINK, D. & WELDHAGEN, G. 2006. Antibiotic susceptibility patterns of anaerobic bacteria isolated in Pretoria, South Africa, during 2003-2004. *South Afr J Epidemiol Infect,* 23**,** 161-163.
MIDDELBERG, A. 2002. Preparative protein refolding. *Trends Biotechnol,* 20**,** 437-43.
MOON, J., HERR, Y., KIM, S. & LEE, J. 2011. In vitro activity of deferoxamine against *Porphyromonas gingivalis*. *FEMS Microbiol Lett,* 323**,** 61-67.
MULLER, M. 1983. mode of action of metronidazole on anaerobic bacteria and protozoa. *Surgery,* 93**,** 165-71.
MULLER, M. & LINDMARK, D. 1976. Uptake of metronidazole and its effect on viability in Trichomonda and *Entamoeba invadens* under anaerobic and aerobic conditions. *Antimicrob Agents Chemother,* 9**,** 696-700.
NAGY, E., DANYI, E. & FOLDES, J. 1990. Plasmid analysis of clinical isolates of *Bacteroides fragilis* group strains. *Acta Microbiol Hung,* 37**,** 367-73.
NAGY, E., URBAN, E. & NORD, C. 2010. Antimicrobial susceptibility of *Bacteroides fragilis* group isolates in Europe; twenty years' experience. *Clinical Microbiology and Infection,* 17**,** 371-379.
NARIKAWA, S., SUZUKI, T., YAMAMOTO, M. & NAKAMURA, M. 1991. Lactate dehydrogenase activity as a cause of metronidazole resistance in *Bacteroides fragilis* NCTC 11295. *J Antimicrob Chemother,* 28**,** 47-53.
NICHOLSON, S. 2012. Molecular characterisation of the *recA* locus in the opportunistic pathogen *Bacteroides fragilis*. *Unpublished PhD thesis,* University of Cape Town.
OLEKHNOVICH, I., GOODWIN, A. & HOFFMAN, P. 2009. Characterization of the NAD(P)H oxidase and metronidazole reductase activities of the RdxA nitroreductase of *Helicobacter pylori*. *FEBS J,* 276**,** 3354-3364.
PAL, S. 2006. A journey across the sequential development of macrolides and ketolides related to erythromycin. *Tetrahedron,* 62**,** 3171-3200.
PARKER, A. & SMITH, J. 2012. Development of an IPTG inducible expression vector adapted for *Bacteroides fragilis*. *Plasmid,* 68**,** 86-92.
PATEL, E., PAUL, L., CASANUEVA, A., PATRICK, S. & ABRATT, V. 2009. Overexpression of the rhamnose catabolism regulatory protein, RhaR: a novel mechanism for metronidazole resistance in *Bacteroides thetaiotaomicron*. *J Antimicrob Chemother,* 64**,** 267-273.
PATRICK, S., BLAKELY, G., HOUSTON, S., MOORE, J., ABRATT, V., BERTALAN, M., CERDENO-TARRAGA, A., QUAIL, M., CORTON, N., CORTON, C., BIGNELL, A., BARRON, A., CLARK, L., BENTLEY, S. & PARKHILL, J. 2010. Twenty-eight divergent polysaccharide loci specifying within-

and amongst-strain capsule diversity in three strains of *Bacteroides fragilis*. *Microbiology,* 156**,** 3255-3269.
PATRICK, S., HOUSTON, S., THACKER, Z. & BLAKELY, G. 2009. Mutational analysis of genes implicated in LPS and capsular polysaccharide biosynthesis in the opportunistic pathogen *Bacteroides fragilis*. *Microbiology,* 155**,** 1039-49.
PAUL, L., PATRICK, S., NORD, C. & ABRATT, V. 2011. The role of *Bacteroides fragilis* RecQ DNA helicases in cell survival after metronidazole exposure. *FEMS Microbiol Lett,* 319**,** 125-32.
PELAEZ, T., CERCENADO, E., ALCALA, L., MARIN, M., MARTIN-LOPEZ, A., MARTINEZ-ALARCON, J., CATALAN, P., SANCHEZ-SOMOLINOS, M. & BOUZA, E. 2008. Metronidazole resistance in *Clostridium difficile* is heterogeneous. *J Clin Microbiol,* 46**,** 3028-3032.
PFAFFL, M. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res,* 29**,** 2002-7.
PODGLAJEN, I., BREUIL, J., BORDON, F., GUTMANN, L. & COLLATZ, E. 1992. A silent carbapenemase gene in strains of *Bacteroides fragilis* can be expressed after a one-step mutation. *FEMS Microbiol Lett,* 70**,** 21-9.
PODGLAJEN, I., BREUIL, J. & COLLATZ, E. 1994. Insertion of a novel DNA sequence, 1S1186, upstream of the silent carbapenemase gene *cfiA*, promotes expression of carbapenem resistance in clinical isolates of *Bacteroides fragilis*. *Mol Microbiol,* 12**,** 105-14.
PODGLAJEN, I., BREUIL, J. & COLLATZ, E. 1995. Genotypic identification of two groups within the species *Bacteroides fragilis* by ribotyping and by analysis of PCR-generated fragment patterns and insertion sequence content. *J Bacteriol,* 177**,** 5270-5.
PODGLAJEN, I., BREUIL, J., ROHAUT, A., MONSEMPES, C. & COLLATZ, E. 2001. Multiple mobile promoter regions for the rare carbapenem resistance gene of *Bacteroides fragilis*. *J Bacteriol,* 183**,** 3531-5.
PRIVITERA, G., DUBLANCHET, A. & SEBALD, M. 1979. Transfer of multiple antibiotic resistances between species of *Bacteroides fragilis*. *J Infect Dis,* 139**,** 97-101.
PUMBWE, L., CHANG, A., SMITH, R. & WEXLER, H. 2006a. Clinical significance of overexpression of multiple RND-family efflux pumps in *Bacteroides fragilis* isolates. *J Antimicrob Chemother,* 58**,** 543-548.
PUMBWE, L., CHANG, A., SMITH, R. & WEXLER, H. 2007a. BmeRABC5 is a multidrug efflux system that can confer metronidazole resistance in *Bacteroides fragilis*. *Microb Drug Resist,* 13**,** 96-101.
PUMBWE, L., GLASS, D. & WEXLER, H. 2006b. Efflux pump overexpression in multiple-antibiotic-resistant mutants of *Bacteroides fragilis*. *Antimicrob Agents Chemother,* 50**,** 3150-3153.
PUMBWE, L., WAREHAM, D., ADUSE-OPOKU, J., BRAZIER, J. & WEXLER, H. 2007b. Genetic analysis of mechanisms of multidrug resistance in a clincal isolate of *Bacteroides fragilis*. *Clin Microbiol Infect,* 13**,** 183-189.
RASMUSSEN, B., BUSH, K. & TALLY, F. 1993. Antimicrobial resistance in *Bacteroides*. *Clin Infect Dis,* 16 Suppl 4**,** S390-400.
RASMUSSEN, B., KEENEY, D., YANG, Y. & BUSH, K. 1994. Cloning and expression of a cloxacillin-hydrolyzing enzyme and a cephalosporinase from *Aeromonas sobria* AER 14M in *Escherichia coli*: requirement for an *E. coli* chromosomal mutation for efficient expression of the class D enzyme. *Antimicrob Agents Chemother,* 38**,** 2078-85.
REID, J. & PATRICK, S. 1984. Phagocytic and serum killing of capsulate and non-capsulate *Bacteroides fragilis*. *J Med Microbiol,* 17**,** 247-57.
REUTER, M., PARRY, F., DRYDEN, D. & BLAKELY, G. 2010. Single-molecule imaging of Bacteroides fragilis AddAB reveals the highly processive translocation of a single motor helicase. *Nucleic Acids Res,* 38**,** 3721-3731.
REYSSET, G. 1996. Genetics of 5-nitroimidazole resistance in *Bacteroides* species. *Anaerobe,* 2**,** 59-69.
SALYERS, A. & AMABILE-CUEVAS, C. 1997. Why are antibiotic resistance genes so resistant to elimination? *Antimicrob Agents Chemother,* 41**,** 2321-5.
SALYERS, A., GUPTA, A. & WANG, Y. 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends Microbiol,* 12**,** 412-6.
SALYERS, A., SHOEMAKER, N., STEVENS, A. & LI, L. 1995. Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. *Microbiol Rev,* 59**,** 579-90.
SALYERS, A., SPEER, B. & SHOEMAKER, N. 1990. A new perspective in tetracycline resistance. *Mol Microbiol,* 4**,** 151-6.
SAMBROOK, J., FRITSCH, E. & MANIATIS, T. 2001. Molecular cloning: a laboratory manual. Cold Spring Harbour, N. Y.
SCHAUER, R. 2004. Sialic acids: fascinating sugars in higher animals and man. *Zoology,* 107.
SEARS, C. 2009. Enterotoxigenic *Bacteroides fragilis*: a rogue among symbiotes. *Clin Microbiol Rev,* 22**,** 349-69.

SELINGER, D., SAXENA, R., CHEUNG, K., CHURCH, G. & ROSENOW, C. 2003. Global RNA half-life analysis in *Escherichia coli* reveals positional patterns of transcript degradation. *Genome Res,* 13**,** 216-23.

SHINN, D. 1962. Metronidazole in acute ulcerative gingivitis. *Lancet,* 279**,** 1191.

SHKOPOROV, A., KHOKHLOVA, E., KULAGINA, E., SMEIANOV, V., KUCHMIY, A., KAFARSKAYA, L. & EFIMOV, B. 2013. Analysis of a novel 8.9kb cryptic plasmid from *Bacteroides uniformis*, its long-term stability and spread within human microbiota. *Plasmid,* 69**,** 146-159.

SHOEMAKER, N., GUTHRIE, E., SALYERS, A. & GARDNER, J. 1985. Evidence that the Clindamycin-Erythromycin resistance gene of *Bacteroides* plasmid pBF4 Is on a transposable element. *J Bacteriol,* 162**,** 626-632.

SHOEMAKER, N., VLAMAKIS, H., HAYES, K. & SALYERS, A. 2001. Evidence for extensive resistance gene transfer among *Bacteroides* spp. and among *Bacteroides* and other genera in the human colon. *Appl Environ Microbiol,* 67**,** 561-8.

SIGETI, J., GUINEY, D. & DAVIS, C. 1983. Mechanism of action of metronidazole on *Bacteroides fragilis*. *J Infect Dis,* 148**,** 1083-9.

SIMON, R., PRIEFER, U. & PUHLER, A. 1983. A broad host range mobilization system for *in vitro* genetic engineering: transposon mutagenesis in gram negative bacteria *J Bacteriol,* 1**,** 784-91.

SINDAR, P., BRITZ, M. & WILKINSON, R. 1982. Isolation and properties of metronidazole-resistant mutants of *Clostridium perfringens*. *J Med Microbiol,* 15**,** 503-9.

SISSON, G., JEONG, J., GOODWIN, A., BRYDEN, L., ROSSIER, N., LIM-MORRISON, S., RAUDONIKIENE, A., BERG, D. & HOFFMAN, P. 2000. Metronidazole activation is mutagenic and causes DNA fragmentation in *Helicobacter pylori* and in *Escherichia coli* containing a cloned *H. pylori rdxA*$^{+}$ (Nitroreductase) gene. *J Bacteriol,* 182**,** 5091-6.

SMITH, C., TRIBBLE, G. & BAYLEY, D. 1998. Genetic elements of *Bacteroides* species: a moving story. *Plasmid,* 40.

SNYDMAN, D., JACOBUS, N., MCDERMOTT, L., GOLAN, Y., HECHT, D., GOLDSTEIN, E., HARRELL, L., JENKINS, S., NEWTON, D., PIERSON, C., RIHS, J., YU, V., VENEZIA, R., FINEGOLD, S., ROSENBLATT, J. & GORBACH, S. 2010. Lessons learned from the anaerobe survey: historical perspective and review of the most recent data (2005-2007). *Clin Infect Dis,* 50 Suppl 1**,** S26-33.

SOKI, J. 2013. Extended role for insertion sequence elements in the antibiotic resistance of *Bacteroides*. *World J Clin Infect Dis,* 3**,** 1-12.

SOKI, J., EDWARDS, R., HEDBERG, M., FANG, H., NAGY, E. & NORD, C. 2006. Examination of *cfiA*-mediated carbapenem resistance in *Bacteroides fragilis* strains from a European antibiotic susceptibility survey. *Int J Antimicrob Agents,* 28**,** 497-502.

SOKI, J., FODOR, E., HECHT, D., EDWARDS, R., ROTIMI, V., KEREKES, I., URBAN, E. & NAGY, E. 2004. Molecular characterization of imipenem-resistant, *cfiA*-positive *Bacteroides fragilis* isolates from the USA, Hungary and Kuwait. *J Med Microbiol,* 53**,** 413-419.

SOKI, J., GONZALEZ, S., URBAN, E., NAGY, E. & AYALA, J. 2011. Molecular analysis of the effector mechanisms of *cefoxitin* resistance among Bacteroides strains. *J Antimicrob Chemother,* 66**,** 2492-2500.

SOKI, J., SZOKE, I. & NAGY, E. 1999. Characterisation of a 5.5-kb cryptic plasmid present in different isolates of *Bacteroides* spp. originating from Hungary. *J Med Microbiol,* 48**,** 25-31.

SPEER, B., SHOEMAKER, N. & SALYERS, A. 1992. Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. *Clin Microbiol Rev,* 5**,** 387-399.

STEFFENS, L., NICHOLSON, S., PAUL, L., NORD, C., PATRICK, S. & ABRATT, V. 2010. *Bacteroides fragilis* RecA protein overexpression causes resistance to metronidazole. *Res Microbiol,* 161**,** 346-354.

STUBBS, S., BRAZIER, J., TALBOT, P. & DUERDEN, B. 2000. PCR-restriction fragment length polymorphism analysis for identification of *Bacteroides* spp. and characterization of nitroimidazole resistance genes. *J Clin Microbiol,* 38**,** 3209-3213.

STUDIER, F. & MOFFATT, B. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol,* 5**,** 113-30.

TALLY, F., SUTTER, V. & FINEGOLD, S. 1972. Metronidazole versus anaerobes. *In vitro* data and initial clinical observations. *Calif Med,* 117**,** 22-6.

TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M. & KUMAR, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol,* 28**,** 2731-9.

TANG, Y. & MALAMY, M. 2000. Isolation of *Bacteroides fragilis* mutants with *In Vivo* growth defects by using Tn4400', a Modified Tn4400 transposition system, and a new screening method. *Infect Immun,* 68**,** 415-419.

THOMPSON, S. & BLASER, M. 1995. Isolation of the *Helicobacter pylori recA* gene and involvement of the *recA* region in resistance to low pH. *Infect Immun,* 63**,** 2185-93.

TRAN, C., TANAKA, K. & WATANABE, K. 2013. PCR-based detection of resistance genes in anaerobic bacteria isolated from intra-abdominal infections. *J Infect Chemother,* 19**,** 279-290.
TRINH, S., HAGGOUD, A., REYSSET, G. & SEBALD, M. 1995. Plasmids plP419 and plP421 from *Bacteroides*: 5-nitroimidazole resistance genes and their upstream insertion sequence elements. *Microbiology,* 141**,** 927-935.
TRINH, S. & REYSSET, G. 1995. Detection by PCR of the *nim* genes encoding 5-nitroimidazole resistance in *Bacteroides* spp. *J Clin Microbiol,* 34**,** 2078-2084.
TRINH, S. & REYSSET, G. 1998. Mutagenic action of 5-nitroimidazoles: in vivo induction of GC-CG transversion in two *Bacteroides fragilis* reporter genes. *Mutat Res,* 398**,** 55-65.
UEDA, O., WEXLER, H., HIRAI, K., SHIBATA, Y., YOSHIMURA, F. & FUJIMURA, S. 2005. Sixteen homologs of the mex-type multidrug resistance efflux pump in *Bacteroides fragilis*. *Antimicrob Agents Chemother,* 49**,** 2807-2815.
UPCROFT, J., DUNN, L. A., WRIGHT, J., BENAKLI, K., UPCROFT, P. & VANELLE, P. 2006. 5-Nitroimidazole drugs effective against metronidazole-resistant *Trichomonas vaginalis* and *Giardia duodenalis*. *Antimicrob Agents Chemother,* 50**,** 344-7.
WANG, Y., ROTMAN, E., SHOEMAKER, N. & SALYERS, A. 2005. Translational control of tetracycline resistance and conjugation in the *Bacteroides* conjugative transposon CTnDOT. *J Bacteriol,* 187**,** 2673-80.
WANG, Y., WANG, G. R., SHELBY, A., SHOEMAKER, N. B. & SALYERS, A. A. 2003. A newly discovered *Bacteroides* conjugative transposon, CTnGERM1, contains genes also found in Gram-positive bacteria. *Appl Environ Microbiol,* 69**,** 4595-603.
WATERS, J. & SALYERS, A. 2013. Regulation of CTnDOT conjugative transfer is a complex and highly coordinated series of events. *MBio,* 4**,** e00569-13.
WEXLER, H. 2007. *Bacteroides*: the good, the bad, and the nitty-gritty. *Clin Microbiol Rev,* 20**,** 593-621.
WEXLER, H. 2012. Pump it up: occurrence and regulation of multi-drug efflux pumps in *Bacteroides fragilis*. *Anaerobe,* 18**,** 200-208.
WHITTLE, G., HUND, B., SHOEMAKER, N. & SALYERS, A. 2001. Characterization of the 13-kilobase *ermF* region of the *Bacteroides* conjugative transposon CTnDOT. *Appl Environ Microbiol,* 67**,** 3488-95.
XU, J., CHIANG, H., BJURSELL, M. & GORDON, J. 2004. Message from a human gut symbiont: sensitivity is a prerequisite for sharing. *Trends Microbiol,* 12**,** 21-8.
XU, J. & GORDON, J. 2003. Honor thy symbionts. *Proc Natl Acad Sci U S A,* 100**,** 10452-9.
YANG, W., MOORE, I., KOTEVA, K., BAREICH, D., HUGHES, D. & WRIGHT, G. 2004. TetX is a flavin-dependent monooxygenase conferring resistance to tetracycline antibiotics. *J Biol Chem,* 279**,** 52346-52.
YANISCH-PERRON, C., VIEIRA, J. & MESSING, J. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene,* 33**,** 103-19.