MOLECULAR CHARACTERIZATION OF ABC-TYPE MULTIDRUG EFFLUX SYSTEMS IN *Bifidobacterium longum* subsp. *longum*^{T} JCM 1217

by

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Assoc. Prof. V. R. Abratt

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List of abbreviations used:

°C	degree(s) Celsius
aa	amino acid
ABC	ATP-binding cassette
Amp	ampicillin
ATCC	American Type Culture Collection
ATP	adenosine 5´-triphosphate
bp	base pair(s)
BSA	bovine serum albumin
cDNA	complementary DNA
CSPD	disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro) tricycle decan]-4-yl) phenyl phosphate
D-	dextrorotatory
d-	distilled
DIG	digoxigenin-11- (2'-deoxyuridine 5'-triphosphate)
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	adenosine, cytosine, guanosine and
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
et al.	*et alia*
g	gram(s)
G+C	guanine and cytosine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HTH	helix-turn-helix
IPTG	isopropyl β-D-1-thiogalactopyranoside
kb	kilobase(s)

KD	Kyte-Doolittle Scale
kDa	kilodalton(s)
L-	levorotatory
M	molar
mg	milligram(s)
MIC	minimum inhibitory concentration
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
pmol	picomole(s)
qRT-PCR	quantitative real time PCR
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse transcriptase PCR
s	second(s)
SDS	sodium dodecyl sulfate
spp.	species
TMD	transmembrane domain
U	unit(s)
v/v	volume per volume
w/v	weight per volume
wHTH	winged helix-turn-helix
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
µF	microfarad(s)
µg	microgram(s)
µl	microlitre(s)
µM	micromolar
V	

CONTENTS

Abstract

A healthy and stable gastrointestinal microbiota is a vital feature of the innate immune system. It affords the host numerous health benefits and acts as a barrier against opportunistic gut infections. Probiotic bacterial supplements are, therefore, widely used in industry to promote good health. There is, however, a need to understand not only the factors underlying the health promoting capabilities of these bacteria, but also the intrinsic antimicrobial resistance mechanisms which these bacteria are known to harbour. These antibiotic resistance traits confer a competitive advantage on these bacteria over other bacterial species where they reside in the gut. It also allows them to survive during antibiotic therapy and they are able to continue conferring health benefits on the host.

To better understand the mechanisms these bacteria utilize in conferring antibiotic resistance, genes which confer multidrug resistance by the active hydrolysis of ATP were studied here. These genes belong to the ATP-binding cassette (ABC) family of efflux transporters. ABC-type transporter genes in other bacteria have previously been shown to confer multidrug resistance to numerous antimicrobial substances. Bioinformatic analysis of the genome of *Bifidobacterium longum* subsp. *longum*^{T} JCM 1217 highlighted the presence of two gene clusters containing two ABC-type transporter genes each, as well as, a putative transcriptional regulator upstream of the structural genes. Reverse transcriptase analysis of these two gene clusters indicated they were arranged in two operons, one comprising three genes, inclusive of the putative regulator and the other comprising two ABC-type transporter genes, excluding the adjacent putative regulator. Cloning and heterologous expression of these genes showed that they were able to confer a multidrug resistance phenotype on the host *Lactococcus lactis* cells. An increase in the minimum inhibitory concentration of between 1.5 – 3 folds to both erythromycin and tetracycline, both of which are known substrates of ABC-type transporters, was observed. Efflux assays, using the substrate Hoechst 33342, gave an indication of the ATP-dependence of these transporters, but, due to the high background efflux activity of the host cells, no definitive conclusions could be made.

Experiments, using multiplex Real Time PCR, were conducted to quantify the gene transcription levels of these transporter genes in *B. longum* cells which had been exposed to erythromycin as compared to the levels of gene expression of cells grown under normal growth conditions. The results indicated an increase in the levels of gene expression for the ABC-type transporters in both gene clusters being studied. In the case of the transporter

genes in the three gene operon, the increase in transcription was shown to be statistically significant.

The presence of the putative transcriptional regulators upstream of both operons was intriguing, since it had previously been shown, in other bacteria, that transcriptional regulators belonging to the MarR family of regulators are able to repress the expression of ABC-type transporter genes under normal growth conditions. Bioinformatic analysis of the two putative regulators in this study showed that the structure of the regulator in the three gene operon, of which it is part, most closely resembled that of other proven MarR-type regulators. To evaluate the role of these putative regulators with respect to the ABC-type transporter genes down-stream of them, electrophoretic mobility shift assays (EMSA) were carried out. The putative regulator genes were cloned, expressed and their proteins purified. The results of the EMSA experiments showed that the putative MarR-type regulator protein, upstream of the three gene operon of which it is part, bound stably, specifically and with high affinity to the DNA sequence directly upstream of the operon. Binding of this regulator protein was not easily de-repressed even in the presence of known inhibitors such as sodium salicylate and it did not bind to other DNA sequences even when presented with a proven promoter region or the upstream DNA sequence of the other operon being studied. No such binding was observed for the second putative regulator, which showed no significant similarity to other MarR-type regulators and was not a part of the operon downstream of it.

The data obtained in this study showed the occurrence of functional ABC-type transporter genes in a probiotic bacterium, and demonstrated that their genome arrangement facilitated simultaneous transcription of these genes. The transcriptional induction of these genes in response to erythromycin exposure was confirmed and the first functional evidence was obtained of a putative MarR-type transcriptional regulator in *B. longum*.

Chapter 1

Literature Review

Contents

1. Introduction

1.1. The gastrointestinal tract

The gastrointestinal tract (GIT) of all animals is known to house a vast and intricate micro-ecosystem of distinct complexity (Holzapfel & Schillinger, 2002). Its mucous covered surface encompasses an area estimated to be approximately 150 – 200 m^2, which when compared to the surface area of the skin covering the entire human body (2 m^2) is rather vast (Waldeck, 1990). The immense surface area available thus enables the immense resident and transient microbiota to adhere to and interact with the host and aids the process of digestion (Holzapfel & Schillinger, 2002). It is estimated that the adult human GIT is colonised with approximately 10^{14} microbes. This implies an important role by these organisms when it is taken into account that there are approximately 10^{13} tissue cells in the entire human body (Ley *et al.*, 2006; Luckey, 1972).

The immense diversity of the GIT differs from one region to the next and its composition is determined by both intrinsic and extrinsic factors such as diet and the physical condition of the host (Holzapfel & Schillinger, 2002). The resident or autochthonous microbiota residing in the GIT has been shown to encompass more than 400 species in diverse bacterial genera. These include Gram positive bacteria, most of which are obligate anaerobes, and which predominate in the large intestine, for example *Eubacterium* and *Bifidobacterium* spp. Gram negative organisms also flourish in this niche, for example; large numbers of *Escherichia coli*, *Bacteroides* and members of the *Enterobacteriaceae* are present (Holzapfel & Schillinger, 2002) (Figure 1.1).

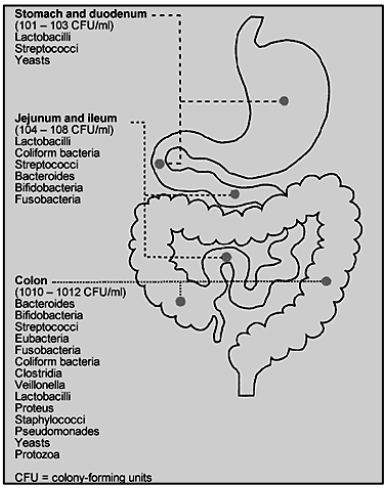


Figure 1.1. Microbial colonization of the human GIT (Kullak, 1997).

The profile of strains of *Bifidobacterium* and *Lactobacillus* residing in a host has been shown to be both unique for every individual (Tannock, 1997), and that the human GIT microbiota differs considerably from that of other mammals (Akkerman *et al.*, 2000). Recent studies using metagenomic approaches to assess the human gut microbiome has shown that even though bacterial profiles may be unique for every individual, the bacterial species present in the human gut and the numbers thereof are largely shared among individuals. These studies also indicate that the major evolutionary driving forces of the diversity of these microbiomes may be as a result of the conditions in the gut as well as human-microbe interactions (Ley *et al.*, 2006; Qin *et al.*, 2010). In the past, little emphasis was placed on the GIT and its resident microbiota since most of the scientific focus was centred on enteropathogens and their pathogenesis leading to dysbiosis. It is now recognised that a healthy and stable GIT microbiota affords a sturdy barrier against any would-be invading pathogens or harmful substances (Holzapfel & Schillinger, 2002). Holzapfel *et al.*, (1998) defined the major functions of the autochthonous biota of the human GIT as barrier function or restoration, immune system stimulation, maintenance of mucosa, nutrition and circulation, production of nutrients and improved bioavailability and the stimulation of bowel motility.

1.2. Colonization and succession

While in the mothers' womb, the developing foetus is maintained in a sterile environment and is thus kept in an aseptic condition since all nutrient metabolism and excretory functions are performed by the mother. It is only during labour that the foetus is, for the first time, exposed to microorganisms. Colonization commences upon contact with the resident bacteria in the birth canal of the mother and subsequently from the surrounding environment. It has been observed that the diet of the baby from this point onwards drastically affects the development of the GIT micro-ecosystem (Palmer *et al.*, 2007). Both breast fed and formula fed babies seemingly start with the same colonising organisms, i.e. *Escherichia coli* and *Streptococcus* predominating. In breast fed babies succession then occurs with a prominent increase in *Bifidobacterium* numbers, coinciding with a decrease in *E. coli* and *Streptococcus* numbers, with *Clostridium* almost never observed (Figure 1.2). It has also been shown that certain *Bifidobacterium* sp. are present in breast milk and are passed from mother to the baby during breast feeding (Gueimonde *et al.*, 2006). 16S rRNA and metagenomic studies are now providing more in-depth analyses on this specialised niche (Koenig *et al.*, 2010).

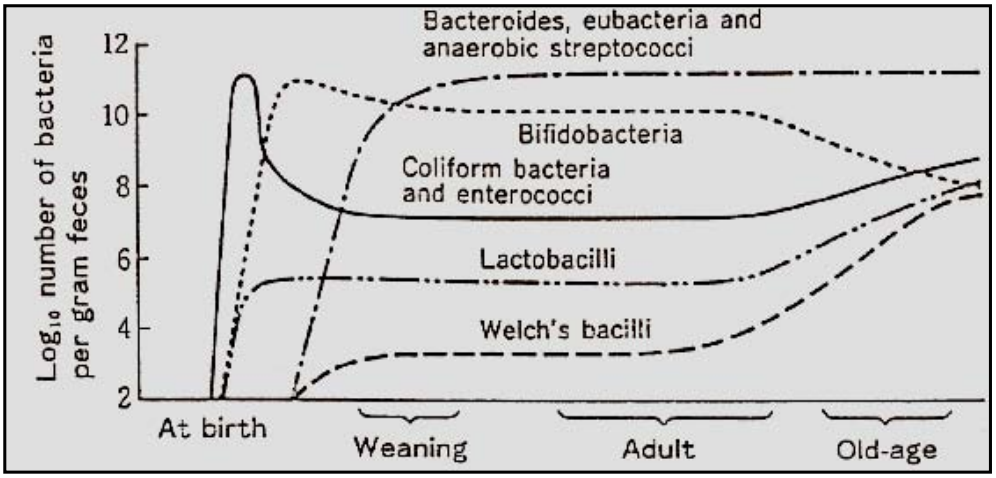


Figure 1.2. Changes in the intestinal microbiota with age (Mitsuoka, 1978).

The situation in formula fed babies is, however, completely different with almost no succession being observed. There are high numbers of *Bacteroides*, *Clostridium* and *Streptococcus* species present, and even though *Bifidobacterium* is found, it is not dominant. When supplements, e.g. iron, are introduced into the diets of breast fed babies, the developing GIT biota resembles that of the formula fed infant, with *Bacteroides* and Gram positive cocci predominating. Once both groups are weaned, the adult GIT microbiota develops, with a decrease in *Streptococcus* and *E. coli* numbers (Bullen *et al.*, 1977; Mevissen-Verhage *et al.*, 1985). Stabilization of the final GIT micro-ecosystem is controlled by the host's immune system and the internal physiology of the GIT itself. These factors cooperatively prevent over or unregulated growth and will determine the final micro-ecosystem. Factors such as pH, enzymes, bile, synergy v/s antagonism and diet, will thus all play a role in determining the final micro-ecosystem of each individual (Koenig *et al.*, 2010; Mitsuoka, 1990; Mitsuoka, 1992), which has been shown to be unique to every individual (Tannock, 1997). Even though the micro-ecosystem of individuals appears to be unique, recent metagenomic studies have shown the occurrence of three so-called 'enterotypes'. These enterotypes identify certain conserved microbial profiles in the gut which are not explained by host properties such as gender, age or weight. Marker genes relating to these host properties are present and can therefore potentially be used in diagnostics (Arumugam *et al.*, 2011). The question, therefore, arises whether to supplement the diet of individuals, who may be pre-disposed to gut disorders, with these health promoting bacteria? For this reason it is necessary that research be conducted to ensure that safe, effective and stable probiotic bacterial supplements are available as treatments to allow for the development of a normal, healthy and beneficial gut microbiota in all individuals who may be deficient.

2. The role of probiotics

2.1. Background and definition

Even though probiotic bacteria encompass a large group of bacterial strains, the important role played by the lactic acid bacteria (LAB) is of predominant interest. As early as 1892, it was suggested that LAB play a beneficial role within the human host (Döderlein, 1892). The origin of the term "probiotic" is believed to have been coined in 1954, in a paper studying the effects of antimicrobials and factors favourable to the gut microbiota, and is derived from Greek, meaning "for life" (Vergio, 1954). One of the current accepted definitions of a probiotic bacterium is that it refers to 'Viable microorganisms that promote or support a beneficial balance of the autochthonous microbial population of the GIT' (Holzapfel *et al.*, 2001; Holzapfel *et al.*, 1998).

2.2. Characterisation of probiotics

Even though probiotics are known to benefit the host, the scientific data to confirm this is still insufficient to provide conclusive proof of their role and involvement in conferring these health benefits. The actual mechanisms underlying the effects observed when probiotic supplements are consumed thus need to be further elucidated. The World Health Organisation (WHO) and the United Nations Food and Agriculture Organisation (UNFAO) have, however, agreed that there is sufficient data to conclude that probiotics do in fact improve human health, and that certain strains are safe for commercial use (UNFAO & WHO, 2001).

In order for a new strain of bacteria to be considered for use as a potential probiotic supplement, there are certain aspects which first need to be evaluated, tested and confirmed before its use can be safely justified. The WHO and FAO have thus formed guidelines for the evaluation of potential probiotic strains. These include: strain identification to species level, using reliable molecular techniques; *in vitro* and *in vivo* studies to confirm the physiological properties of the strain, such as adhesion and pathogen inhibition capabilities; safety assessment of the strain, especially in immunocompromised patients where septicaemia may occur; phase 2 clinical trials, to compare the efficacy of the probiotic in comparison to a placebo; phase 3 clinical trials, to compare the efficacy of the potential

probiotic to that of a current treatment of the same type; and finally, accurate health claims and labelling of the new product to inform the consumer of the actual proven health benefits (UNFAO & WHO, 2002).

For the effective use of probiotics in industry, they must possess certain features to ensure the best possible results for the manufacturer and consumer. There are, therefore, three main characteristics which are considered desirable for a probiotic bacterium. They should be non-pathogenic, able to survive the hosts' immune response and be genetically stable. They should be culturable in large scale applications for industrial processing of the final product. They should offer numerous beneficial properties *in vivo* (Havenaar *et al.,* 1992).

One of the most valuable contributions conferred by the presence of these organisms in the GIT is the competitive exclusion of pathogenic organisms. It has been proven that certain strains of *Bifidobacterium* are able to bind to the surfaces of most regions in the intestines where they reside, as a result of their hydrophobicity profiles and autoaggregative properties (Bibiloni *et al.*, 2001; Del Re *et al.*, 2000). In so doing they are proposed to reinforce the GIT barrier, thus preventing the binding of pathogenic organisms. They have also been shown to secrete a protein which inhibits the binding of certain pathogens such as enterotoxigenic *E. coli* (Fujiwara *et al.*, 1997). Other antimicrobial substances are also known to be produced by certain strains of *Lactococcus lactis* (nisin) and *Bifidobacterium,* and when coupled with the secretion of lactic acid by these bacteria and the subsequent lowering of the surrounding pH, the outer membrane of Gram negative cells is significantly weakened, thus potentiating the effect of these and other antimicrobial substances present (Alakomi *et al.*, 2000; Liévin *et al.*, 2000; Mierau & Kleerebezem, 2005). The most noticeable pathogen exclusion model is, however, the exclusion of these organisms by competitive binding to enterocyte cell surface receptors for attachment or stearic hindrance, as was demonstrated with *Lactobacillus* species (Chan *et al.*, 1985). The pathogen inhibiting properties of lactic acid bacteria once again strengthens the case for their use in the pharmaceutical industry, as their health promoting activity seems to outweigh their potential negative properties.

2.3. Prebiotics and synbiotics

The term 'prebiotic' is alleged to have been coined in 1995 and was defined as "A non-digestible food ingredient that beneficially affects the host by selectively stimulating the

growth and/or activity of one or a limited number of bacteria in the colon" (Gibson & Roberfroid, 1995). These substances include indigestible but fermentable sugars, usually short chain carbohydrates (SSC) or low-digestible carbohydrates, which stimulate the growth of certain bacterial groups. These prebiotic substances are generally shown to increase sugar digestion and absorption in the bowel, affect glucose and lipid metabolism and protect against certain risk factors of cardiovascular disease. They also produce short-chain fatty acids in the colon which is considered to be a factor in the prevention of colorectal cancer (Scheppach *et al.*, 2001).

When a probiotic bacterium and a prebiotic substrate are combined in a single product or supplement the term 'synbiotic' is applied. Since the term alludes to synergism, it should be reserved for situations where the prebiotic substrate favours the growth of the probiotic bacterium it is combined with (Schrezenmeir & de Vrese, 2001). Synbiotics can be specifically designed to affect or target an isolated region of the GIT or even more than one region (Holzapfel & Schillinger, 2002). It has been proven that a synbiotic can confer benefits to the host which are above that of either one on its own (Rowland *et al.*, 1998).

3. Bifidobacteria

3.1. Background and morphology

Bifidobacteria are human gut commensals and can be regularly isolated from the GIT of humans and animals. They play a major role in maintaining the homeostatic balance required in the GIT. They are obligate anaerobes, and are classified as high G+C Gram positive bacteria in the Actinomycetales branch, grouping them with the Corynebacteria, Mycobacteria, and Streptomycetes. Even though most of the approximately 32 species of *Bifidobacterium* identified to date are isolated from the GIT, a small number are found in the human vagina and oral cavity as normal flora in healthy individuals and recent studies using non-culture dependent techniques have identified new species in the GIT which were previously not isolated (Biavati & Mattarelli, 2001; Turroni *et al.*, 2009). The morphology of bifidobacteria were described in 1889 as being "curved rods and rods with ends split to give the characteristic Y-shape which led to the designation of 'bifid'" (Tissier, 1889). They were subsequently further characterised as non-motile, non-spore forming rods which may vary in morphology depending on the growth medium used (Rasić & Kurmann, 1983). These

researchers' found that in media rich in the necessary nutrients, bifidobacteria occur as curved or clubbed shaped, uniform or branched, bifurcated Y and V forms, and may also be spatulate. In nutrient limited media, the cells are highly pleomorphic and branched, but are mostly rod shaped in their natural environment.

3.2. Growth requirements

The bifidobacteria are classified as obligate anaerobes (Scardovi, 1984), but a few species are able to tolerate oxygen (Simpson *et al.*, 2004). The optimum growth temperature of human isolates of bifidobacteria is between 36 – 38°C, and that of animal strains, between 41 – 43°C, but, can be as high as 49.5°C for the thermophile *Bifidobacterium thermoacidophilum* or as low as 4°C for *Bifidobacterium psychraeophilum* (Dong *et al.*, 2000; Simpson *et al.*, 2004). Bifidobacteria are considered to be acid tolerant microbes and have an optimum growth pH of between 6.5 and 7.0. Some strains can grow at a pH of 3.5 (Matsumoto *et al.*, 2004) but none survive if cultured at pH 8.5 or above (Biavati *et al.*, 2000).

Due to the similar niche occupied by bifidobacteria and lactobacilli, a semi-synthetic supplemented growth medium, which stimulates the growth of lactobacilli, was initially used to culture bifidobacteria (Norris *et al.*, 1950). It was however only in 1951, when studies on the properties of the medium above showed that a simple medium, supplemented with cysteine, could support the growth of bifidobacteria, as they would utilise ammonium salts as a source of nitrogen (Hassinen *et al.*, 1954). It is only *Bifidobacterium bifidum* that requires the further addition of *N*-acetyl-D-glucosamine-containing saccharides, which facilitate cell wall synthesis (Glick *et al.*, 1960; O'Brien *et al.*, 1960).

3.3. Classification

Most Gram positive, non-spore forming, branched or unbranched rods, not yet classified in the early 1900's, were initially assigned to the genus *Bacillus* and then subsequently to the genus *Lactobacillus*. Because of this, references to Gram positive rods in earlier literature could have referred to a number of different genera recognised today as separate (Poupard *et al.*, 1973). Tissier's description in 1899 – 1900, of irregular, Gram positive, Y-shaped, non-gas producing, anaerobic bacteria, which may be bifurcated, isolated from the stools of breast-fed babies, as *B. bifidum*, marked an initial endeavour to classify these organisms. The genus *Bifidobacterium* was defined in 1924, when Tissier proposed that *B. bifidum* should in

fact be classified in its own genus which he designated as *Bifidobacterium* (Orla-Jensen, 1924). This is currently the accepted nomenclature for members of this family.

Today the genus is represented by approximately 37 species, isolated mainly from the GIT's of humans and animals (Turroni *et al.*, 2011). The genus is anchored in the branch of Actinomycetales encompassing the high G+C Gram positive bacteria (Klein *et al.*, 1998).

4. Antibiotics and resistance

4.1. Background

With the advent of antibiotics in the 1940s, it was believed that bacterial infections and mortalities due to such infections were to become a thing of the past and focus was thus aimed at researching new and more complex diseases. This was, however, not the case. Instead of the expected decline in the incidence of bacterial infections and the successful treatment thereof, incidences of infection due to resistance to the current therapies increased readily and drug resistance is now a major concern. It is only recently that the reasons for antimicrobial resistance to these compounds have been researched and scientists in this field now know that bacteria have always been faced with unfavourable conditions and exposure to toxic compounds in their natural environments. This has consequently lead to the development of mechanisms, by these organisms, to counteract the negative effects of these molecules (Salyers & Amábile-Cuevas, 1997). These ancient resistance mechanisms may be the underlying cellular machinery, which bacteria have adapted to become resistant to modern antimicrobials.

4.2. Mechanisms

Bacteria have evolved numerous mechanisms which they use to prevent the damage inflicted by exposure to antimicrobial and toxic substances. All of these mechanisms can be grouped into four basic systems (Figure 1.3); drug inactivation, target alteration, inhibition of drug influx and active extrusion of these compounds from the inside to the outside (Putman *et al.*, 2000). Research focus in the past was mainly on the first three mechanisms listed, as these were likely targets for the development of new generations of antibiotic compounds. It is now, however, realised that bacteria have the ability to develop resistance mechanisms to newly designed drugs in short periods of time, and that new targets need to be exploited.

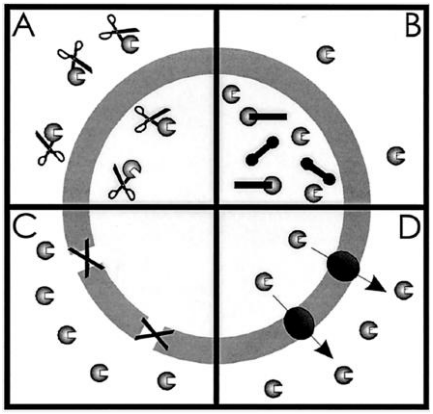


Figure 1.3. The four antimicrobial resistance mechanisms in bacteria. (A) Drug inactivation, (B) target alteration, (C) inhibition of influx, and (D) extrusion (Putman *et al.*, 2000).

Since drug efflux mechanisms in bacteria allow them to prevent the entry of toxic molecules into the cell, as well as, the occurrence of multidrug efflux transporters, drug extrusion systems have become a focus of antimicrobial research (Borges-Walmsley *et al.*, 2003). The first three mechanisms above, i.e. inactivation, alteration and preventing influx, are limited in their efficacy and range as they are often specific for the relevant substrates they act on or against. The active extrusion of toxic compounds, facilitated by efflux pumps, is not limited in this respect. Even though substrate specific pumps abound in bacteria, there are a large number of multidrug transporters that are able to actively efflux a vast number of unrelated antimicrobial compounds (Borges-Walmsley *et al.*, 2003). This mechanism of multidrug resistance, conferred by a single protein, is only now being studied in much greater detail.

4.3. Resistance mechanisms in *Bifidobacterium*

Probiotic bacteria such as *Bifidobacterium* confer health benefits on the host and are not considered to be pathogenic under normal conditions – both of which are criteria for their selection as probiotics. It is, therefore, not a concern that these bacteria are in fact intrinsically resistant to numerous antimicrobial compounds which are routinely administered as treatments for disease conditions. Even though the presence of efflux proteins in these bacteria is alarming and may indicate that organisms harbouring such systems should be avoided, the presence of these systems in non-pathogenic organisms, especially probiotic organisms is largely ignored. When this is coupled with the lack of understanding on the underlying mechanisms of resistance, it may appear safe to use these bacteria as probiotic supplements to alleviate certain gut disorders or generally improve health.

4.4. Gene transfer

If these probiotic strains do in fact pose no risk to the human host directly, the question regarding the possibility of the dissemination of these multidrug resistance genes via horizontal gene transfer to other potential pathogens in the GIT cannot be ignored (Figure 1.4). These genes would allow opportunistic pathogens to become hardy colonisers and extremely difficult to control, or even more dangerous, pathogenic strains may become completely untreatable. The topic of horizontal gene transfer (HGT), (transfer of genetic material from one organism to another) is considered a common occurrence in bacteria, due to the current profusion of phylogenetic information available due to the sequencing of entire genomes. This abundance of information has highlighted the presence of more and more homologous genetic material in unrelated species, which does not appear to be a part of the hosts' original genome. Because of this, geneticists and phylogeneticists agree that HGT is in fact widespread amongst prokaryotes and has been occurring for many years, and is believed to be one of the driving forces of evolution in the prokaryote kingdom (Salyers *et al.*, 2004).

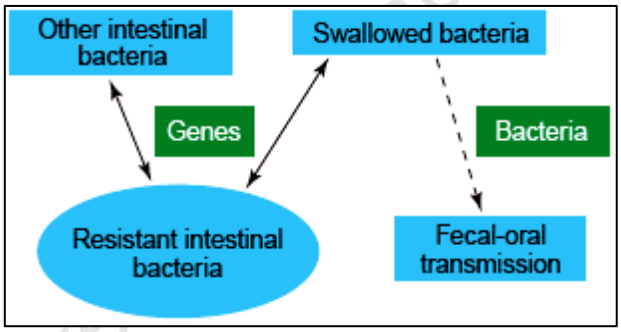


Figure 1.4. Schematic representation of possible resistance gene transfer mechanisms (Salyers *et al.*, 2004).

In order for a gene to be transferred from one organism to another, a transfer mechanism is needed. There are three generally excepted methods for HGT to occur in nature, namely: transformation (uptake of DNA from the surrounding environment), transduction (transfer of DNA from one cell to another using a bacteriophage) and conjugation (the transfer of DNA from one organism to another using a pilus). Each of these mechanisms has its drawbacks, such as a narrow host range, the presence of DNA degrading enzymes and the need for sequence homology for integration, but, it appears that nature always seems to find a way, especially if sufficient selective pressure is applied and the cells are forced to adapt or die.

It has been shown that antibiotic resistance genes transferred from Gram positive bacteria via conjugation, are readily expressed in Gram negative bacteria while the reverse is rarely

observed. Because of this, Gram negative cells are able to access the diverse genetic pool of Gram positive organisms. Even though most plasmids of Gram positive origin are known to be unstable when expressed in Gram negative cells, conjugation is known to occur and the evidence of plasmids of Gram positive origin are still isolated from Gram negative organisms, as is the case with the transfer of the *erm*B gene from *Enterococcus faecalis* to *E. coli* in the human GIT (Arthur *et al.*, 1987).

Another mechanism often overlooked is that of transposition. In 1991 it was proven that a conjugative transposon could in fact be transferred from Gram positive to Gram negative bacteria (Bertram *et al.*, 1991). Since transposons are able to integrate into the hosts' genome, the question of plasmid instability is no longer a problem. Once integrated, the genetic material is subsequently replicated and transferred to all the progeny of this mother cell.

This concept of HGT is quite alarming when one considers the vast numbers of bacteria residing in the human GIT. Of all the colonized areas in and on the body, the human GIT is one of the richest and most diverse, and also plays host to the vast numbers of transient bacteria which inadvertently pass through the system. The concept that a reservoir of antibiotic genes found in the micro-ecosystem of the human GIT, which is shared among members of this niche and its transient microbiota, is thus not that farfetched (Simonsen *et al.*, 1998; Teuber *et al.*, 1999; Van Den Braak *et al.*, 1998). Evidence of this transfer is becoming abundant, with specific emphasis on the transfer of resistance genes between Gram positive and Gram negative bacteria in the human GIT (Figure 1.5).

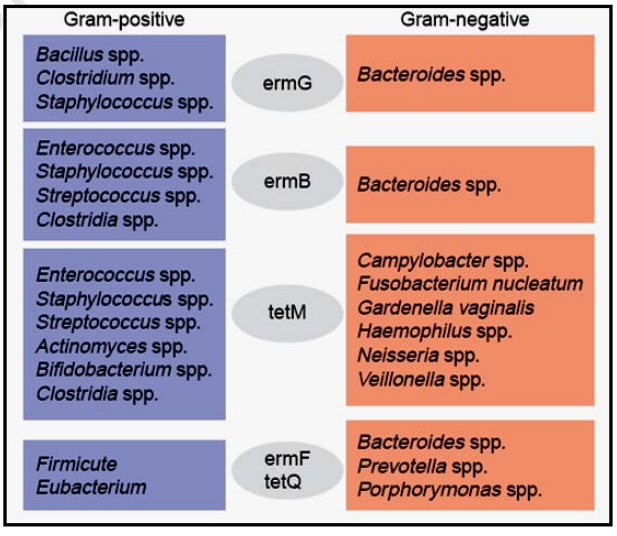


Figure 1.5. Resistance genes with shared homology in Gram negative and Gram positive bacteria (Salyers *et al.*, 2004).

It is not surprising that *Bifidobacterium* spp. are included in the Figure above. These bacteria are a dominant species in the infant GIT, are present in large numbers in the adult GIT and are common probiotic cultures. They have also been shown to harbour the *tet*M gene (Aires & Butel, 2007). It would, therefore, be unlikely that they did not participate in this exchange of genetic material. Research is, however, still lacking in this regard as the extent to which these organisms participate in this process is still unknown. It is only recently been shown that strains of *Bifidobacterium* are capable of suppressing the transfer of certain antibiotic genes *in vitro* and *in vivo.* However, it has also been shown that this effect is strain specific (Moubareck *et al.*, 2007).

It was believed that bifidobacteria did not harbour any plasmids since none were isolated from any strains. Studies have now indicated the presence of plasmids in numerous strains of bifidobacteria including, *Bifidobacterium asteroides*, *Bifidobacterium breve*, *Bifidobacterium globosum*, *Bifidobacterium indicum*, *Bifidobacterium pseudocatenulatum* and *Bifidobacterium longum*; where *B. longum* is now most frequently found to harbour plasmids (Álvarez-Martín *et al.*, 2007; Iwata & Morishita, 1989; Sgorbati *et al.*, 1982). It has also been shown that the plasmids in *B. longum* show evidence of molecular re-arrangements, indicating their ability to share genetic material with other bacteria (Lee & O'Sullivan, 2006).

The need to understand the basis of efflux systems and gene transfer of these systems would therefore allow for the selection of safe probiotic bacterial strains, since strains which are known to be drug resistant and which carry such determinants on mobile transmissible elements can be avoided. Strains which stably maintain these genes on the chromosome can then be preferentially used since they can survive antibiotic therapy and are highly unlikely to transfer these genes to other bacteria.

5. Efflux transporters

5.1. Background

Efflux pumps are integrated membrane proteins which actively pump unwanted or toxic compounds out of the cell (Borges-Walmsley & Walmsley, 2001). Since bacteria are believed to have evolved in a 'primordial soup', the cellular membrane must be a relatively old organelle. This membrane served as a compartment between the complex internal

cellular constituents of the bacterium and its surrounding environment. In this way the cell would be protected from its harsh, unfavourable surroundings, and would also be allotted a region within which it could perform its cellular reactions in a closed and regulated system (Van Bambeke *et al.*, 2000).

These efflux transporter proteins form an integral constituent of bacterial cell membranes and based on sequence homology, it has been calculated that approximately 15 – 20% of the *E. coli* genome may encode this type of protein (Paulsen *et al.*, 1998). Even though efflux pumps are considered to be a major resistance mechanism, it must be noted that their activity is limited in terms of the levels of resistance they confer to the host. Most efflux systems confer an increase of between 1 – 64 folds in the minimum inhibitory concentration (MIC) of any given organism to a particular compound (Lomovskaya *et al.*, 1999; Mazzariol *et al.*, 2000). It must also be noted that resistance to a specific compound may be due to more than a single factor. For example, the intrinsic resistance of many organisms to a substrate may be dependent on the constitutive or induced expression of a single or numerous efflux pumps (Li *et al.*, 1995). The expression of numerous different pumps may indicate a high level of resistance to a specific compound, which is in fact targeted by all these expressed systems co-operatively (Lee *et al.*, 2000). Efflux systems may also work in conjunction with other systems such as enzymes, to rid the cell of a toxic substance. For example, a β-lactamase coupled with an efflux pump to protect the cell from β-lactam type drugs (Okamoto *et al.*, 2001). Some efflux pumps are regulated by the binding of certain antimicrobials to gene regulators and can in this way be induced to efflux this and/or other antimicrobials (Roberts, 1996). Mutations to these regulators or their DNA-binding sites may result in the efflux system being constitutively over-expressed (Poole, 2000), and if this is part of a global regulation system it may result in the over-expression of a number of efflux systems simultaneously (George, 1996). Currently the most startling observation is that these efflux proteins are readily exchanged and transferred, even amongst distantly related bacterial genera (Del Grosso *et al.*, 2002).

5.2. Efflux pumps in probiotic bacteria

Probiotic bacteria such as *Bifidobacterium* and *Lactobacillus* have been shown to be intrinsically resistant to numerous antimicrobial compounds (Aimmo *et al.*, 2007; Masco *et al.*, 2006). These resistance properties are considered beneficial when selecting commercial strains for probiotic supplements, since they are able to survive antibiotic therapy and can

continue providing their beneficial roles in the GIT, they may also prevent secondary infections in the gut. Some of these resistance profiles are conferred by the presence of drug-specific resistance genes such as *tet*W/M/O, which confers resistance to only tetracycline (Aires & Butel, 2007). The most interesting contribution to resistance in probiotic bacteria is, however, the presence of efflux transporters, since these transporters are known to confer multidrug resistance (MDR) phenotype on the host cell, where a single protein is able to simultaneously confer resistance to numerous structurally unrelated antimicrobial substrates. MDR transporters are ubiquitous in bacteria and have been identified in numerous probiotic bacteria such as *L. lactis* (Florez *et al.*, 2006) and *B. breve* (Margolles *et al.*, 2006). By expressing the proteins, these bacteria are able to remain viable during antibiotic therapy with numerous different antibiotics.

5.3. Classification of bacterial transporters

Due to ubiquity of these transporters, coupled with a lack of detailed phylogenetic data, a classification system based on primarily functional relatedness has been proposed. The classification of drug transporters is thus based primarily on three criteria: the energy source used for driving it, its preliminary phylogenetic data, and its substrate specificity. It has been proposed that a four-digit code be used to classify such transporters. In this system the first digit represents the mode of transport and energy source used to drive the efflux pump; the second and third digits, refer to its phylogeny; and the fourth digit the substrate specificity of the transporters (Paulsen *et al.*, 1998).

Bacterial drug transporters have, therefore, been broadly classified into five families. These familial systems are classified based on the three primary criteria above, but include the criterion of the presence of certain conserved amino acid sequences within the genes in question (Table 1.1). The five families are the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the resistance-nodulation-cell division (RND) family, the multidrug and toxic compound extrusion (MATE) family and finally the ATP-binding cassette (ABC) family (Putman *et al.*, 2000). Together these families encompass all of the active efflux systems identified to date. Despite the fact that these systems are grouped so closely to one another, they are in fact extremely diverse in both structure and function, and have evolved intriguing and diverse mechanisms to purge unwanted substances from the tightly controlled interior of the cell.

Table 1.1. Amino acid consensus sequences of the conserved motifs in the MFS, SMR, and RND families (Putman *et al.*, 2000).

Motif	Consensus sequence[a]	Location
MFS, both 12- and 14-TMS cluster		
A	GxLaDrxGrkxx(x)l	Loop between TMS 2 and 3
B	lxxxRxxqGxgaa	TMS 4
C	gxxxGPxxGGxl	End of TMS 5
MFS, 12-TMS cluster		
D2	lgxxxxxPvxP	End of TMS 1
G	GxxxGPL	End of TMS 11
MFS, 14-TMS cluster		
D1	lDxTvxnvAlP	End of TMS 1
E	DxxGxxL	TMS 7
F	lgxxxGxavxgxl	TMS 13
H	WxwxFllNvPig	TMS 6
SMR family		
A	WixlviAillEV	TMS 1
B	KxseGFtrlxPS	Loop between TMS 1 and 2
C	PvGtAYAvWtGlG	Start of TMS 3
RND family		
A	GxsxvTvxFxxgtDxxxAqvqVqnkLqxAxpxLPxxVqxqgxxvxk	Loop between TMS 1 and 2
B	alvlsaVFlPmaffgGxtGxiyrqfsiTxvsAmalSvxvaltltPAlcA	TMS 6
C	xxxGkxlxeAxxxaaxxRLRPlLMTsLafilGvlPlaiatGxAGa	TMS 11
D	SiNtlTlfglvlaiGLlvDDAlVvVENveRvlae	TMS 4

[a] The motifs were identified by alignment of amino acid sequences. (x) any amino acid, (Capitalised) amino acid occurs in >70% of sequences, (lowercase) amino acid occurs in < 40%, [(x)] amino acid not always present.

The five families are foremost separated from one another based on the energy source used to drive them, i.e. either a proton gradient or by the hydrolysis of ATP. Subsequently, the two classes are then further divided into the five respective families differentiated by the presence of specific conserved amino acid sequences in the coding regions of the genes. In this way, four of the five families are clearly distinguishable from one another. The MATE family merely encompasses those transporters which do not fit into any of the other four families based on the classification criteria. There may thus be several new families classified in the near future which may selectively include some of these transporters (Putman *et al.*, 2000). Figure 1.6 outlines the current relationships of the multidrug transporters found primarily in bacteria, based on their shared characteristics.

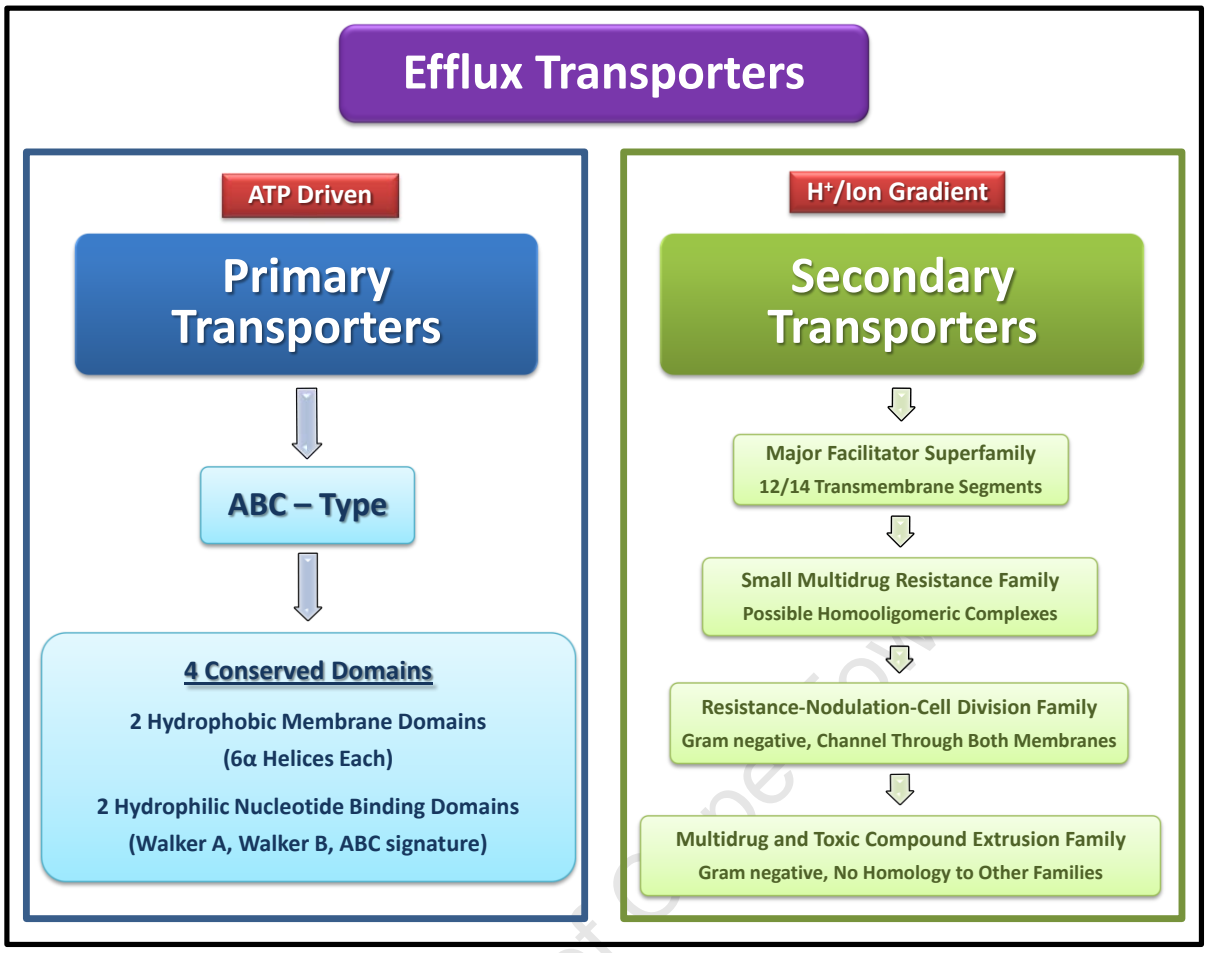


Figure 1.6. Concise description of efflux transporter classification.

5.4. Secondary multidrug transporters

This class of transporters includes the MFS, SMR, RND and MATE families. These transporters share a common feature, i.e. they are energised by an ion gradient between the internal cellular milieu and the external environment surrounding the cell. This gradient is usually formed by a differential concentration of Na^+ or H^+ ions between the inside and outside of the cell.

5.4.1 The major facilitator superfamily (MFS)

The MFS comprises a family made up of those transporters which have transmembrane proteins capable of symport, antiport and uniport of various compounds into and out of the cell. These membrane proteins are able to transport substances such as sugars and antibiotics, and are found in both prokaryotes and higher eukaryotes (Marger & Saier, 1993). Based on sequence alignments it has been found that this family can be divided into two clusters, i.e.

those with 12 transmembrane segments (TMS) and those with 14 TMS's. This family contains transporters present in both Gram positive and Gram negative bacteria, e.g. Bcr in *E. coli* and Bmr in *Bacillus subtilis* (Paulsen & Skurray, 1993).

5.4.2 The small multidrug resistance (SMR) family

These transporters are some of the smallest proteins with efflux function isolated thus far. They are approximately 107 amino acids long and are presumed to be arranged in a four-helix, antiparallel bundle which is tightly packed (Putman *et al.*, 2000). Because of the small size of these transporters, function would only be feasible if a multi-unit complex is formed. It is thus believed that the transporters found in the SMR family occur as homooligomeric complexes of the single units fused to one another to form a complete functional unit (Paulsen *et al.*, 1995).

5.4.3 The resistance-nodulation-cell division (RND) family

These transporters are present only in Gram negative bacteria. Since the Gram negative bacterial cell envelope is composed of a thin peptidoglycan layer, surrounded by a thick outer membrane, these transporters need to transport compounds across not just one, but in fact two membranes. To facilitate this, these transporters make use of a membrane fusion protein as well as an outer membrane protein to transport substances out of the cell directly into the surrounding medium (Putman *et al.*, 2000). It has been proposed that these transporters are composed of 12 TMS with large loops occurring between segments 1 and 2, and 7 and 8 (Paulsen *et al.*, 1996). The membrane fusion proteins are proposed to fuse the inner and outer membrane by using either a single N-terminal transmembrane segment and a C-terminal periplasmic domain (Dinh *et al.*, 1994), or they are able to form a channel between the two membranes, in the periplasmic space (Zgurskaya & Nikaido, 1999).

5.4.4 The multidrug and toxic compound extrusion (MATE) family

This family encompasses all the multidrug transporters identified to date which do not fit the classification criteria of any of the other families. Initial comparative analysis of 30 transporter proteins in this family alluded to a grouping of 3 clusters (Brown *et al.*, 1999), but, more recent studies using a PSI-blast search have identified > 800 related sequences from all three kingdoms of life (Omote *et al.*, 2006). Phylogenetic analysis of this data has alluded to three large subfamilies which are then further divided into fourteen sub-groups.

5.5 Primary multidrug transporters

All of the transporters in this family are driven by the hydrolysis of ATP which supplies the energy for translocating substrates out of the cell. All of the ATP-dependent transporters which have thus far been identified are members of the ABC superfamily (Higgins, 1992).

5.5.1 The ATP-binding cassette (ABC) family

The principal ABC-type MDR transporter identified in a LAB was isolated from *L. lactis* and the gene coding for it was subsequently designated *lmrA* (Bolhuis *et al.*, 1994). Since then, it has been noted in the quaternary amino acid structure, that members of this family of transporters comprise four distinct domains (Figure 1.7). They have two hydrophobic membrane domains and two hydrophilic cytoplasmic domains. The two hydrophobic membrane domains are composed of 6 α-helix transmembrane segments and are thought to form a one-way channel through the membrane. The two hydrophilic cytoplasmic domains are exploited for ATP binding and hydrolysis and are thus referred to as the nucleotide binding domains (NBD). These NBD's contain the conserved sequences characteristic of this family, i.e. the Walker A, Walker B, and the ABC signature motifs' (Hyde *et al.*, 1990; Walker *et al.*, 1982). It has been established, that a single gene does, in most cases, code for all four of these domains, as is the case with the well-studied human P-glycoprotein genotype, which is coded for in the order TMD-NBD-TMD-NBD (Gottesman *et al.*, 1996).

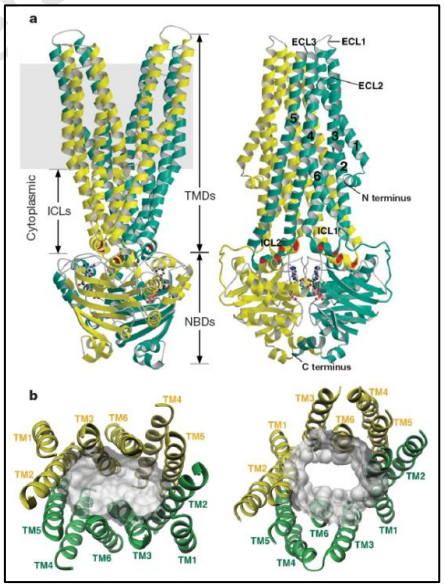


Figure 1.7. Crystal structure of Sav1866 from *Staphylococcus aureus* (Dawson & Locher, 2006). (a) Structure viewed perpendicular to the cell membrane lipid bilayer (grey box) in two orientations at right angles to each other. ICL's indicate intracellular loops. (b) Structure in the plane of the cell membrane showing the substrate translocation pathway from the intracellular (left) and extracellular (right) faces of the membrane. TM indicates the transmembrane helices and the translocation channel is in grey shading (Higgins, 2007).

The LmrA protein isolated from *L. lactis* has been the model protein for research in this field. The protein is 590 amino acids in length and is a membrane protein containing the four conserved domains of ABC transporters. This protein, LmrA, is confirmed to be homologous to each of the two halves of the human P-glycoprotein, but, is functional as a homodimer (Veen *et al.*, 1996). When LmrA is substituted for P-glycoprotein in human lung fibroblast cells, it is able to complement the function of the other, and thus proves that this type of transporter is conserved between prokaryotes and eukaryotes (van Veen *et al.*, 2000). ATP-dependent transporters have also been isolated from Archaea (*Haloferax volcanii*), illustrating their ubiquity in nature (Kaidoh *et al.*, 1996).

5.6 Modes of transport

Even though a great deal of research has been done on these multidrug resistance proteins, there still remains much controversy on the actual mode of action. Questions regarding how they recognise and efflux such a vast array of unrelated compounds and how the molecules are actually transported out, are only now beginning to be answered. Current research proposes three basic models, i.e. the hydrophobic vacuum cleaner model, the flippase model and the periplasmic vacuum cleaner model.

In both the hydrophobic vacuum cleaner model and the flippase model, the efflux protein is proposed to bind to the substrate in the inner leaflet of the lipid bilayer, and it is then subsequently removed from the cell. The difference in the two models being that in the hydrophobic vacuum cleaner model, the bound substrate is actively 'sucked' from the inner leaflet of the membrane and then expelled into the surrounding medium. With the proposed flippase model, the bound substrate is merely thought to be 'flipped' from the inner leaflet to the outer leaflet and is then removed by diffusion into the external medium. The variation regarding the proposed periplasmic vacuum cleaner model arises when applied to Gram negative organisms. In this model, the substrate is once more expelled using the vacuum cleaner approach, but, substrates are now removed not only from the inner leaflet of the lipid bilayer but also from the periplasmic space between the inner and outer membranes in Gram negative cells, and may utilise the action of another protein to transport the substrate from the periplasmic space to the external medium, as is the case with the *acr*AB-t*ol*C system in *E.*

coli (Figure 1.8) (Fralick, 1996; Gottesman & Pastan, 1993; Higgins & Gottesman, 1992; Murakami *et al.*, 2002; Murakami & Yamaguchi, 2003; Zheleznova *et al.*, 2000).

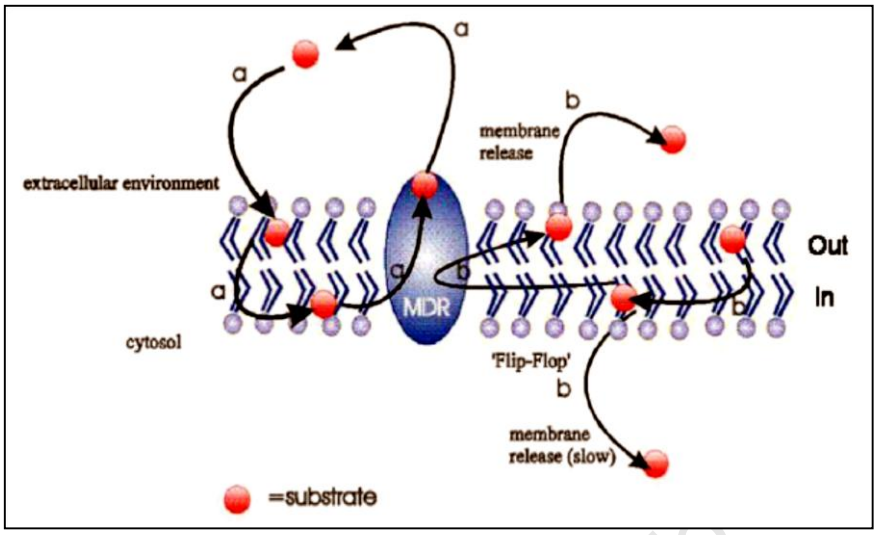


Figure 1.8. Schematic representations of the proposed mechanisms of efflux via ABC-type transporters. (a) efflux into the external medium using the vacuum model, or, (b) flipped into the outer membrane of the lipid bilayer using the flippase model (Langton *et al.*, 2005).

5.7 ABC-type multidrug efflux in lactic acid bacteria

Numerous ABC-type multidrug efflux proteins have been discovered in probiotic bacteria which confer multidrug resistance phenotypes such as the *Lactococcal lmr*A and *lmr*CD genes and the *hor*A system in *Lactobacillus* (Lubelski & Driessen, 2007). These systems have also been identified in *Bifidobacterium* strains (Margolles et al., 2006). Recently the crystal structures of ABC-type transporters have been elucidated, such as Sav1866 from *Staphylococcus aureus*, which is homologous to *lmr*A in *L. lactis* (Dawson & Locher, 2006). These crystal structures have provided much needed insight into the arrangement of these membrane proteins, and have confirmed the nature of the half transporter and full transporter which comprises a homodimer of two protein molecules joined to form one transporter (Dawson & Locher, 2006; Pleban *et al.*, 2004). The crystal structures of the vitamin B12 transporters ButCD and BtuF are now shedding light on the actual arrangement of the NBD's and on the actual mechanism of ATP-binding and hydrolysis (Locher & Borths, 2004).

5.8 Putative regulators

Since MDR efflux transporters are not essential for bacterial growth under conditions where the antimicrobial substrates are absent, the transcriptional induction of these genes in the

presence of toxic molecules is another intriguing research question. The role of transcriptional regulators in controlling the expression of these genes in bacteria has been investigated and research has shown that regulators of bacterial MDR transporter genes belong to four main families, namely: AraC, MarR, MerR and TetR, which all possess the Helix-Turn-Helix (HTH) domain used for DNA-binding. All four families are highly complex with varying and complicated regulatory mechanisms (Lubelski & Driessen, 2007). Of particular interest with regards to ABC-type MDR transporters is that of the negative transcriptional regulator MarR, since it is well characterised and is known to be one of the primary regulators of MDR transporters in *E. coli*. Crystal structures have also recently become available for this protein and have shown the presence of the winged-Helix-Turn-Helix (wHTH) domain, which is the characterising feature of this family of transcriptional regulators (Nichols *et al.*, 2009). These regulators are also known to auto-regulate their own transcription by binding to DNA sequences (two direct repeats) upstream of the coding gene (Alekshun & Levy, 1997; Nichols *et al.*, 2009). Since these regulators are known to play a role in regulating bacterial MDR transporters, further research into their occurrence in probiotic bacteria needs to be conducted.

6. Project aims

From the information presented here, it is clear that probiotic strains play a significant role in promoting good health, as well as, in maintaining homeostasis in the human GIT. There is, however, a great deal of research needed to bridge the gap between the observed effects they produce and the elucidation of the molecular basis for this, as well as, the underlying mechanisms of drug resistance they harbour. This study is thus aimed at characterising both structurally and functionally, two putative ABC-type multidrug efflux gene clusters, identified in a healthy human gut isolate of *Bifidobacterium longum* subsp. *longum*^{T} JCM 1217, to illustrate the ubiquity and importance of these systems in conferring multidrug antibiotic resistance to members of the prokaryotic kingdom. This will be accomplished using three main experimental approaches:

1. Bioinformatic analysis to identify genes of interest in the genome of *Bifidobacterium longum* subsp. *longum*^{T} JCM 1217 which may confer a MDR phenotype. These

genes will be heterologously expressed to observe any MDR function, as well as, the ATP-dependence of the transporters needed for active transport.

2. Confirmation of the role of the ABC-type transporter genes using antibiotic induction studies. This will be investigated by studying possibility of inducing an increase in resistance to certain antibiotics in *B. longum* by first pre-exposing the cells to that antibiotic. Any observed induction in the levels of transcription of these ABC-type transporter genes will then be measured using RNA-hybridization and quantitative Real Time PCR analysis.

3. Finally, the role of putative transcriptional regulators which may regulate these genes will be investigated using electrophoretic mobility shift assays. This may indicate the way in which the transcription of these genes is upregulated in response to antimicrobial exposure.

CHAPTER 2

Gene cloning and functional expression

Contents

1. Summary

Bifidobacterium longum is a gut commensal in the human gastrointestinal tract. In this environment it is able to confer certain health benefits on the host. In order for this continued interaction the bacterium must bind to and colonise the gut. The use of antibiotic treatments to treat bacterial infections often causes significant changes in the gut biota of humans' since the beneficial bacteria are simultaneously eliminated in the process, and this may lead to secondary infections by opportunistic bacteria in the gut, which would otherwise not be able to bind to and colonise the gut. Bifidobacteria are, however, intrinsically resistant to numerous antimicrobial substances and are therefore usually able to survive antibiotic therapy. The mechanisms underlying this innate resistance are poorly characterised and research must be conducted to confirm the mechanisms as well as the genetic basis for it. Identifying resistant strains of *B. longum* may allow for the development of therapeutic strains to be administered as prophylactics concomitantly with antibiotic therapy. In this Chapter, bioinformatic analysis is presented which shows the occurrence of ABC-type multidrug efflux proteins in the probiotic bacterium *Bifidobacterium longum* subsp. *longum* JCM 1217. Four such proteins, grouped into two gene clusters were selected for further investigation. RT-PCR experiments showed that the genes are clustered into 2 operons. When these proteins were heterologously expressed in *Lactococcus lactis,* they were able to confer a multidrug resistance phenotype on the host cells, with a 1.5 – 3 fold increase in resistance to erythromycin and a 2 – 3 fold increase in resistance to tetracycline. There was also an indication of the inducibility of these genes when they were exposed to erythromycin for prolonged periods as observed in the RT-PCR experiments. When the heterologously expressed proteins were exposed to the fluorescent chemical – Hoechst 33342, a known substrate of ABC-type transporters, an increase in the rate of efflux of this molecule was observed. This data indicates the presence of functional ABC-type transporters in a gut commensal bacterium.

2. Introduction

Bacteria utilise transporter proteins to carry useful molecules into the cell and unwanted or waste molecules out of the cell. The advent of antibiotic therapy to treat bacterial infections inadvertently gave rise to the development of a number of antibiotic resistance mechanisms

specifically directed towards these toxic molecules such as membrane alterations to reduce permeability, enzymes to degrade the foreign molecules and alteration of the drug binding site to prevent the desired mode of action. The recruitment of transporter proteins to this end is thus not surprising as they allow the bacterium to rid the cell of the toxic molecule before it is able to carry out its toxic effect inside the cell. Even though specific transporters exist which target only one molecule, one of the most important developments was the evolution of multidrug transporters which are able to confer resistance to numerous, structurally unrelated molecules at the same time, using only one or two protein molecules to pump the antimicrobial substance out of the cell. This confers a great competitive advantage to the bacterium by increasing its pathogenicity and virulence, however, the potential for disseminating these genes to other would be pathogens cannot be ignored. Even though numerous families of bacterial transporters have been identified, such as the major facilitator superfamily, the resistance-nodulation-cell division family, etc, the most intriguing are the ATP-binding cassette type family of transporters (ABC-type) as they are able to actively extrude toxic molecules with the concomitant hydrolysis of ATP. These transporters present an excellent target for drug design, as specifically inhibiting the ATP binding ability of these transporters would render the bacterium sensitive to numerous antimicrobials and would thus allow for treatment with antibiotics which may have become ineffectual (Putman *et al.*, 2000).

Various mechanisms are employed by bacteria to confer antibiotic resistance on the cell, but the multidrug efflux system is of particular interest. This mechanism allows the bacterium to be simultaneously resistant to numerous structurally unrelated antimicrobial substances using only one or two proteins. The primary transporters of the ATP-binding cassette (ABC) family are integral membrane proteins known to encode multidrug resistance proteins (MDR). This family of transporters relies on the active hydrolysis of ATP to facilitate transport of antimicrobial substance across the cell membrane. All ABC-type transporters possess the Walker domains and six transmembrane domains as a half-transporter. Full ABC-type transporters function as homodimers comprising two protein polypeptides bound to each other. The Walker domains comprise three conserved motifs used to bind and hydrolyse ATP once an antimicrobial substance has been trapped in the transmembrane domains. Hydrolysis of the bound ATP facilitates the active extrusion of the antimicrobial substances from the cell membrane back into the surrounding medium. The final full-transporter structure necessitates the binding and hydrolysis of two ATP molecules to allow

for active transport through the channel formed by twelve transmembrane segments (Davidson *et al.*, 2008; Higgins, 2001).

Cloning and functional heterologous expression of proteins requires specialised systems to allow for stable production of the foreign molecule. This is even more complex when membrane proteins are expressed, since any changes to the bacterial membrane composition could destabilise it and allow for uncontrolled movement of molecules across the membrane. Not all MDR proteins are constitutively expressed and they often require specific inducing conditions or the presence of an inducer molecule to induce their transcription. Once transcription is induced, it must be tightly controlled since uncontrolled expression could compromise the integrity of the bacterial cell membrane. The choice of a heterologous expression system, therefore, must thus take into consideration all these factors to provide the optimal conditions for stable gene expression. The *Escherichia coli* KAM3 and KAM32 (Chen *et al.*, 2002; Morita *et al.*, 1998) gene expression systems allow for the heterologous expression of efflux transporters since these host cells have been mutated in the major *E. coli* efflux pump, *acr*AB, rendering it defunct. This allows easier observation of any heterologous contribution by the heterologous efflux proteins.

The Nisin Controlled Expression (NICE) system is one of the most widely used expression systems for the heterologous expression of Gram positive membrane proteins, especially those from lactic acid bacteria and allows for tight control of expression using the nisin inducible promoter (Mierau & Kleerebezem, 2005). It comprises a system of two host strains of *L. lactis*: *L. lactis* NZ9000 and *L. lactis* NZ9700, which are both resistant to nisin – an antimicrobial peptide produced by *L. lactis* NZ9700. *L. lactis* NZ900 has been genetically engineered to be deficient in nisin production, but, still remain resistant to it. A plasmid was isolated from *L. lactis* MG1363, the wild type parental strain of NZ9000, and two expression vectors were created for expressing genes in the host strains pNZ8048 (Figure 2.1) and pNZE8048.

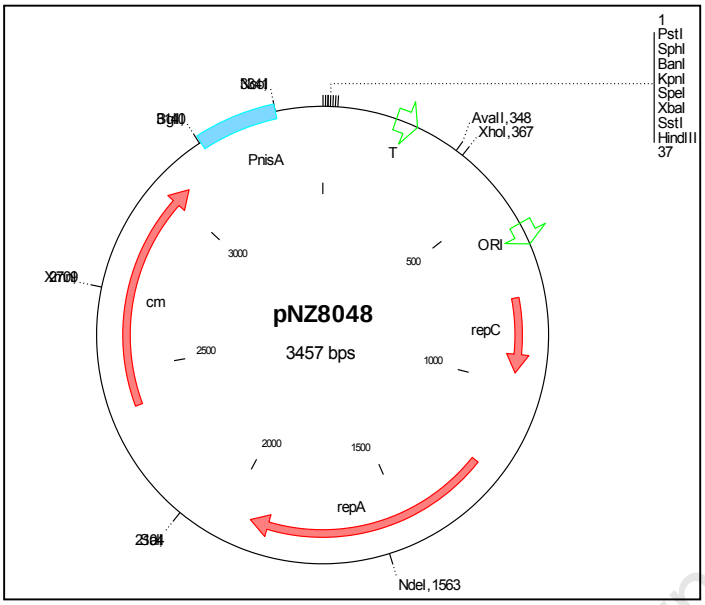


Figure 2.1. Vector diagram of pNZ8048 used for heterologous gene expression in *L. lactis*.

The vectors contain a nisin inducible promoter, which allows for transcription of exogenous genes when cloned in frame at the transcription start site, i.e. transcriptional fusion. The vector pNZ8048 has a chloramphenicol resistance cassette to allow for antibiotic selection of recombinants. There is also a small multiple cloning site, a transcription terminator and an origin of replication. These vectors have been successfully used to functionally express other exogenous membrane proteins (Mierau & Kleerebezem, 2005). For this reason the NICE system was chosen as the ideal system for functionally characterising the selected *Bifidobacterium longum* JCM 1217 ABC-type transporters in this study.

The active extrusion of antimicrobial substances from the cell requires that the substance be recognised by the transporter and bound before being actively pumped out of the cell. To measure this process in real time necessitates a traceable molecule which is a substrate of the transporter being studied. Measuring the rate of efflux of a substrate requires that a known substrate of the transporter in question be labelled in such a manner as to allow for tracking of the molecule. Different approaches can be used to label a substrate, but, altering the structure of a molecule by the addition of an adjunct label may alter its chemical properties significantly. The use of naturally fluorescent molecules is thus preferred. Ethidium bromide is a naturally fluorescent molecule and has previously been used for investigating efflux mechanisms in *L. lactis* (Bolhuis *et al.*, 1996). Ethidium bromide is, however, known to be carcinogenic and alternate substrates are preferable. Hoechst 33342 is another useful molecule for this purpose since it is a fluorescent molecule and a known substrate for ABC-

type transporters which only produces a signal when bound to DNA. Efflux assays can thus carried out using this substrate to confirm the ATP-dependent mechanism employed by bacteria to confer resistance by measuring the active efflux of a substance from a cell mediated by an efflux transporter (Van Den Berg Van Saparoea *et al.*, 2005).

Since Hoechst 33342 is a lipophilic molecule, it is able to passively diffuse across bacterial membranes. When de-energised bacteria are exposed to Hoechst 33342 in solution, the substrate passively crosses the bacterial envelope and floods into the cells, due to the osmotic difference. Hoechst 33342 is essentially non-fluorescent in aqueous solutions and fluoresces only when bound to nucleic acids (Lalande *et al.*, 1981). This property leads to a measurable increase in fluorescence once inside bacterial cells. When the cells are once more energised by the addition of a carbon source and the subsequent production of ATP, efflux pumps that are present which recognise this substrate will cause the Hoechst 33342 to be actively pumped out of the cell. This efflux is observed as a decrease in fluorescence as the Hoechst 33342 is pumped back into the surrounding medium, where it no longer produces a fluorescent signal. The addition of ortho-vanadate, a known ATPase inhibitor, would at this point inhibit all cellular ATPases, including ABC-type efflux pumps. This would result in either a dramatic increase in fluorescence as the Hoechst 33342 floods back into the cells, or a levelling off of (Lalande *et al.*, 1981)the fluorescence level as a steady state is reached, or a marked decrease in the rate of efflux as non ATP-dependent pumps still pump out the Hoechst 33342 unaided by the ATP-dependent pumps (Bolhuis *et al.*, 1994; Van Den Berg Van Saparoea *et al.*, 2005; Venter *et al.*, 2008).

In this chapter the functional characterization of selected ABC-type transporters will be presented using bioinformatic analyses, heterologous gene expression systems and active efflux measurements.

3. Methods and Materials

3.1. Bacterial strains and plasmids

Bifidobacterium longum subsp. *longum*^{T} JCM 1217 (NC_015067.1) was obtained from the NCIMB culture collection (NCIMB 702259), U.K. *B. longum* JCM 1217 cultures were propagated in BY medium (Degnan & Macfarlane, 1993) supplemented with 1% Glucose (Sigma) (BYG medium), incubated at 37°C in an anaerobic chamber (Forma Scientific, Model 1024), in an atmosphere of 5% H_2, 10% CO_2 and 85% N_2, for 24-48 hours. *Lactococcus lactis* NZ9000 and NZ9700 and the expression vectors pNZ8048 and pNZE8048 were obtained from Abelardo Margolles, Spain. *L. lactis* cells were propagated in 1% GSM17 (M17 medium (Sigma Aldrich) supplemented with 1% Glucose and Sucrose (Sigma)) and grown aerobically at 30°C without agitation, for 24 hours, with or without the addition of 5 µg/ml Chloramphenicol (Chl) for plasmid selection and recombinant screening.

3.2. Genomic and plasmid DNA extractions

B. longum subsp. *longum* JCM 1217 genomic DNA was isolated from cultures grown for 18 hours in BYG medium. Briefly, 2 – 4 ml of the cultures was centrifuged and the pellets treated with 200 µl of pre-lysis buffer [20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1.2 % Triton X-100, 20 mg/ml Lysozyme (Sigma)] before incubating at 37°C for 30 min with agitation. Proteinase K (Fermentas) (1 µl) was added and the reaction re-incubated at 37°C with agitation for 30 min. The genomic DNA was purified using the Genomic DNA purification kit (Fermentas) as per the manufacturer's instructions with the addition of 0.5 µl RNaseA (Fermentas) to the final resuspension.

Gene cloning and expression was carried out using the Nisin Controlled Expression System (NICE) (de Ruyter *et al.*, 1998). For cloning, pNZ8048 plasmid DNA was isolated from 18 hr cultures of NZ9000 (pNZ8048), grown aerobically in 1 %GM17 + 5 µg/ml Chl at 30°C. Cells were treated using the same pre-lysis step as described for the genomic DNA extraction and then using the alkaline lysis method (Ish-Horowicz & Burke, 1981) with the addition of 0.5 µl RNaseA (Fermentas) to the final resuspension step.

3.3. *B. longum* erythromycin induction and total cellular RNA extraction

Total cellular RNA was extracted from *B. longum* JCM 1217 cells grown using standard growth conditions (uninduced) as well as from cells grown in standard conditions with the addition of erythromycin (erythromycin induced) using the gradient plate method (Szybalski, 1952). For the bottom layer of the plates 10 ml of BYG agar containing 1 µg/ml erythromycin was used. The top layer comprised 10 ml of BYG agar without erythromycin. The prepared gradient plate was reduced in the anaerobic chamber for 4 hours before spread plating 200 µl of an 18 hour culture of *B. longum* on the surface. The plate was incubated anaerobically at 37°C for 3 – 5 days until a lawn of bacteria had grown. The lawn was then washed off in BYG broth and the cells replated sequentially on fresh gradient plates of 3, 5 or 7 µg/ml erythromycin. The final culture was then washed into 70 ml reduced BYG containing 0.1 µg/ml erythromycin and grown anaerobically for 18 hrs at 37°C.

For extracting total cellular RNA from *B. longum* cells the standard protocol for Gram positive lactic acid bacteria was adapted to work on these cells (Magni *et al.*, 1995). The following protocol was designed. The 70 ml induced and uninduced 18 hr cultures were centrifuged at 10 000g for 15 min at 4°C. Cell pellets were resuspended in a solution containing 200 µl 10 % SDS (Sigma); 2 ml water-equilibrated 1:1 phenol-chloroform isoamyl-alcohol (chloroform:isoamyl-alcohol 24:1 $^{v}/_{v}$ + phenol (pH 5.5) equal volume); 2 ml of resuspension buffer (for 100 ml: 0.272 g Na-acetate (Sigma), 0.2 ml 0.5 M EDTA, pH 5.5 with glacial acetic acid (Sigma)). The resuspended pellets were then incubated at 70°C for 10 min, centrifuged at 6000g for 5 min and the pellets discarded. This procedure was repeated twice on the supernatant. The remaining total cellular RNA was precipitated by the addition of 1:10 volume of 3 M Na-acetate (pH 5.5) and 2.5x the volume of cold, absolute ethanol (abs EtOH), and incubated for up to 18 hrs at - 20°C. The tubes were then centrifuged for 30 min at 14 000g at 4°C. The resulting pellets were air-dried and resuspended in 200 µl sterile, distilled water (dH_2O). The residual genomic DNA was removed by treating with DNase (Roche) as per kit instructions (1 U/µg nucleic acid, 18 hrs at 37°C). The DNase was removed by resuspending the reaction mix in an equal volume of phenol-chloroform isoamyl-alcohol then centrifuging at 14 000g for 10 min. The remaining phenol was removed by adding an equal volume of choloform isoamyl-alcohol (24:1) and then centrifuging at 6000g for 5 min. The remaining pure, total cellular RNA was precipitated for up to 18 hrs at - 20°C as described above, followed by centrifugation at 14 000g for 30 min at 4°C and the resulting pellet was resuspended in 50 µl sterile dH_2O and

stored at - 80°C. All RNA samples were checked for contaminating DNA by direct PCR using the *Bifidobacterium* sp. specific 16S rRNA gene primers (Table 2.1).

3.4. cDNA conversions

All cDNA conversions were carried out as per kit instructions using the ImProm-II Reverse Transcription System (A3800) (Promega) using the following conditions: 1 µg RNA, 0.5 µg random hexamer primers, 6 mM $MgCl_2$, incubation at 42°C for 2 hours. All conversions were carried out in duplicate and pooled after confirming the conversion by PCR analysis using the *Bifidobacterium* sp. specific 16S rRNA primers (Table 2.1). All PCR products, DNA, RNA and plasmid extractions, as well as, restriction enzyme digest were checked by electrophoresing through a 0.8% agarose gel at 80 V with ethidium bromide (EtBr) staining and were visualised on a Gel Doc System (BioRad).

Table 2.1. Oligonucleotide primers used.

Primer name	Primer sequence (5' – 3')	Region targeted	Size	Source
BLLJ_1838-7F	CTGAACAAGTGCGTGAGGC	BLLJ_1838-7 Intergenic	410 bp	This study
BLLJ_1838-7R	CCAGCGTGGTGATGTCTG			
BLLJ_1837-6F	GCGAAGACGAGCTGATAAGCG	BLLJ_1837-6 Intergenic	420 bp	This study
BLLJ_1837-6R	CCAAAGTCCAGGCGATGTG			
BLLJ_1496-5F	GTGCACAACGCGAATTTGAAC	BLLJ_1496-5 Intergenic	350 bp	This study
BLLJ_1496-5R	AGCAGAATCACGCCGAACTTG			
BLLJ_1495-4F	TACACCAACGACACCGATACG	BLLJ_1495-4 Intergenic	413 bp	This study
BLLJ_1495-4R	CAGGATGTAGAGCAGGTAGCCC			
BLLJ_0511IF	GCCGACTTCGAAGGTGATG	BLLJ_0511 Internal Fragment	469 bp	This study
BLLJ_0511IR	CCTCCAACGTGATGAGCTACC			
Bif 164F	CATCCGGCATTACCACCC	*Bifidobacterium* sp. spec 16S rRNA gene	520 bp	(Langendijk *et al.*, 1995)
Bif 662R	CCACCGTTACACCGGGAA			
BLLJ_1837LF	GGAAACACATGTTTCGCATCATG	BLLJ_1837 and BLLJ_1836 genes	3968 bp	This study
BLLJ_1836LR	CGAGTAAAGCTTATCCGCTAATC			
BLLJ_1495LF	AAGGAGGAGAACCCATGGGCGATACC	BLLJ_1495 gene	1994 bp	This study
BLLJ_1495LR	TTCTTGAAACACTAGTCGTTGCTCATCG			

* Underlined nucleotides indicate restriction site listed in the section 3.6.

3.5. Reverse transcriptase – polymerase chain reaction

All the oligonucleotide primers used in this experiment (Table 2.1) were designed using the genome sequence of *B. longum* NCC2705 (NC_004307) to target the gene homologues in *B. longum* JCM 1217. The PCR reactions were carried out using the GoTaq PCR kit (Promega) with the following conditions for a 25 µl reaction: 0.4 mM dNTP mix (Promega), 1.5 mM

$MgCl_2$, 0.5 mM of each oligonucleotide primer, 0.5 U GoTaq, 2 µl cDNA as template or 100 ng genomic DNA for the genomic DNA control, or 500 ng RNA for the RNA controls. The PCR reactions were amplified using the following conditions in a GeneAmp 9700 thermocycler (Applied biosystems): Initial denaturation (95°C for 5 min) followed by amplification using 30 cycles of denaturation (95°C for 30 sec); primer annealing (60°C for 30 sec); elongation (72°C for 1 min); and a final elongation (72°C for 10 min). All PCR products were electrophoresed through a 2 % agarose gel at 80 V and were visualised on a Gel Doc System (BioRad) using EtBr staining.

3.6. Gene cloning and expression

Escherichia coli KAM3 (Morita *et al.*, 1998), an *acr*AB mutant derivative of *E. coli* TG1 and *E. coli* KAM32 (Chen *et al.*, 2002) a mutant derivative of KAM3 lacking the *ydh*E gene, were propagated in Luria-Bertani broth at 37 °C with lateral shaking (Sambrook & Russell, 2001). Agar (Merck) was incorporated into solid media at a concentration of 1.5% ($^{w}/_{v}$) for plating. pTZ57R/T (Fermentas) was used as a cloning vector and expressed in these strains.

The putative MDR operon BLLJ_1837-1836 was PCR amplified using the High Fidelity PCR Enzyme Mix (Fermentas), from *B. longum* JCM 1217 genomic DNA using the oligonucleotide primers BLLJ_1837LF and BLLJ_1836LR which include PscI and HindIII restriction sites respectively (Table 2.1) to facilitate cloning into pNZ8048 by translational fusion to the nisin inducible promoter. The following PCR conditions were used: Initial denaturation (95°C for 5 min), followed by amplification for 10 cycles: Denaturation (95°C for 30 sec); primer annealing (53°C for 30 sec); elongation (68°C for 4 min); then amplification for 20 cycles; Denaturation (95°C for 30 sec); primer annealing (54°C for 30 sec); elongation (68°C for 4 min + 10sec/cycle); and a final elongation step (68°C for 10 min). The BLLJ_1837-6 PCR fragment was purified using the PCR cleanup kit (Biospin), simultaneously digested for 18 hrs at 37°C as per kit instructions with PscI and HindIII (Fermentas), and purified again. The pNZ8048 vector was simultaneously digested for 18 hrs with NcoI and HindIII (Fermentas) and purified using the PCR cleanup kit. The prepared insert and vector were ligated for 18 hrs at 22°C using T4 DNA ligase (Fermentas) as per the manufacturer's instructions.

Competent NZ9000 cells were prepared for electroporation. Cells were electrotransformed using the following conditions: 10^{10} cells/ml (≈ 100 µl of prepared cells); a single pulse at: 2.5 KV (E=12.4 KV/cm), 200 Ω, 25 µF (Pulse length of 4.6 ms), in 2 mm cuvettes (Eurogentec), using 1 µg of plasmid DNA. Cells were immediately resuspended in 5 ml of pre-warmed 1% GSM17 broth containing 0.1 % nisin supernatant and incubated at 30°C for 1 hr. Chloramphenicol (5 µg/ml) was then added and the culture incubated for another hour at 30°C. Cultures were then centrifuged and plated on 1% GSM17 agar including 5 µg/ml chloramphenicol and 0.1 % nisin supernatant, and incubated at 30°C for 24 – 48 hours (Papagianni *et al.*, 2007). Functional clones were selected by first screening for the correct insert using colony PCR and subsequently spotting cell suspensions onto 1% GSM17 agar including 0.1 % nisin supernatant with various sub-lethal concentrations of erythromycin, tetracycline or streptomycin.

The gene BLLJ_1495 was cloned in the same manner as described above, using the primers BLLJ_1495LF and BLLJ_1495LR to target the BLLJ_1495 gene in *B. longum* JCM 1217. The primers contained NcoI and SpeI restriction sites respectively (Table 2.1) to facilitate cloning into pNZ8048 by translational fusion to the nisin inducible promoter. The target fragment was PCR amplified using Supertherm Taq (Southern BioCross) using the kit instructions and including 4 mM $MgCl_2$. The amplification reaction was carried out using the following conditions: Initial denaturation (95°C for 5 min), amplification using 30 cycles of: Denaturation (95°C for 30 sec); primer annealing (56°C for 30 sec); elongation (72°C for 1 min 30 sec); and a final elongation step (72°C for 10 min). The resulting 2 kb band was electrophoresed through a 0.8 % agarose gel, excised and purified using the Gel extraction kit (Biospin). The product was ligated into the pTZ57R/T vector using the InsTAclone kit (Fermentas) and transformed into competent *E. coli* JM109 cells (Dagert & Ehrlich, 1979). Plasmid DNA was extracted from all resulting clones using the alkaline lysis (Ish-Horowicz & Burke, 1981). The resulting clone bank was linearised by an SpeI restriction digest (Fermentas). Since the BLLJ_1495 gene contains internal NcoI restriction sites, the resulting plasmid DNA was partially digested with NcoI and the resulting 1996 bp band was excised from a 0.8 % agarose gel and purified using the Gel extraction kit (Biospin). The vector pNZ8048 was digested with NcoI and SpeI and purified using the PCR cleanup kit (Biospin) before ligating the BLLJ_1495 gene into it using T4 DNA ligase (Fermentas), for 18 hrs at 22°C. The ligation reactions were electrotransformed into *L. lactis* as previously described.

The clones were first screened for insert by colony PCR analysis and then for function by spotting on antibiotic containing plates, as described above.

To construct a single gene clone of BLLJ_1837, a deletion from the two gene construct previously made (BLLJ_1837-6) was carried out. A restriction map of the two gene operon showed that a KspAI and HindIII digest would excise 1614 bp of the 1830 bp BLLJ_1836 gene from the two gene construct, leaving only 200 bp of the BLLJ_1836 gene at the 5' end. The double digest was carried out and the resulting fragment containing only BLLJ_1837 and 200 bp of BLLJ_1836 was gel excised, purified, the sticky ends polished with T4 DNA polymerase (Fermentas) and then religated using T4 DNA ligase (Fermentas). The pNZ8048 containing BLLJ_1837 plasmid was electrotransformed, as previously described, into competent *L. lactis* NZ9000 cells and clones were checked and screened as previously described.

3.7. E-Tests

E-tests on the *L. lactis* cells expressing the heterologous genes (AB Biodisk, Solna, Sweden) were carried out as per the manufacturer's instructions using the protocol outlined by Margolles *et al*., (2005) with the following changes: 0.1 % culture supernatant from the nisin producing strain *L. lactis* NZ9700 was added to induce transcription and incubated for 60 min at 30°C; the cultures were diluted back to $OD_{660} \approx 0.4$ and 600 µl of each culture was added to 18 ml of 1% GSM17 sloppy agar before layering on top of 30 ml 1% GSM17 agar + 0.1 % nisin containing supernatant. E-test strips were applied to the surface of the plates and the plates incubated for 48 hrs at 30°C before reading the results as per the manufacturer's instructions (Margolles *et al.*, 2005).

3.8. Efflux assays

The efflux assays were carried out by modifying the various protocols commonly used for efflux assays using whole *L. lactis* cells (Bolhuis *et al.*, 1994; Bolhuis *et al.*, 1995; Van Den Berg Van Saparoea *et al.*, 2005). Pre-cultures of the *L. lactis* cells harbouring the various gene constructs were grown for 18 hrs in 10 ml of 1 % GM17 + 5 µg/ml Chl, at 30°C. These cultures were then re-inoculated into 100 ml of 1 % GM17 + 5 µg/ml Chl to a starting OD_{660}

of ≈ 0.1. The cultures were grown at 30°C until an OD_{660} of 0.6 – 0.8 was reached. At this point the cultures were split into 2 X 40 ml cultures and 40 µl of nisin containing culture supernatant (0.1%) from the nisin producing strain *L. lactis* NZ9700 was added to induce transcription (Van Den Berg Van Saparoea *et al.*, 2005) was added to one of each culture. For the control cultures, no nisin containing supernatant was added (1 nisin induced and 1 uninduced). Both cultures were grown for another 90 min at 30°C to allow for gene expression. The cells were then harvested by centrifugation at 4000 rpm for 5 min and resuspending the pellets in 10 ml 50 mM Potassium-HEPES (pH 7.0) + 5mM $MgSO_4$ (Sigma). To de-energise the cells, 50 µl of a 100 mM 2,4-Dinitrophenol solution (0.5 mM) (Sigma) was added and the cells were incubated for 30 min at 30°C. To remove the dinitrophenol the cells were washed four times with 5 ml with 50mM Potassium-HEPES (pH7.0) + 5mM $MgSO_4$, with centrifugation at 4000 rpm for 5 min between each step. To measure the rate of efflux the washed cells were diluted to an OD_{660} of ≈ 0.5 in 15 ml of HEPES buffer and 22.48 µl of Bisbenzimide Hoechst 33342 (1 µM) (Sigma) was added to each culture. Aliquots (200 µl) of each strain were added to the wells in black microtitre plates (Amersham). Excitation (365 nm) and emission (457 nm) measurements were recorded at 2 min intervals for a total of 20 min using the UV optical kit in the Modulus Microplate Fluorometer 9300 (Turner BioSystems). The cells were re-energised at this point by the addition of 10 µl of a 25 mM glucose solution was and measurements were recorded for 6 min at 2 min intervals. Finally, 1.05 µl of 100 mM *ortho*-vanadate (0.5 mM final) (Sigma) was added to each well to inhibit all cellular ATPases and measurements were recorded for 20 min at 2 min intervals. The same protocol was used for ethidium bromide as a substrate at 10 µM and using the blue optical kit (Margolles *et al.*, 1999).

4. Results and Discussion

4.1. Bioinformatics

The first *B. longum* subsp. *longum* genome to be sequenced and published in the public domain was that of *Bifidobacterium longum* subsp. *longum* NCC2705. For the selection of genes of interest to be included in this study a fully sequenced genome would have been preferable, however, the genome sequence of *Bifidobacterium longum* subsp. *longum*^{T} JCM 1217, the strain used in this study, has only recently become available (Fukuda *et al.*, 2011). As an alternative, a list of all the putative transporters in *Bifidobacterium longum* subsp. *longum* NCC2705 was consulted at http://www.membranetransport.org/. This list (Appendix Figure A2.1) identifies genes which may code for transporter proteins based on conserved domain identity. Since this study is focussed on ABC-type transporters, only those transporters in the list were considered for future work. Of particular interest were the two gene clusters identified as BL0160-2-3 and BL1765-6-7 (Table 2.2). Both of these gene clusters contain a putative transcriptional regulator and two adjacent genes, which are each classified as putative multidrug resistance genes which contain both an ATP-binding cassette (ABC) and a membrane domain as one polypeptide.

Table 2.2. Open reading frames (ORF) investigated in this study.

ORF in *B. longum* NCC2705	Accession number in *B. longum* NCC2705	ORF in *B. longum* JCM 1217	Accession number in *B. longum* JCM 1217
BL0160	NP_695379.1	BLLJ_1838	YP_004221594.1
BL0162	NP_695381.1	BLLJ_1837	YP_004221593.1
BL0163	NP_695382.1	BLLJ_1836	YP_004221592.1
BL1765	NP_696912.1	BLLJ_1496	YP_004221255.1
BL1766	NP_696913.1	BLLJ_1495	YP_004221254.1
BL1767	NP_696914.1	BLLJ_1494	YP_004221253.1

Since MarR-type transcriptional regulators have previously been shown to regulate ABC-type transporters (Lubelski & Driessen, 2007; Putman *et al.*, 2000), it is intriguing that the genome arrangement for both gene clusters is identical with two ABC-type transporter genes and a putative MarR-type transcriptional regulator upstream. Using the BLASTP algorithm at the National Center for Biotechnology Information at www.ncbi.nlm.nih.gov and the newly published genome sequence for JCM 1217, the gene homologues for the gene clusters BL0160-2-3 and BL1765-6-7 (including the putative regulators) were identified, as

BLLJ_1838-7-6 and BLLJ_1496-5-4 respectively (Figure 2.2) (Table 2.2) and were used as the genes of interest in this study.

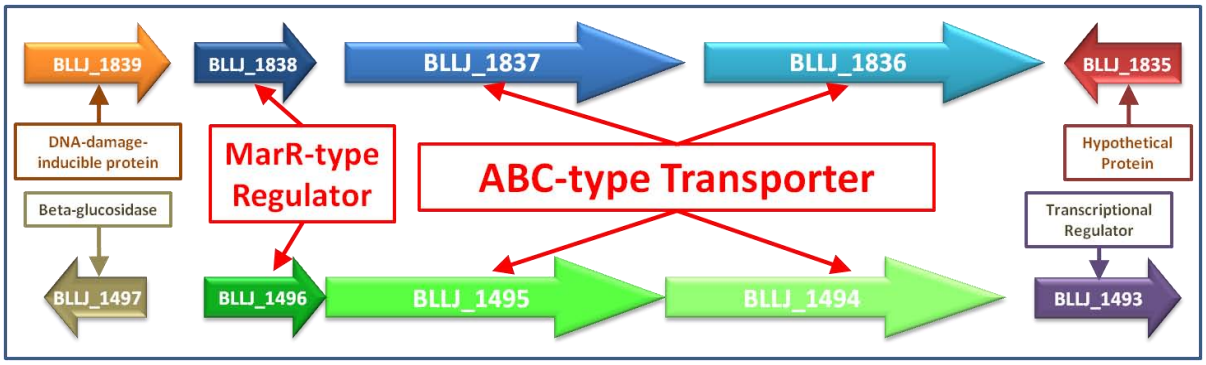


Figure 2.2. Genome arrangement of the gene clusters BLLJ_1838-7-6 and BLLJ_1496-5-4, in *B. longum* JCM 1217, used in this study.

To confirm whether these genes possessed the requisite domains necessary to function as ABC-type transporters, further bioinformatic analysis was carried out. Each of the structural genes were subjected to a conserved domain search at www.ncbi.nlm.nih.gov using the CDD algorithm (Marchler-Bauer *et al.*, 2009; Marchler-Bauer *et al.*, 2011) (Appendix Figure A2.2). The results of the CDD search showed that all four of the selected genes individually possessed all of the conserved domains needed to be functional transporters. To determine the conservation of these domains a multiple protein sequence alignment of all four ABC-type transporters was constructed using ClustalW (Thompson *et al.*, 1994) (Figure 2.3).

The results of the conserved domain architecture search, as well as, the multiple sequence alignments showed that all four of these genes individually possessed the Walker A (GXXXXGK(T/S)), Walker B (hhhhD) and the ABC-signature motif (LSGGQ) (Rees *et al.*, 2009; Walker *et al.*, 1982). The Walker A and B motifs allow for the binding and subsequent hydrolysis of ATP, and the signature motif is conserved in all ABC-type transporters. To identify potential transmembrane domains (TMD), of which ABC-type transporters have 6 each, necessary to form the channel where antimicrobial substances are bound and then transported out of the cell. Hydropathy plots were, therefore, constructed using the KD-scale (Kyte & Doolittle, 1982) (Figure 2.4) to identify any potential transmembrane domains based on the hydropathy of the amino acid sequence and composition. Possession of all these domains would putatively allow each gene to function as a full ABC-type transporter individually (Higgins, 1992). Since most functional ABC-transporters function as dimers (van Veen *et al.*, 2000), it is possible that two structural genes would lie next to each other

and their resulting proteins combine to form one functional transporter comprising 12 TMD's and 2 ATPase domains.

The results from these analyses showed that all four genes possessed all the necessary conserved domains to function independently as ABC-type transporters. Even though these genes individually possessed all the domains needed to function as independent transporters the multiple protein sequence alignment indicated that they are all significantly different in their amino acid composition and are therefore not merely duplications of each other, but, are four different ABC-type transporters, each with the potential to function as a transporter in their own right.

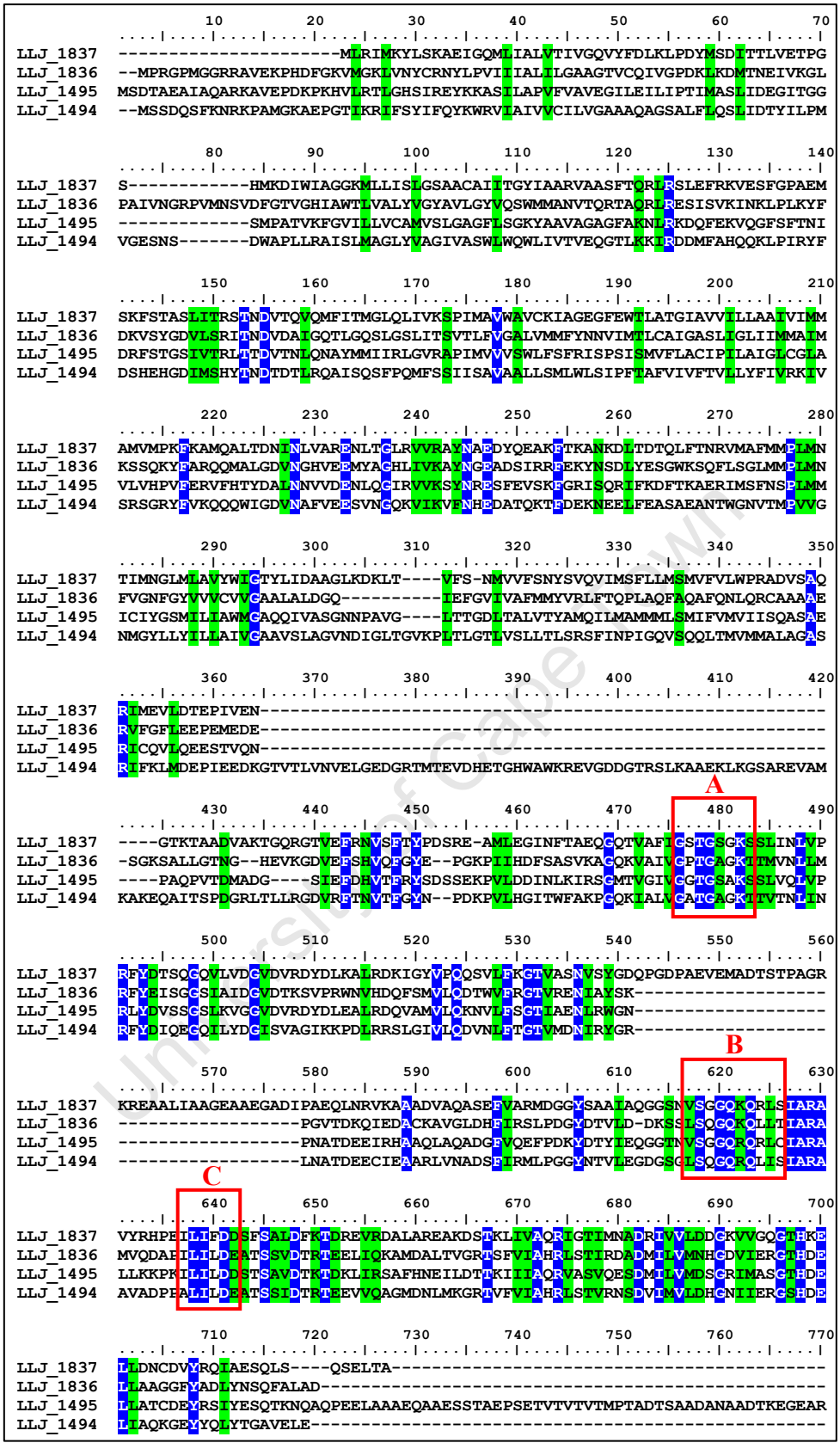


Figure 2.3. Multiple protein sequence alignment of BLLJ_1837, 1836, 1495, 1494 using Clustal W. Similar amino acids are shaded green and identical amino acids are shaded blue. The characteristic ABC-transporter conserved domains are indicated in red boxes. (A) Walker A domain, (B) ABC signature motif, (C) Walker B domain.

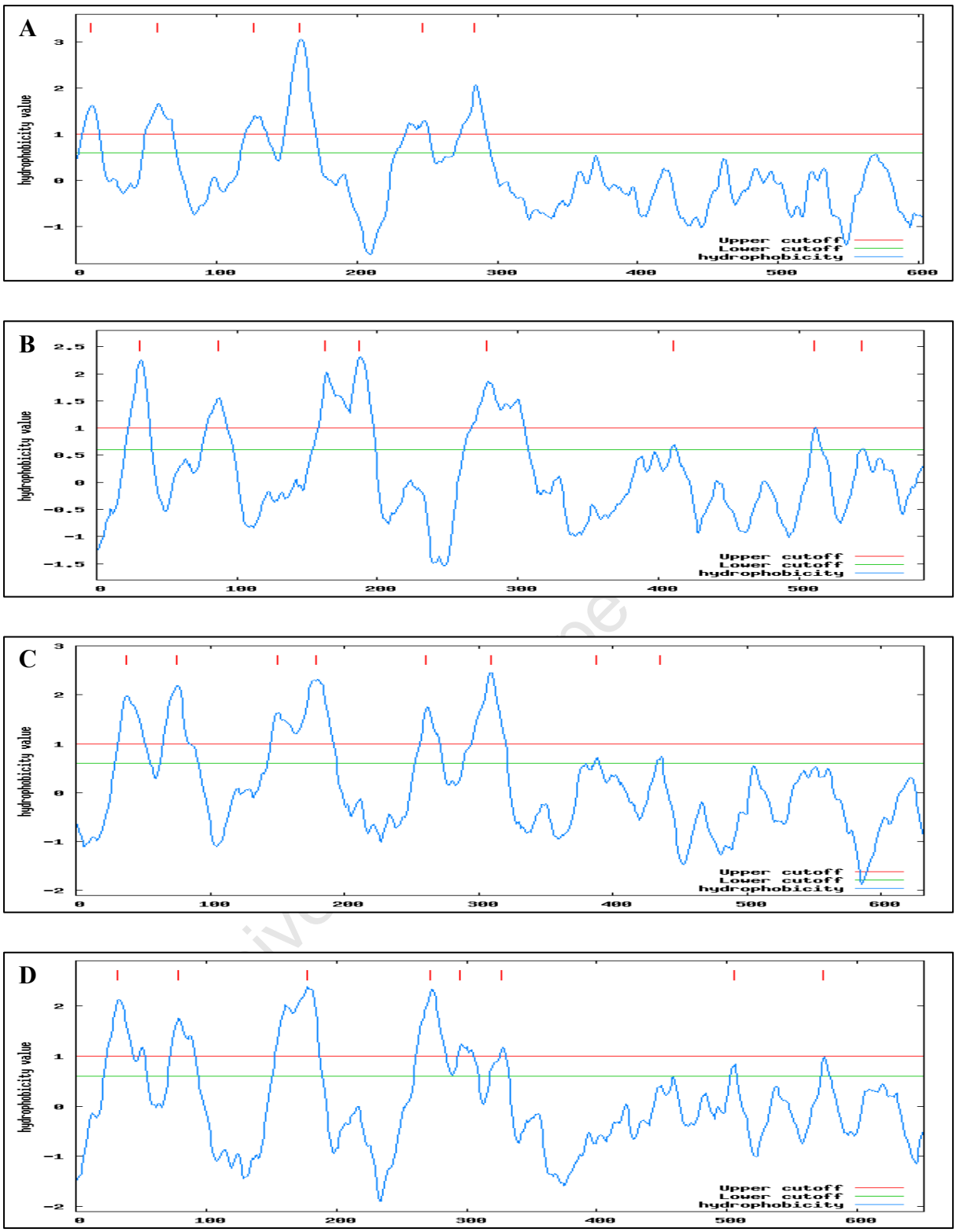


Figure 2.4. Hydrophobicity plots of (A) BLLJ_1837, (B) BLLJ_1836, (C) BLLJ_1495, (D) BLLJ_1494. Red bars above the major peaks indicated the probable TMD's. Plots were constructed using the KD-scale plot (Kyte & Doolittle, 1982), using TopPred 0.01 software.

4.2. Genome arrangement

The observed genome arrangement of both gene clusters is interesting as they both include a putative transcriptional regulator and two structural genes – each of which could possibly act independently as an efflux transporter. This arrangement of functionally related genes being clustered together with a putative regulator is indicative of a possible operon-like structure. Reverse transcriptase – polymerase chain reaction (RT-PCR) experiments were conducted to investigate the possibility that these genes are transcriptionally linked as an operon (transcribed as a single transcript).

For this experiment it is important that the conditions under which these genes are investigated be reflective of the conditions under which these genes would be transcribed *in vivo*. Since these genes are putative multidrug resistance genes, their transcription may not be carried out under normal cellular growth conditions free from antimicrobial challenge. Previous experiments on this strain (Price *et al.*, 2006) showed that pre-exposure of *B. longum* JCM 1217 to erythromycin lead to a significant increase in its minimum inhibitory concentration (MIC) of this bacterium to erythromycin on subsequent challenge. In this study total, cellular RNA was, therefore, harvested from cells grown under normal cellular growth conditions, as well as, cells grown with prolonged exposure to erythromycin. This RNA was subsequently converted to cDNA for RT-PCR analysis. PCR primers were designed, for both gene clusters, to amplify the intergenic region between the putative regulator and the first ABC-type structural gene, as well as, the intergenic regions between the two structural genes (Figure 2.5). In a previous study by Price *et al.*, (2006), the *ctr* gene (BL1102), belonging to the family of secondary transporters, was identified and functionally classified as a Na^+-deoxycholate transporter, which specifically transports only Na^+-deoxycholate. The *ctr* gene should not respond to erythromycin challenge since erythromycin is not a substrate for this transporter. To ensure that any transcriptional changes which may occur as a result of erythromycin challenge are specific in response to this erythromycin exposure, the gene homologue of BL1102 in *B. longum* JCM 1217 (BLLJ_0511) was included as a comparative control in this experiment. The RT-PCR was conducted using the following nucleic acid targets: genomic DNA extracted from JCM 1217, as well as, the cDNA produced from the uninduced and the erythromycin induced total RNA extractions (Figure 2.6).

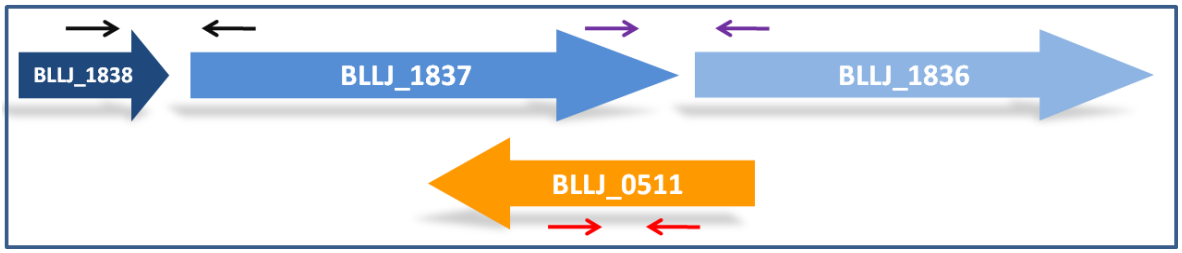


Figure 2.5. Position of primers designed to amplify the intergenic regions between the genes of interest, as well as, the internal fragment region of the control *ctr* gene. The black and purple arrows indicate the two intergenic regions targeted and the red arrows indicate the internal fragment of the *ctr* gene.

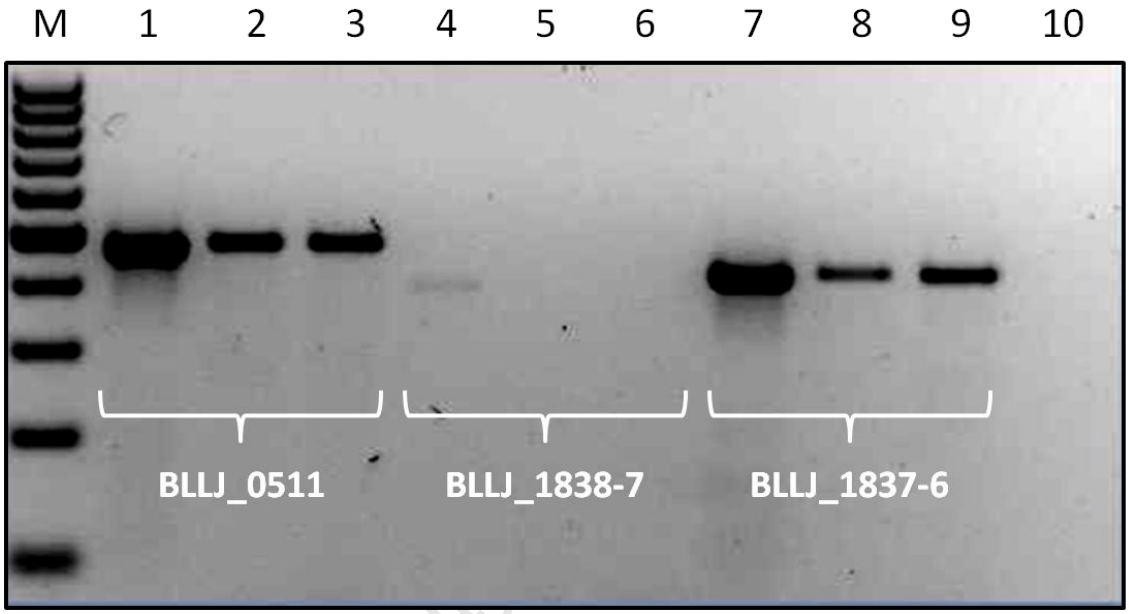


Figure 2.6. RT-PCR of the intergenic regions BLLJ_1838-7 and BLLJ_1837-6 and the *ctr* gene fragment. Lanes 1, 4, 7, genomic DNA target; lanes 2, 5, 8, uninduced cDNA target; lanes 3, 6, 9, erythromycin induced cDNA target; lane 10, no template control.

Since no amplicon was generated from the BLLJ_1838-7 intergenic PCR, the results obtained clearly indicated that that the gene cluster BLLJ_1838-7-6 is transcribed as a two gene operon comprising the two structural genes BLLJ_1837-6, excluding the putative transcriptional regulator BLLJ_1838. The BLLJ_1838-7 intergenic PCR was repeated with double the amount of cDNA to ensure there was no error due to primer specificity, but, even using these highly favourable conditions, no amplicons were observed. The operon was constitutively transcribed under both normal growth conditions and erythromycin induced conditions. Equal molar concentrations of RNA were converted to cDNA for both the uninduced and the erythromycin induced conditions in duplicate, and equal volumes of cDNA were used for the RT-PCR, as well as, equal loading of PCR products on gels. Therefore there appeared to be a slight semi-quantitative increase in transcription levels of this operon under erythromycin induced conditions (Figure 2.6, lane 9), which could be seen

as a visible increase in band intensity when compared to the uninduced condition (Figure 2.6, lane 8). These results were reinforced by the unchanged transcription levels of the *ctr* gene, even in the erythromycin induced condition (Figure 2.6, lanes 2 and 3). To ensure these results were accurate, control experiments were conducted to ensure the extracted RNA was free of residual DNA. These experiments showed no presence of DNA even when 500 ng of RNA was used as template (data not shown). Internal fragment primers were also designed to ensure all the genes being studied were represented in the cDNA produced from the extracted RNA. These experiments showed that all the genes were present in sufficient concentrations to be detectable (data not shown).

For the second gene cluster (BLLJ_1496-5-4) the same procedure as described above was followed. Figure 2.7 shows the primer positions of the intergenic regions and Figure 2.8, the RT-PCR results of this region.

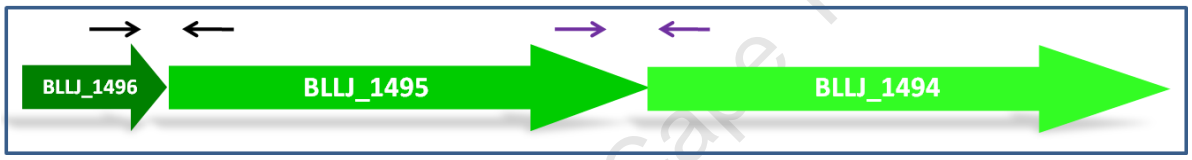


Figure 2.7. Position of primers designed to amplify the intergenic regions for BLLJ_1496-5-4. The black and purple arrows indicate the two intergenic regions targeted.

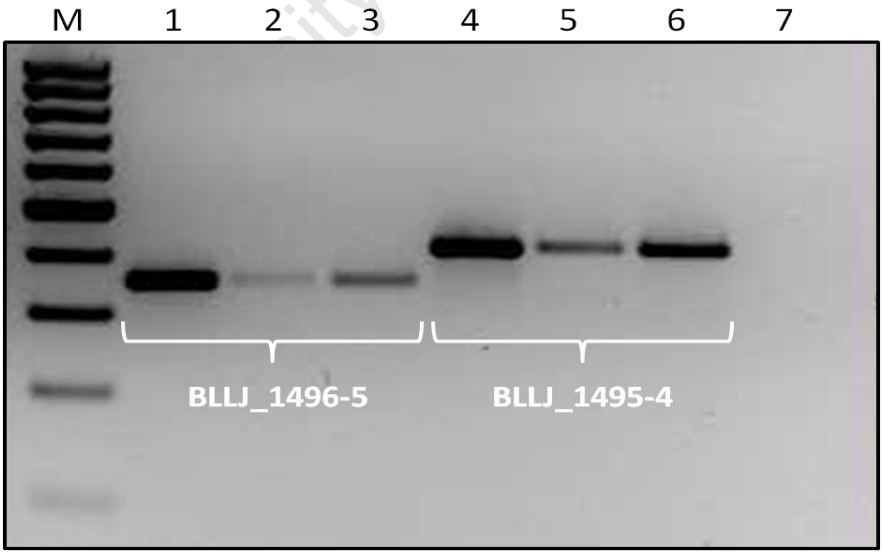


Figure 2.8. RT-PCR of the intergenic regions between BLLJ_1496-5 and BLLJ_1495-4. Lanes 1 and 4, genomic DNA target; lanes 2 and 5, uninduced cDNA target; lanes 3 and 6, erythromycin induced cDNA target; lane 7, no template control.

Since amplicons are produced from both intergenic region PCR reactions, the results indicated that the gene cluster BLLJ_1496-5-4 was transcribed as a three gene operon

comprising the two ABC-type structural genes BLLJ_1495-4 and including the putative transcriptional regulator BLLJ_1496. The operon was transcribed under both normal growth conditions and erythromycin induced growth conditions. As was observed in the first RT-PCR results, there appeared to be a slight semi-quantitative increase in transcription levels of this operon under erythromycin induced conditions (Figure 2.8 lanes 3 and 6), which could be seen as a visible in increase in band intensity compared to the uninduced condition (Figure 2.8 lanes 2 and 5).

4.3. Cloning of putative MDR genes and functional heterologous expression

Heterologous expression of membrane proteins is not easily accomplished and requires systems that can accommodate the added load on the host cell, as well as the necessary cellular machinery to functionally express the heterologous proteins and stably integrate them into the membrane. Initial attempts at cloning and functionally expressing these genes in systems such as *E. coli* KAM3 and KAM32 (Chen *et al.*, 2002; Morita *et al.*, 1998) were undertaken but were unsuccessful, even though numerous strategies were employed to improve stability and expression. This was possibly due to the heterologous expression of Gram positive membrane proteins in a Gram negative host. To increase the probability of obtaining a functional clone a Gram positive expression system was chosen.

Cloning the BLLJ_1837-1836 two gene operon into pNZ8048 was accomplished using translational fusion, by exploiting the ATG codon in the NcoI restriction enzyme site in the pNZ8048 vector (Figure 2.1). Since the BLLJ_1837-1836 gene sequence contains an internal NcoI restriction site, the forward primer used for PCR amplifying the gene from the *B. longum* JCM 1217 genomic DNA was designed to include a PscI restriction site, at the ATG start codon of the BLLJ_1837 gene, which produces complementary ends for cloning into a NcoI site. A HindIII restriction site was included in the reverse primer to facilitate directional cloning into the vector. Positive recombinant clones were screened for function by plating serial dilutions of these cultures on 1 % GM17 + 0.1 % nisin plates with varying sub-lethal concentrations of antibiotics, and the clone with the highest increase in resistance compared to the control (NZ9000 + pNZ8048) was selected for further work. This clone (**Clone 23)** (NZ9000 + pNZ8048:1837-6) was checked for the correct insert with restriction enzyme digests (Figure 2.9) (Table 2.2) and the DNA sequence verified with DNA sequencing analysis.

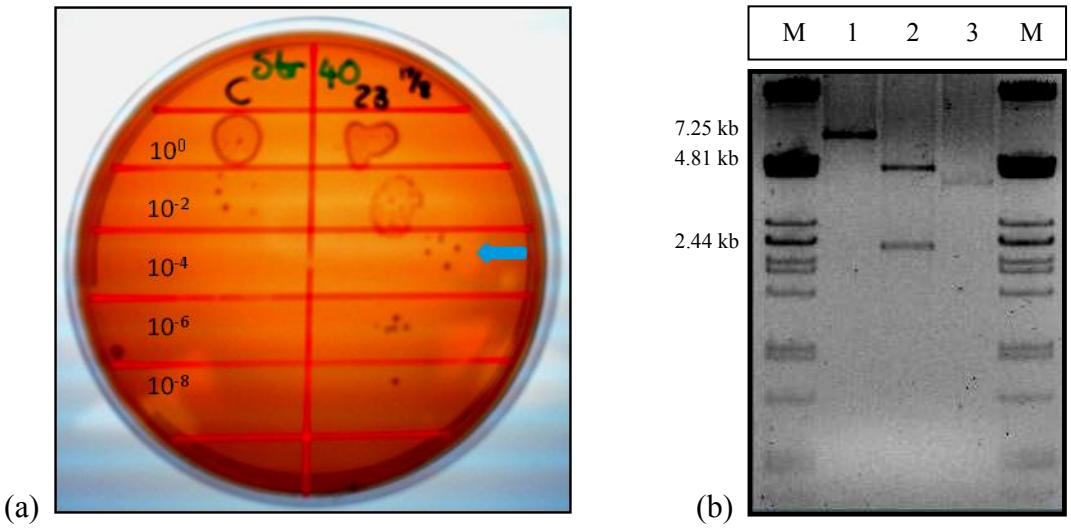


Figure 2.9. Functional clone selection and insert confirmation with restriction digests. (a) Example of plate spotting a serial dilution of bacteria (10^{0} – 10^{-8}) showing increased MIC levels of the selected **Clone 23** (blue arrow) compared to the control (C) plated on 1 % GM17 + 0.1 % nisin with 40 µg/ml streptomycin (Str 40). (b) Restriction digests of the selected **Clone 23**; M, λ DNA – PstI digest molecular weight marker, lane 1, XhoI digest; lane 2, SalI digest; lane 3, undigested pNZ8048 + BLLJ_1837-6 **Clone 23** plasmid DNA.

Table 2.2. List of recombinant gene constructs for heterologous expression in *L. lactis*.

Name of construct	*B. longum* JCM 1217 ORF being expressed
pNZ8048	None (vector without insert)
pNZ1837-6	BLLJ_1837 and BLLJ_1836
pNZ1837Δ6	BLLJ_1837 and (200bp 5' BLLJ1836)
pNZ1495	BLLJ_1495

To determine the individual contribution of each gene of the cluster BLLJ_1837-6 to the observed increase in MIC to antibiotics, a deletion of the BLLJ_1836 gene from the two gene construct already produced was carried out (Figure 2.10). Restriction enzyme digestion was used to excise all but 200 bp of BLLJ_1836 at the 5' end of the gene. This would produce a clone which is non-functional in BLLJ_1836 expression since it would lack 5 of the TMD's, as well as, both of the ATP-binding domains. Any MDR function observed from this construct would then be attributed to the activity of the BLLJ_1837 protein alone.

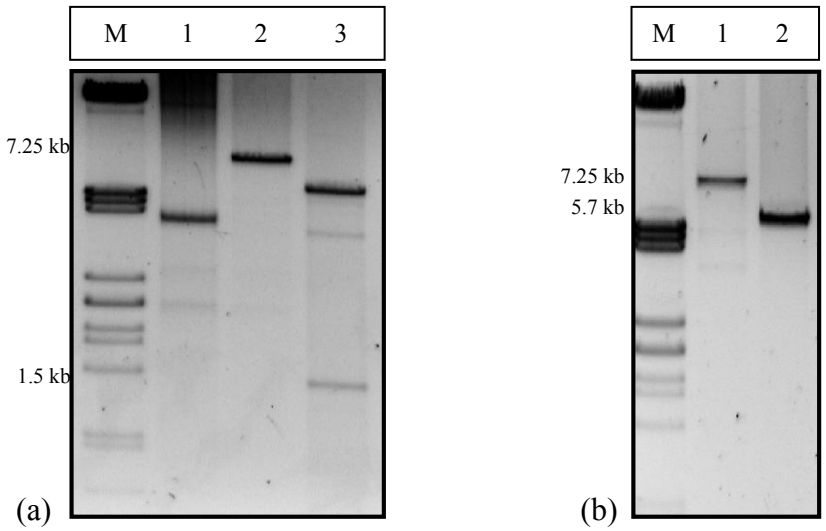


Figure 2.10. Agarose gels showing the construction of pNZ8048+BLLJ_1837Δ6. (a) Plasmid DNA showing the restriction digestion of BLLJ_1836. M, λ DNA – PstI digest molecular weight marker; lane 1, pNZ8048+BLLJ_1837-6 super-coiled plasmid DNA; lane 2, pNZ8048+BLLJ_1837-6 digested with KspAI to linearise (7.25 kb); lane 3, pNZ8048+BLLJ_1837-6 digested with HindIII and KspAI to excise the ≈ 1.5 kb fragment of BLLJ_1836 DNA. (b) Plasmid extraction from positive clones of BLLJ_1837. M, λ DNA – PstI digest molecular weight marker; lane 1, pNZ8048+BLLJ_1837-6 XhoI digest (linearised); lane 2, positive clone of pNZ8048+BLLJ_1837Δ6 (≈ 1.5 kb smaller).

From the above result, it can be seen that the BLLJ_1836 gene product was successfully deleted from the two gene construct and a stable, positive clone (NZ9000 + pNZ1837Δ6) was isolated.

Attempts to clone the two gene operon comprising BLLJ_1495-4 were conducted using a similar approach as above. Recombinant clones were obtained and were confirmed by colony PCR. The clones were sub-cultured but all exogenous DNA was subsequently lost from the clones. Repeated attempts at improving the clone stability, such as plating on antibiotics had no effect on increasing the insert stability. In an attempt to produce a stable clone of these genes, only one of the genes in the operon (BLLJ_1495) was cloned and expressed in this system. A stable, functional clone (NZ9000 + pZN1495) was isolated and the insert confirmed with colony PCR, restriction digests and DNA sequencing analysis as for all the other gene constructs. All of the gene constructs produced were subsequently screened for multidrug resistance function using the E-test method.

4.4. Functional expression of ABC-type transporters and multidrug resistance

To conclusively prove that the recombinant constructs produced in this study did in fact confer multidrug resistance (MDR) on the host, the ABC-type transporter genes were tested to ascertain if they conferred increased resistance to various structurally unrelated antimicrobial substances *in vivo*. To test this hypothesis, the constructs described above were all subjected to minimum inhibitory concentration analysis using the E-test method (Figure 2.11) (Margolles *et al.*, 2005). The antibiotics selected for this were erythromycin (macrolide), tetracycline (polyketide) and streptomycin (aminoglycoside) as they have previously been shown to be substrates of ABC-type transporters (Margolles *et al.*, 2005). An increase in MIC in response to two or more of these antibiotics would indicate an MDR phenotype.

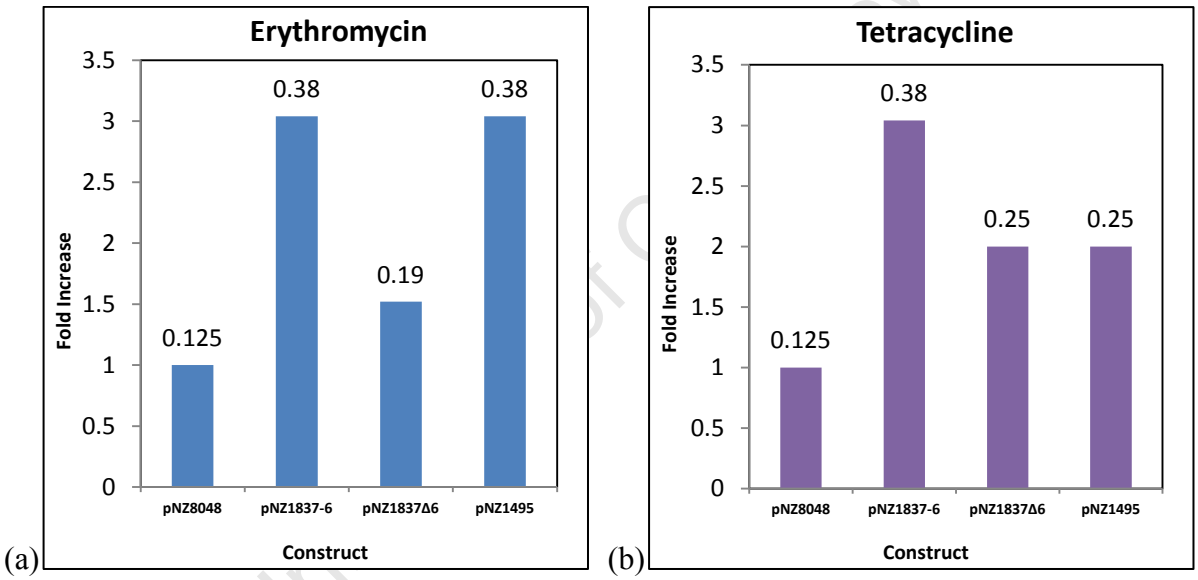


Figure 2.11. E-test MIC determinations of *L. lactis* cells harbouring the various gene constructs in this study compared to the host harbouring the vector without insert (pNZ8048). The Fold increase of all clones in response to (a) erythromycin challenge and (b) tetracycline challenge is plotted. Numbers above each bar indicate the MIC in µg/ml.

These results (Figure 2.11) conclusively show a MDR phenotype, as all of the constructs increase the resistance of the host cell to both erythromycin and tetracycline by 1.5 – 3 folds. It is interesting that the two gene construct BLLJ_1837-6 produces the same MIC as the single gene construct BLLJ_1495 in response to erythromycin challenge. The single gene construct BLLJ_1837Δ6, in response to erythromycin, produced an MIC which is exactly half that of the two gene parent construct, indicating that the phenotype produced is as a result of the equal contribution of both genes to the increased MIC. In response to tetracycline, the two gene construct BLLJ_1837-6 and the single gene construct BLLJ_1495 did not produce

the same level of resistance as was observed in response to erythromycin challenge. This indicated the poly-specific nature of MDR transporters for their substrates (Aller *et al.*, 2009; Davidson *et al.*, 2008). Even though MDR transporters have diverse substrate profiles the specificity of each transporter binding each substrate will determine the level of efflux, as well as, which transporters are recruited in response to antimicrobial challenge. In response to tetracycline, the single gene construct, BLLJ_1837Δ6, had an MIC which was lower by one fold compared to that of the two gene parent construct, BLLJ_1837-6. This showed that the phenotype results from a dual effect produced unequally by the contribution of both genes in this cluster. No increase in MIC was observed in response to challenge with streptomycin (results not shown), indicating it is not a substrate for any of these proteins.

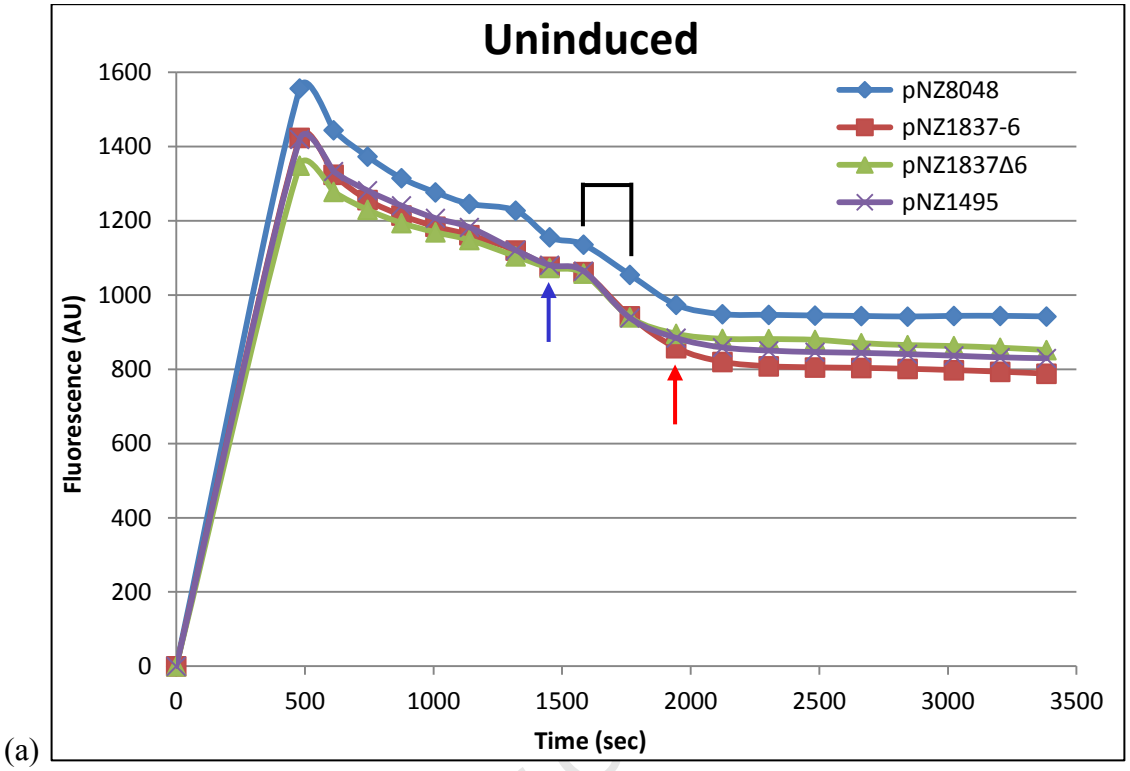
4.5. Active efflux

Efflux assays were designed and carried out on all the constructs produced in this study to ascertain whether the rate of active ATP-dependent efflux was increased in the presence of the heterologous *B. longum* putative ABC-type efflux proteins when expressed in *L. lactis*. The presence of the nisin inducible promoter allowed for the direct comparison of the cells harbouring the pNZ8048 vector with the respective ABC-type transporter genes in the induced and uninduced states (Figure 2.12).

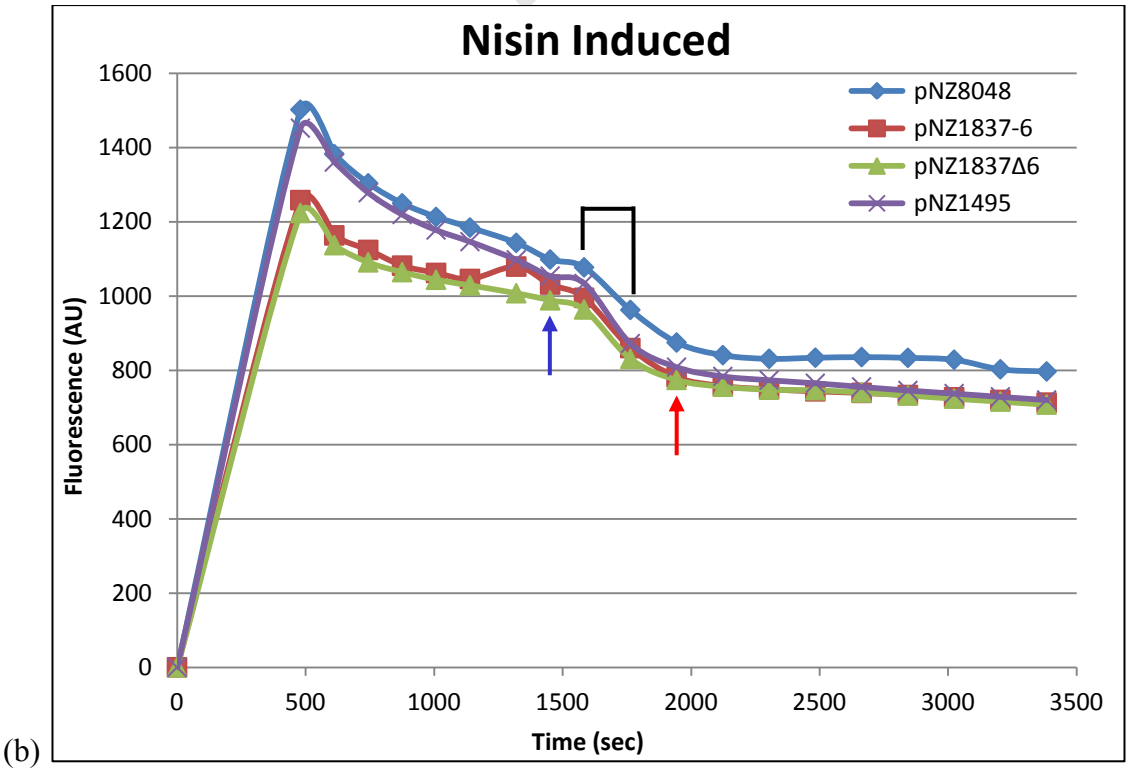
No visual increase in the levels of efflux can be directly observed from the results obtained. The primary reason for this is the high background of ATP-dependent efflux activity in the *L. lactis* expression system, which is known to possess highly efficient efflux systems (Bolhuis *et al.*, 1996; Margolles *et al.*, 1999; Zaidi *et al.*, 2008). In this background, it becomes difficult to note the contribution of the exogenous *B. longum* ABC-type transporters to the basal rate of efflux, even though it has previously been proven that they are functional and confer a MDR phenotype on these cells.

A notable decrease in the maximum accumulation of Hoechst 33342, by approximately 200 units observed at 480 seconds, can be seen in cells harbouring the exogenous ABC-type transporters BLLJ_1837-6 and BLLJ_1387Δ6, in the nisin induced condition. This may be due to some residual efflux of Hoechst 33342 during the uptake phase of the assay the ABC transporters themselves, which may still have some bound ATP. Alternatively, it may be due to the fact that the expression of genes encoding membrane proteins could alter the host

membrane structure and may structurally impede the influx of Hoechst 33342 in to cells to some extent which may differ from the host cells not expressing these exogenous genes.



(a)



(b)

Figure 2.12. Hoechst 33342 efflux from whole *L. lactis* NZ9000 cells harbouring the ABC-type constructs. (a) Cells not induced by nisin; (b) cells induced with nisin. Fluorescence values are represented in arbitrary units (AU). Blue arrows indicate the addition of glucose. Red arrows indicate the addition of ortho-vanadate. Black boxes indicate the time points used for calculating the rates of efflux.

Another interesting observation was that even though the cells were de-energised before the addition of the Hoechst 33342, there was a steady decrease in the fluorescence levels for all the constructs from the outset of the experiment. This may have been due to some residual efflux of the substrate by transporters which may still have had some energy reserves available or by transporters which were not solely reliant on ATP for their activity. This highlighted the high levels of constitutive background efflux present in this system. The subsequent addition of ortho-vanadate, a known ATPase inhibitor, lead to a decrease in the overall rate of efflux and the establishment of an eventual steady state by all the ATP-dependent transporters both endogenous and exogenous, in *L. lactis*.

To mathematically determine the empirical rates of efflux, the slope of each graph was calculated during the energised efflux phase between T = 1452 and T = 1584 (Table 2.3). The rates of active efflux during the glucose re-energising phase showed a notable increase in efflux for cells harbouring the exogenous ABC-type transporters.

Table 2.3. Rates of active efflux from *L. lactis* harbouring ABC-type constructs in nisin uninduced (w/o) and nisin induced (w) conditions.

Construct	Slope	% Difference
pNZ8048 w/o	-0.1508	**- 1.003**
pNZ8048 w	-0.1609	
pNZ1837-6 w/o	-0.1077	**- 16.387**
pNZ1837-6 w	-0.2716	
pNZ1837Δ6 w/o	-0.1154	**- 7.134**
pNZ1837Δ6 w	-0.1867	
pNZ1495 w/o	-0.1227	**- 2.843**
pNZ1495 w	-0.1512	

From these calculated values it became evident that expression of the exogenous ABC-type transporters made a contribution to the rate of efflux when the *L. lactis* host cells were re-energised by the addition of glucose. The difference in the rates of efflux between nisin uninduced and nisin induced cells harbouring the pNZ8048 vector without any insert was an increase of 1 %. This indicated that the empty vector's contribution to efflux was negligible even when the host cells were grown in nisin inducing conditions. This was not the case for the cells harbouring the ABC-type transporters BLLJ_1837-6 (pNZ1837-6) and BLLJ_1837Δ6 (pNZ1837Δ6). Expression of the two gene operon BLLJ_1837-6 lead to a 16.4 % increase in the rate of active efflux from the host cells in the nisin induced condition when compared to its uninduced counterpart. The single gene construct BLLJ_1837Δ6 produced an increase in efflux of 7.1 % which was approximately half that of the two gene construct BLLJ_1837-6.

The same trend was observed for BLLJ_1495 (pNZ1495) expression in the nisin induced condition, where a 2.8 % increase in efflux was observed compared to the uninduced culture. Even though the increase in the rate of efflux was much lower than that of either of the two previously described constructs, it was still higher than the background seen with the empty vector, and therefore shows that this protein is an ABC-type transporter, which actively exports Hoechst 33342 by the hydrolysis of ATP.

Similar results were obtained when this experiment was repeated with ethidium bromide, another fluorescent molecule known to be a substrate of ABC-type transporters (Bolhuis *et al.*, 1994; Bolhuis *et al.*, 1995). Using this substrate, no significant increase in the levels of efflux could be observed due to background efflux activity of the *L. lactis* cells (results not shown). In other studies characterising Hoechst 33342 and ethidium bromide efflux, using *L. lactis* cells, much stronger evidence of active extrusion was observed. This was due to the fact that the genes being studied (*lmr*A and *lmr*P) were in fact genes which occurred in the host *L. lactis* cells. This expression system allowed for over-expression of genes already present (Margolles *et al.*, 1999) and also for the construction of knock-out mutants, lacking the genes being characterised, which then allowed for the characterisation of the gene of interest when the mutant host cell was complemented with the gene being expressed on a plasmid (Bolhuis *et al.*, 1995).

The results obtained here indicated that the heterologously expressed genes may in fact be ATP binding cassette type transporters, as indicated by the complete inhibition of ATP-dependent efflux when the ATPase inhibitor ortho-vanadate was added. There was also an indication that the cells expressing the heterologous *B. longum* ABC-type proteins appeared to actively extrude Hoechst 33342 by the hydrolysis of ATP, although, that a better expression system is required to prove this definitively.

5. Conclusions

This chapter confirms the presence of two putative multidrug efflux systems in *B. longum* JCM 1217. The two MDR systems are transcribed as operons, one composed of two ABC-type efflux genes with a separate putative transcriptional regulator, and the other comprising two ABC-type efflux genes inclusive of a putative transcriptional regulator. The ABC-type efflux genes were shown to confer an MDR phenotype when expressed in *L. lactis* and contribute to the increase in resistance to both erythromycin and tetracycline. The rate of efflux of a fluorescent substrate, Hoechst 33342, appeared to be increased when these genes were heterologously expressed in *L. lactis*. Unfortunately, due to the high background efflux in the *L. lactis* NICE system this could not be proven conclusively and should in the future be carried out in a system where the major efflux systems have been mutated to be dysfunctional, or where deletions of the actual gene homologues being studied can be carried out. These results strongly indicate that the genes BLLJ_1837, 1836 and 1495 are ABC-type MDR genes, which appear to actively extrude antimicrobial substances by the hydrolysis of ATP.

From the results obtained in the RT-PCR, the genes also appeared to be transcriptionally upregulated when the host was exposed to erythromycin, a known substrate of these types of efflux genes. These results were merely qualitative and the inducibility of these genes merits further investigation. This will be covered in Chapter 3 of the study. The presence of two putative transcriptional regulators in these gene clusters is also intriguing and their roles will be further investigated in Chapter 4 of the study.

CHAPTER 3

Transcriptional induction of ABC-type transporter genes in response to antibiotic challenge

Contents

1. Summary

The advent and administration of antibiotic treatment has allowed for a significant decrease in the morbidity due to bacterial infections. Unfortunately, the irresponsible use of antibiotics and the rapid evolutionary adaptations by bacteria to these drugs has lead to a situation where multidrug resistance is a common and ever increasing problem which necessitates the constant development of new drugs to treat 'old bacteria'. This Chapter presents experimental data showing the inducibility of antibiotic resistance in *B. longum* JCM 1217 when pre-exposed to erythromycin. The results showed a dramatic increase (> 200 fold) in the MIC to erythromycin. Using quantitative Real Time PCR, the transcriptional profiles of the ABC-type multidrug efflux genes identified and characterised in Chapter 2, are also presented. This data indicated that these genes are transcriptionally up-regulated in response to erythromycin exposure, by ≈ 1.5 – 3 folds, and may therefore play a role in conferring resistance to this antibiotic.

2. Introduction

In Chapter 2 of this study, ABC-type transporter genes were identified in the genome of *B. longum* JCM 1217. Using various experimental techniques they were proven to be transcriptionally linked and conferred a multidrug resistance phenotype on the host when heterologously expressed in *L. lactis* cells. There was an indication that they were able to actively extrude Hoechst 33342, a fluorescent substrate of known ABC-type transporters, and this was done in a manner which appeared to be dependent on ATP hydrolysis. An interesting finding was the indication of an increase in the gene transcription levels of these ABC-type transporters genes when the cells were grown with prolonged exposure to erythromycin, corresponding to a concomitant increase in the erythromycin MIC. Since these ABC-type transporter genes directly confer resistance to erythromycin when heterologously expressed, the upregulation of their transcription in response to erythromycin is not surprising. It is, however, important to show that this upregulation is not a general cellular stress response, but rather a response by specific antibiotic resistance genes to erythromycin exposure.

The data presented thus far merely gave a qualitative indication of gene upregulation. It is, therefore, important to measure the change in the transcription levels of these genes using a

method which can accurately determine any changes in the gene transcription levels in response to this erythromycin exposure. The advent of quantitative real-time PCR (qRT-PCR) indicated a milestone in molecular science, since this method allowed for the indirect determination of the levels of gene transcription based on their respective mRNA levels, in real time. This was accomplished by harvesting the total cellular RNA, converting it to cDNA, and using this cDNA as a template for PCR to measure the amount of starting material, with the aid of a standard curve of known concentrations, based on the increase in fluorescence as more ethidium bromide bound to the double stranded DNA being formed (Higuchi *et al.*, 1993). Since then, a number of advances in this science have refined it, allowing it to be used to accurately and reliably quantify unknown DNA concentrations. The development of TaqMan based qRT-PCR made it even more sensitive than the commonly used SYBR green technology and even more reliable since primer-dimers produce no signal, a common artefact of SYBR green based qRT-PCR. TaqMan based qRT-PCR relies on the 5' exonuclease activity of *Taq* polymerase to hydrolyse a DNA bound oligonucleotide probe, thus releasing the attached fluorophore and producing a signal (Holland *et al.*, 1991).

This Chapter describes experiments conducted to determine the impact of exposure to erythromycin on the transcriptional levels of the *B. longum* subsp. *longum*^{T} JCM 1217 ABC-type transporters being investigated.

3. Methods and Materials

3.1. Gradient plate inductions

Induction of *B. longum* subsp. *longum* JCM 1217 by erythromycin exposure was carried out using the gradient plate method as described in Chapter 2. The erythromycin MIC of the cells was determined before exposure (basal level) and after each passage on a higher concentration of erythromycin, using the E-test method. The E-tests were carried out as per the manufacturer's instructions by plating a 1 McFarland (McFarland, 1907) cell suspension on the surface of a reduced, BYG agar plate before applying the E-test strip and then incubating anaerobically, at 37°C for 48 hrs, before reading the results.

3.2. RNA hybridization analysis

To investigate the genetic response of *B. longum* JCM 1217 in response to erythromycin exposure, RNA hybridization experiments were conducted. *B. longum* cells were exposed to erythromycin using the gradient plate method (3.1) and total cellular RNA was extracted from cells grown in erythromycin induced and uninduced conditions, as is described in Chapter 2.

DIG-labelled, DNA oligonucleotide probes were used to determine semi-quantitatively whether a change in the transcription levels of each gene in this study had occurred under erythromycin induced growth conditions. The DNA oligonucleotide probes were constructed by PCR amplification of a 1 kb fragment of DNA from the middle region of each gene being investigated, using the PCR primers listed in Table 3.1. The *B. longum* JCM 1217 16S rRNA gene was used as a reference. These DNA fragments were purified using the PCR cleanup kit (BioSpin) and subsequently DIG-labelled using the DIG-PCR labelling kit (Roche) as per the manufacturer's instructions.

RNA hybridization experiments were carried out by spotting 8 µg of total cellular RNA from erythromycin induced and uninduced *B. longum* JCM 1217 cells onto a nitrocellulose membrane (Amersham). The RNA was then UV crosslinked and probed individually with each individual DNA oligonucleotide probe constructed using the primer sets in Table 3.1. The membranes were then visualised as described in the DIG-manual (Roche).

Table 3.1. List of oligonucleotide primers used for RNA hybridization experiments.

Primer name	Primer sequence (5' – 3')	Region targeted	Size	Source
BLLJ_1837PF	CATCGTCGGGCAGGTCTACTTC	BLLJ_1837 Internal Fragment	1 Kb	This study
BLLJ_1837PR	GCGAATCCGGGTAGGTGAAG			
BLLJ_1836PF	CGTGAACTACTGCCGCAACTATC	BLLJ_1836 Internal Fragment	1 Kb	This study
BLLJ_1836PR	CAAAGCGGACTTGTCGGATTCG			
BLLJ_0511PF	GCCTTTGCAGATTGGCTGA	BLLJ_0511 Internal Fragment	1 Kb	This study
BLLJ_0511PR	CCGCTGAAACGGTGCTCTT			
BLLJ_1495PF	TGGTGGTCGTATCCTGGC	BLLJ_1495 Internal Fragment	1 Kb	This study
BLLJ_1495PR	TACCGCCCTGCTCGATG			
BLLJ_1494PF	GCTCTACATCCTGCTTGCC	BLLJ_1494 Internal Fragment	1 Kb	This study
BLLJ_1494PR	CCAGCTTGCACAACCTCTTC			
Lm26F	GATTCTGGCTCAGGATGAACG	*B. longum* specific 16S rRNA	1.35 Kb	(Kaufmann *et al.*, 1997)
Lm3R	CGGGTGCT***I***CCCACTTTCATG			
Bif164F	CATCCGGCATTACCACCC	*Bifidobacterium* specific 16S rRNA gene	520 bp	(Langendijk *et al.*, 1995)
Bif662R	CCACCGTTACACCGGGAA			

I = Inosine, matches all four nucleotides (A, C, G, and T)

3.3. Determination of sub-lethal erythromycin inducing conditions

B. longum JCM 1217 cells grown in BYG broth were exposed to various concentrations of erythromycin to determine the sub-lethal concentration of erythromycin and the effect on their growth rate was monitored. *B. longum* JCM 1217 cells were grown for 18 hours in 10 ml of reduced BYG broth as described in Chapter 2. This culture was re-inoculated into 100 ml of reduced BYG broth and grown anaerobically until an $OD_{600nm} \approx 0.3$, corresponding to mid-log growth phase, was reached. At this point, varying concentrations of erythromycin (0.0/ 0.5/ 1.0/ 2.0/ 4.0 µg/ml) were added to each culture and the incubation continued with the optical density measured every hour for a total of 28 hours.

3.4. Quantitative Real Time – PCR (qRT-PCR)

Changes in the transcription levels of the selected genes, after erythromycin exposure, were quantified using quantitative Real Time PCR (qRT-PCR). *B. longum* JCM 1217 cells were induced by growing them as described in 3.3, with the addition of 0.0, 1.0 or 2.0 µg/ml erythromycin at mid-log phase ($OD_{600nm} \approx 0.3$). After 2 hours of growth under these conditions, total cellular RNA was extracted from all of the cultures using the method described in Chapter 2 with the addition of a final purification step using the RNeasy kit (Roche) as per the manufacturer's instructions. All experiments were conducted in triplicate.

cDNA conversions were carried out using the Bioline cDNA conversion kit as per the manufacturer's instructions using 2 µg of RNA per 20 µl reaction. The random hexamer primers were annealed to the RNA at 65°C for 10 min before incubating the reactions at 42°C for 18 hours to allow for complete cDNA synthesis of all cellular RNA. The reactions were heat inactivated at 70°C for 15 min. To reduce experimental error during the cDNA conversions, all the reactions were carried out in duplicate and checked for the production of cDNA, using the *Bifidobacterium* sp. specific primers Bif164F and Bif662R (Table 3.1), before pooling the duplicate reactions.

To accurately determine the change in the gene expression levels during erythromycin exposure, qRT-PCR analysis was conducted using the oligonucleotide primers in Table 3.2 and the oligonucleotide probes in Table 3.3. All the primers and probes used were designed using the Beacon Designer 7.21 software (PREMIER Biosoft International) to be compatible for a multiplex PCR. The resulting primer/probe sets with the highest compatibility were selected. DNA oligonucleotide primers and probes were designed to amplify an internal

fragment of BLLJ_1495 and BLLJ_1837 using the Taqman method of qRT-PCR. All the reactions were setup as multiplex PCR reactions which included the 16S rRNA primers and probe, as well as, the gene of interest (GOI) primers and probe. Since the BLLJ_0511 *ctr* gene showed no discernible change in transcription when exposed to erythromycin (Chapter 2) it was also included as a comparative gene of interest to determine if any cross-induction could be observed using a more sensitive technique.

A typical SYBR green qRT-PCR reaction was as follows. For a 50 µl reaction using the 2 X SensiMix dT Kit (Quantace): 200 nM of each primer, 1 X SensiMix, 1 µl SYBRO, 100 ng *B. longum* genomic DNA as template for optimization. All reactions were conducted on the Rotor-Gene 6000 Series (Corbett Life Science) using the software version 1.7.87. The following cycling conditions were used: Initial activation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 10 sec, primer annealing at 60°C for 15 sec, and elongation at 72°C for 15 sec. A DNA melt curve was added at the end of this by ramping the temperature from 67°C – 95°C at 1°C/cycle.

Table 3.2. List of oligonucleotide primers used for qRT-PCR experiments.

Primer name	Primer sequence (5' – 3')	Region targeted	Size	Source
16S1495F	AGTGAGTTTACCCGTTGAATAAGC	16S rRNA gene	150 bp	This study
16S1495R	CACCGTTAAGCGATGGACTTTC			
Q1495F	CCATGTCACCTTCCGCTACTC	BLLJ_1495 Internal Fragment	137 bp	This study
Q1495R	GCACGAGCTGTACCAGAGAC			
16S1837F	AGTGAGTTTACCCGTTGAATAAGC	16S rRNA gene	150 bp	This study
16S1837R	CACCGTTAAGCGATGGACTTTC			
Q1837F	GAGGCCAAGGATTCCACCAAG	BLLJ_1837 Internal Fragment	134 bp	This study
Q1837R	TCGCAGTTGTCGAGCAGTTC			
16S0511F	AAGAACCTTACCTGGGCTTGAC	16S rRNA gene	127 bp	This study
16S0511R	TGCGGGACTTAACCCAACATC			
Q0511F	AGATCATGTGGCAGATCACACC	BLLJ_0511 Internal Fragment	103 bp	This study
Q0511R	CTCGCTTCTCAGTACGTGTCC			

Table 3.3. List of oligonucleotide probes used for qRT-PCR experiments.

Probe name	Primer sequence (5' – 3')	Region targeted	Size	Source
P16S1495	CGACGAACCGCCTACGAGCCCTTT	16S rRNA Internal Probe	24 bp	This study
P1495	AACCAGTACCGCCGACGATGCCG	BLLJ_1495 Internal Probe	23 bp	This study
P16S1837	CGACGAACCGCCTACGAGCCCTTT	16S rRNA Internal Probe	24 bp	This study
P1837	TGCCCTGACCCACGACCTTGCC	BLLJ_1837 Internal Probe	22 bp	This study
P16S0511	CGAGCTGACGACGACCATGCACCA	16S rRNA Internal Probe	24 bp	This study
P0511	CGGGAAGAGCACCACCTTGACGGC	BLLJ_0511 Internal Probe	24 bp	This study

- All 16s rRNA internal probes were 5' labelled with HEX (*6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein)*
- All GOI internal probes were 5' labelled with FAM (6-carboxyfluorescein)
- All probes were 3' labelled with DABCYL (dimethylaminoazosulfonic acid)

A typical TaqMan multiplex qRT-PCR reaction was as follows. For a 12.5 µl reaction using the 2 X SensiMix Probe Kit (Quantace): 1 X SensiMix, 200 nM of each oligonucleotide primer (16S rRNA and GOI), 66.67 nM of each fluorescently labelled TaqMan probe (16S rRNA and GOI), as template, 25 ng *B. longum* genomic DNA for optimization or 1 µl of uninduced or erythromycin induced cDNA (diluted 1:10 in sterile dH_2O) for quantitation. For no template controls (NTC) 1 µl sterile dH_2O was used instead of DNA template and for RNA controls 20 ng of the extracted total cellular RNA was used as template to ensure no false positives were obtained as a result of residual genomic DNA in the sample. The following cycling conditions were used: Initial activation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 10 sec, coupled primer annealing and elongation at 60°C for 25 sec, acquiring to the green and yellow channels after each cycle. For standard curve construction, equal volumes of uninduced and induced cDNA were pooled and then serially diluted from $10^0 – 10^{-4}$ in sterile dH_2O. All measurements were conducted in triplicate and each replicate experiment was measured in the same run using the Rotor-Gene 6000 Series (Corbett Life Science) using the software version 1.7.87.

4. Results and Discussion

4.1. Erythromycin gradient plate inductions

The gradient plate method was used to study the effect of erythromycin on the MIC of *B. longum* JCM 1217 cells. This method allows for long term exposure of the cells to the antibiotic with the result that the bacteria have time to adjust to and stabilize in the presence of the antibiotic. The drawback of this technique is that over time the bacteria may change the mechanisms employed to deal with the antimicrobial challenge and may lead to the development of persister cells or mutants (Bigger, 1944). Figure 3.1 shows the fold increase in MIC of *B. longum* to erythromycin over four passages of growth on an erythromycin gradient.

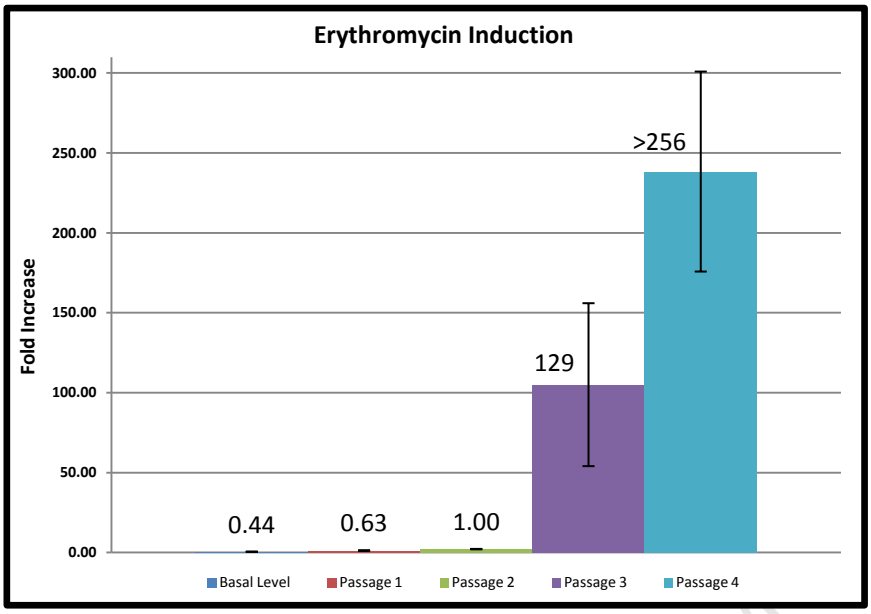


Figure 3.1. Trend graph showing the fold increase in MIC of *B. longum* exposed to erythromycin. The numbers above each bar indicate the actual MIC in µg/ml. Error bars indicate the standard error of the mean of 3 experiments.

The results showed a significant increase in the MIC of *B. longum* JCM 1217 to erythromycin (≈ 240 fold) after four passages of erythromycin exposure. Even though the results obtained gave a strong indication that these bacteria possessed mechanisms which allowed them to survive antibiotic exposure, the large error observed indicated that long term exposure may result in the bacterium changing the mechanisms which are recruited in response to antimicrobial challenge and also the extent to which they are used. The development of persister cells which may become transiently resistant to the antibiotic due to the long term exposure is also possible (Gefen & Balaban, 2009; Keren *et al.*, 2004).

The persister cells which remain after the initial erythromycin exposure have been reported not to become resistant to erythromycin, but rather to tolerate it, and thus survive to form a new population of bacteria in subsequent passages of this culture onto higher concentrations (Gefen & Balaban, 2009). The development of a slow growing persister population of bacteria would allow the culture to survive this long term antibiotic exposure and may allow for the changing of resistance mechanisms and the levels at which they are employed. In this way the bacterium is able to still survive the antimicrobial challenge, but, may not produce the same MIC each time as the levels of tolerance developed may change with each subsequent experiment.

This result indicated a cellular response by the bacteria to erythromycin exposure, but did not indicate whether the ABC-type transporter genes being studied here were in fact recruited in

response to these erythromycin inducing conditions. In order to investigate whether this increase in MIC is in part due to the action of the ABC-type transporter genes being studied, the actual transcriptional changes of the ABC-type transporters need to be measured and compared to the uninduced condition.

4.2. RNA hybridization analysis

In Chapter 2 of this study RT-PCR experiments revealed a semi-quantitative increase in gene transcription levels of the ABC-type transporters genes being investigated, in *B. longum* cells exposed to erythromycin. This result merely presented an indication of upregulation of the genes as clusters, since the region targeted by the RT-PCR was the intergenic region spanning the genes in the operon. In order to determine whether the observed induction occurs for each gene being studied, RNA hybridization by means of dot blot analysis was employed. This technique gives a qualitative indication of the gene expression levels when compared to a gene which remains stably expressed under the same conditions being tested, e.g. the 16S rRNA gene. For this purpose, gene-specific DNA oligonucleotide probes were designed to bind to the RNA extracted from cells grown in either erythromycin inducing conditions or from cells grown in normal growth conditions, to compare their transcription levels (Figure 3.2).

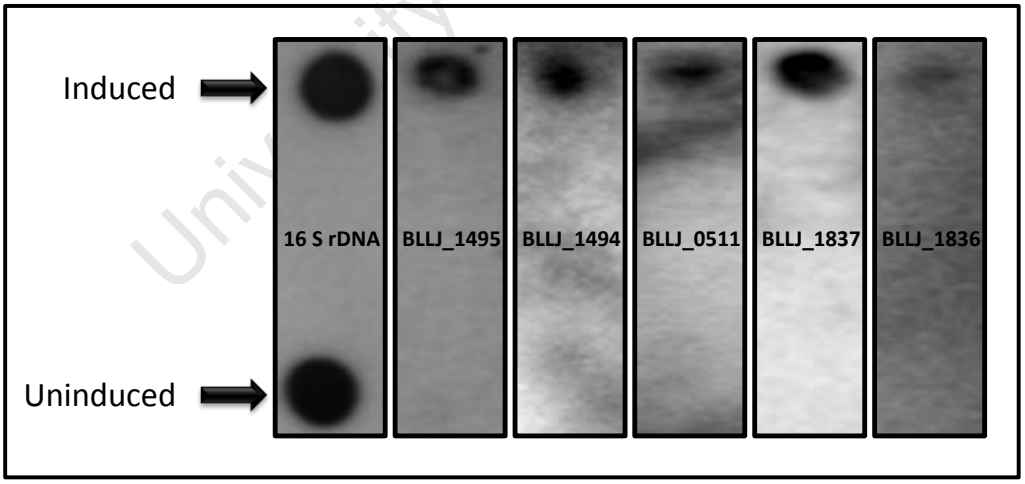


Figure 3.2. RNA hybridization analysis of erythromycin induced and uninduced *B. longum* cells.

The results obtained indicated that all the genes being investigated here were up-regulated in response to erythromycin exposure, when compared to the uninduced condition. Since these genes have been proven to be functional multidrug efflux genes (Chapter 2), their up-regulation is not surprising when they are presented with a known substrate of ABC-type

transporters (Margolles *et al.*, 2005; Woebking *et al.*, 2005). The up-regulation of these genes in response to antimicrobial challenge once more strengthens their classification as multidrug efflux, ABC-type transporters, which contribute to the cells intrinsic resistance.

An interesting outcome of this experiment is the moderate up-regulation of the *ctr* gene homologue BLLJ_0511, a Na^+ dependent nucleoside transporter. In Chapter 2, the RT-PCR analysis showed no visible increase in the transcription levels of this gene, but the use of a more sensitive technique such as RNA hybridization analysis shows a slight increase. This gene is a proven cholate-specific transporter and has previously been shown to be cross-induced by exposure to bile salts, leading to a increase in its resistance to erythromycin (Price *et al.*, 2006). Here it is shown that prolonged exposure of *B. longum* JCM 1217 cells to erythromycin lead to an increase in the transcriptional levels of the ABC-type transporter genes BLLJ_1495/4, 1837/6 and the cholate transporter gene BLLJ_0511. These results indicate a probable role of these genes in erythromycin resistance since the genes being upregulated are proven multidrug resistance genes, belonging to the ABC-type family. No quantitative comparison of the spot intensities could be carried out due to the differing probe efficiencies for each gene. qRT-PCR will be used to quantify the gene expression levels.

This increase in the transcription levels of MDR transporter genes by long term exposure to antimicrobials is not uncommon and has previously been observed in *Staphylococcus aureus* which, when exposed to biocides and other toxic chemicals commonly used in the hospital environment, showed the development of mutants which had significantly increased MIC's to numerous antimicrobials (Huet *et al.*, 2008). In *Salmonella typhimurium* it was observed that exposure to sub-inhibitory levels of antibiotics, such as erythromycin, lead to an increase in the transcription of various genes by the modulation of numerous bacterial promoters (Goh *et al.*, 2002). These studies correlate well with this study, showing that long term exposure to antimicrobials can lead to changes in the transcriptional levels of numerous genes related to the tolerance of the antimicrobial substance.

4.3. Determination of the sub-lethal concentration of erythromycin

Since the gradient plated method was used with several passages on increasing concentrations of erythromycin, the results above indicate the effect of prolonged erythromycin exposure on *B. longum* JCM 1217 cells. In order to determine the immediate physiological impact of

erythromycin exposure on the transcriptional levels of the genes being studied, a more specific exposure condition must be used. For this purpose, growth studies were conducted on *B. longum* JCM 1217 grown in BYG broth supplemented with various concentrations of erythromycin, and the effect on growth was determined by measuring the change in optical density of the culture over time (Figure 3.3). These conditions allowed for a direct measure of the impact on cell growth by a short exposure to erythromycin, and allowed for the determination of the sub-lethal concentration of erythromycin which produced a physiological response.

The data obtained from this experiment indicated that the cells did respond to the addition of erythromycin, to varying degrees. It is interesting to note that even though the basal MIC obtained for the gradient plate inductions was only 0.44 µg/ml, the bacteria were able to tolerate concentrations of 1.0 µg/ml of erythromycin, in broth cultures, when in mid-log phase and exhibited no significant reduction in their growth rate at these concentrations.

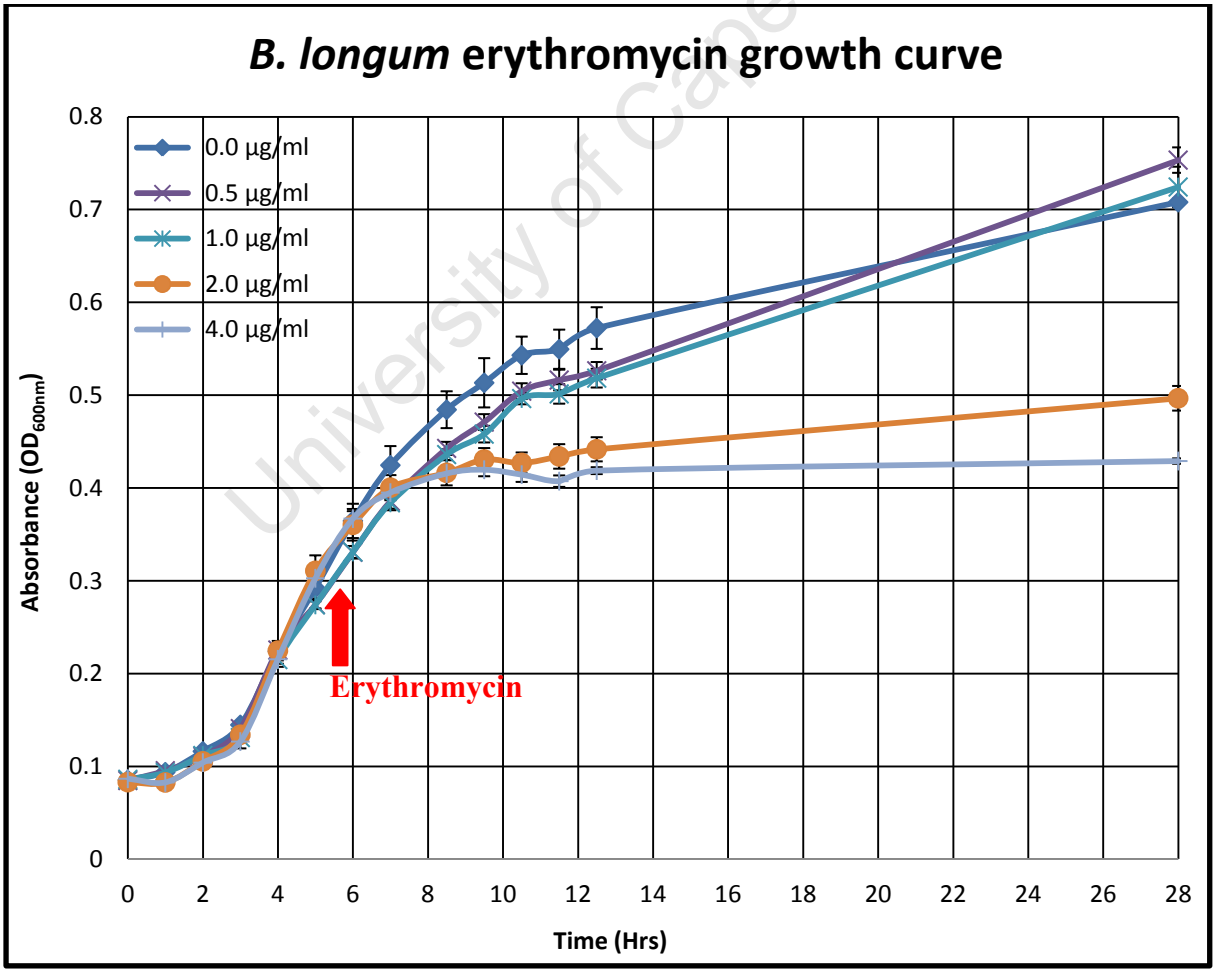


Figure 3.3. Growth curves showing the result of erythromycin addition on the growth of *B. longum* JCM 1217. Error bars indicated the standard error of the mean of three independent experiments.

When concentrations exceeding 2 µg/ml of erythromycin were added, the cells showed a marked decrease in their growth rate, as well as, in the final optical density they reached. At 1 µg/ml of erythromycin the final OD_{600} was ≈ 0.7 whereas for 2 µg/ml it only reached a final OD_{600} of ≈ 0.5. This may have been as a result of a shift in the transcriptome of the cells by the induction of resistance genes which were recruited to detoxify the cell, a phenomenon observed for *B. animalis* cells in response to bile salts which lead to a shift in the transcriptome of the cells to tolerate the bile salts stress, which included the upregulation of membrane bound transporter proteins (Lin *et al.*, 2005; Ruiz *et al.*, 2009; Sánchez *et al.*, 2007). The fact that there is no overall decrease in the optical density after this point may indicate a steady state where the cells are able to tolerate the added erythromycin and cell death is averted. However, since no viable plating was carried out, this cannot be confirmed. The plating of cells on erythromycin gradient plates does however give an indication that if not all cells, then a group of persister cells may remain viable leading to the observed phenotype of a significantly more resistant population of bacteria.

When 4 µg/ml of erythromycin was added, another marked decrease in the final OD_{600} reached (≈ 0.4) compared to that obtained for 2 µg/ml, was observed. It can be inferred that a maximum level of tolerance is reached at 1 µg/ml and that bacterial growth is significantly impaired at higher concentrations. These results indicate a response by *B. longum* JCM 1217 to erythromycin exposure as indicated by an altered growth response in its presence. The sub-lethal dose of erythromycin for *B. longum* JCM 1217 growing in BYG broth was established to be 1 µg/ml and significant reduction in the growth rate was observed at 2 µg/ml. Using this data, the levels of gene transcription could be measured at the concentrations which lead to a change in the growth dynamics of the cells and any changes observed may be linked to the tolerance of the erythromycin.

4.4. Quantitative Real Time – PCR (qRT-PCR)

Quantitative Real Time PCR (qRT-PCR) was used to quantify the effect of erythromycin on the transcription of the ABC-type transporter genes being studied here. RT-PCR experiments in Chapter 2 and the RNA hybridization experiments above gave a semi-quantitative indication that all the genes being investigated were up-regulated in response to erythromycin challenge. In order to quantify the transcriptional levels of the ABC-type transporter genes of cells exposed to erythromycin, physiological conditions had to be selected which would

reflect the conditions where the transcriptome might be altered to cope with the antibiotic challenge. Growth curve experiments showed that the addition of 1 µg/ml of erythromycin had no significant influence on the growth dynamics of mid-log *B. longum* cells, whereas, 2 µg/ml of erythromycin greatly reduced the growth of the cells. To accurately determine whether these cells do in fact recruit the ABC-type transporter genes under investigation here in response to erythromycin challenge, qRT-PCR experiments were carried out on cDNA produced from the total cellular RNA harvested from mid-log *B. longum* cells exposed to 1 µg/ml or 2 µg/ml of erythromycin for two hours.

Since both gene clusters being studied here are transcribed as operons (Chapter 2), this experiment was designed to determine the transcription levels of only the upstream gene of each gene cluster (BLLJ_1495 and BLLJ_1837) as an indication of the levels of gene transcription for the entire operon, in relation to the 16S rRNA gene. The BLLJ_0511 gene was also included in this experiment to determine if cross induction of this gene also occurs as was previously observed to a lesser extent, in the RT-PCR and RNA hybridization experiments.

TaqMan based qRT-PCR was used to quantify the gene expression levels (Holland *et al.*, 1991). TaqMan qRT-PCR allows for multiplexing of reactions in qRT-PCR and does not produce false results associated with primer-dimer formation; it also does not require post reaction processing as only the correct primer-probe interaction with the template DNA produces a fluorescent signal (Li *et al.*, 2003; Nazarenko *et al.*, 2002). DNA oligonucleotide primers and probes were designed to target an internal fragment of each gene of interest (GOI), as well as the 16S rRNA gene, as a reference gene. To reduce the inter- and intra-reaction variation commonly associated with qRT-PCR, the experiment was designed to be performed as a multiplex qRT-PCR, which would incorporate the GOI and 16S rRNA primer/probe sets in one reaction. Since each GOI probe and each 16S rRNA probe was labelled with different fluorescent dyes, they could be carried out in the same reaction tube and their respective products measured separately at the same time (Nazarenko *et al.*, 2002). This approach greatly reduces the technical, as well as, reaction error since both reactions are carried out in the same reaction environment and all conditions are exact for both measurements. To test the specificity of the oligonucleotide primers designed for this experiment, a basic PCR was first conducted (Figure 3.4).

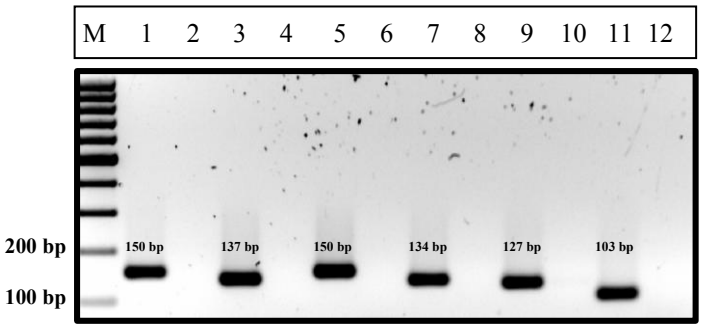


Figure 3.4. qRT-PCR primer specificity using standard PCR analysis. M = molecular weight marker. Lane 1, 16S1495F/R; lane 3, Q1495F/R; lane 5, 16S1837F/R; lane 7, Q1837F/R; lane 9, 16S0511F/R; lane 11, Q0511F/R. Lanes 2, 4, 6, 8, 10, 12, no template control for each respective primer pair.

The results of the PCR analysis on each of the primer pairs designed for qRT-PCR experiments showed that a single specific amplicon of the correct size (Table 3.2) was produced for each reaction.

To confirm whether a single product was produced using qRT-PCR conditions, the primer pairs were then tested using SYBR green based qRT-PCR (Figure 3.5) (Morrison *et al.*, 1998; Vitzthum *et al.*, 1999). This technique allowed for the construction of a melt curve at the end of the reaction, which indicated the presence of all products in the reaction.

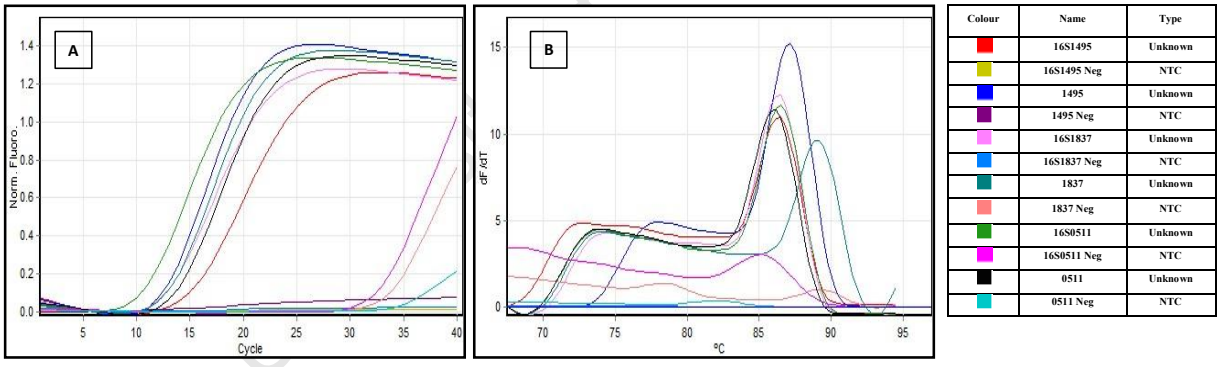
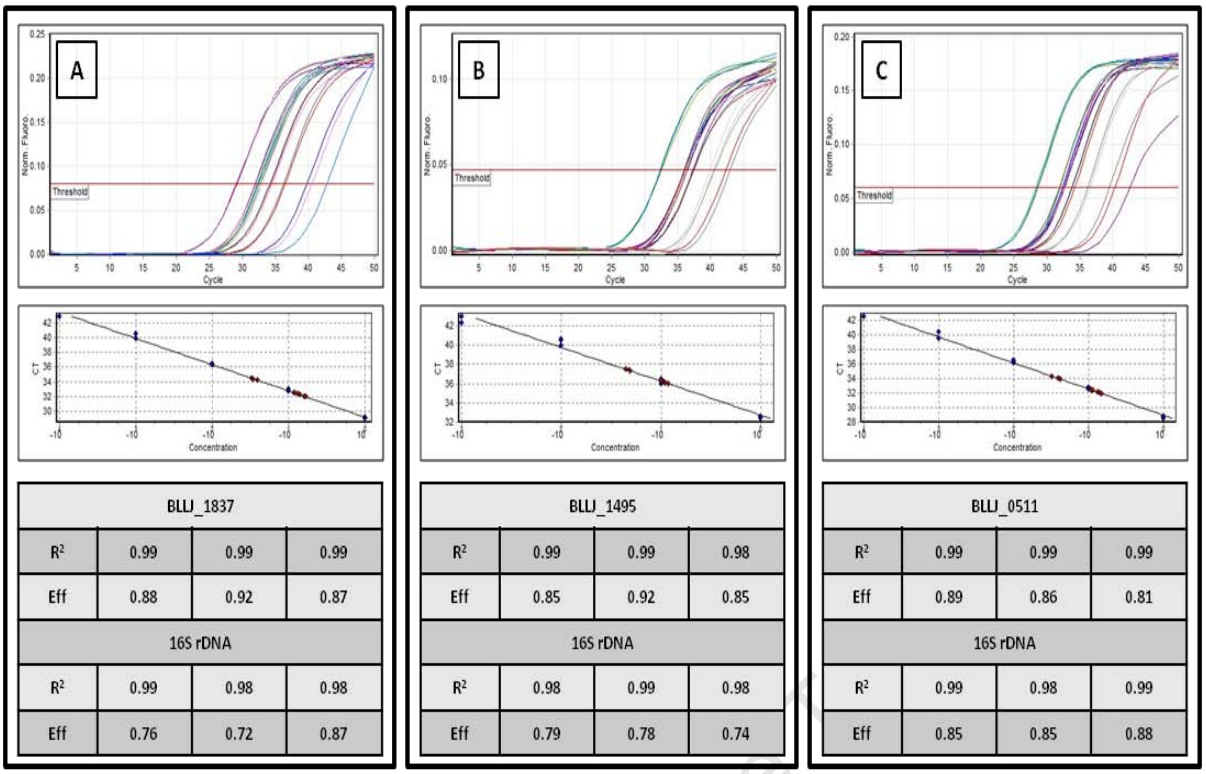


Figure 3.5. SYBR green qRT-PCR analysis of primer specificity. (A) Amplification curves of individual primer pair reactions. (B) Melt curves of individual primer pair reactions. Type = type of sample. Unknown = sample to be assayed. NTC = no template control.

The SYBR green analysis of the primers showed that during normal qRT-PCR amplification a single amplification curve was generated for each primer pair, when tested individually. By heating the reaction products in a step-wise manner to complete denaturation, the DNA-binding dye SYBR green, incorporated during the amplification, was released and a curve was constructed showing the inverse of the resulting decrease in fluorescence. Since each product of varying length denatured at a different temperature, related to its length, the presence of non-specific products would be visualised when more than one peak is produced during the melt curve (Ririe *et al.*, 1997). The data obtained above showed that a single

product was being specifically amplified in each reaction when genomic DNA was used as a template. The resulting melt curve analysis also showed that a single product was produced for each primer pair and that the no template controls were well below the detectable level. A shoulder was also visible in all of the primer melt curves, however, this is a common occurrence in SYBR green based qRT-PCR and is an indication of the formation of primer-dimers during amplification. This is often a problem using this technique and reduces the sensitivity of the measurement since all values must be normalised to account for this background increase in fluorescence. This is not a phenomenon that occurs in TaqMan based qRT-PCR since a fluorescent signal is only produced when the probe binds to its complimentary DNA sequence and is subsequently hydrolysed by the DNA polymerase enzyme, releasing the probe-bound fluorophore (Bustin, 2000). This data showed that the designed primers were specific for the DNA targets they were designed to amplify and did not produce non-specific products.

Using the conditions tested thus far, multiplex qRT-PCR reactions were carried out for all the primer-probe sets which included the GOI and 16S rRNA primer sets. Optimal reaction conditions were obtained using a 60°C primer annealing/elongation step. Figure 3.6 shows the results obtained in the final multiplex qRT-PCR reactions used to quantify the gene expression levels of BLLJ_1837, BLLJ_1495 and BLLJ_0511. A typical quantitation and standard curve is shown for each gene being quantified, as well as, the reaction data obtained for each replicate experiment.



BLLJ_1837			
R^2	0.99	0.99	0.99
Eff	0.88	0.92	0.87
16S rDNA			
R^2	0.99	0.98	0.98
Eff	0.76	0.72	0.87

BLLJ_1495			
R^2	0.99	0.99	0.98
Eff	0.85	0.92	0.85
16S rDNA			
R^2	0.98	0.99	0.98
Eff	0.79	0.78	0.74

BLLJ_0511			
R^2	0.99	0.99	0.99
Eff	0.89	0.86	0.81
16S rDNA			
R^2	0.99	0.98	0.99
Eff	0.85	0.85	0.88

Figure 3.6. Data obtained for all replicates of multiplex qRT-PCR of (A) BLLJ_1837, (B) BLLJ_1495 and (C) BLLJ_0511. R^2 = best fit regression analysis, Eff = efficiency of each reaction.

The data above indicated that a successful amplification plot and reliable reaction data for each run was obtained. The reaction efficiencies obtained were somewhat lower than those obtained using standard SYBR green qRT-PCR. This is due to the multiplexing of the reactions using TaqMan probes to quantify the gene transcription levels. Since two reactions are taking place simultaneously in the same tube the reaction efficiencies are lower since they may impact each other during amplification. The efficiencies were still within the acceptable range and the reduction in reaction error obtained by multiplexing far outweighed the slight reduction in reaction efficiencies. Based on this the levels of gene transcription for the ABC-type transporters obtained were quantified.

4.5. Quantification of transcriptional levels

To quantify the data obtained from these experiments, it is important to take into consideration the difference in reaction efficiency obtained for each GOI and its respective 16S rRNA reference gene. Changes in reaction efficiencies reflect the difference in the rates

of DNA amplification during each run and must be taken into consideration during analysis to ensure the data is relatable and to ensure there is no bias for one product or the other.

The aim of this experiment was to determine whether the genes being investigated were transcriptionally up-regulated in response to erythromycin exposure. The final answer only requires that the ratio of gene expression between the reference gene (16S rRNA) and the GOI be determined. The outcome of this is thus relative and not absolute. The Pfaffl method of data analysis takes all of these criteria into account in one algorithm (Pfaffl, 2001). This formula presents the ratio of gene expression of the GOI relative to the reference gene and incorporates the reaction efficiencies of each run in the calculation as well.

Using the Pfaffl equation, the ratios of gene expression relative to the reference gene can be obtained, but this ratio does not reflect the statistical significance of the change in expression. To determine the statistical relevance of the data obtained as it refers to the ratios obtained using the Pfaffl method, further statistical analysis needed to be conducted. Traditional methods for the comparison of multiple groups would involve either the use of multiple t-tests (with appropriately adjusted p-values) or ANOVA methods (were the data normally distributed), or the equivalent nonparametric tests: the Wilcoxon-Rank Sum or Kruskal-Wallis tests. Since the ratios obtained here are non-symmetrically distributed, and are calculated using the Pfaffl methodology the above methods are not appropriate. For this reason we adjusted the standard methodology to obtain the same ratios as those found with the Pfaffl method which allowed us to statistically test the data obtained.

Since the Pfaffl method assumes a normally distributed data set, the statistical analysis must also be conducted using methods which apply to a normally distributed data set. The data set obtained from this experiment, as is the case with most biological data, is not normally distributed due to the high degree of variability inherent in all living organisms. For this reason, statistical relevance can only be inferred if the data set is mathematically adjusted, in a linear form, to generate a normally distributed version of the data set. Using this approach the data is not changed since the same adjustment is equally applied to each value. This is accomplished by calculating the Log_{10} equivalent of each value obtained. Since the Pfaffl method shows the ratio of GOI/REF = A/B, and mathematically A/B = $\mathrm{Exp}[\ln(A) - \ln(B)]$, taking the natural log of each value still gives the same ratio as does the Pfaffl method. When this approach is used, a normally distribute data set is obtained. Statistical analysis done on this normalised data can be directly applied to the Pfaffl ratios calculated, since the

data has not been changed and the normalisation preserves the Pfaffl ratios already calculated. Using this normalised data set, a regression analysis was carried out to determine the average ratio obtained for each gene at each condition tested. This regression indicated the statistical significance of the changes observed and were identical to those obtained using the Pfaffl equation and could therefore be applied here (Table 3.4).

Table 3.4. Statistical significance, indicated by probability values, obtained from the regression analysis on the normalised qRT-PCR data.

Condition	BLLJ_1837	BLLJ_1495	BLLJ_0511
1 µg/ml	0.018	0.294	0.244
2 µg/ml	0.003	0.304	0.102

This analysis showed that for both conditions tested (1 µg/ml or 2 µg/ml of erythromycin), only BLLJ_1837 showed statistically significant differences in gene expression. For BLLJ_1837 exposed to 1 µg/ml of erythromycin, the 3.33 fold increase in gene transcription is statistically significant with a p-value of 0.018, and at 2 µg/ml of erythromycin, the 3.29 fold increase in gene transcription is statistically significant with a p-value of 0.003. Figure 3.7 shows the changes in gene expression, during erythromycin challenge of the ABC-type transporter genes using the Pfaffl method of quantitation, as well as, the statistically significant changes obtained from the regression analysis.

The results obtained from the multiplex qRT-PCR showed an increase in the transcription levels of all the genes assayed. This correlates well with the data obtained from the RT-PCR in Chapter 2, as well as, the RNA hybridization analysis presented in this Chapter. The most notable increase was obtained for BLLJ_1837, which exhibited greater than 3-fold increase in response to both 1 and 2 µg/ml of erythromycin, and is statistically significant. There is no significant difference in the transcription levels of BLLJ_1837 between the two erythromycin concentrations tested. This could indicate a maximum gene response at 1 µg/ml erythromycin. The slight decrease observed at 2 µg/ml of erythromycin may be indicative of the reduction in the growth rate observed in the growth curve analysis, and strengthens the argument that a maximum response is elicited at 1 µg/ml.

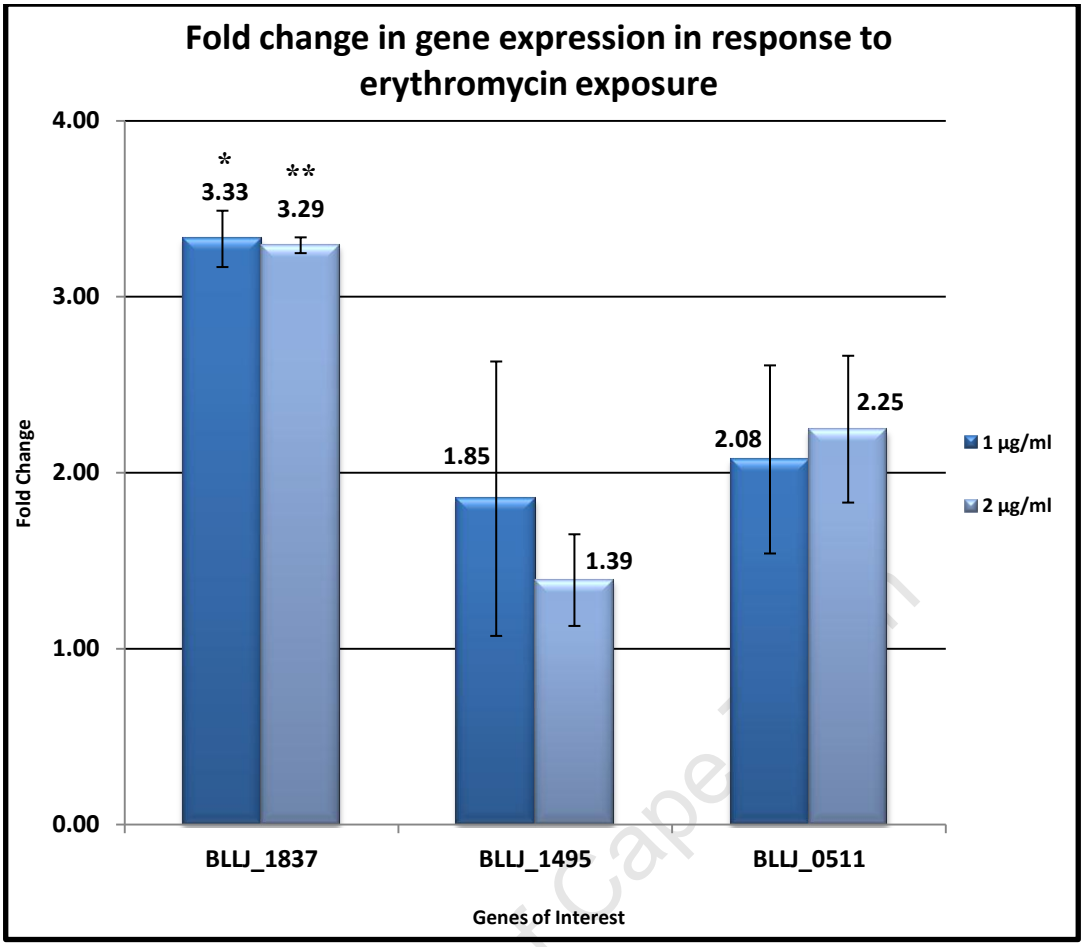


Figure 3.7. Bar graph showing the fold change in the gene expression levels of BLLJ_1837, 1495, 0511 in response to erythromycin exposure. Error bars represent the standard error of three independent biological replicates. Asterisks' indicate the statistical significance (*, P = 0.018; **, P = 0.003).

For BLLJ_1495, the increase observed was not as dramatic, and even though it is not statistically significant, it was still high enough to indicate a change in transcription, in response to erythromycin exposure. Once again a slight decrease was observed at 2 µg/ml erythromycin, this may also have indicated that a maximum in the gene expression levels may have been reached at 1 µg/ml and that the cells may be severely compromised at concentrations above that, as was observed in the growth curve experiments. This data correlates well with other studies which have shown that bacteria exposed to sub-inhibitory concentrations of antibiotics exhibited increased levels of gene transcription, including those involving multidrug efflux systems (Goh *et al.*, 2002; Huet *et al.*, 2008; Lin *et al.*, 2005).

When the gene expression levels obtained here were compared to the E-test MIC levels obtained in Chapter 2, where the genes were heterologously expressed in *L lactis*, the results

showed the inverse situation. The MIC obtained for BLLJ_1837 and BLLJ_1495 was 0.19 μg/ml and 0.38 μg/ml respectively. Since the gene expression levels obtained are inversely proportionate to their MIC values, it again highlights the multifaceted mechanism these transporters play in conferring multidrug resistance. Not only do they simultaneously confer resistance to multiple, structurally unrelated antimicrobial substances; they do this in a poly specific manner which determines to what extent they are able to bind to and subsequently expel these substances from the cell (Lubelski & Driessen, 2007). Therefore, even though the gene expression level of BLLJ_1495 was half that of BLLJ_1837 when exposed to 1 – 2 μg/ml of erythromycin, it still conferred a level of resistance to erythromycin which was double that of BLLJ_1837, when measured using the E-test method. This may have indicated that BLLJ_1495 may have had an enhanced responsivity towards erythromycin and could thus possibly expel it to a greater extent, than could BLLJ_1837. This may also explain the large error obtained in the gradient plate inductions, since whichever transporter is recruited, and to what extent it is used in response to antibiotic challenge, will determine the MIC at that concentration. This would thus result in different values being obtained during long term exposure, as the cells may switch from one transporter to another depending the energy requirements and efficiency of each transporter.

The increase in the gene expression levels observed for BLLJ_0511 indicates a minor response to erythromycin exposure. This was observed in RNA hybridization experiments where a slight increase in the gene expression levels was obtained. Even though this gene is specific to cholate transport it has been shown to be cross induced to a higher erythromycin MIC by pre-exposure to cholate (Price *et al.*, 2006). It is, therefore, possible that exposure to erythromycin may lead to a slight increase in the levels of BLLJ_0511 transcription as well. However, whether this increase in gene transcription levels leads to an increase in cholate efflux still needs to be experimentally determined.

The presence of a putative transcriptional regulator in each gene cluster being studied (Chapter 2) presents another question regarding the transcription of these genes. Transcriptional regulators can significantly alter the transcriptional profiles of genes in response to external stimuli. This aspect will be further investigated in Chapter 4 of this study.

5. Conclusions

The experimental data presented in this Chapter gave strong evidence of the inducibility of these ABC-type transporter genes. The long term exposure of the bacteria to erythromycin lead to a dramatic increase in the MIC with a concomitant increase in the gene expression levels of the genes being investigated. Exposure of the bacteria to specific concentrations of erythromycin also resulted in a physiological decrease in their growth rate and a depression in the final population levels. The levels of gene transcription for all the genes being studied also showed a marked increase in response to these specific growth conditions, which indicated a mechanism of directed gene up-regulation by *B. longum* JCM 1217, in response to erythromycin challenge.

The influence of the putative transcriptional regulators identified upstream of each operon, warrants further investigation. Since all of the genes in both operons being studied are simultaneously up-regulated in response to erythromycin exposure, it is possible that such a regulatory system may be responsible for the upregulation of all these resistance genes by simultaneously up-regulating their expression through the recruitment of individual gene-specific regulators. This would allow for specific and controlled induction of numerous genes to detoxify the cell during antibiotic exposure. The role of the putative regulators in these gene clusters must thus be investigated to ascertain their roles in regulating the expression of these genes, and will be addressed in Chapter 4 of this study.

CHAPTER 4

Regulator Studies

Contents

1. Summary

The genome arrangement of the two gene clusters under investigation here revealed the presence of two putative MarR-type transcriptional regulators upstream of the ABC-type transporter genes being investigated. This Chapter presents the first evidence of the possible transcriptional regulation of bacterial gene expression in *B. longum* JCM 1217 by a MarR-type regulator. Both of the putative regulators, BLLJ_1496 and BLLJ_1838, were cloned and expressed in the soluble fraction of *E. coli* BL21pLysS (DE3) cells. Using these purified proteins in electrophoretic mobility shift assays, it was shown that the BLLJ_1496 protein bound stably, specifically and with high affinity to a single DNA fragment upstream of the operon, even in the presence of sodium salicylate (a known inhibitor of MarR protein binding) and when presented with other potential DNA targets. The role of erythromycin as an effector molecule was also investigated, but showed no significant function in disrupting the observed regulator binding. The purified protein, BLLJ_1838, did not exhibit any binding ability to any of the potential DNA target fragments presented.

2. Introduction

The regulation of gene expression at the transcriptional level is a vital mechanism employed by all organisms to facilitate control of cellular energy and resources. Unorganised or unnecessary expression of genes would deplete cellular resources and lead to cell death. Since most prokaryotes rely on fluctuating external energy sources, tight control is needed to prevent wastage of important cellular reserves. One of the most efficient mechanisms in regulating gene expression at a transcriptional level involves the use of regulatory proteins binding to DNA to up- or down-regulate gene expression in response to various stimuli (Huffman & Brennan, 2002). Since membrane proteins underpin the structure of the cell wall, it is vital to control the amount of various proteins present in the phospholipid bilayer comprising the bacterial cell membrane. ABC-type multidrug efflux transporters are integral membrane proteins and are only required when active transport of specific compounds is needed. Their expression thus requires tight regulation with prompt response with changing cellular conditions (Grkovic *et al.*, 2001).

The transcriptional regulation of multidrug resistance pumps is not uncommon and, in particular, ABC-type efflux transporters have been shown to be regulated by MarR-type regulators (Lubelski & Driessen, 2007; Putman *et al.*, 2000). MarR-type regulators are negative transcriptional regulators which down-regulate gene expression when bound to palindromic or pseudopalindromic sequences upstream of structural genes and may also auto-regulate their own production (Wilkinson & Grove, 2001). In Chapter 2 of this study, two putative transcriptional regulator proteins (BLLJ_1838 and BLLJ_1496) were identified upstream of the two operons being studied here using the genome sequence of *B. longum* JCM 1217 obtained from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The gene annotation data for this bacterium classifies BLLJ_1496, part of the three gene operon identified in Chapter 2, as a transcriptional regulator belonging to the GntR family of regulators, classified by a helix-turn-helix domain. This family of regulators has also been shown to regulate the transcription of ABC-type transporters (Hillerich & Westpheling, 2006; Rigali *et al.*, 2002). The second putative regulator, BLLJ_1838, upstream of the two gene operon identified, is classified as a transcriptional regulator with no family classification stipulated. Based on their sequence homology to other transcriptional regulators, these two putative transcriptional regulators do, however, resemble the MarR family of transcriptional regulators, most closely. Their potential for regulating the ABC-type multidrug efflux genes downstream is investigated in this Chapter.

An important first step in proving gene regulation by DNA binding proteins is to show the proposed binding reaction in vitro. Specific binding of a regulator to a DNA sequence implies a role in regulating the genes that follow. Experimental techniques such as x-ray crystallography and DNase footprinting are technically demanding and rely on DNA labelling with radioactive molecules, as well as, running complex sequencing electrophoresis gels (Martin & Rosner, 1995). Electrophoretic mobility shift assays (EMSA) offer a simpler and less toxic approach, and allow for the indirect identification of the DNA-protein interaction as a retardation of the DNA mobility in a native polyacrylamide gel when bound to a protein (Martin & Rosner, 1995).

In this chapter we investigate the binding of the two putative transcriptional regulator proteins, BLLJ_1496 and BLLJ_1838, to proposed target DNA sequences, using EMSA. The classification of BLLJ_1496 as a member of the MarR-type family of regulators, as opposed to the GntR-type family of regulators, will also be clarified.

3. Methods and Materials

3.1. Bacterial strains and media and plasmids

Escherichia coli DH5α cells were propagated in Luria-Bertani broth at 37 °C with lateral shaking (Sambrook & Russell, 2001). Agar (Merck) was incorporated into broth media at a concentration of 1.5% ($^{w}/_{v}$) for plating. When necessary, Ampicillin (Amp) was used at 50 μg ml^{-1}, Chloramphenicol (Chl) at 34 μg ml^{-1} (Sigma-Aldrich), IPTG at 1 mM and X-gal at 50 μg ml^{-1} (Bioline).

3.2. Preparation and manipulation of DNA

Plasmid DNA was routinely isolated from *E. coli* DH5α using the alkaline lysis method (Ish-Horowicz & Burke, 1981). Plasmid DNA for use in cloning or sequencing was isolated from *E. coli* DH5α cells using the EZNA Plasmid Miniprep Kit (Peqlab Biotechologie GmbH) as per the manufacturer's protocols. *E. coli* DH5α competent cells were prepared by $CaCl_2$ treatment and transformed using the heat shock approach (Dagert & Ehrlich, 1979). Chromosomal DNA was extracted from *B. longum* JCM 1217 cells as described in Chapter 2. DNA fragments were routinely analysed by electrophoresis through either 0.8% ($^{w}/_{v}$) or 2.0% ($^{w}/_{v}$) agarose gels in TAE buffer (40 mM Tris-acetate, 0.5 mM EDTA), visualised using ethidium bromide staining and a Gel Doc-XR UV System (Bio-Rad) and, when required for cloning, purified using the Biospin PCR Purification Kit (Bioflux). Lambda bacteriophage DNA (Promega) digested with PstI was routinely used as a DNA molecular size marker. DNA quality and concentration were measured with a *ND*-1000 spectrophotometer (NanoDrop Technologies). Restriction digests and ligations were performed according to standard methods (Sambrook & Russell, 2001). All restriction enzymes were obtained from Fermentas. DNA samples were stored at - 20 °C in sterile dH_2O.

3.3. DNA amplification using the polymerase chain reaction and DNA sequencing

Polymerase chain reaction (PCR) mixtures (50 μl) typically consisted of (final concentrations indicated in parentheses): 2 μl *B. longum* genomic or plasmid template DNA (4 ng μl^{-1}), 2.5 μl of each primer (0.5 μM), 25 μl of 2 × KAPATaq ReadyMix (10 mM Tris-HCl [pH 8.6], 50 mM KCl, 0.05% [v/v] Tween 20, 0.5 mM DTT, 5% [v/v] glycerol, 1.5 mM $MgCl_2$, 1.25 U KAPATaq and 0.2 mM of each dNTP) (Kapa Biosystems) and 18 μl of sterile dH_2O. The thermal cycling for each PCR was carried out using either GeneAmp PCR System 9700

(Applied Biosystems) or an Advanced Primus 25 Thermal Cycler (Peqlab Biotechologie GmbH). DNA fragments used for EMSA were amplified using KAPATaq Readymix. To PCR amplify target DNA for protein expression, the High Fidelity PCR System (Fermentas) was used.

The identities of the purified target DNA fragments for EMSA were verified by DNA sequencing. For protein expression, the DNA fragments were first ligated into pET22b (+) (Novagen), transformed into DH5α cells and the plasmids harvested and purified before submitting for sequencing. The reaction products were sequenced by Macrogen (South Korea) and the sequence data analysed using the BLAST algorithm at NCBI.

3.4. Cloning and protein expression

To construct a system where the putative regulator proteins could be over-expressed for EMSA, the open reading frames encoding the genes BLLJ_1838 and BLLJ_1496 were PCR amplified using HiFi Taq (Fermentas) and the primers in Table 4.1, from genomic DNA isolated from *B. longum* JCM 1217. The primer pairs (Table 4.1) contained restriction enzyme sites to facilitate cloning into, as well as, read-through for the incorporation of the histidine tag at the 3' end of the pET22b (+) expression vector. The resulting PCR products were digested with the relevant enzymes and then ligated into the pET22b (+) expression vector digested with the same enzymes and transformed into competent *E. coli* DH5α cells. The resulting plasmid DNA was harvested and the constructs confirmed for the presence of the correct insert DNA by DNA sequencing. The constructs containing the correct DNA fragments were retransformed into the competent expression host cells, *E. coli* BL21 (pLysS) (Novagen).

3.5. Over-expression and purification of recombinant proteins

To produce sufficient quantities of recombinant protein for EMSA, the two recombinant putative regulator proteins were over-expressed as per the pET System Manual (Novagen). The following changes were made to facilitate soluble protein production: a 10 ml pre-culture of *E. coli* BL21 (pLysS) expressing either the recombinant protein BLLJ_1838 or BLLJ_1496 was grown overnight with Amp and Chl and was then used to inoculate 500 ml of LB broth containing Amp and Chl. The culture was grown aerobically at 37 °C with lateral shaking in a baffled flask and the optical density measured until an $OD_{600} \approx 0.6$ was reached. At this point IPTG was added to 1 mM to induce protein expression and the culture

was further incubated for 4 hours at the same conditions to allow for protein production. The cells were harvested by centrifugation (20 min, 4000 x g, 4 °C). Cell pellets were stored at -20 °C for up to 18 hours when necessary.

Protein purification of the expressed proteins as accomplished using the HIS-Select nickel affinity agarose column (Sigma-Aldrich). Cell pellets were resuspended in 10 ml equilibration/wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, pH 8.0 with NaOH) (Sigma). Lysozyme (Sigma) was added to a final concentration of 1 $mg.ml^{-1}$ and the mixture was incubated on ice for 30 min. Cells were disrupted by sonication using the microtip on the Virsonic Digital 600 sonicator (Virtis) with the following program: 5 min (30 sec pulse, 20 sec cooling in an ice-alcohol bath) at Level 3 power output. The resulting lysate was clarified by centrifugation (30 min, 10 000 rpm, 4 °C). The supernatant was loaded onto a prepared Ni^{+} affinity column as per the manual instructions (Sigma). The bound column was washed with approximately 50 ml of equilibration/wash buffer and the remaining bound protein was eluted using 5 ml elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole, pH 8.0 with NaOH) (Sigma), and stored at -80 °C. The eluted protein was quantitated using Coomassie Brilliant Blue G-250 dye (Bio-rad) and Bovine Serum Albumin (Fermentas) for the construction of a calibration curve (Bradford, 1976).

Prior to electrophoretic separation, protein samples were denatured in the presence of an equal volume of loading buffer (125 mM Tris-HCl [pH 6.8], 2.5% [w/v] SDS, 20% [v/v] glycerol, 0.2 % [w/v] bromophenol blue) by heating at 100 °C for 5 min. Proteins were then separated according to molecular weight by SDS-polyacrylamide (37:1) gel electrophoresis (PAGE) using a Mini-PROTEAN Electrophoresis System (Bio-Rad) with a 6% stacking gel and a 15% resolving gel as described by (Laemmli, 1970). The prestained PageRuler protein molecular weight marker (Fermentas) was used to determine the apparent molecular weights of the separated proteins. Hexahistidine-tagged proteins were detected according to the standard Western blot method using an anti-6-His rabbit antibody (1:5000 dilution) and a horse radish peroxidise-conjugated anti-rabbit goat secondary antibody (1:5000 dilution) in conjunction with the chromogenic TMB membrane peroxidase substrate (all manufactured by GeneTex) (Sambrook & Russell, 2001).

Table 4.1. Primer sequences and features for putative regulator cloning and EMSA DNA targets

Primer name	Primer Sequence (5' – 3')	Purpose	Features	Size
BLLJ_1838PEF	GGAATGGATGGTGCTCACATGTTATTTGAG	Expressing	PstI restriction site	563 bp
BLLJ_1838PER	GCCAGCGGGAGGAAGCTTGTCTCGCTC	BLLJ_1838	HindIII restriction site	
BLLJ_1496PEF	CGGAGGACCATGGATCAGC	Expressing	NcoI restriction site	555 bp
BLLJ_1496PER	CTGCGGTATCTCGAGTTCG	BLLJ_1496	XhoI restriction site	
BL1518F	CAGGAAGATCTATATCGGCACCATCGCGTTC	BL1518	(Klijn *et al.*, 2006)	291 bp
BL1518R	GCTGCCCTCGAGCATATTGTCTCCTTCGAC	promoter region		
BLLJ_1839GF	TGTCTTCATCCGCCTTTTCGACC	EMSA	This study	252 bp
BLLJ_1838GR	AGAGCACCATCCATTCCCATTCG	DNA Target 2	Figure 4.7	
BLLJ_1838GF	AGCGCGCGGAGAACTAATCGG	EMSA	This study	264 bp
BLLJ_1837GR	ACCAGAGCGATGAGCATCTGACC	DNA Target 3	Figure 4.7	
BLLJ_1837GF	TCAGCCAGTCGGAGCTTACGG	EMSA	This study	250 bp
BLLJ_1836GR	CACGCCTGCCGCCCATTGG	DNA Target 4	Figure 4.7	
BLLJ_1497GF	TGGTTGACGAGTACGCCAAGC	EMSA	This study	270 bp
BLLJ_1496GR	TGAGAATATGCTGATCCATTGTCC	DNA Target 1	Figure 4.7	

3.6. Electrophoretic mobility shift assays (EMSA)

For EMSA visualisation, the target DNA fragments were 3' labelled with a DIG-molecule using the DIG Gel Shift Kit, 2nd Generation (Roche) by means of PCR amplification using the primer pairs in Table 4.1. The fragments were gel excised, purified and confirmed with DNA sequencing. A typical labelling reaction was performed as follows: 3.85 pmol DNA in 10 µl TEN buffer (10 mM Tris, 1 mM EDTA, 0.1 M NaCl, pH 8.0) was heated to 95 °C for 10 min then cooled to 20 °C for 2 min. 4 µl 5 X Labelling buffer (1 X), 4 µl $CoCl_2$ solution, 1 µl DIG-ddUTP solution, 1 µl Terminal Transferase was then added to the DNA to a final volume 20 µl. The mixture was incubated at 37 °C for 15 min after which 2 µl of 0.2 M EDTA pH 8.0 (2 mM) as added to stop the reaction. The final reaction volume was adjusted to 25 µl by adding 3 µl of sterile dH_2O and the labelled DNA was stored at -20 °C.

To determine whether binding of the putative regulator proteins to their target DNA sequences had occurred, a binding reaction was conducted *in vitro* to mimic the *in vivo* reaction. Protein DNA-binding reactions were prepared on ice using the DIG Gel Shift Kit, 2nd Generation (Roche). A 20 µl reaction typically included 4 µl of 5 X binding buffer (100 mM HEPES [pH 7.6], 5 mM EDTA, 50 mM $(NH_4)_2SO_4$, 5 mM DTT, 1% [w/v] Tween 20, 150 mM KCl). Poly-(d[I-C]) and poly-L-lysine solutions were added (1 $\mu g.\mu l^{-1}$ each). Finally, 2 µl labelled DNA (3.1 fmol) and 3 µg purified protein were added and the reaction volume adjusted to 20 µl with sterile dH_2O. The reaction mix was incubated at 25 °C for 15

min, and then placed on ice. Varying concentrations of erythromycin (1, 5, 10, 20, 50, 100 mM) and Na-salicylate (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 1.2 M) (Sigma) were tested for their potential to inhibit binding.

3.7. Native Polyacrylamide Gel Electrophoresis

Protein DNA-binding reactions were analyzed by running on a native 6% (w/v) polyacrylamide gel (30%, 29:1 acrylamide mix; Sigma-Aldrich; 0.25 × TBE buffer; 0.1% APS; 0.1 % TEMED) at 100 V using a Mini-PROTEAN Electrophoresis System (Bio-Rad) at 4 °C. Prior to the separation of unbound DNA and DNA-protein complexes by native PAGE, 2.5 µl of loading buffer (0.25 × TBE buffer [22.25 mM Tris-borate, 0.5 mM EDTA], 60% [w/v] glycerol, 0.2% [w/v] bromophenol blue) was added to each reaction. Prior to loading, native PAGE gels were pre-run for 1 hr at 100 V. Experimental gels were typically run for approximately 2 hrs until the loading dye had run off the bottom of the gel. The binding reactions were then transferred from the native polyacrylamide gels to a Hybond-N^+ nylon membrane (Amersham Biosciences) via electroblotting (DIG 2^{nd} Generation Gel Shift Kit manual), and then UV-crosslinked. Transferred labelled nucleic acid was finally visualised using the chemiluminescent CSPD substrate (Roche) as outlined in the DIG Application Manual for Filter Hybridisation (Roche).

4. Results and Discussion

4.1. Bioinformatics

The bioinformatic analysis of the two ABC-type multidrug efflux transporter gene clusters being studied here was reported in Chapter 2 of this thesis. Upon closer inspection of these clusters in *B. longum* NC27705, the presence of two putative MarR-type transcriptional regulators upstream of both of the selected efflux gene clusters was noted. When compared to their homologues in *B. longum* JCM 1217 the same genome arrangement was observed, with the exception that BLLJ_1496 was annotated as a GntR-type transcriptional regulator (Figure 4.1).

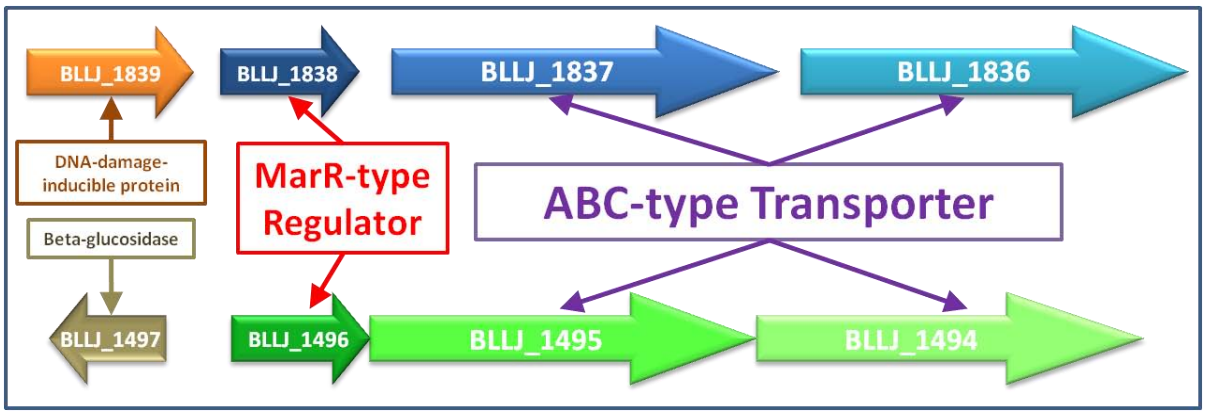


Figure 4.1. Genome arrangement of both *B. longum* JCM 1217 gene clusters in this study, showing the position of the putative transcriptional regulator genes relative to their adjacent genes.

Since BLLJ_1838 was annotated in the genome as a 'transcriptional regulator' with no family classification, whereas BLLJ_1496 is annotated as belonging to the GntR family of transcriptional regulators in the *B. longum* JCM 1217 genome sequence at NCBI, further bioinformatic analysis was conducted to confirm their identities. A BLASTP search was done against all prokaryote genomes in the BLAST database (Altschul *et al.*, 1997). The results showed that both putative regulators had very high similarities to other putative MarR-type regulators (Appendix Table A4.1 and A4.2). When a multiple protein sequence alignment (DNAMAN, Version 4.13, Lynnon Biosoft) was carried out using the top five BLASTP results, it was observed that the their combined sequence identity was > 95 % for BLLJ_1496 and > 91 % for BLLJ_1838 (Appendix Figure A4.1 and A4.2).

To further investigate the classification of these proteins, more reliable bioinformatic analysis was carried out. The GntR family of transcriptional regulators are characterised by their helix-turn-helix (HTH) domain which is known to be a tertiary protein structure used for DNA binding (Pabo & Sauer, 1992). The MarR family of transcriptional regulators are characterised by DNA binding with a winged-helix-turn-helix (WHTH) domain (Wilkinson & Grove, 2001). A multiple protein sequence alignment was constructed, using ClustalW, of BLLJ_1496 and BLLJ_1838 with the proven MarR regulators: MarR from *Escherichia coli*, MprA from *E. coli*, MexR from *Pseudomonas aeruginosa* and SlyA from *Salmonella tyhphimurium*. The results of this alignment showed that there was a 26.87% overall sequence identity for BLLJ_1496 (Figure 4.2 A) and a 23.82% overall sequence identity for BLLJ_1838 (Figure 4.2 B), when compared to the proven MarR regulators used for this analysis.

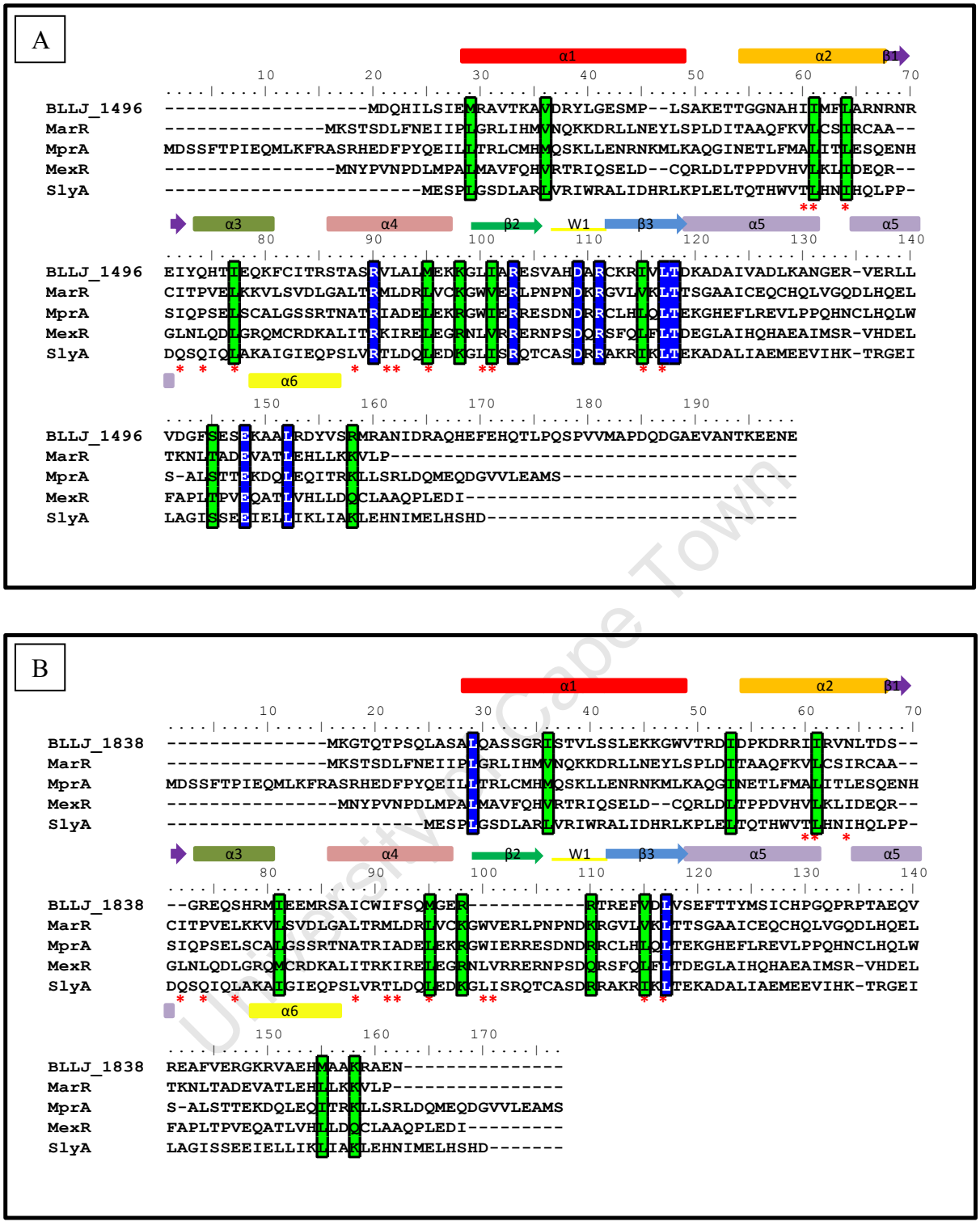


Figure 4.2. ClustalW multiple sequence alignment of (A) BLLJ_1496 and (B) BLLJ_1838 with known MarR-type proteins. Secondary structure annotations adapted from (Alekshun *et al.*, 2001). Residues that are identical in all homologues are coloured blue and similar residues are coloured green. The MarR secondary structure elements are illustrated as boxes for the α-helices (α), arrows for the β-sheets (β) and the single winged region as a line (W1). The red asterisks below the sequence designate residues identified from the MarR crystal structure to form the hydrophobic core of the monomeric DNA binding domain.

The multiple sequence alignment of BLLJ_1496 with four functionally proven MarR regulators shows 8 identical residues (90, 103, 109, 111, 117, 118, 148, 152) in the C-terminal half of the proteins. The helix-turn-helix domain is formed by the helices α-3 and α-4 and the 4 residues between them. This is then flanked by the "wing motif" comprising β-2, W1 and β-3. Of the identical residues, 1 (residue 90) occurs in the α-4 helix, known as the recognition helix for DNA binding and which makes physical contact with the DNA. The next 5 identical residues (103, 109, 111, 117, 118) occur in the wing motif of MarR, and of the final two identical residues, 1 (148) occurs just before and 1 (152) inside the final α-6 helix. Of the 14 residues comprising the hydrophobic core of MarR, 6 are conserved and 1 is identical in BLLJ_1496 (Gajiwala & Burley, 2000; Huffman & Brennan, 2002). Based on this and the strong similarity between BLLJ_1496 and other proven MarR regulators, as well as the presence of the wing motif, it seems highly probable that BLLJ_1496 is in fact a MarR-type transcriptional regulator, as it is annotated in the genome sequence of *B. longum* NC27705, and not a GntR-type regulator as it is annotated in *B. longum* JCM 1217, since GntR-type regulators lack the 'wing motif'.

The analysis of BLLJ_1838 does not present such compelling evidence that it is a MarR-type regulator. Most notably is the absence of the wing motif in BLLJ_1838. The helix-turn-helix structure appears to exist, but there is a gap where the β-2 and W1 regions exist in the MarR structure. There are only 2 identical residues in the entire sequence analysis, of which only residue 118 lies in the tail end of the β-3 sheet region. Of the 14 hydrophobic core residues in MarR, only 3 are conserved and 1 is identical. This analysis suggests the possibility for DNA binding using the HTH motif, and possibly gene regulation, but it does not favour reclassification of BLLJ_1838 as a MarR-type regulator protein.

Based on these analyses it was decided that EMSA would be conducted, using the purified proteins BLLJ_1496 and BLLJ_1838, to determine whether they were able to specifically bind to cognate DNA sequences flanking these putative regulators and the structural genes downstream. Binding of these regulator proteins to these DNA sequences would suggest a role in regulating their transcription in response to certain environmental and/or cellular changes.

4.2. Protein expression and purification

Purified proteins are preferred for electrophoretic mobility shift assays (EMSA) since they produce less background interference, as well as, greatly reduce the possibility of false positive results. For this reason, both putative regulator proteins were cloned and expressed, using the pET Expression System (Novagen). Optimum expression of the 19.8 kDa, histidine tagged BLLJ_1838 protein in the soluble fraction was obtained after expressing for 2 – 4 hours after induction with 1mM IPTG (Figure 4.3).

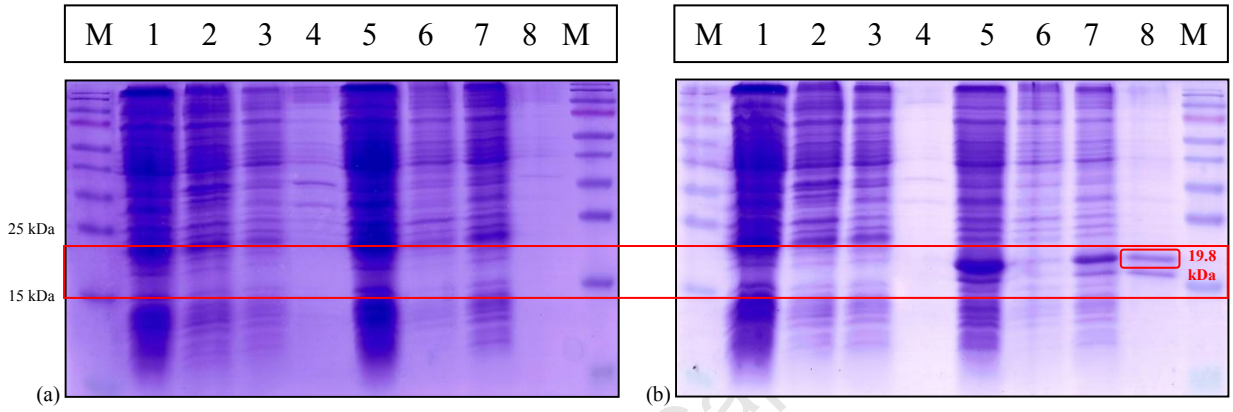


Figure 4.3. SDS PAGE of BLLJ_1838 expression after 2 hours of IPTG induction. Lanes 1 and 5, total cell protein; lanes 2 and 6, periplasmic fraction; lanes 3 and 7, soluble fraction; lanes 4 and 8, insoluble fraction; M, molecular weight marker. (a) pET22b without insert, (b) pET22b containing BLLJ_1838. Lanes 1-4, no IPTG added; lanes 5-8, 1mM IPTG added.

Optimum expression of the 23.1 kDa, histidine tagged BLLJ_1496 protein in the soluble fraction was obtained by expressing for 4 – 6 hours after induction with 1mM IPTG (Figure 4.4).

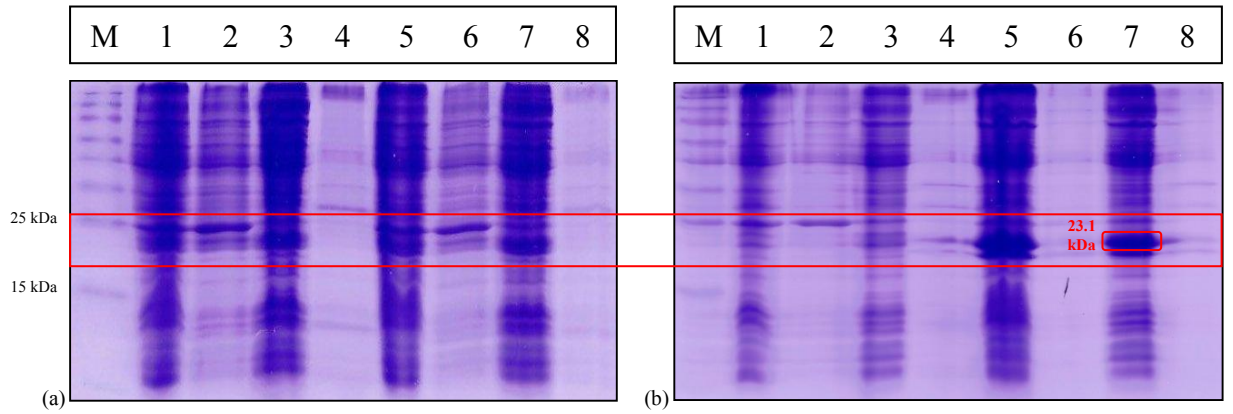


Figure 4.4. SDS PAGE of BLLJ_1496 expression after 6 hours of IPTG induction. Lanes 1 and 5, total cell protein; lanes 2 and 6, periplasmic fraction; lanes 3 and 7, soluble fraction; lanes 4 and 8, insoluble fraction; M, molecular weight marker. (a) pET22b without insert, (b) pET22b containing BLLJ_1496. Lanes 1-4, no IPTG added; lanes 5-8, 1mM IPTG added.

The addition of the C-terminal hexahistidine fusion tag allowed for purification by affinity chromatography. The N-terminal PelB leader sequence provides the potential for periplasmic re-localisation of recombinant protein in *E. coli* BL21 (DE3) cells if needed (Georgiou & Segatori, 2005; Wolfe *et al.*, 1983).

Once both proteins were optimally expressed in the soluble fraction they were purified using a Ni^+ Affinity column (Roche) and detected using an anti-Histidine Western blot. The blot showed that the correct proteins were bound and eluted off the Ni^+ affinity column (Figure 4.5).

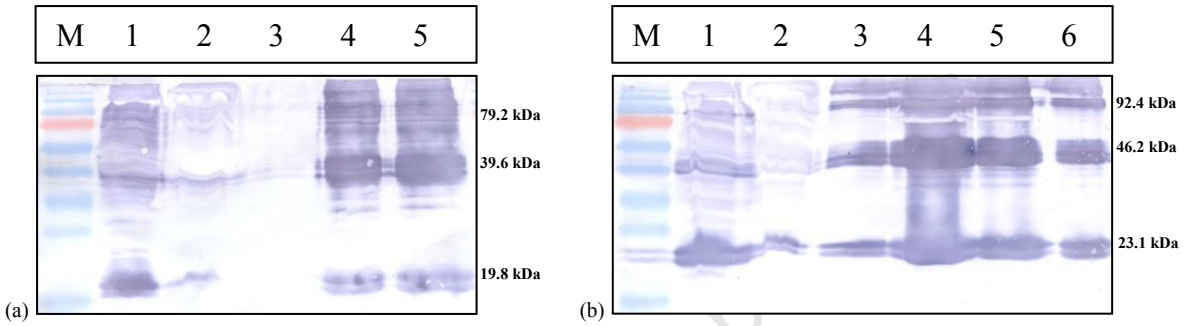


Figure 4.5. Anti-His Western blot of purified proteins. (a) BLLJ_1838 anti-histidine western blot, (b) BLLJ_1496 anti-histidine Western blot. Lane 1, total cell protein after IPTG induction; lane 2, cleared lysate after column binding; lane 3, wash buffer; lane 4, 5, 6(b), elution's 1, 2 and 3 respectively.

The protein expression and purification results clearly show that both putative regulators BLLJ_1838 and BLLJ_1496 were successfully purified from the total cellular protein milieu. All of the SDS-PAGE gels run for these experiments produced a doublet at the expected size, as well as, bands at increasing increments of double the expected protein size. Previous work on MarR-type regulators has shown that these regulators act as homodimers on their target DNA comprising two molecules of protein for every binding site (Alekshun *et al.*, 2001). This would explain the presence of bands at approximately double the expected protein size, as *in vitro* the purified proteins would be able to dimerise in a repeating manner. To confirm this, another SDS-PAGE gel was run which included β-mercaptoethanol, which resolves quaternary proteins structures. The resulting gels clearly show the resolution of these bands into one band of the correct size (Figure 4.6).

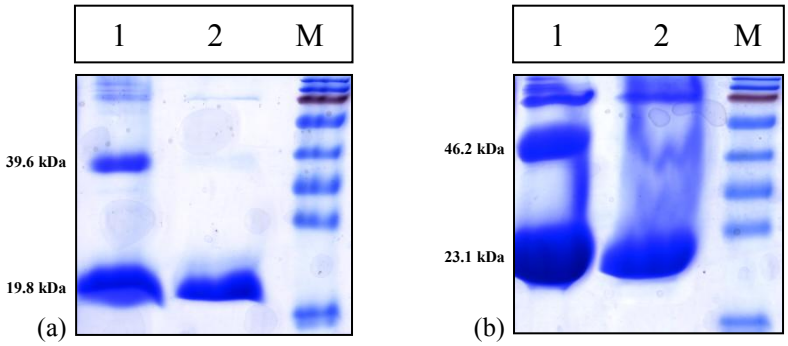


Figure 4.6. SDS-PAGE of purified the proteins (a) BLLJ_1838, (b) BLLJ_1496. Lane 1, purified protein without β-mercaptoethanol; lane 2, purified protein with added β-mercaptoethanol.

4.3. Electrophoretic mobility shift assays (EMSA)

EMSA was used to determine whether regulation of the two operons under investigation is in fact controlled by the putative regulators identified upstream of each one. Binding of a regulator protein to a DNA sequence upstream of a structural gene gives a strong indication of a regulatory function on that gene. To ascertain whether this is the case here, the BLLJ_1838 and 1496 genes were cloned, the proteins expressed and purified and were used in *in vitro* binding experiments.

To determine whether binding occurs, the purified proteins must be presented with possible target DNA sequences. DNA targets of approximately 250 – 300 bp upstream of each putative regulator as well as regions between the regulators and the structural genes in the operons were selected as putative regulator binding sites (Figure 4.7). Since MarR-type transcriptional regulators bind to promoters or just upstream of promoter regions (Martin & Rosner, 1995), these DNA target sequences were PCR amplified, gel excised and DIG-labelled for EMSA experiments.

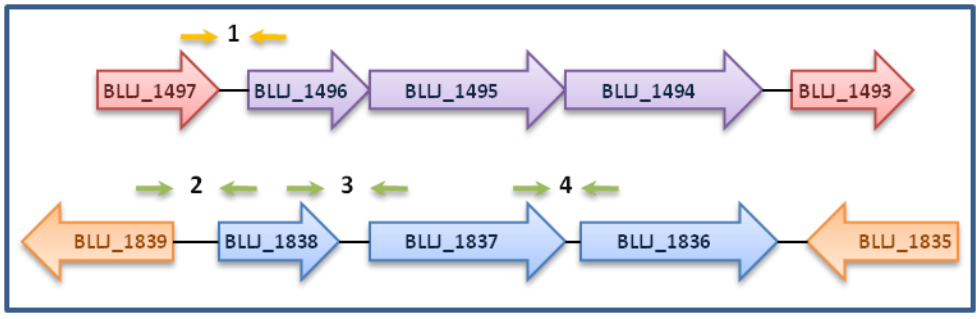
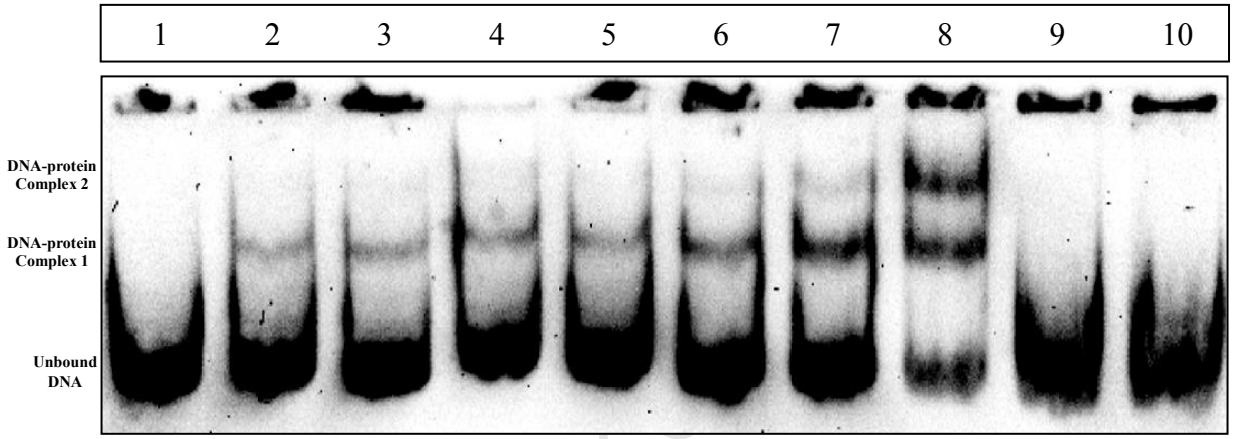


Figure 4.7. Figure showing the regions of DNA targeted in EMSA experiments. The numbers 1 – 4 indicate the various DNA target fragments, PCR amplified for EMSA. Numbering corresponds to the primer pairs in Table 4.1.

Based on the bioinformatic analysis and genome arrangement of the operon BLLJ_1496-5-4 the only probable DNA binding region for the putative transcriptional regulator was the region of DNA upstream of the 3-gene operon (Figure 4.7, region 1). When the purified BLLJ_1496 protein was added to this target DNA a notable shift in the migration of the bound DNA was observed when run on a native PAGE gel (Figure 4.8). Poly-(d[I-C]) and poly-L-lysine, which have been shown to reduce non-specific DNA-protein interactions and enhance the stability of various DNA-protein interactions were included in all the binding reactions (Kozmík *et al.*, 1990).



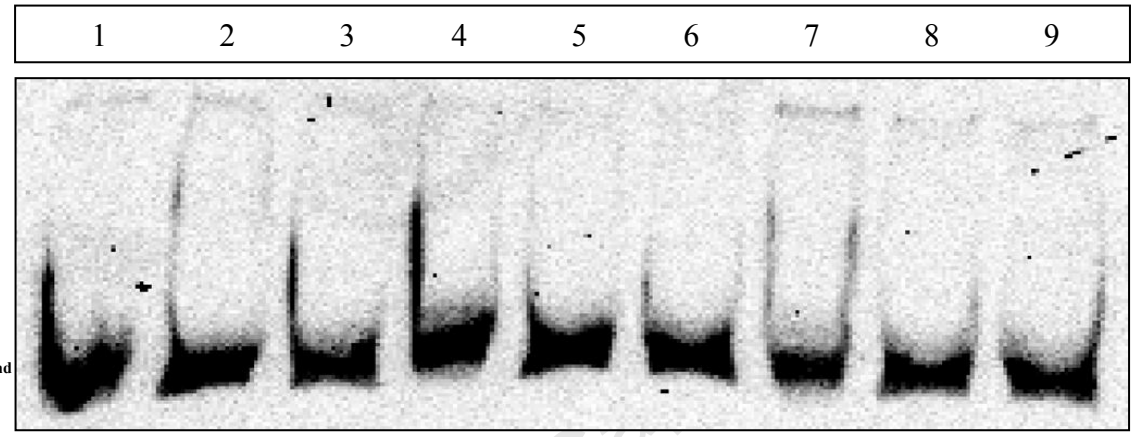
DNA	Target 1	Target 1	Target 1	Target 1	Target 1	Target 1	Target 1	Target 1	Target 1	Target 1
Protein	-	0.2 µg	0.4 µg	0.6 µg	0.8 µg	1.0 µg	2.0 µg	3.0 µg	1.0 µg	2.0 µg
Comp	-	-	-	-	-	-	-	-	100 ng	100 ng

Figure 4.8. Native PAGE gel of EMSA of BLLJ_1497-6 Target 1 DNA fragment with purified BLLJ_1496 protein. Lane 1, BLLJ_1497-96 DIG labelled DNA, lanes 2-8 labelled DNA with increasing amounts of purified BLLJ_1496 protein. Lanes 9 and 10, same as 6 and 7 except with 100 ng unlabelled DNA added as competitor (Comp).

The binding of the protein to the target DNA fragment and subsequent reduction in mobility observed above proved to be concentration dependent and at high concentrations (2 – 3 µg) of added protein, a "super-shift" is observed (lanes 7 and 8). This super-shift may indicate the presence of a second binding site in this region, which is commonly observed for MarR-type DNA-protein binding reactions (Martin & Rosner, 1995). Binding of the putative regulator protein BLLJ_1496 to this region of DNA, as well as the 'super-shift' observed here gives a strong indication that this may in fact be the transcriptional regulator of this operon, and that BLLJ_1496 may well belong to MarR family of transcriptional regulators, based on the observed 'super-shift'.

For the operon BLLJ_1837-6, based on the bioinformatic analysis and genome arrangement, several candidate DNA regions for regulator binding are possible. The region upstream of

the putative regulator (Figure 7, region 2) would be the most likely candidate based on the binding observed for BLLJ_1496 to the DNA fragment upstream of the operon, but, the region between the putative regulator and the two gene operon downstream (Figure 7, region 3), as well as, the region between the two structural genes in the operon (Figure 7, region 4) cannot be ignored as potential regulator binding sites. For this reason an EMSA was carried out using the purified BLLJ_1838 putative transcriptional regulator protein and target regions from all these selected regions of DNA (Figure 4.9).



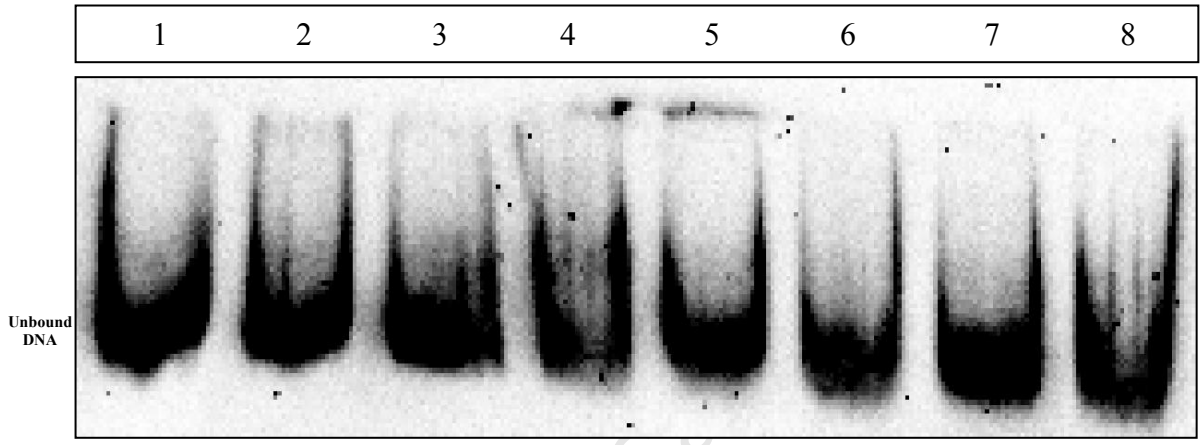
DNA	Target 2	Target 2	Target 2	Target 3	Target 3	Target 3	Target 4	Target 4	Target 4
Protein	-	3.0 µg	3.0 µg	-	3.0 µg	3.0 µg	-	3.0 µg	3.0 µg
Comp	-	-	100 ng	-	-	100 ng	-	-	100 ng

Figure 4.9. PAGE gel of EMSA of BLLJ_1839-8 Target 2, 38-7 Target 3 and 37-6 Target 4 DNA fragments with purified BLLJ_1838 protein.

No binding was observed for this putative regulator protein with any of the proposed DNA targets using the *in vitro* conditions tested. Based on this it is unlikely to be involved in regulating its own transcription or the two gene ABC-type transporter operon downstream. Further experiments should be conducted using conditions which mimic the *in vivo* conditions more accurately, such as changing the ionic profile used, as well as, the use of other stabilising adjuncts, to conclusively rule out the involvement of this protein in regulating the transcription of these genes. It is also not possible to infer any family classification of the putative regulator since no functional relationship has been proven here, even though the BLAST analysis showed that it has > 91% similarity to other MarR-type transcriptional regulators.

While binding of the putative MarR-type regulator BLLJ_1496 to the target DNA sequence upstream of the operon has been demonstrated here (Figure 4.8), it is important to show that this binding is specific for the target sequence presented and that the regulator does not bind

to any possible promoter region. To confirm the specificity of this regulator's binding, the reactions were repeated with target DNA obtained by PCR amplifying a region of DNA from *B. longum* JCM 1217 which includes a proven functional promoter sequence in *B. longum* NC27705 (Klijn *et al.*, 2006). Purified BLLJ_1496 protein was added to this DNA using the same conditions which produced the successful binding reaction. We also tested the purified BLLJ_1838 protein in this experiment to determine its specificity even though it did not bind to any of the likely target DNA sequences presented before (Figure 4.10).



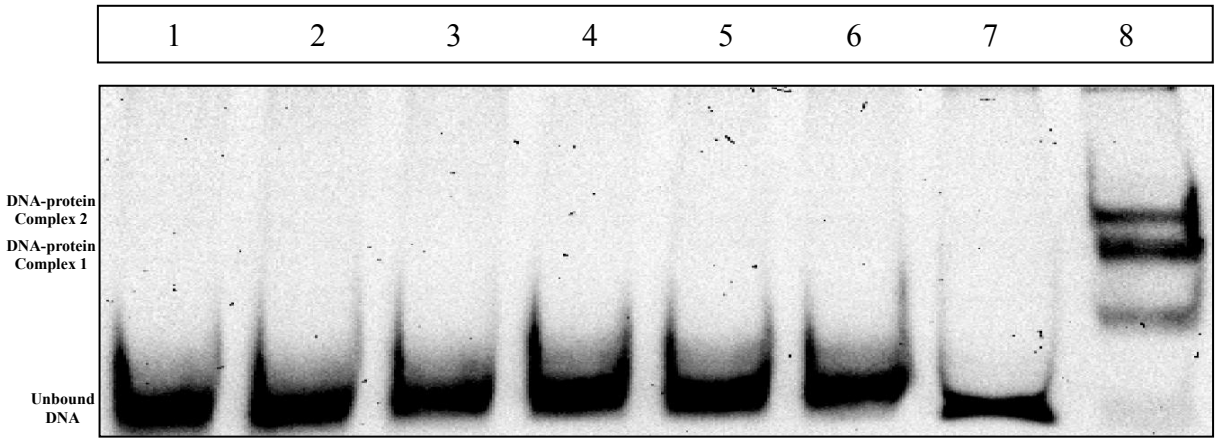
DNA	BL1518	BL1518	BL1518	BL1518	BL1518	BL1518	BL1518	BL1518
Protein	-	BLLJ_1496	BLLJ_1496	BLLJ_1496	-	BLLJ_1838	BLLJ_1838	BLLJ_1838
Comp	-	-	BL1518	Target 1	-	100 ng	BL1518	Target 2

Figure 4.10. Native PAGE of EMSA with a proven promoter region as DNA target. Lanes 1-8 contain BL1518 labelled DNA; lanes 2-4, 3 µg BLLJ_1496 purified protein; lanes 6-8, 3 µg BLLJ_1838 purified protein; lanes 3 and 7, unlabelled BL1518 DNA; lane 4, unlabelled BLLJ_1497-6 DNA as competitor; lane 8, unlabelled BLLJ_1839-8 DNA as competitor (Comp).

Since no binding was observed in any of these reactions it can be concluded that the binding of BLLJ_1496 to the Target 1 DNA sequence is specific and it is, therefore, most probably the regulator of this operon. Functional regulator studies, where the regulator protein is expressed *in vivo* with the ABC-type transporter genes, are required to conclusively prove this. For the putative regulator BLLJ_1838, no binding was observed for any of the target DNA sequences provided, including that of the control promoter region, this leads to the conclusion that it is most likely not the regulator of the operon downstream. It also did not bind to the control DNA form the proven promoter region, even though it possesses the HTH motif for DNA binding.

Another question that arises regarding binding specificity of these regulators is whether they are specific for regulating only the gene expression of their own gene clusters, or whether they are able to bind to and regulate other cognate ABC-type transporter genes. Since MarR-

type regulators are part of a regulon in *E. coli* and *S. typhimurium* (Alekshun & Levy, 1997), they may recognise similar binding sites for other promoters included in this regulon. To ascertain whether this occurs, with these regulator proteins, in *B. longum* JCM 1217, an EMSA was conducted where the purified proteins were presented with the target DNA fragments of the alternate operon being studied (Figure 4.11).



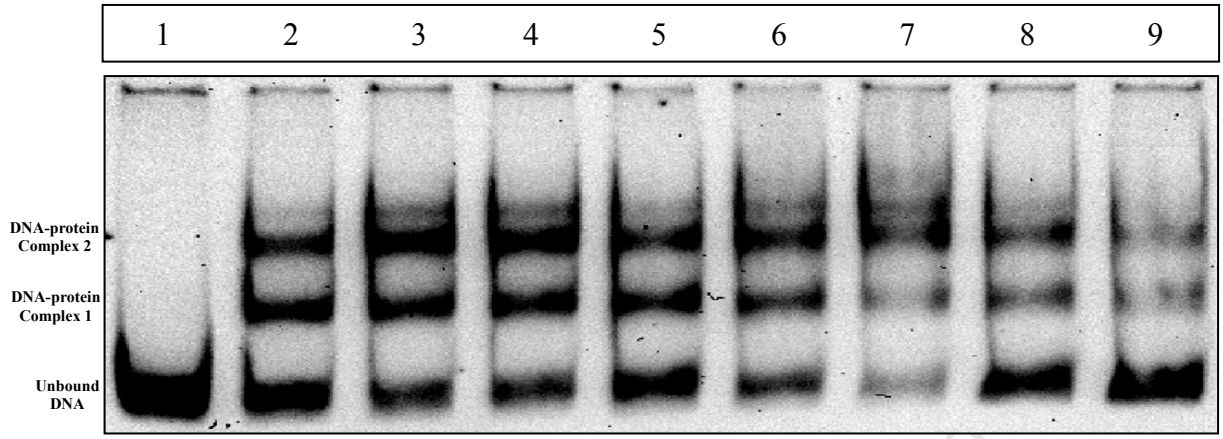
DNA	Target 2	Target 2	Target 2	Target 3	Target 3	Target 3	Target 1	Target 1
Protein	-	BLLJ_1496	BLLJ_1496	-	BLLJ_1496	BLLJ_1496	BLLJ_1838	BLLJ_1496

Figure 4.11. Native PAGE of EMSA testing cross binding. Lanes 1-3 contain BLLJ_1839-8 labelled DNA; lanes 4-6 contain BLLJ_1838-7 labelled DNA; lanes 7 and 8 contain BLLJ_1497-6 labelled DNA; lanes 2, 3, 5, 6, 8, contain 3 µg purified BLLJ_1496 protein; lane 7, contains 3 µg purified BLLJ_1838 protein.

The data obtained from this experiment reaffirms the previous results which showed that the binding of the regulator protein BLLJ_1496 to its target DNA fragment was highly specific and that even when presented with another DNA fragment upstream of another ABC-type transporter, no binding was observed. If BLLJ_1496 is indeed a MarR-type transcriptional regulator, it shows that even though MarR-type regulators are part of a global regulatory system in other bacteria, the binding of the regulator tested here was highly specific for its respective DNA sequence. This binding specificity would allow the cell to tightly control which operons are upregulated in response to challenge by external stimuli even though a global response may be initiated.

Since MarR-type regulators are classified as negative transcriptional regulators, initiation of gene transcription would require the presence of an inducer molecule to de-repress the regulatory function of these proteins. Sodium salicylate is a molecule previously shown to disrupt binding of MarR-type transcriptional regulators to their cognate DNA sequences, thus inducing gene expression (Martin & Rosner, 1995; Saridakis *et al.*, 2008). To investigate further whether the putative regulator protein BLLJ_1496 may belong to the MarR-type

family of regulators, an EMSA was conducted with the addition of Na-salicylate (Figure 4.12).

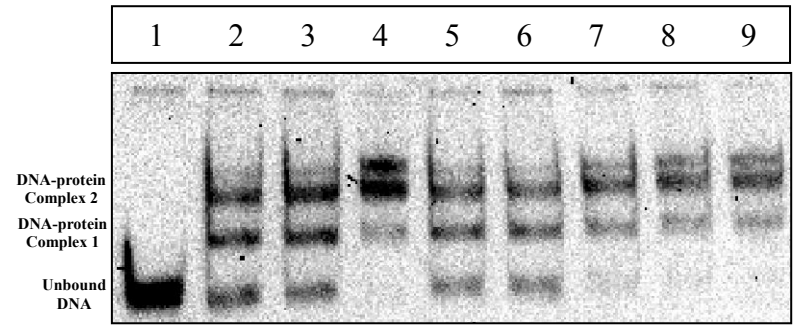


DNA	Target 1	Target 1	Target 1	Target 1	Target 1	Target 1	Target 1	Target 1	Target 1
Protein	-	3 µg	3 µg	3 µg	3 µg	3 µg	3 µg	3 µg	3 µg
Na-Sal	-	0.01 M	0.02 M	0.05 M	0.1 M	0.2 M	0.5 M	1.0 M	1.2 M

Figure 4.12. Native PAGE of EMSA with sodium salicylate. Lanes 1-9 contain BLLJ_1497-6 labelled DNA with lanes 2 – 9, 3 µg of purified BLLJ_1496 protein; lanes 2-9 have increasing concentrations of sodium salicylate.

The results obtained showed that sodium salicylate appeared to have a moderate effect on regulator binding, since, the shifted bands decreased in intensity only when very high concentrations (1 and 1.2 M) were added. This indicated that sodium salicylate had little effect on disrupting the regulator binding observed in its absence (Nichols *et al.*, 2009). It may also indicate the high affinity with which the protein bound to the DNA since it was not easily displaced by exposure to sodium salicylate.

In the work presented in Chapters 2 and 3 of this thesis it was noted that the exposure of *B. longum* JCM 1217 to sub-lethal concentrations of erythromycin resulted in an increase in the transcription levels of genes downstream of the MarR-type transcriptional regulator BLLJ_1496. This raises the question as to whether erythromycin might act as an effector molecule, and as such, whether it might have any effect on disrupting the regulator's ability to bind to the target DNA sequence, thereby inducing transcription of the structural genes downstream. To test this hypothesis we conducted another EMSA using the Target 1 DNA fragment with 3 µg of the purified BLLJ_1496 protein, with the addition of various amounts of erythromycin (Figure 4.13).



DNA	Target 1	Target 1	Target 1	Target 1	Target 1	Target 1	Target 1	Target 1	Target 1
Protein	-	3 µg	3 µg	3 µg	3 µg	3 µg	3 µg	3 µg	3 µg
Ery	-	-	1 mM	5 mM	10 mM	20 mM	50 mM	100 mM	EtOH

Figure 4.13. Native PAGE of EMSA with erythromycin. Lanes 1-9 contain BLLJ_1497-6 labelled DNA and 3 µg of purified BLLJ_1496 protein; lanes 3-8 have increasing concentrations of erythromycin. Lane 9, 5 µl of 70 % EtOH as vehicle control.

The results showed no discernible reduction in binding efficiency, but rather the opposite in that an increase in the binding efficiency appears to occur in the presence of erythromycin. However, this is an artefact since the vehicle control in lane 9 (5 µl 70% EtOH) produces the same effect as that observed in lane 4 (5 mM Ery in 5 µl 70% EtOH). It appears that ethanol may increase binding affinity in this situation, although the reason for this has not yet been established.

5. Conclusions

This work reports the first step in determining whether two putative regulator proteins have the ability to bind DNA sequences adjacent to the genes they are proposed to regulate. The data obtained showed that the putative transcriptional regulator BLLJ_1496 bound to a region of DNA upstream of the three gene operon in which it lies. This binding occurs in a manner similar to that of known MarR-type regulators, where a 'super-shift' is often observed when the regulator which acts as a dimer binds to two sites in the promoter region (Martin & Rosner, 1995). The observed binding occurred with a strong affinity for the target sequence and was not significantly reduced when sodium salicylate, a known inhibitor, was present. Binding was also shown to be very specific for the target DNA sequence since no binding is observed when other potential DNA targets are presented. No DNA binding was observed for the putative regulator BLLJ_1838 to any of the DNA targets presented. No conclusions can be drawn regarding the function or classification of this protein.

Disparity regarding the classification of the putative regulator BLLJ_1496 as a GntR-type transcriptional regulator was presented here and even though strong bioinformatic and experimental evidence was presented to motivate its re-classification as a MarR-type regulator instead, further bioinformatic and experimental analysis proving its function needs to be conducted to conclusively prove this. To confirm its role as a repressor of these ABC-type transporter genes the regulator protein could be expressed, *in trans*, with the ABC-type transporter genes it is proposed to regulate, in the same host cell. No resulting increase in the MIC to erythromycin and tetracycline would indicate its ability to repress the expression of these genes, since, when the ABC-type transporters are expressed in its absence an MDR phenotype is observed in *L. lactis*.

This data shows the first evidence of a probable MarR-type transcriptional regulator in *B. longum* JCM 1217, which may regulate its own transcription as well as an ABC-type multidrug efflux transporter system.

CHAPTER 5

General Conclusions

Probiotic bacteria are a major constituent of the gut microbiota of both humans and animals. Their presence constitutes a positive influence by conferring numerous health benefits to the host. Even though the presence of these bacteria is advantageous, they are known to be intrinsically drug resistant and harbour genes coding for ABC-type multidrug efflux systems. These systems are beneficial to these bacteria since they confer a competitive advantage during antibiotic therapy and also to the host since the bacteria are able to continue providing their health benefits during periods of disease. Regrettably, with the current increase in antibiotic resistance in prokaryotes, the possibility of transfer of these genes via HGT cannot be ignored. This study thus aimed to characterise these ABC-type multidrug systems in an effort to better understand the ubiquity, range and mechanisms underlying these genes in the probiotic bacterium *B. longum* subsp. *longum*^{T} JCM 1217.

In Chapter 2 of this study, bioinformatic analysis showed the ubiquity of these types of efflux transporters in *B. longum* JCM 1217, as was highlighted in the membrane protein database. Using this database, two putative multidrug efflux systems were identified in the genome of *B. longum* JCM 1217 for further analysis. These genes exhibited an operon-like structure as both clusters were composed of two putative ABC-type transporter genes, as well as, a putative transcriptional regulator situated upstream of the two structural genes. Reverse transcriptase PCR experiments showed that these two gene clusters were in fact transcribed as two operons; one comprising three genes – inclusive of the putative transcriptional regulator, and the other comprising two genes – excluding the putative transcriptional regulator. An interesting outcome of this experiment was the indication of a semi-quantitative increase in the transcription levels of these genes when the *B. longum* cells were grown in the presence of erythromycin. These results together showed that these genes do in fact not only reside in the genome of *B. longum*, but also that they are arranged in a manner which facilitates the simultaneous transcription of two ABC-type transporter genes. The

transcription of these genes also appeared to be up-regulated when erythromycin, a known substrate of ABC-type transporters, was present. The clustering of two genes encoding different transporter proteins is interesting, as on exposure to antibiotics, cellular resources would be minimized since a single transcriptional event is carried out which results in two different resistance proteins being produced. Since these transporters are known to function as a homodimer comprising two transporter protein molecules, this would result in a full transporter being produced for each transcriptional event, since a number of polypeptides are produce for every mRNA transcript produced. Even though the research to-date has showed that these transporters usually function as homodimers, a heterodimer arrangement is probable in this case if the two different polypeptides produced are compatible. This would increase the substrate range of the transporter even further as each half-protein, comprising one polypeptide, would provide an overlapping, but possibly variant substrate range. To confirm this, assays identifying *in vivo* protein-protein interactions need to be carried out, and should be considered for future work.

When these ABC-type transporter genes were cloned and heterologously expressed in *L. lactis* cells, they were shown to confer an MDR phenotype on the host by increased resistance to erythromycin and tetracycline. Even though this provides unequivocal proof of the MDR resistance function of these genes, further experiments are needed to elucidate the full substrate range of each gene under investigation. An indication of the ATP-dependence of these proteins, when active, was also shown using the substrate Hoechst 33342. These results conclusively showed that these genes are functional multidrug resistance genes and when the Hoechst 33342 efflux is coupled with the bioinformatic analysis, it presents a strong indication that these genes belong to the ATP-binding cassette family of transporters. Unfortunately, due to the high background efflux activity in the host *L. lactis* cells, a definitive conclusion regarding their contribution to efflux, could not be established. Future work must, therefore, include experiments aimed at conclusively proving the ATP-dependence of these genes for active efflux. This can be accomplished be expressing the genes in a systems which lack or are deficient in efflux transporter proteins. The most reliable method would, however, be by expressing these genes in a homologous host system where their gene homologues have been deleted. Any observed increase in the rate of efflux would thus be attributable to the expression of the transporter genes, *in trans*. Even though the ATP-dependence of these genes could not be conclusively prove here, there is sufficient

data to indicate their classification as functional ABC-type multidrug efflux transporter genes.

The semi-quantitative induction of these genes observed in the RT-PCR analysis indicated their ability to be induced to higher levels of resistance by their exposure to antimicrobial substances. To further investigate this, qRT-PCR experiments were conducted. These experiments showed that when *B. longum* cells were exposed to sub-lethal concentrations of erythromycin, a notable increase in the transcription levels of these genes was observed. This data directly links the transcription of these genes to the observed resistance phenotype in this bacterium since these genes are transcriptionally up-regulated in response to erythromycin exposure, and confirms the observed MDR phenotype to both erythromycin and tetracycline when heterologously expressed in *L. lactis* cells. Further experiments are needed to show whether this increase in the transcription levels of these genes occurs as a result of the cells' exposure to any antimicrobial substance, or, whether it is in fact due to the presence of erythromycin specifically. This can be accomplished by repeating this experiment with other substrates of these efflux genes, as well as, other antimicrobial or toxic compounds. If the response is indeed an antibiotic specific phenomenon, this would highlight the poly-specific binding of the transporters for their substrates, which has previously been shown. The use of microarrays can also be used to assay if the observed gene up-regulation is in fact confined to these ABC-type transporters in response to antimicrobial exposure, or, whether a more global cellular response is initiated.

The influence of a global regulatory mechanism is possible here, since putative transcriptional regulators, upstream of each operon, were identified in Chapter 2. Since all of the genes in both operons being studied here are simultaneously up-regulated in response to erythromycin exposure, it is probable that a global regulon may be responsible for the upregulation of all these resistance genes by simultaneously up-regulating their expression through the recruitment of individual gene-specific transcriptional regulators. This would allow for specific and controlled induction of numerous genes to detoxify the cell during antibiotic exposure. The regulator experiments presented in Chapter 4 of this study gives a strong indication of not only a gene-specific regulatory mechanism, since both operons being studied here were simultaneously up-regulated in response to erythromycin exposure, but

also, an indication that they may belong to a global regulatory system, such as the *mar* regulon, since a protein, BLLJ_1496, with a strong homology to proven MarR-type transcriptional regulators, was shown to bind specifically and with high affinity, to a DNA sequence upstream of the operon of which it is part, in a manner similar to that of other proven MarR-type regulator proteins. Since the data presented here showed that erythromycin was not a direct effector molecule of the MarR-type regulator identified, up-regulation of the levels of gene transcription is probably due to the indirect action of erythromycin on another cellular target. The *mar* regulon is known to lead to the up-regulation of multidrug resistance genes in numerous bacteria through the use of MarA, the *mar* activator protein. This protein is a part of the *mar*RAB locus first identified in *E. coli*, and has been identified as the transcriptional activator of the *mar* regulon by de-repressing binding of *mar*R repressor proteins to their specific DNA sequences (Alekshun & Levy, 1997; Alekshun & Levy, 1999). Whether MarA is the transcriptional activator responsible for leading to the transcriptional up-regulation of the ABC-type transporter genes identified in this study needs to be determined. No such *mar*A gene homolog has been identified in *B. longum* JCM 1217 and an in-depth bioinformatic analysis is required to identify any potential *mar*A homologues. If such a homolog is indeed identified, it should be functionally characterised and its role in de-repressing the MarR-type transcriptional regulator identified here should be confirmed.

The work presented in this study elucidates the occurrence of ABC-type MDR genes in a probiotic bacterium, which is widely used in industry as a dietary supplement, as well as, a culture strain in the food industry. These genes could potentially afford a competitive advantage to the bacterium, and may therefore be exploited as a beneficial characteristic when new probiotic strains are selected for use in industry. The potential for transmission of these genes to other gut bacteria cannot be ignored, however, the data presented here gives as indication as to the stringent conditions required for heterologous expression of these genes. This may indicate the limitation of HGT in a natural setting, since these genes are not easily expressed in other commonly used laboratory bacteria such as *E. coli*. Future work should therefore also look at the dissemination of these genes to other gut bacteria using techniques such as co-culture and subsequent genome-genome comparative analysis.

Appendix

Chapter 2

Figure A2.1. List of putative ABC-type transporters in *B. longum* NCC2705 at http://www.membranetransport.org/

Transporter Proteins in *Bifidobacterium longum* NCC2705

ATP-Dependent Transporters

The ATP-binding Cassette (ABC) Superfamily

PROTEIN ABC	membrane	binding protein	SUBSTRATE
BL0064	BL0063*		?
BL0154	BL0155		?
BL0197	BL0198?		?
	BL0267* BL0268 BL0269		?
BL0450	BL0451 BL0452*		?
BL0887	BL0888*		?
BL0932	BL0931		?
	BL1127	BL1126	?
BL1135	BL1133*		?
BL1277	BL1278?		?
	BL1369*		?
BL0870	BL0871* BL0872		? (Fe-S assembly/SufBCD system)
BL0299			? (Uup homolog/duplicated ATPase)
BL0689			? (Uup homolog/duplicated ATPase)
BL1688			? (Uup homolog/duplicated ATPase)
BL1717 BL1718	BL1715 BL1716	BL1714	branched-chain amino acid
BL1183	BL1182		cell division
BL0043*			cobalt
BL0693	BL0694		cobalt
BL1070	BL1069		cobalt
BL0264			CydD homolog
BL0956	BL0955	BL0957	D-methionine
BL1677	BL1678?		daunorubicin
BL0901	BL0900		daunorubicin?
		BL0394	dipeptide
		BL0829	dipeptide/oligopeptide
BL1157 BL1158	BL1159 BL1160	BL1161	dipeptide/oligopeptide
BL1348	BL1346 BL1347	BL1345	dipeptide/oligopeptide
BL1390	BL1387 BL1389	BL1386	dipeptide/oligopeptide
		BL1449	glutamate
BL0021	BL0023 BL0024	BL0022	glutamate/glutamine
BL0076	BL0074** BL0075	BL0077	glutamine
BL1120	BL1119**	BL1118	glutamine
BL1176	BL1177	BL1178 BL1179	glutamine
BL0995	BL0996	BL0994	manganese/zinc ion
	BL0162* BL0163*		multidrug
	BL0179* BL0180*		multidrug
BL1041			multidrug
	BL1766* BL1767*		multidrug
	BL0104	BL0103	nitrate/sulfonate/taurine
BL0312	BL0313 BL0314	BL0315	phosphate
BL0207	BL0208		polysaccharide
BL0034	BL0035 BL0036	BL0033	ribose
BL0341	BL0342 BL0343	BL0344	spermidine/putrescine/iron(III)
	BL0055		sugar
	BL0143 BL0144	BL0141	sugar
	BL0189 BL0190	BL0188	sugar
	BL0260 BL0261	BL0262	sugar
	BL0423 BL0424	BL0425	sugar
	BL0524		sugar

BL0673			sugar
	BL1331 BL1332	BL1330	sugar
	BL1522 BL1523	BL1521	sugar
	BL1639 BL1640	BL1638	sugar
BL1692 BL1695	BL1696 BL1706	BL1694	sugar
	BL1169 BL1170	BL1163 BL1164 BL1165	sugar/solute

* contains both an ABC and a membrane domain as one polypeptide

** contains both a membrane domain and a binding protein domain as one polypeptide

Figure A2.2. CDD search at NCBI, showing all the ABC transporter conserved domains.

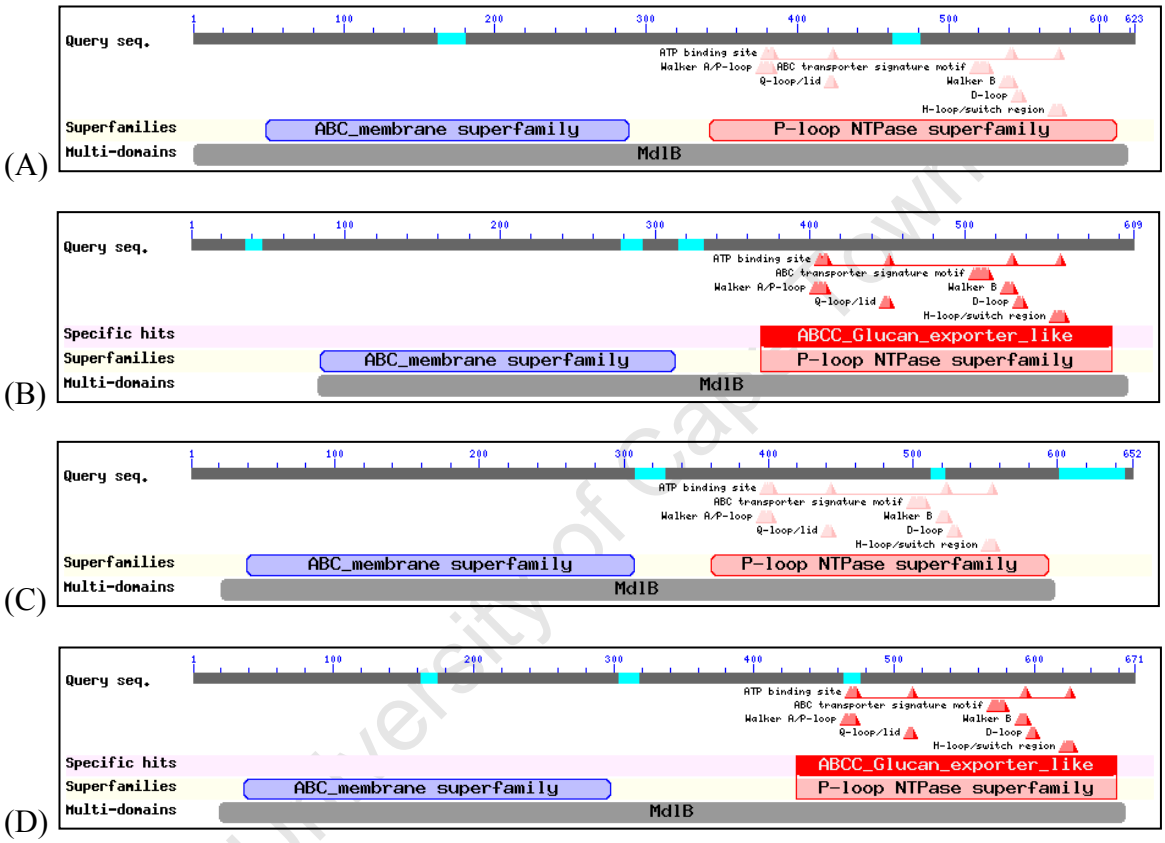


Figure 2. Results of conserved domain architecture analysis of (A) BLLJ_1837, (B) BLLJ_1836, (C) BLLJ_1495, (D) BLLJ_1494. The blue boxes indicate the ABC membrane superfamily transmembrane region comprising six transmembrane helices. The pink boxes represent the P-loop NTPases family which contains the all the domains required for ATP hydrolyses. The grey boxes show the MdlB domain which encompasses the ABC-type multidrug transport system ATPase and permease components.

Chapter 4

BLASTP analysis of BLLJ_1496 and BLLJ_1838 against bacterial genomes

Table A4.1. Top 10 hits of BLASTP search

BLLJ_1496 as query sequence

Accession	Description	Max	Total	Coverage	E-Value

		Score	Score		
ZP_06596777.1	transcriptional regulator, MarR family [Bifidobacterium breve DSM 20213] >ref|ZP_07940798.1| MarR family protein [Bifidobacterium sp. 12_1_47BFAA] >ref|YP_004221255.1| transcriptional regulator [Bifidobacterium longum subsp. longum JCM 1217] >gb|EFE88740.1| transcriptional regulator, MarR family [Bifidobacterium breve DSM 20213] >gb|EFV38184.1| MarR family protein [Bifidobacterium sp. 12_1_47BFAA]	365	365	100%	5.00E-100
ZP_04664729.1	transcriptional regulator [Bifidobacterium longum subsp. infantis CCUG 52486] >gb|EEQ55440.1| transcriptional regulator [Bifidobacterium longum subsp. infantis CCUG 52486]	365	365	100%	6.00E-100
YP_004209465.1	transcriptional regulator [Bifidobacterium longum subsp. infantis 157F]	362	362	100%	8.00E-99
ZP_03976718.1	transcriptional regulator [Bifidobacterium longum subsp. infantis ATCC 55813] >gb|EEI80865.1| transcriptional regulator [Bifidobacterium longum subsp. infantis ATCC 55813]	347	347	100%	2.00E-94
ZP_00120756.2	COG1846: Transcriptional regulators [Bifidobacterium longum DJO10A] >ref|YP_001955889.1| MarR family transcriptional regulator [Bifidobacterium longum DJO10A]	347	347	100%	3.00E-94
NP_696912.1	MarR-type transcriptional regulator [Bifidobacterium longum NCC2705] >ref|YP_004001394.1| marr-type transcriptional regulator [Bifidobacterium longum subsp. longum BBMN68]	342	342	100%	7.00E-93
ZP_03645867.1	MarR-type transcriptional regulator [Bifidobacterium bifidum NCIMB 41171] >ref|YP_003938265.1| transcriptional regulator, MarR family [Bifidobacterium bifidum S17]	309	309	100%	4.00E-83
ZP_07801892.1	transcriptional regulator [Bifidobacterium bifidum NCIMB 41171] >gb|EFR49826.1| transcriptional regulator [Bifidobacterium bifidum NCIMB 41171]	309	309	100%	6.00E-83
YP_003970605.1	MarR family transcriptional regulator [Bifidobacterium bifidum PRL2010]	274	274	86%	2.00E-72
ZP_02029901.1	hypothetical protein BIFADO_02362 [Bifidobacterium adolescentis L2-32] >gb|EDN82234.1| hypothetical protein BIFADO_02362 [Bifidobacterium adolescentis L2-32]	121	121	76%	2.00E-26

Table A4.2. Top 10 hits of BLASTP search

BLLJ_1838 as query sequence

Accession	Description	Max Score	Total Score	Coverage	E-Value
YP_004209819.1	transcriptional regulator [Bifidobacterium longum subsp. infantis 157F]	222	222	100%	8.00E-57
YP_004221594.1	transcriptional regulator [Bifidobacterium longum subsp. longum JCM 1217]	221	221	100%	1.00E-56
ZP_03976952.1	MarR family transcriptional regulator [Bifidobacterium longum subsp. infantis ATCC 55813] >gb|EEI80471.1| MarR family transcriptional regulator [Bifidobacterium longum subsp. infantis ATCC 55813]	218	218	100%	8.00E-56
ZP_04664129.1	conserved hypothetical protein [Bifidobacterium longum subsp. infantis CCUG 52486] >ref|YP_004001056.1| marr-type transcriptional regulator [Bifidobacterium longum subsp. longum BBMN68] >gb|EEQ55887.1| conserved hypothetical protein [Bifidobacterium longum subsp. infantis CCUG 52486]	217	217	100%	2.00E-55
ZP_07941654.1	MarR family protein [Bifidobacterium sp. 12_1_47BFAA] >gb|EFV37294.1| MarR family protein [Bifidobacterium sp. 12_1_47BFAA]	217	217	100%	2.00E-55
YP_003662205.1	MarR family transcriptional regulator [Bifidobacterium longum subsp. longum JDM301]	216	216	100%	5.00E-55
NP_695379.1	MarR-type transcriptional regulator [Bifidobacterium longum NCC2705]	214	214	99%	1.00E-54
YP_001955466.1	MarR family transcriptional regulator [Bifidobacterium longum DJO10A]	211	211	99%	2.00E-53
ZP_06596358.1	transcriptional regulator, MarR family [Bifidobacterium breve DSM 20213] >gb|EFE88897.1| transcriptional regulator, MarR family [Bifidobacterium breve DSM 20213]	195	195	100%	9.00E-49
YP_002323867.1	transcriptional regulator, MarR family [Bifidobacterium longum subsp. infantis ATCC 15697]	186	186	98%	3.00E-46

Figures A4.1 and A4.2 showing the multiple protein sequence alignments of the top 5 hits

Figure A4.1. Multiple protein sequence alignment of BLLJ_1496 with the top 5 BLASTP hits.

Figure A4.2. Multiple protein sequence alignment of BLLJ_1838 with the top 5 BLASTP hits.

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