Resistance to Antimicrobial Agents in Bifidobacteria

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CONTENTS

ABSTRACT

Bifidobacteria are administered in large quantities as probiotics, because they have many beneficial effects on human health. The growing interest in the health benefits of bifidobacteria has prompted the inclusion of these organisms in many dairy foods leading to an increase in their consumption. The survival of ingested microorganisms in the GIT influences the efficacy of probiotics. For bifidobacteria to survive and achieve colonisation, they have to interact with inhibitory host-produced substances such as bile salts. Another aspect which should be studied is the safety of the probiotic bacterium and risks of acquisition of genes for resistance to antimicrobial agents. Although bifidobacteria exhibit resistance to a wide range of antibiotics, little is known about the molecular basis for this resistance. The aim of this project was, therefore, to investigate the molecular mechanisms responsible for the resistance to antibiotics and bile salts observed in bifidobacteria, and more specifically, to determine whether efflux systems are involved in this resistance.

Five *Bifidobacterium* spp. were exposed to a range of antimicrobial agents. These included ethidium bromide, the bile salt sodium glycocholate, and a range of antibiotics. Survival curves as well as intrinsic MIC values were determined in the presence of the antimicrobial agents. It was then determined whether pre-exposure to the antimicrobial agents resulted in an increased MIC value for the antibiotic tested (adaptive MIC value). The resistance profiles displayed a great amount of interstrain and interspecies variation in both the intrinsic and adaptive MIC values. Adaptation to at least one of the antimicrobial agents tested was observed in every one of the bifidobacterial species.

To identify genes encoding antimicrobial efflux proteins, a genomic library of *Bifidobacterium lactis* hosted in the hypersusceptible strain *Escherichia coli* KAM3 was screened on tetracycline and ethidium bromide for colonies with increased resistance to either antimicrobial agent. Ten recombinant plasmids were isolated that conferred elevated levels of resistance to ethidium bromide. Sequence analysis was performed on all 10 plasmids. A putative ABC transporter system was isolated and further characterized. Attempts to measure the ethidium efflux of the transporter system proved unsuccessful in *E. coli* KAM3. The transporter was, therefore, subcloned into a *Lactococcus lactis* expression system. Expression of the ABC transporter system in a drug-sensitive *L. lactis* strain did not complement the phenotype.

Analysis of the *Bifidobacterium longum* NCC2705 genome sequence allowed the identification of putative efflux genes based on sequence similarity to known genes. Using this method, a putative sodium/bile symporter was identified in *B. longum*. The gene, BL1102, was isolated using a PCR amplification approach. BL1102 was subcloned and expressed in *E. coli* KAM3, an efflux negative mutant. The construct was shown to confer greatly elevated levels of resistance to sodium glycocholate (16-fold). Energy-dependent efflux of the bile salt was demonstrated using radiolabelled cholate. This is the first bile transporter described in bifidobacteria. The conditions under which BL1102 is expressed could shed light on the role of this gene in the resistance of *B. longum* to bile in the human GIT.

ABBREVIATIONS

A	adenosine
aa	amino acids
ATP	adenosine triphosphate
BSA	bovine serum albumin
bp	base pair(s)
COG	conserved orthologous domains
C	cytosine
C-	carboxy-(terminal)
cDNA	complementary DNA
CFU	colony forming units
DNA	deoxyribonucleic acid
dNTP	deoxynulceotide triphosphate
G	guanosine
g	gram
h	hour(s)
HTH	helix-turn-helix
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pairs
LB	Luria-Bertani medium
log	logarithmic
min	minutes
mRNA	messenger RNA
M	molar
mg	milligram
MIC	minimum inhibitory concentration
MW	molecular weight
N-	amino-(terminal)
NCBI	National Centre for Biotechnology Information
nm	nanometers
OD_x	optical density at x nm
ORF	open reading frame
ori	origin of replication
p	plasmid
PCR	polymerase chain reaction
R	(superscript) resistance
RNA	ribonuclease
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase PCR
SDS	sodium dodecyl sulphate
sec	seconds
T	(superscript) type strain
T	thymidine
Tris	tri(hydroxymethyl)aminomethane
UV	ultraviolet
w/v	weight per volume
α	alpha
β	beta
Δ	delta
μ	micro

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION TO BIFIDOBACTERIA

Bifidobacteria are natural inhabitants of the intestines of warm-blooded animals and are also major components of the human intestinal microflora [Simon & Gorbach, 1984]. They are anaerobic, Gram-positive, rod like organisms with a characteristic Y- or V-shaped end [Scardovi, 1986]. Bifidobacteria were first isolated from the stools of breast-fed infants. They are more prevalent in the intestines of infants and children than adults with a decrease in numbers of this microorganism with age [Hopkins *et al.*, 2001].

1.1.1 Classification of bifidobacteria

Traditionally, bifidobacteria are considered to be lactic acid bacteria, since they produce acetic and lactic acid as the end products of glucose metabolism. This classification has not, however, been unanimously accepted. The genus *Bifidobacterium* is a member of the family *Bifidobacteriaceae*. The phylogenetic analysis of the genus is an ongoing area of investigation. Currently there are 35 species, including 6 subspecies assigned to the genus. When the 16S rRNA gene sequences of 31 of the type strains were analysed, almost all of the species formed a compact cluster with identity levels ranging from 93 to 99%. Only 2 species, namely *Bifidobacterium inopinatum* and *Bifidobacterium denticolens*, did not share the high degree of sequence identity [Miyake *et al.*, 1998]. Other gene sequences have been investigated for use as alternatives to 16S rRNA gene sequences in the phylogenetic analysis of the genus *Bifidobacterium*. One such gene is the highly conserved and ubiquitous HSP60 gene [Jian *et al.*, 2001]. The partial HSP60 DNA sequence identity of the 36 *Bifidobacterium* strains studied ranged from 73 to 96%. Once again, *B. inopinatum* and *B. denticolens* did not fall into this range. These 2 species have been subsequently transferred to new genera, namely, *Scardovia* and *Parascardovia* respectively [Jian and Dong, 2002]. The taxonomic

standing of *Bifidobacterium lactis* and *Bifidobacterium animalis* is a subject of ongoing investigation. Some studies have concluded that *B. lactis* and *B. animalis* are to be considered one single species. Other studies using methods such as protein profiling, atpD and groEL gene sequence analysis, BOX-PCR fingerprinting, and Fluorescent Amplified Fragment Length Polymorphism (FAFLP), have demonstrated that representatives of *B. animalis* and *B. lactis* constitute two separate subgroups [Marco *et al.*, 2004]. It has been suggested that *B. animalis* be renamed *Bifidobacterium animalis* subsp. *animalis* subsp. nov. and *B. lactis*, *Bifidobacterium animalis* subsp. *lactis* subsp. nov. Such studies highlight that bacterial classification is in a state of flux. In this study the name *B. lactis* is used.

1.2 GENETIC MODIFICATION OF BIFIDOBACTERIA

The genetic manipulation of bifidobacteria is not widely practised due to limited suitable replicating vectors and difficulties in transformation of the bacteria. In addition, the DNA of bifidobacteria has a high mole percent G+C, ranging from 55-64%.

1.2.1 Vectors for Bifidobacteria

Bifidobacterium longum and *Bifidobacterium breve* [Iwata and Morishita, 1989] are the only bifidobacteria of human origin that have been shown to harbour plasmids, for example pMB1 from *B. longum* [Rossi *et al.*, 1996]. The replicon from pMB1 has subsequently been used to construct improved cloning vectors, such as pDG7, as well as *Escherichia coli-Bifidobacterium* shuttle vectors, such as pLF5 [Rossi *et al.*, 1998]. A problem associated with pDG7 was that the success of transformation depended on the species of bifidobacteria used, the origin of the plasmid DNA, and the gene subcloned into the plasmid. For example, the cholesterol oxidase gene of *Streptomyces* spp. was cloned into pDG7, resulting in pDC07. Transformation of *B. animalis* with plasmid isolated from *E. coli* was unsuccessful.

However, when pDC07 was purified from *B. infantis*, *B. animalis* was efficiently transformed [Rossi *et al.*, 1998]. Other plasmids constructed from pDG7, purified from *E. coli*, did efficiently transform all *Bifidobacterium* spp. tested. The *Escherichia coli-Bifidobacterium* shuttle vectors constructed by Rossi *et al.* (1996) were able to express all the different antibiotic resistance genes used as markers.

Other potential types of vectors that could be used in bifidobacteria would be those derived from closely related bacteria, or broad host-range plasmids. Argani *et al.* (1996) found that 2 plasmids from the genus *Corynebacterium* replicated in bifidobacteria. They also tested 2 broad-host range plasmids from *Lactobacillus* spp. and *Lactococcus* spp., but found that they did not replicate in bifidobacteria. This could be due to the AT-rich DNA of the microorganisms [Argani *et al.*, 1996].

Chemical mutagenesis with ethyl methanesulphonate (EMS) and N-methyl-N'-nitro-N-nitroguanidine (MNNG) has been performed on bifidobacteria to increase β-galactosidase production [Ibrahim and O'Sullivan, 2000]. However, there has been no published data on gene deletions or site-directed mutagenesis of any bifidobacteria.

1.2.2 Transformation of bifidobacteria

The uptake of exogenous DNA molecules can be prevented by the presence of a thick cell wall such as those possessed by bifidobacteria. The cell wall of bifidobacteria is composed of peptidoglycan layers, polysaccharides, lipoteichoic acids and proteins [Bezkorovainy, 1989]. The earliest successful protocol provided used electroporation, with competent cells needing to be frozen at -135°C for 1 hour [Missich *et al.*, 1994]. A more reliable and efficient technique was later described, using an electroporation buffer containing ammonium citrate and sucrose. The cells were made competent by preincubation in electroporation buffer for 4

hours at 4°C [Argani *et al.*, 1996]. A high sucrose concentration in the growth media as well as the electroporation buffer was shown to be essential. Subsequent procedures have been developed at the TNO Nutrition and Food Research, but have been patented (Patent Application WO 95/35389) [Van der Werf and Venema, 2001] and are not widely available for research use.

1.3 BENEFICIAL EFFECTS OF BIFIDOBACTERIA

Bifidobacteria have been used successfully to prevent post-antibiotic complications, as well as intestinal diseases in bottle-fed infants who have been unable to establish normal intestinal flora [Saavedra *et al.*, 1994]. This is of particular importance in South Africa (SA) where bottle-feeding of infants is becoming more prevalent in response to fears of mother-to-child HIV transmission through breast-feeding.

There are also claims that bifidobacteria have other beneficial effects on human health, in that they may play a role in preventing cancer [Wolloski *et al.*, 1999], and in lowering blood cholesterol levels [Tahri *et al.*, 1995], which are prevalent as medical problems in SA. Bifidobacteria have been shown to inhibit the growth of pathogens, such as *Escherichia coli* O157:H7 [Kim *et al.*, 2001] as well enhance the immune system of the host [Ouwehand *et al.*, 2002]. They are able to repress rotavirus infections [Saavedra *et al.*, 1994] and have positive effects on antibiotic-associated diarrhoea and restoration of gut flora after antibiotic therapy [Linskens *et al.*, 2001]. They have been to shown to alleviate lactose intolerance through the production of β-galactosidases [Jiang *et al.*, 1996].

1.4 PROBIOTICS, PREBIOTICS AND SYNBIOTICS

1.4.1 Probiotics

Bifidobacteria are generally considered to be good probiotic candidates. The use of live microbes as probiotics is a subject of intense and growing interest [Kullen *et al.*, 1997]. Although many different definitions of a probiotic have been proposed the most widely used, scientifically valid version is that of Fuller, 1991: "a live microbial food supplement that beneficially affects the host animal by improving its intestinal microbial balance".

During the last 15 years the growing interest in the health benefits of bifidobacteria has prompted the inclusion of these organisms in many dairy foods leading to an increase in their consumption.

An effective probiotic should

- exert a beneficial effect on the host
- be non-pathogenic and non-toxic
- contain a large number of viable cells
- remain viable during storage and use
- have good sensory products
- be isolated from the same species as its intended host
- have colonisation potential in the human gastrointestinal tract (GIT)
- be metabolically active within the GIT

[Kailasapathy & Chin, 2000; Collins & Gibson, 1999]

1.4.2 Prebiotics

A prebiotic is 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. It modifies the composition of the colonic microflora in such a way that a few potentially health-promoting bacteria (especially, but not exclusively lactobacilli and bifidobacteria) become predominant, thereby benefiting the host [Kolisam *et al.*, 2002]. Raffinose, lactulose, stachyose, fructo-, isomalto- and galacto-oligosaccharides have all been reported to be effective in the proliferation of resident or implanted bifidobacteria [Crittenden & Playne, 1996].

Analysis of the *Bifiobacterium longum* genome, revealed that approximately 8.5% of the total predicted proteins are assigned to COGs in the carbohydrate transport metabolism [Schell *et al.*, 2002]. This suggests that the ability of *B. longum* to utilize non-digested carbohydrates in the human GIT is more extensive than previously anticipated. Genomic analysis data may, therefore, be used in the identification of possible prebiotics to stimulate the growth of bifidobacteria in the human GIT.

1.4.3 Synbiotics

A synbiotic is a mixture of a probiotic and a prebiotic. This beneficially affects the host by improving the survival and establishment of live microbial dietary supplements in the GIT [Kailasapathy & Chin, 2000]. The combination improves the survival of the bacterial strain by providing a specific substrate for its fermentation. For example, a fructooligosaccharide can be used in conjunction with a bifidobacterial strain.

1.5 SELECTION OF SUITABLE PROBIOTIC STRAINS: SURVIVAL AND SAFETY IN THE HUMAN GIT

Survival in the human GIT, and safety concerns regarding the consumption of probiotic-containing food products, are important factors in the selection of suitable candidate strains for use as probiotics. Other factors include the intrinsic properties of the strain, activity in the intestines, dose-response relationships, faecal and mucosal recovery, and the interactions between the strain and the host [Salminen *et al*, 1998].

1.5.1 Survival in the human GIT

The survival of a probiotic can be compromised in the supplemented product before ingestion, for example during storage, and even more so in the host after ingestion. The bacteria are confronted by many physiological conditions that may adversely influence its viability, including gastric acid and secretions of the small intestines such as bile salts and pancreatic enzymes [Collins & Gibson, 1999]. In addition, the bacteria must compete effectively with a complex and metabolically active indigenous flora present in the large intestine. The administration of antibiotic treatment directed at disease-causing bacteria can also compromise the survival of probiotic bacteria in the GIT.

1.5.2 Safety concerns with the administration of probiotic bacteria

Traditional dairy strains of lactic acid bacteria, including bifidobacteria, have a long history of safe use. However, new species and more specific strains of probiotic bacteria are constantly being identified, and it cannot be assumed that these strains share the same safety characteristics as other tested and traditional strains [Salminen *et al*, 1998]. Even closely

related strains may have heterogenous qualities [Sullivan & Nord, 2002]. Therefore, safety aspects always have to be considered and constantly evaluated [Salminen *et al*, 1998], and it is important to develop a science-driven, evidence based overview of the safety of bifidobacteria used as probiotics in food products [Borriello *et al*, 2003]. One of the aspects which should be studied is the safety and risks of acquisition of resistance to antimicrobial agents [Sullivan & Nord, 2002].

1.5.3 Antimicrobial susceptibility of probiotic strains

The antibiotic susceptibility of the probiotic is important for 3 main reasons; namely: the requirements for sustained survival during antibiotic treatment directed against disease-causing organisms; the treatment of rare opportunistic infections; and also to evaluate the risk of drug resistance transfer to other colonic bacteria.

The administration of antimicrobial substances can cause the suppression of certain beneficial bacterial groups, including bifidobacteria, which promote natural resistance of the host against infection [Lim *et al.*, 1993]. Current evidence suggests that the risk of infection with probiotic lactobacilli or bifidobacteria is similar to that of infection with commensal strains. However, it has been suggested that probiotic strains should be susceptible to at least 2 major antibiotics so as to treat the rare cases of probiotic-associated infections [Borriello *et al*, 2003].

There are also safety concerns regarding the administration of bifidobacteria in high numbers in food products when such antibiotic resistance genes are present. It is currently difficult to interpret studies of gene transfer *in vivo*, and further methods need to be developed. Also, the focus should be on possible gene transfer to pathogenic bacteria such as *Staphylococcus aureus*, rather than on homologous gene transfer [Borriello *et al*, 2003].

1.6 ANTIBIOTIC RESISTANCE

It has been shown that bifidobacteria exhibit resistance to amikacin, aztreonam, cefoxitin, colistin sulphate, fusidic acid, gentamycin, kanamycin, metronidazole, naladixic acid, neomycin, norflaxin, paromomycin sulphate, polymyxin B, streptomycin, trimethoprim and vancomycin. Resistance to tetracycline and nitrofurantoin in the strains studied was variable [Charteris *et al*, 1998; Lim *et al*, 1993]

Although bifidobacteria exhibit resistance to a wide range of antibiotics, little is know about the molecular basis for this resistance.

1.6.1 Mechanisms of Antibiotic Resistance

There are four major mechanisms by which bacteria in general can resist the effect of antibiotics, as illustrated in Figure 1.1 [Levy, 1998; Neu, 1992]; namely: inactivation of the antibiotic by destruction or modification of the antibiotic (A), alteration of the target site of the antibiotic (B), prevention of drug influx (C), and active extrusion of the drug from the cell (D).

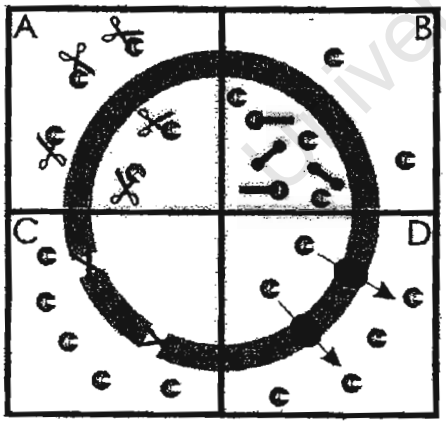


FIGURE 1.1 Resistance mechanisms in bacteria comprise (A) drug inactivation, (B) target alteration, (C) prevention of drug influx, and (D) active extrusion of drug from cell [Putman *et al.*, 2000].

Well-known examples of enzymes that inactivate antibiotics are β-lactamases. These enzymes are located in the periplasm and confer resistance to β-lactams such as penicillin and ampicillin [Reviewed in Shah, 2004]. A single base change in a gene encoding a bacterial β-lactamase can change the substrate specificity of the enzyme [Davies, 1994]. These changes occur frequently, especially in *Enterobacteriaceae* species. This cycle of natural protein engineering changes in response to antibiotic-selection pressure.

The most common mechanism of resistance is the development of altered forms of the normal targets that have increased resistance to antibiotics. Such resistance may involve the acquisition of new genes carried on plasmids or transposons that result in the enzymatic modification of the normal target. Resistance to macrolide antibiotics, such as erythromycin, is caused by methylation of 23S ribosomal RNA [Thakker-Varia, *et al.*, 1985].

The prevention of access of an antibiotic to the target by a barrier and/or the active transport functions of bacterial membranes confers a more general mechanism of drug resistance [Reviewed in Nikaido, 1994]. The bacterium can surround itself with a barrier of low permeability to decrease the influx of the drug into the cell. Gram-negative bacteria, such as *Escherichia coli* have the outer membrane, which functions as an effective barrier to antibiotics [Nikaido & Vaara, 1985].

In addition or alternatively to the prevention of drug influx, the antimicrobial agent can be pumped out of the cell before it can act. The resistance caused by energy-dependent transmembrane efflux of antimicrobial agents was first observed in *E. coli* with plasmid-encoded tetracycline resistance [Speer *et al.*, 1992]. These efflux systems will form the main focus of this review.

1.7 MULTIDRUG EFFLUX SYSTEMS

Unlike transporters such as the tetracycline efflux proteins [Speer *et al.*, 1992], which are dedicated systems mediating the extrusion of a given drug or class of drug, multidrug transporters can handle a wide variety of structurally dissimilar compounds. Bacterial multidrug efflux systems are a serious problem in the pharmacological treatment of patients with infectious diseases, since the substrate spectra of many multidrug transporters include clinically relevant antibiotics [Putman *et al.*, 2000].

Active multidrug efflux systems in bacteria can be divided into two major classes on the basis of bioenergetic and structural criteria [Putman *et al.*, 2000]. These classes are:

- The secondary multidrug transporters which extrude drugs using the proton motive force (PMF), and
- The ATP-dependent multidrug transporters which are driven by the free energy of ATP hydrolysis.

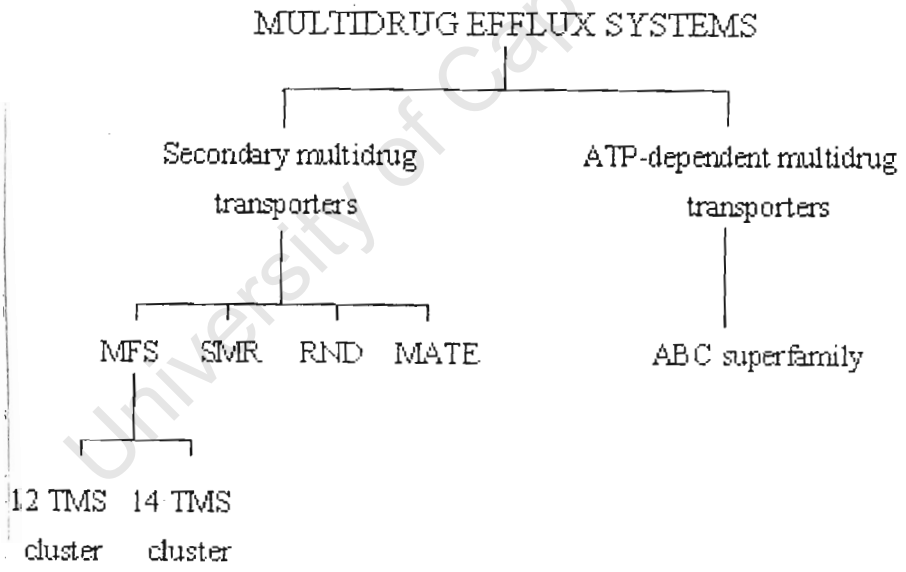


FIGURE 1.2 Classification of multidrug transporters into classes based on bioenergetic and structural criteria, and families based on supramolecular assembly mechanism and sequence homology. The secondary multidrug transporter class is divided into the Major Facilitator Superfamily (MFS), Small Multidrug Resistance Family (SMR), Resistance-Nodulation-Cell Division Superfamily (RND), Multidrug and Toxic Compound Extrusion Family (MATE). The MFS is further divided into the 12 and 14 transmembrane segment (TMS) clusters. All ABC transporters belong to the ATP-binding cassette (ABC) superfamily [Nikaido, 1994; Putman *et al.*, 2000].

As illustrated in Figure 1.2, these classes are further divided into families and clusters on the basis of supramolecular assembly and sequence similarity. The drug resistance profiles of the multidrug transporters vary strongly, and family-specific profiles are not found.

1.7.1 Secondary multidrug transporters

1.7.1.1 Major Facilitator Superfamily (MFS)

The MFS consists of membrane transport proteins which are found in many organisms, from bacteria to higher eukaryotes. They are involved in the symport, antiport or uniport of various substances such as sugars, Krebs cycle intermediates, phosphate esters, oligosaccharides and antibiotics [Marger & Saier, 1993]. The MFS proteins can be subdivided into two separate clusters, with either 12 or 14 transmembrane segments (TMS) as shown in Figure 1.2 and Table 1.1 [Paulsen & Skurray, 1993]. Figure 1.3 shows a model of the structure of the 12 TMS multidrug transporters of the MFS.

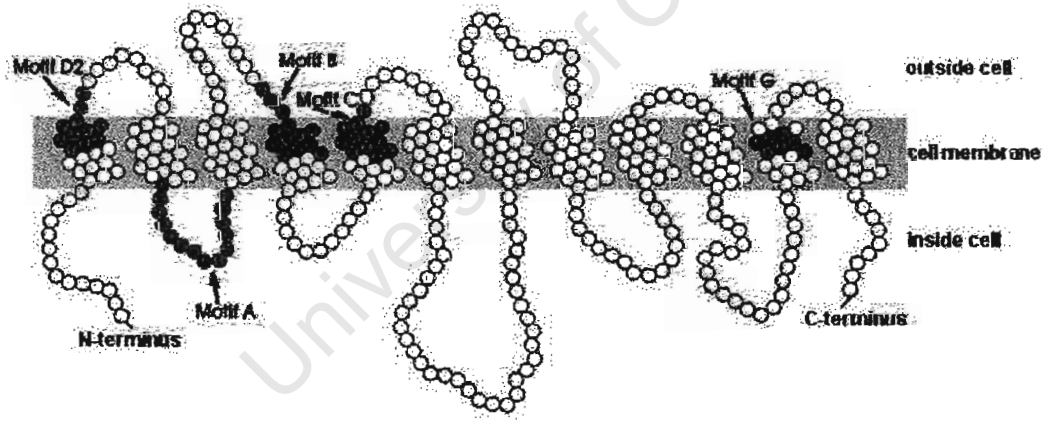


FIGURE 1.3 Structural model for the 12-TMS multidrug transporters of the MFS. The residues constituting the conserved sequence motifs, A, B, C, D2, and G, are shaded [adapted from Putman *et al.*, 2000].

1.7.1.2 Small Multidrug Resistance Family (SMR)

Multidrug transporters of the SMR family are most likely composed of a tightly packed four-helix antiparallel bundle (Table 1.1) [Yerushalmi *et al.*, 1996]. The protein sequences in the

SMR family are highly hydrophobic with very few charged residues most of which are found within putative loops [Paulsen *et al.*, 1996]. The family members bear few distinct signature sequence elements and several fully conserved residues. In some of the conserved residues, Ala-10, Glu-14, Tyr-60 and Trp-63, even replacements with a related amino acid bring about a loss of function. Glu-14, which is the only conserved charged residue embedded in the membrane, is directly involved in the transporter coupling mechanism [Paulsen *et al.*, 1996]. It has been proposed that the SMR family be placed in the drug/metabolite transporter superfamily. This superfamily consists of 14 families of which SMR is the best characterized drug transporter [Saier & Paulsen, 2001].

1.7.1.3 Resistance-Nodulation-Cell Division Family (RND)

In Gram-negative bacteria, RND efflux pumps occur as part of a three-component system, where they are linked to an outer membrane channel protein by a membrane fusion protein, possibly via the two large periplasmic loops (Table 1.1). This protein complex allows the bacteria to extrude substrates directly into the external medium, bypassing the periplasm [Guan *et al.*, 1999].

1.7.1.4 Multidrug and Toxic Compound Extrusion Family (MATE)

In 1998, NorM was identified as a putative multidrug efflux protein in *Vibrio cholerae* [Morita *et al.*, 2000]. This protein was shown to mediate norfloxacin efflux. Although hydropathy analysis suggested the presence of 12 TMS, it did not exhibit any of the signature sequences. NorM was later identified as the prototype of a new family of transporters, the MATE family [Brown *et al.*, 1999]. However, this family's contribution to drug resistance has been shown only for a few isolated cases, for example BexA in *Bacteriodes thetaiotaomicron* [Miyamae *et al.*, 2001].

TABLE 1.1 Classification of multidrug transporters according to sequence similarity and membrane topology

Class	**ATP-dependent**	**Secondary**				
Family	ABC	MFS		SMR	MATE	RND
Cluster		12-TMS cluster	14-TMS cluster			
Reference to structure	[Hyde *et al.*, 1990; Walker *et al.*, 1982]	[Marger & Saier, 1993; Paulsen & Skurray, 1993]	[Marger & Saier, 1993; Paulsen & Skurray, 1993]	[Paulsen *et al.*, 1996; SteinerMordoch *et al.*, 1999]	[Morita *et al.*, 1998]	[Fralick, 1996; Saier *et al*, 1994]
Structure	Four domains: two highly hydrophobic membrane domains, consisting of six trans-membrane helices each, and two hydrophilic nucleotide-binding domains (NBDs)	Two homologous, each containing six trans-membrane α-helices	Two homologous, each containing seven trans-membrane α- helices	Four transmembrane domains with short loops in between	12 transmembrane domains	Two homologous, each containing six trans-membrane α-helices with two large cytoplasmic loops Occur as part of three-component system in Gram-negative bacteria
Consensus sequences of conserved motifs	NBDs contain Walker A and B motifs and ABC signature	**A**: G x L a D r x G r k x x (x) l **B**: l x x x R x x q G x g a a **C**: g x x x G P x x G G x x G G x l **D2**: l g x x x x x P v x P **G**: G x x x G P L	**A**: G x L a D r x G r k x x (x) l **B**: l x x x R x x q G x g a a **C**: g x x x G P x x G G x x G G x l **D1**: l D x T v x n v A l P **E**: D x x G x x L **F**: l g x x x G x a v x g x l **H**: W x w x F l l N v P i g	**A**: W i x l v l l l E V **B**: K x s e G F t r l x P S **C**: P v G t A Y A v W t G l G		**A**: G x s x v T v x F x x g t D x x x A q v q V q n k L q x x L P x x V q x q g x x v x k **B**: a l v l s a V F l P m a f f g G x t G x i y r q f s l T x v s A m a l S v x v a l t l t P A l c A **C**: x x x G k x l x e A x x x a a x x R L R P I L M T s L a f l I G v l P l a i a t G x A G a **D**: S l N t l T l f g l v l a i G L l v D D A l V v V E N v e R v l a e
Size	590 aa residues	338 aa residues	513 aa residues	107 aa residues	403 aa residues	1 000 aa residues

1.7.2 ATP-dependent multidrug transporters

Unlike most bacterial multidrug transporters that utilise the PMF or sodium ions for the extrusion of antimicrobial agents, some drug efflux systems are driven by the free energy of ATP hydrolysis. All ATP-dependent drug efflux proteins known to date are members of the ATP-binding cassette (ABC) superfamily [Higgins, 1992]. ABC transporters form one of the largest of all protein families with substrate specificities ranging from the uptake of sugars, amino acids and inorganic ions to the export of large protein toxins [Bolhuis *et al.*, 1994]. The ABC superfamily currently includes 21 families of prokaryotic efflux systems [Saier & Paulsen, 2001]. Two well-characterized ABC multidrug transporters are found in the Gram-positive lactic acid bacteria *Lactococcus lactis* and *Lactobacillus brevis* (Table 1.2).

TABLE 1.2: Reported drug resistance profiles of ATP-dependent multidrug transporters [a] [adapted from Putman *et al.*, 2000].

	ABC transporters	
Drugs	HorA *Lactobacillus brevis* [Sakamoto *et al.*, 2001]	LmrA *Lactococcus. lactis* [Poelerands *et al.*, 2000]
Aminoglycosides		+
β -Lactams		
Carbapenems		-
Cephalosporins		+
Penicillins		+
Chloramphenicol	-	+
Glycopeptides		-
Hop compounds	+	
Lincosamides		+
Macrolides		
14-membered	-	+
15-membered		+
Monovalent cations	+	+
Novobiocin	+	
Quinolones		
Hydrophilic		+
Hydrophobic		+
Tetracyclines	-	+
Trimethoprim		-

[a] +, resistance; -, no resistance

The transporters have an N-terminal hydrophobic domain consisting of six putative transmembrane helices and a C-terminal hydrophilic domain, containing the ATP-binding cassette (Table 1.1). This latter domain contains features diagnostic of an ATP-type ATPase, such as the ABC signature sequence and the Walker A and B motifs [Walker *et al.*, 1982].

1.7.3 Origin and regulation of multidrug transporter genes

Multidrug transporters are associated with both intrinsic and acquired resistance to antibiotics. Acquired multidrug resistance can arise via three mechanisms: (i) amplification and mutation of genes encoding multidrug transporters, changing the expression level or activity [Putman *et al.*, 2000], (ii) mutations in specific or global regulatory genes which lead to the increased expression of multidrug transporters [Poole *et al.*, 1996], and (iii) intercellular transfer of resistance genes on transposons or plasmids [Kazama *et al.*, 1998]. In addition, the expression of several genes encoding multidrug efflux pumps is inducible by the drugs to which they offer protection [Zheleznova *et al.*, 1999]. The expression of some multidrug transporters can also be modulated in response to environmental conditions [Baranova *et al.*, 1999].

Addition of the compounds exported by the multidrug transporters themselves actually enhances the expression levels of multidrug transporters. This has been shown for both Bmr from *Bacillus subtilis* [Ahmed *et al.*, 1994] and QacA from *Staphylococcus aureus* [Grkovic *et al.*, 1998]. These observations indicate that the expression of multidrug transporters is controlled by regulatory proteins which, like the transporters, are capable of recognizing structurally diverse compounds.

A comparable ability to bind diverse hydrophobic cationic drugs is characteristic of the *B. subtilis* transcription regulator, BmrR, which upon binding activates expression of the

multidrug transporter, Bmr [Zheleznova *et al.*, 1999]. BmrR, which is encoded by a gene immediately downstream of *bmr*, belongs to the MerR family of regulators and has strong homology with other members of the family only in the N-terminal helix-turn-helix DNA-binding domain [Ahmed *et al.*, 1994]. The inducer-binding, C-terminal domain of MerR-type proteins are dissimilar, which allows them to participate in a wide variety of responses, for example, to mercury ions (MerR), flavonoids (NolA) and oxidative stress (SoxR). The C-terminal of BmrR binds a variety of structurally diverse hydrophobic cations, including many toxins transported by Bmr, thus allowing the expression of the transporter to be enhanced by many of its toxic compounds [Ahmed *et al.*, 1994]. Crystal structures of the multidrug-binding domain of BmrR, and of its complex with the drug tetraphenylphosphonium (TPP^+), revealed a drug-induced unfolding and relocation of an alpha helix, which exposes an internal drug-binding pocket. TPP^+ binding is mediated by stacking and van der Waals contacts with multiple hydrophobic residues of the pocket, and by an electrostatic interaction between the positively charged drug and a buried glutamate residue, which is the key to cation selectivity. Similar binding principles may be used by other multidrug-binding proteins [Zheleznova *et al.*, 1999].

Rhodamine 6G is a substrate of Bmr. Binding of one rhodamine 6G molecule to a dimer of BmrR increases the affinity of BmrR for the *bmr* promoter, resulting in enhanced transcription of the *bmr* gene [Ahmed *et al.*, 1994]. The individually expressed C-terminal region of BmrR forms dimers and binds a broad range of drugs with the same affinity as full-length BmrR [Zheleznova *et al.*, 1999].

B. subtilis also expresses another multidrug transporter, Blt, which is specifically controlled by another MerR-type regulator, BltR. Both BmrR [Zheleznova *et al.*, 1999] and BltR [Baranova *et al.*, 1999] are encoded in the vicinity of their corresponding transporter genes,.

Specific regulatory mechanisms that affect the expression of single multidrug transporters are not the only way of controlling the level of gene expression. Induction of multidrug efflux systems can also result from global regulatory mechanisms. In addition to regulation by BltR and BmrR, both Blt and Bmr are controlled by a global MerR-type regulator, Mta [Baranova *et al.*, 1999]. The N-terminal domain of Mta (MtaN), when expressed separately, acts as a constitutive activator of the transcription of *bmr* and *blt* genes as well as of its own transcription and the transcription of at least one other gene, *ydfK*. The full-length Mta does not possess such an activity. It has been suggested that the separation of the N-terminal domain of Mta from its C-terminal domain mimics the activation of Mta occurring naturally upon binding of its hypothetical inducer, which has yet to be identified. Mta-mediated regulation could either provide protection from a specific environmental toxin or control normal bacterial physiological processes [Baranova *et al.*, 1999]. The control of Bmr and Blt expression by at least two independent regulatory mechanisms is similar to the control of the *E. coli* multidrug transporter, AcrAB, by a specific regulator, AcrR, and by two global regulators, MarA and SoxS [Okusu *et al.*, 1996].

The multiplicity of control mechanisms suggests that the bacterial cell takes advantage of the broad substrate specificity of each multidrug transporter and uses it to transport different substrates, depending on the physiological needs of the cell [Baranova *et al.*, 1999]. The multiplicity of similar multidrug transporters in the same cell may originate from the recruitment of duplicated copies of an earlier multidrug transporter gene into different operons, in which they are placed under different regulatory controls and become involved in different functions, as seen with Bmr and Blt. The existence of a global regulator, Mta,

indicates that at least some of the functions of these transporters remain either identical or closely related.

1.8 RESISTANCE TO BILE

The survival of ingested microorganisms in the GIT influences the efficacy of probiotics. For bifidobacteria to survive and achieve colonisation, they have to interact with inhibitory host-produced substances such as bile salts [Margolles *et al.*, 2003]. Bile concentrations range from 0.5% to 2.0% in the first hour of digestion and the levels may decrease during the second hour [Kailasapathy & Chin, 2000]. Bile is a digestive secretion that plays a major role in the dispersion and absorption of fat-soluble vitamins, lipids and cholesterol. The composition of bile is complex, but can be thought of as lipid rich and protein poor. Lipid molecules found in bile include cholesterol, phospholipids and bile acids, while immunoglobulins are one of the major protein constituents [Hofmann, 1998].

Bile acids are synthesized from cholesterol in the liver and secreted into bile. This is stored in the gallbladder until ingestion of a fatty meal when they enter into the lumen of the duodenum. Most of the bile salts are absorbed from the duodenum and returned to the liver via the portal circulation [Hofmann, 1984]. The remaining part of the bile salts is deconjugated by the action of bile salt hydrolases present in enteric bacteria, with the formation of the corresponding free bile acids such as cholate and chenodeoxycholate [Doerner *et al.*, 1997]. The free bile acids are further metabolized by some members of the intestinal bacteria into secondary bile acids such as deoxycholate and lithocholate [Franklund *et al.*, 1993].

Bile acids are surface active, amphipathic molecules, and are, therefore, a type of detergent. As such, they possess potent antimicrobial activity as they disaggregate the ordered structure

of biological membranes. However, enteric flora and pathogens have developed mechanisms to resist the action of bile [Gunn, 2000; Yokota *et al.*, 2000].

Bile resistance is of importance to many fields of interest since the survival of ingested microorganisms in the gastrointestinal tract influences the risk of food-bourne infections and the efficacy of probiotics and orally dosed live vaccines [Marteau *et al.*, 1997]. The resistance of enteric bacteria to bile has been known for decades, however, relatively little is known about its molecular basis. The effect of bile on Gram-positive bacteria is not well understood, but some of the resistance mechanisms found in Gram-negative bacteria are likely to be found in Gram-positive enteric bacteria as well [Gunn, 2000]. Some of the major mechanisms are reviewed below.

1.8.1 Efflux pumps

Efflux of bile salts from the bacterial cytoplasm directly out of the cell is the best-characterized mechanism of bile salt resistance. The secondary multidrug transporters AcrB and EmrB are involved in bile salt resistance in *E. coli* [Okusu *et al.*, 1996; Lomovskaya & Lewis, 1992]. Similar efflux systems have been identified in other bacteria, including *Salmonella typhimurium* [Lacroix *et al.*, 1996], *Pseudomonas aeruginosa* [Li *et al.*, 1995] and *Neisseria gonorrhoeae* [Hagman *et al.*, 1995].

Cholate resistance in *L. lactis* is mediated by an ATP-dependent multispecific organic anion transporter. This transporter does not, however, confer resistance to deoxycholate or cationic drugs, such as ethidium bromide and rhodamine 6G, which are typical substrates of other multidrug transporters such as LmrP and LmrA in *L. lactis* [Yokota *et al.*, 2000].

1.8.2 Lipopolysaccharide (LPS)

The LPS of Gram-negative bacteria constitutes the majority of the outer leaflet of the outer membrane. While the lipid A portion of the LPS molecule anchors it in the membrane, the O-antigen protrudes from the surface of the membrane and provides the major barrier to external compounds. A loss of the O-antigen creates a "rough" phenotype which results in a decreased resistance to bile as demonstrated in *S. typhimurium* [Prouty *et al.*, 2002].

1.8.3 Bile salt hydrolases

In the intestine, the amino acid may be hydrolysed from the conjugated bile salt by bacterial enzymes known as bile salt hydrolases (BSHs). There are two main hypotheses as to the function of BSHs in enteric bacteria. One hypothesis is that bacteria that are able to deconjugate bile salts use the amino acid taurine as an electron acceptor. Evidence to support this hypothesis has been obtained for some *Clostridium* species [Gopal-Srivastava & Hylemon, 1988]. The second hypothesis states that BSHs decrease the toxicity of conjugated bile acids for bacteria. However, studies in lactobacilli have shown that BSH activity and resistance to the toxicity of conjugated bile salts are not related [Moser & Savage, 2001]. The addition of ionophore nigericin to BSH-producing *Lactobacillus amylovorus,* to dissipate the ΔpH, decreased the viability of the bacterium in the presence of conjugated bile salts. It has been hypothesised that after deconjugation, the deconjugated bile salts may be transported out of the cell using the PMF [Grill *et al.*, 2000].

1.8.4 Affect of bile on antibiotic resistance in bifidobacteria

An interesting observation is that conjugated bile salt-tolerant bifidobacteria have an altered antibiotic susceptibility pattern in the presence of these bile salts [Charteris *et al.*, 2000]. The

bile salt oxgall diminished the intrinsic resistance of all strains of bifidobacteria to polymyxin B and co-trimoxazole, while aminoglycoside resistance was lost. The extrinsic resistance of bifidobacteria to ampicillin, chloramphenicol, erythromcyin, penicillinG, rifampicin and tetracycline was also affected by the presence of oxgall but in a strain- and source-dependent manner [Charteris *et al.*, 2000].

1.9 PROJECT AIMS

The aim of this project was to investigate the molecular mechanisms responsible for the resistance against antibiotics and bile salts observed in bifidobacteria, and more specifically, to determine whether efflux systems are involved in this resistance.

This thesis describes the investigation into the intrinsic resistance of *Bifidobacterium* spp to a wide range of toxic compounds and antibiotics. The question whether the bacteria are able to adapt to the presence of toxic compounds to become more resistant, was also investigated.

Evaluation of the functionality of putative transporter genes was investigated using complementation in the drug hypersusceptible *Escherichia coli* strain, KAM3 [Morita *et al.*, 1998] as well as bioinformatic analysis of the sequences and possible membrane topologies.

In addition, analysis of the *Bifidobacterium longum* genome was used to identify putative efflux genes based on sequence similarity to known genes. Putative transporter genes were further investigated and characterized by way of efflux assays.

CHAPTER 2

PHYSIOLOGICAL ANALYSIS OF RESISTANCE TO ANTIMICROBIAL AGENTS IN *BIFIDOBACTERIUM* SPP.

2.1 SUMMARY

Five *Bifidobacterium* spp. were exposed to a range of antimicrobial agents. These included ethidium bromide, the bile salt sodium glycocholate and a range of antibiotics. Survival curves as well as intrinsic MIC values were determined in the presence of the antimicrobial agents. It was then determined whether pre-exposure to the antimicrobial agents resulted in an increased MIC value for the antibiotic tested (adaptive MIC value). The resistance profiles displayed a great amount of interstrain and interspecies variation in both the intrinsic and adaptive MIC values. Adaptation to at least one of the antimicrobial agents tested was observed in every one of the bifidobacterial species.

2.2 INTRODUCTION

There are many factors to consider in the selection of suitable bifidobacterial strains for use as probiotics. Survival in the human GIT, and safety concerns with the consumption of probiotic-containing food products are important factors to consider. As mentioned in section 3 of chapter 1, the administration of antibiotic treatment directed at disease-causing bacteria can also compromise the survival of probiotic bacteria in the GIT. This can result in antibiotic-associated diarrhea. While resistance to antimicrobial agents may increase the survival rate of bifidobacteria in the human GIT, one of the aspects which should be studied is the safety and risks of acquisition of genes for the resistance to antimicrobial agents [Sullivan & Nord, 2002], especially if these bacteria are to be added in high numbers to food products.

Minimum inhibitory concentration (MIC) values for a number of bifidobacterial strains have been reported [Charteris *et al*, 1998; Lim *et al*, 1993]. A great amount of interstrain and interspecies variation has been reported. It has been shown that some strains of bifidobacteria are resistant to up to 10 antibiotics [Yazid *et al.*, 2000]. Although bifidobacteria exhibit resistance to a wide range of antibiotics, little is know about the molecular basis for this resistance. There are also no studies investigating whether bifidobacteria are able to adapt in the presence of antibiotics. As reviewed in Chapter 1, active drug efflux can contribute to antibiotic resistance, possibly through the activity of multidrug transport proteins. There are a number of antimicrobial agents that are transported by such multidrug transport proteins.

The mutagen ethidium bromide is a substrate common to many multidrug transporters [Miyamae *et al.*, 2001; De Rossi *et al.*, 1998]. Ethidium readily crosses the membrane by passive diffusion and intercalates between the bases of DNA, thereby exerting its growth-inhibitory and mutagenic

effects. The presently characterized bacterial ethidium resistance mechanisms are all membrane-located extrusion systems [Bolhuis *et al.*, 1994]. Ethidium efflux systems have different specificities, but all confer cross-resistance to, or catalyze efflux of, unrelated compounds, like acriflavine, phosphonium ions, quaternary ammonium ions, aminoglycosides, rhodamine 6G and chloramphenicol [Bolhuis *et al.*, 1994]. The physiological function of the ethidium extrusion systems could be related to a need for the organism to extrude toxic compounds encountered in the natural habitat, such as toxins produced by microorganisms or plants. Many of these compounds are both water and lipid soluble [Bolhuis *et al.*, 1994].

The presence of ethidium bromide resistance can therefore be used as an indicator of the presence of a multidrug efflux system(s). As mentioned in section 1.7.3 of Chapter 1, the expression of several genes encoding multidrug efflux pumps is inducible by the drugs to which they offer protection [Zheleznova *et al.,* 1999]. The ability of bacterial cells to become more resistant to ethidium bromide after pre-exposure to the compound, can also be evidence of the presence of a multidrug efflux system(s).

The active efflux of bile salts such as sodium glycocholate is a common mechanism of bile salt resistance found in Gram-negative bacteria [Gunn, 2000] and has been described for the Gram-positive *L. lactis*. Cholic acid is the main free bile acid produced by bile salt hydrolase (BSH) activity in the intestine and is one of the most abundant salts in bile [Schultz *et al.*, 1991].

As mentioned in section 1.8.3 of Chapter 1, there is still much debate as to the role of bile salt hydrolases (BSH) in resistance to bile salts. The BSH isolated from bifidobacteria has been shown not to be induced by any extracellular factors including bile salts [Grill *et al.*, 1995]. A change in the ability of bifidobacteria to withstand the effects of bile salts following pre-exposure to the bile salts would, therefore, not likely to be due to a change in the expression of BSH.

Since many multidrug transporters are regulated by the substrates they transport, an increase in the level of bile resistance following exposure to the toxic compound would indicate that such a transporter may be present.

The aim of this study was, therefore, to ascertain whether the bifidobacterial strains studied were noticeably more resistant to certain antibiotics, ethidium bromide and/or the bile salt, sodium glycocholate. Also, whether the resistance profiles were similar to those reported in the literature since a great amount of interstrain and interspecies variation has been reported. In addition, it was of interest whether pre-exposure to antimicrobials would result in an increase in the resistance observed, since multidrug transporters are regulated by the compounds they transport [Baranova *et al.*, 1999].

The comparison of MIC values can be difficult due to differing techniques and growth conditions. For this reason, the NCCLS has standardized techniques to determine MIC values for both aerobic and anaerobic bacteria. The MIC values in this study were, therefore, all determined using the same methodology and values were compared to those reported by the NCCLS. In this way, the values could be interpreted as to their significance.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial strains, plasmids and culture conditions

Bifidobacterium bifidum NCIMB 702203^T (formerly NCFB 2203), *Bifidobacterium breve* NCIMB 702257^T (formerly NCFB 2257), and *Bifidobacterium longum* NCIMB 702259^T (formerly NCFB 2259) were obtained from the NCIMB culture collection, UK. *Bifidobacterium lactis* DSM 10140^T was isolated from freeze-dried yoghurt starter culture (Darleon Distribution)

(courtesy of Dr M. I. Trindade). *B. bifidum* strain W was obtained from a 6 day old baby (courtesy of Dr L. Brown, Food Technology, Cape Technikon). Bifidobacterial cultures were propagated anaerobically 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. Bifidobacteria were cultured in BYG medium (Appendix A.2). Glycerol stocks were prepared from cells grown in BHI broth (Appendix A.1) and stored at -70°C [Sambrook *et al.*, 1989].

2.3.2 Resistance to ethidium bromide

MIC values of *Bifidobacterium* spp to ethidium bromide were determined by a standard broth dilution method carried out using a two-fold dilution of the toxic compound [Koneman *et al.*, 1988]. Briefly, bifidobacterial strains grown for 16 h in BYG, were diluted to an OD_{600} of approximately 0.25 (Beckman DU64 spectrophotometer) and transferred to BYG broth containing ethidium bromide (Sigma) at concentrations ranging from 12.5 to 400 µM. Following incubation for 16 h, the OD_{600} of the culture was measured. The MIC values obtained are referred to as "intrinsic" values. Cells from the culture tube just below the MIC were diluted to an OD_{600} of 0.25 and used to re-inoculate fresh BYG containing ethidium bromide concentrations in the same range as previously described. This second series was incubated under the same conditions and OD_{600} readings taken. The MIC values obtained are referred to as "adaptive" values. The MIC values were defined as the lowest concentration of the toxic compound that inhibited growth. Complete experiments were completed in triplicate.

2.3.3 Resistance to sodium glycocholate

Resistance of *Bifidobacterium* spp to sodium glycocholate (Difco) was investigated using the same methods as described for ethidium bromide except that plate counts were used instead of OD_{600} to evaluate growth. The pK_a of cholate is 6.4, causing cholate precipitation in liquid media, thus making absorbance readings inaccurate. The appropriate dilutions were made in duplicate and the experiments were repeated in triplicate.

2.3.4 Resistance to antibiotics

Antibiotics were supplied as laboratory standard powders (all Sigma). Intrinsic MIC values of *Bifidobacterium* spp. to ampicillin, chloramphenicol, erythromycin, and tetracycline was determined by a standard broth dilution method carried out using a two-fold dilution of each antibiotic [Koneman *et al.*, 1988]. Adaptation to the antibiotics was tested using a method adapted from Carsenti-Etesse *et al* (1999). Briefly, cells grown for 16 h were streaked onto gradient plates containing various concentrations of the antibiotic tested. At each passage, the sub-MIC culture (colonies nearest the inhibition zone) was selected and streaked onto other gradient plates containing higher concentrations of the antibiotic. This was repeated for 4 passages and these MIC values obtained are referred to as "adaptive" values. A cell suspension was made from the adapted cells and inoculated into a broth dilution series of the antibiotic being tested. After 16 h, 10 µl of the culture was spotted onto plates to determine cell viability. Gradient plates were not used until at least 4 hours after being made, to ensure diffusion of the antibiotic. Complete experiments were performed in triplicate.

2.3.5 Stability of adaptive resistance

B. bifidum and *B. lactis* cells adapted to tetracycline were tested for stability of the phenotype in the absence of the antibiotic. Pre-adapted cells were passaged 4 consecutive times on antibiotic-free agar plates. A cell suspension was made from these cells and inoculated into a two-fold dilution series of the antibiotic being tested. The MIC values were measured as described in section 2.3.4. Complete experiments were performed in triplicate.

2.4 RESULTS AND DISCUSSION

2.4.1 Resistance to ethidium bromide

In BYG, laboratory strains of bifidobacteria grew to an OD_{600} of approximately 1 after 16 h, while the natural isolate, *B. bifidum* strain W only grew to approximately 0.35.

As shown in Figure 2.1 A, *B. bifidum* was able to grow in the presence of up to 50 µM ethidium bromide. Following exposure to the toxic compound it grew at 100 µM ethidium bromide. Neither *B. breve* nor *B. longum* showed any ability to resist the toxic affects of ethidium bromide, both showing intrinsic MIC values of 12.5 µM (Figure 2.1 B and D).

Although *B. lactis* displays a greater ability than *B. breve* to grow in ethidium bromide, it is not significantly resistant to the compound. Pre-exposure of *B. lactis* to ethidium bromide did not result in an increased ability to resist the effects of this toxic compound, with the MIC value remaining at 50 µM (Figure 2.1 C).

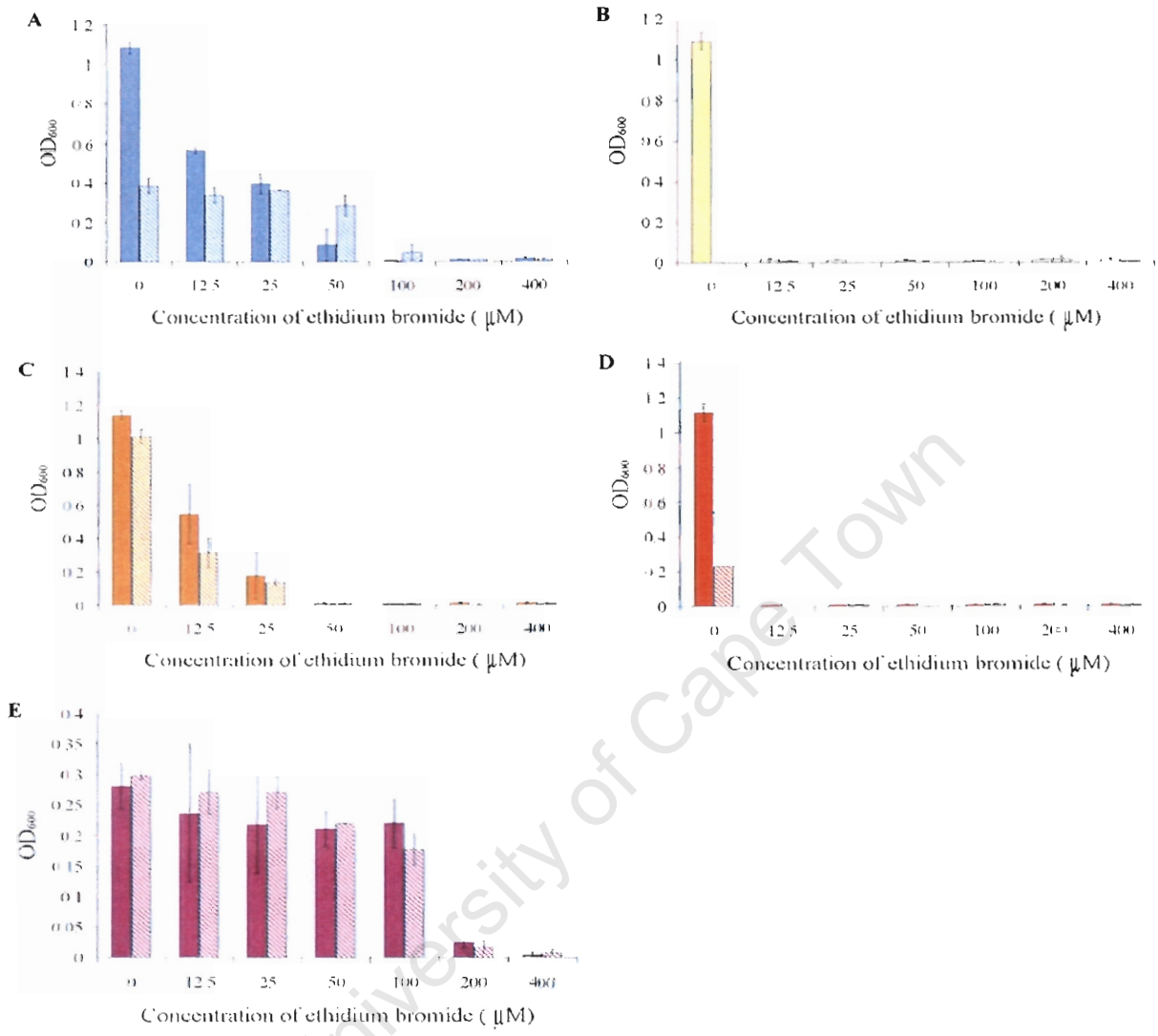


Figure 2.1 Resistance to ethidium bromide of the bifidobacteria as determined by the double dilution method. Growth was monitored by measuring the OD_{600} after overnight growth. The solid bars represent initial growth in ethidium bromide, and hatched bars growth following exposure to ethidium bromide. A: *B. bifidum*, B: *B. breve*, C: *B. lactis*, D: *B. longum*, E: *B. bifidum* strain W. All experiments were repeated in triplicate, with the variation in the OD_{600} expressed in the Y-error bars.

Growth in the presence of ethidium bromide revealed that *B. bifidum* strain W possessed a higher level of intrinsic resistance than any of the other strains (Figure 2.1 E). The strain grew relatively well in ethidium bromide concentrations up to 100 µM and weakly up to 200 µM. Similar results

have been described for *Lactococcus lactis* subsp *lactis* isolates expressing the multidrug transporter, LmrA, which were found to grow weakly in 200 μM ethidium [Bolhuis *et al.*, 1994]. The growth did not appear to be significantly different following exposure to ethidium bromide. The MIC values reported are summarized in Table 2.1.

As previously mentioned, all ethidium bromide resistance mechanisms known to date are membrane-located transport systems. The results reported here show that further investigation into the area of drug transporters in bifidobacteria is warranted.

2.4.2 Resistance to sodium glycocholate

Two different growth patterns were observed when unadapted bifidobacteria were grown in the presence of sodium glycocholate. *B. bifidum* and *B. lactis* grew in concentrations up to 14 % w/v (Figure 2.2 A and B). In both species, there was a rapid decrease in viability in growth of the bacteria in cholate concentrations up to about 4 %, followed by a gradual decrease in viability until no growth was observed.

These observations suggest the presence of a subpopulation of cells that are more resistant to the effects of sodium glycocholate. In any population of bacteria, there is a small fraction of cells that are not killed by the presence of an antimicrobial agent. These cells are named "persisters". Persisters are not resistant to antimicrobial agents and do not exhibit increased MIC values [Lewis, 2000]. They are not mutants and their increased survival is not transferred to their progeny [Keren *et al.*, 2003]. It has been shown that cells grown in the presence of antibiotics and then inoculated into fresh medium displayed the same growth pattern in the presence of the antibiotic tested as the original population. The growth of a subpopulation of *B. bifidum* and *B. lactis* up to 14 % w/v is, therefore, not due to the presence of persister cells. After pre-

exposure to cholate, the growth pattern suggests that it is this subpopulation that grows up to 18 % since the same level of growth is observed.

A different pattern of growth was observed with *B. longum* and *B. bifidum* strain W (Figure 2.2 C and D). These species grew only in concentrations of cholate up to 0.4 %. The cessation of bacterial growth occurred quickly with no subpopulation able to resist the effects of the cholate.

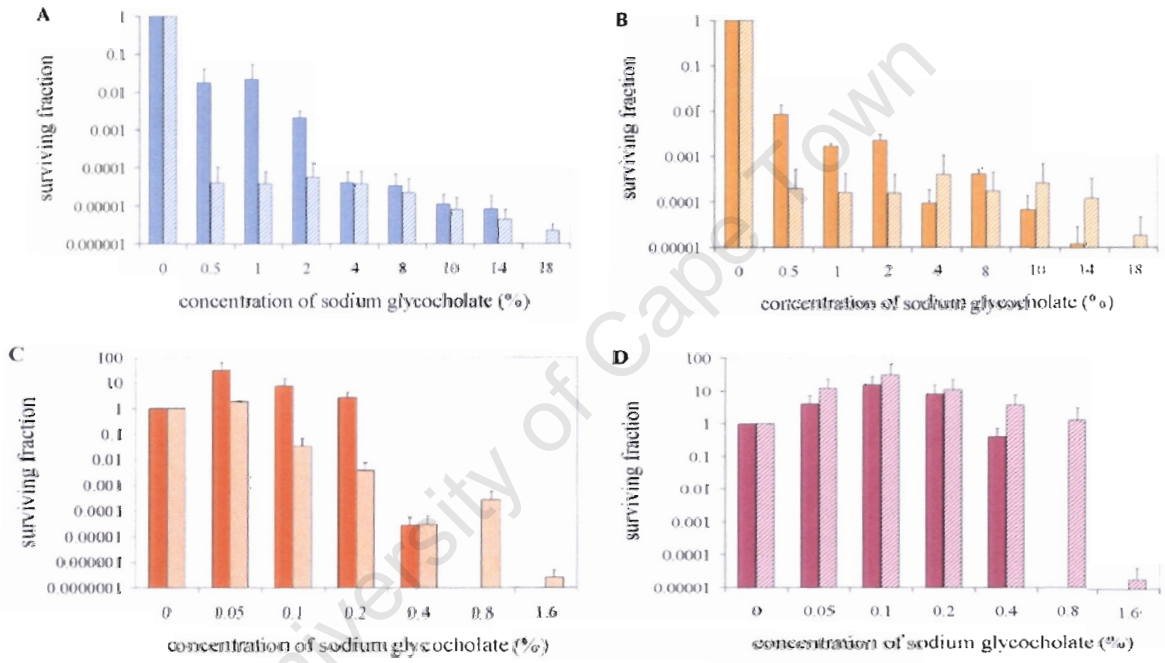


Figure 2.2 Resistance to sodium glycocholate of the bifidobacteria as determined by the double dilution method. Growth was monitored by measuring viable cell count after overnight growth. The solid bars represent initial growth in sodium glycocholate (parent cells), and hatched bars growth after pre-exposure to low levels of sodium glycocholate (pre-exposed cells). A: *B. bifidum,* B: *B. lactis* C: *B. longum,* D: *B. bifidum* strain W. All experiments were repeated in triplicate, with the variation in the results expressed in the Y-error bars.

Following pre-exposure to the compound, *B. longum* is able to grow in concentrations up to 1.6 %. Cell viability decreased quickly as the non-exposed cells did, but growth occurred up to 1.6 %. A slightly different growth pattern was observed with *B. bifidum* strain W. This strain

grew in up to 0.4 % as with *B. longum*. Following pre-exposure to cholate, the strain grows well in concentrations of cholate up to 0.8 %, after which viability decreases rapidly. This pattern of growth does not suggest the presence of a subpopulation of slow-growing cells [Keren *et al.,* 2003]. The MIC values reported are summarized in Table 2.1.

2.4.3 Resistance to common antibiotics

Antibiotics with a variety of structures and targets were tested. Gram-negative spectrum antibiotics, for example naladixic acid, were not used since they have been reported to be ineffectual against bifidobacteria [Lim *et al.,* 1993]. The comparison of MIC values can be difficult due to differing techniques and growth conditions. For this reason, the NCCLS has standardized techniques to determine MIC values for both aerobic and anaerobic bacteria. In this way, the values could be interpreted as to their significance. According to the criteria set out in the NCCLS, MIC values can be divided into 3 interpretive categories, namely susceptible, intermediate and resistant (Table 2.2).

2.4.3.1 β-lactam

As shown in Table 2.1, *B. longum* was the only species studied that displayed an adaptation to ampicillin exposure with a 2 fold increase in the MIC value of the adapted cells. The MIC values go from a susceptible category to an intermediate one following exposure to ampicillin [NCCLS, 1985] (Table 2.2).

TABLE 2.1 MIC values of antimicrobial agents tested against 5 bifidobacterial strains as determined by the double dilution method. An increase in the MIC value following growth in the antimicrobial is indicated in bold. The antimicrobial agents used were, ampicillin (Amp), chloramphenicol (Chl), erythromycin (Em), Tetracycline (Tet), ethidium bromide (EtBr), and cholate.

Antimicrobial agent	***Bifidobacterium* spp.**				
	B. bifidum	*B. bifidum* strain W	*B. lactis*	*B. breve*	*B. longum*
Amp (µg/ml)					
intrinsic	6.25	0.8	12.5	1.6	12.5
adaptive	6.25	0.8	12.5	1.6	25
Fold increase	1	1	1	1	**2**
Chl (µg/ml)					
intrinsic	6.25	1.6	12.5	3.12	3.12
adaptive	6.25	1.6	12.5	6.25	3.12
Fold increase	1	1	1	**2**	1
Em (µg/ml)					
intrinsic	0.8	0.8	0.8	1.6	0.8
adaptive	1.6	1.6	1.6	6.25	3.12
Fold increase	**2**	**2**	**2**	**4**	**4**
Tet (µg/ml)					
intrinsic	12.5	10	25	6.25	6.25
adaptive	50	10	100	6.25	6.25
Fold increase	**4**	1	**4**	1	1
EtBr (µM)					
intrinsic	100	400	50	12.5	12.5
adaptive	200	400	50	12.5	12.5
Fold increase	**2**	1	1	1	1
cholate (% w/v)					
intrinsic	16	ND	16	0.6	0.6
adaptive	20	ND	20	1.8	1.8
Fold increase	1.25	ND	1.25	**3**	**3**

TABLE 2.2 Interpretive categories and MIC values against anaerobic bacteria. Adapted from National Committee for Clinical Laboratory Standards (1985).

	MIC (µg/ml)		
Antibiotic	**Susceptible**	**Intermediate**	**Resistant**
Ampicillin	<0.5	1	>2
Chloramphenicol	8	16	>32
Tetracycline	<4	8	>16

2.4.3.2 Gram-positive spectrum

All species studied were able to adapt to increasing concentrations of erythromycin, with *B. breve* and *B. longum* showing the greatest increase in erythromycin resistance (Table 2.1). Erythromycin has been reported to be the most active agent against bifidobacteria [Matteuzzi *et al.,* 1983; Lim *et al.,* 1993] but is not tested against anaerobic bacteria in the NCCLS.

2.4.3.3 Broad-spectrum antibiotics

B. breve showed a 2 fold increase in the MIC value following pre-exposure to chloramphenicol (Table 2.1). While *B. lactis* did not exhibit the ability to adapt to increasing concentrations of the antibiotic, it did display a highest the intrinsic MIC value to chloramphenicol than any of the other species studied.

Chloramphenicol is generally effective against bifidobacteria [Matteuzzi *et al.,* 1983]. Although there is strain variation, the bifidobacteria in this study can be classified as susceptible to chloraphenicol (Table 2.2).

Following pre-exposure to tetracycline, both *B. bifidum* and *B. lactis* showed increased MIC values to the antibiotic. The intrinsic resistance of *B. bifidum* to tetracycline can be classified as intermediate (Table 2.2). However, after exposure to the antibiotic, it becomes resistant to tetracycline. *B. lactis* showed the highest intrinsic MIC value to tetracycline, and becomes greatly resistant to the antibiotic following pre-exposure with a 4 fold increase in the MIC value (Table 2.1).

In general *B. bifidum* strain W was the most susceptible to the antibiotics tested, while *B. lactis* showed the greatest ability to grow in the presence of the antibiotics tested.

Importantly, the development of increased resistance to the various antibiotics and toxic compounds was highly reproducible upon repeat experimentation.

2.4.4 Stability of adaptive resistance

Both *B. bifidum* and *B. lactis* adapted cells did not lose their ability to withstand higher levels of tetracycline after repeated subculturing in the absence of the antibiotic.

These findings are similar to those found in *E. coli* and *Salmonella enterica* cells adapted to detergents and antibiotics [Braoudaki & Hilton, 2004]. In that study, the adaptive resistance remained stable even after 30 days of passage in the absence of the detergents or antibiotics.

2.5 CONCLUSIONS

As mentioned in section 2.2, there is great interspecies and interstrain variation in the antibiotic profiles of bifidobacteria. This was once again displayed in the results presented in section 2.4. Adaptation to at least one of the antimicrobial agents tested was observed in every one of the bifidobacterial species. A level of resistance below the criteria as set by NCCLS would still be important, since even a small change in the susceptibility of a strain to any given antibiotic and/or antimicrobial may confer a growth advantage on it.

Bifidobacteria are resistant to a number of different antimicrobials with different targets and modes of action. Antibiotic resistance in bacteria can be achieved via modification of the target of action, direct inactivation of the antibiotic, changes in the cell permeability and multidrug drug efflux pumps.

The permeability of the bacterial cell envelope greatly influences the ability of antibiotics to enter the cell. However, in contrast to mycobacteria and Gram-negative microorganisms, most Gram-positive organisms are not well protected from penetration by antibiotics [Lambert, 2002]. The thick peptidoglycan cell wall does not impede the movement of molecules less than of 50 kDa [Hogan & Kolter, 2002]. It is, therefore, unlikely that the resistance seen in this study is as a result of drug exclusion.

The upregulation of multidrug efflux systems can provide cross-resistance to a number of antibiotics and/or antimicrobial agents [Levy, 2002]. In order to determine whether this is the case in bifidobacteria, future experiments should include the investigation as to whether pre-exposure to one compound results in the increased resistance to another/other antimicrobial(s). Cross-resistance towards various antibiotics could be determined by streaking parent and adapted cells onto gradient plates each containing different antibiotics. Any differences observed would then be confirmed using the broth dilution method. This would allow the quick screening for cross-resistance.

Of particular interest would be whether the cells pre-exposed to cholate displayed an altered range of MIC values to other antimicrobial agents. The bile salt, oxgall, has been shown to alter the susceptibility of bifidobacteria to all the antibiotics tested in this study [Charteris *et al.*, 2000]. In addition, antibiotics designed against a spectrum of Gram-negative bacteria were found to be more effective against some bifidobacterial strains in the presence of bile salts. The mechanism by which this occurs is unknown.

The ability of the *Bifidobacterium* strains tested to adapt to a number of unrelated antimicrobials was considered enough evidence to warrant further investigation into the possible molecular basis for the physiological characteristics observed and reported in this chapter.

CHAPTER 3

ISOLATION, SEQUENCING AND CHARACTERIZATION OF ANTIMICROBIAL EFFLUX GENES FROM *BIFIDOBACTERIUM LACTIS*

3.1 SUMMARY

A genomic library of *Bifidobacterium lactis* hosted in the hypersusceptible strain, *Escherichia coli* KAM3, was screened on tetracycline and ethidium bromide for colonies with increased resistance to either antimicrobial agent. Ten colonies were isolated which showed elevated levels of resistance to ethidium bromide. Sequence analysis was performed on all 10 recombinant plasmids. A putative ABC transporter system was identified and further characterized. Attempts to measure the ethidium efflux activity of the cloned transporter system proved unsuccessful in *E. coli* KAM3. The transporter was, therefore, subcloned into a *Lactococcus lactis* expression system. Expression of the *B. lactis* ABC transporter system in a drug-sensitive *L. lactis* strain did not complement the phenotype.

3.2 INTRODUCTION

The identification of genes encoding antimicrobial efflux proteins has been achieved using two main approaches. The first is the screening of a genomic library in a bacterial host for clones conferring an increased level of resistance to the host. This method has been used extensively in the identification of antimicrobial efflux proteins in many different bacteria, for example *Vibrio parahaemolyticus* [Morita *et al.,* 1998] and *Enterococcus faecalis* [Lee *et al.,* 2003]. With the advent of whole genome sequencing, complete genome sequences can be systematically analyzed and predicted antimicrobial efflux proteins can be identified. The function of the predicted transport proteins would then be confirmed through expression in a bacterial host. This method was used by Nishino and Yamaguchi (2001) to analyze 37 ORFs in *Escherichia coli* assumed to be antimicrobial efflux proteins by sequence similarities to known proteins in the databases. In both approaches a stable, drug- sensitive bacterial host is necessary. In this study, *E. coli* KAM3 [Morita *et al.,* 1998] was used for the screening of a *Bifidobacterium lactis* genomic library and the subsequent characterization of putative antimicrobial efflux proteins. This hypersusceptible strain has been used in the screening of bacterial genomic libraries [Morita *et al.,* 1998], the expression of genes predicted to encode antimicrobial efflux proteins by sequence similarity [Nishino & Yamaguchi, 2001], and the characterization of putative efflux proteins by ethidium bromide transport assays [Morita *et al.,* 1998]. *E. coli* KAM3 has even been used in the isolation of a multidrug efflux protein in *Arabidopsis thaliana* through the screening of a cDNA library [Li *et al.,* 2002].

Morita *et al.,* (1998) constructed *E. coli* KAM3 from the K-12 derivative TG1 (Appendix B). *E. coli* TG1 cells were infected with the Mud(Amp^R *lac*) phage in order to disrupt the

chromosomal *acrAB* genes, which code for the major multidrug efflux system in *E. coli.* The Mu phage region was then removed from the chromosome by heat induction at 42°C. The resulting cells were designated KAM3 and were shown to be sensitive to many drugs that are known substrates of the AcrAB system. The strain lacks a DNA restriction modification system making it ideal for the expression of heterologous genes.

E. coli KAM3 is sensitive to the cationic dye ethidium bromide [Nishino & Yamaguchi, 2001]. As already mentioned, ethidium bromide is widely used to detect the presence and activity of multidrug transporter proteins. Ethidium bromide is also a useful substrate in the identification of antimicrobial efflux proteins as it can be used in the subsequent characterization of the protein. Ethidium bromide becomes fluorescent upon intercalation with polynucleotides, making the measurement of its influx/efflux into/out of bacterial cells convenient to monitor. Other commonly used compounds include Hoescht 33342, the hydrophobic ester BCEF-AM and the anti-tumour drug, daunomycin.

In Chapter 2, it was reported that *B. lactis* DSM 10140^{T} was in general more resistant to a number of antimicrobial agents when compared to the other strains tested. This bifidobacterial strain was isolated from a dairy starter culture that is currently used in food products [Courtesy of Dr. M. I. Trindade]. Since the aims of the thesis were to investigate the presence of antimicrobial efflux systems in bifidobacteria, with the view to evaluate the safety of the administration of these probiotic strains, a commercial strain was chosen for further study. A genomic library of *B. lactis* DSM 10140 ^{T} was, therefore, used for screening and isolation of genes possibly encoding for antimicrobial efflux proteins.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial strains, plasmids and culture conditions

E. coli KAM3 served as the cloning and expression host for the introduction of recombinant plasmids [Morita *et al.,* 1998]. *E. coli* KAM3 was cultured in Luria-Bertani (LB) medium at 37°C. Ampicillin (100 µg/ml) was added to media for the growth of cells containing plasmid. Plasmids M13-derived Bluescript SK (pSK) (Stratagene, La Jolla, California, USA), and pEcoR251 [Zabeau & Stanley, 1982] were used for subcloning and sequencing. Plasmid pMT104, in which the *Eco*RI endonuclease has been insertionally inactivated [Wehnert *et al.,* 1990], was used as a negative control vector in the characterization of recombinant plasmids.

3.3.2 General recombinant DNA procedures

Plasmid DNA was isolated by the alkali lysis method of Ish-Horowicz and Burke (1981) or with the QIAGEN Plasmid Midi Kit. All DNA modifications were performed according to standard procedures [Sambrook *et al.*, 1989]. All restriction and modifying enzymes were obtained from Roche Diagnostics and used according to general manufacturer's instructions. Competent *E. coli* KAM3 cells were prepared using the rubidium chloride method [Armitage *et al.,* 1988] for use in subcloning. Electrocompetent *E. coli* KAM3 cells were prepared using the method described by Tung and Chow (1995) for use in the screening of the *B. lactis* genomic library. Electroporation was carried out using Gene pulser apparatus (Bio-Rad laboratories, Richmond, Calif.) at 2.5 kV, 200 Ω, and 25 µF.

3.3.3 Screening of the *B. lactis* genomic library

A *B. lactis Sau3A* genebank (Courtesy of Dr. M. I. Trindade) was transformed into *E. coli* KAM3. Transformants were screened for increased resistance to ethidium bromide or tetracycline on LB agar (1.5 % w/v agar) containing ampicillin and ethidium bromide or tetracycline. Concentration ranges of 10 to 25 µg/ml ethidium bromide and 0.2 to 0.8 µg/ml tetracycline were used for the screening of the genomic library. Plasmid DNA was isolated from resistant colonies and used to retransform parental *E. coli* KAM3 cells to confirm the resistant phenotype.

3.3.4 Nucleotide sequencing and sequence analysis

Nucleotide sequences were determined by the fluorescent dideoxynucleotide chain termination method of Sanger *et al.* (1977) using the DYEnamic ET Dye Terminator Cycle sequencing Kit from MegaBACE (Molecular Dynamics). All reactions were performed according to the manufacturer's instructions and cycle sequenced on a GeneAmp PCR system 9700 (Perkin Elmer, Applied Biosystems). The sequencing reaction products were analyzed on the MegaBACE 500 sequencer (AmershamPharmacia Biotech). The nucleotide sequences were analyzed using the MegaBACE 500 Sequence Analyser v2 and the DNAMAN software packages. Nucleotide and amino acid homology searches were performed, using the BLAST algorithm using the databases contained at NCBI [Altschul *et al.*, 1997].

3.3.5 Characterization of antibiotic resistance profiles of putative transporter proteins

The MIC values for a range of antimicrobial agents were determined using the broth dilution method of Koneman *et al.* (1988). These included acriflavine orange (Merck) ethidium bromide

(Sigma), erythromycin (Sigma), SDS (Merck), sodium glycocholate (Difco), and tetracycline (Sigma). The experiments were repeated in triplicate.

3.3.6 Transport assays

The efflux of ethidium bromide by *E. coli* KAM3 cells containing a putative transporter protein was performed using the method adapted from van Veen *et al.* (1996). Exponentially growing *E. coli* KAM3 cells (culture volume of 100 ml) were harvested and washed twice with 50 mM potassium phosphate buffer containing 1 mM $MgSO_4$ pH 7.0 (KPi). The cells were resuspended to an OD_{600} of 5 in KPi and placed on ice. The cells were diluted 1/10 in pre-warmed KPi for fluorescence measurements. Ethidium bromide was added to concentrations of 10 μM to 50 μM and allowed to accumulate. Glucose was then added to a concentration of 10 mM. To preload the cells with ethidium bromide, harvested and washed cells were incubated in the presence of ethidium bromide for 20 min at 37°C. The cells were washed and concentrated as described above. The cells were diluted 1/10 into pre-warmed KPi for fluorescence measurements. Glucose was added to a concentration of 10 mM. The fluorescence the ethidium bromide complex with polynucleotides was measured at excitation and emission wavelengths 500 and 580 nm, respectively [Bolhuis *et al.*, 1994]. For Hoescht 33342 (Molecular Probes), the compound was added to a concentration of 0.5 μM to washed cells followed by the addition of glucose to a concentration of 10 mM. Hoescht fluorescence was measured at excitation and emission wavelengths 355 and 457 nm, respectively. All measurements were performed in the PerkinElmer Life Sciences model 50B fluorimeter with magnetic stirring holder at 37°C.

3.3.7 Expression of putative ABC transporter in *Lactococcus lactis*

The ORF containing the putative ABC transporter was subcloned in the lactococcal vector pNG8048E (NIZO Food Research, The Netherlands) (Appendix C). The ABC transporter gene was amplified by PCR from plasmid pEcoB4.23. *NcoI* and *XbaI* restriction sites were introduced flanking the genes to enable cloning into pNG8048E, resulting in pNGABC. This plasmid contains the putative ABC transporter gene under the control of the nisin-inducible promoter, Pnis. Primers (ABC-F and ABC-R) used are shown in Table 3.1. PCR amplification using pEcoB4.23 as a template was performed with Taq Polymerase (0.25 U) (Supertherm) in a 50 µl reaction mixture containing: 10 ng plasmid DNA; 0.5 mM of each of ABC-F and ABC-R; 0.5 mM $MgCl_2$; and 200 µM dNTP's. The amplification cycles were as follows: 96°C for 5 min; 25 cycles of 96°C for 1 min, 52°C for 45 s, and 72°C for 1 min, and finally 72°C for 5 min.

PCR products were purified using the High Pure PCR Product Purification kit (Roche).

TABLE 3.1 Primers used for the subcloning of ORF into pNG8048E. Restriction sites included are underlined.

Primer name	Primer sequence
ABC-F	NcoI 5′-AGTTCCATGGTGCATCGGCTGAGTTC-3′
ABC-R	XbaI 5′-TTGAGATCTAGATTTAATGGCTGCTGGTAATG-3′

L. lactis NZ9000 served as the cloning host for the introduction of recombinant plasmids. Electrocompetent *Lactococcus lactis* NZ9000 cells were prepared using the method described by Holo and Nes (1995). Electroporation was carried out using the Gene pulser apparatus (Bio-Rad

laboratories, Richmond, Calif.) at 2.5 kV, 600 Ω, and 25 μF. *L. lactis* was grown at 30°C in M17 medium (Difco) supplemented with 5 g glucose per litre and 5 μg/ml chloramphenicol when necessary.

3.3.8 Expression in *Lactococcus lactis ΔlmrCD*

Lactococcus lactis ΔlmrCD (Courtesy of J. Lubelski and R. van Merkerk, Department of Microbiology, University of Groningen) (Appendix B.1) was transformed with pNGABC. This lactococcal strain is sensitive to a number of antimicrobial agents including ethidium bromide and cholate due to the deletion of the ABC multidrug transporter LmrCD (J. Lubelski and R. van Merkerk, unpublished data). MIC values were determined by a standard broth dilution method carried out using a two-fold dilution of each antibiotic [Koneman *et al.*, 1988]. NisinA (Sigma) was added to a final concentration of 10 ng/ml to induce the expression of the putative ABC transporter.

3.4 RESULTS AND DISCUSSION

3.4.1 Identification and sequence analysis of potential transport proteins

Screening of *E. coli* KAM3 transformed with the *B. lactis* genomic library on tetracycline did not yield any colonies with a greater level of resistance to the antibiotic. However, screening of the genomic library on ethidium bromide in *E. coli* KAM3 yielded 10 colonies exhibiting increased levels of resistance to the toxic compound (Table 3.2). Sequence analysis was performed on all recombinant plasmids (Table 3.2) and ORFs that showed sequence similarity to membrane extrusion systems were chosen for further investigation. Recombinant plasmid pEcoB1.3 showed a low level of sequence similarity to a cell wall anchor domain protein found in *B. longum* NCC2705. Sequence analysis of the insert DNA of pEcoB1.5 revealed 4 putative

ORFs. These ORFs coded for the following putative proteins: a membrane protein with sequence similarity to colicin uptake proteins such as TolA; a metalloendopeptidase; an acetyltransferase; and a putative molecular chaperone (Figure 3.1). Recombinant plasmid pEcoB1.7 contained only one ORF on the insert DNA, which showed sequence similarity to an aminopeptidase. Analysis of the insert DNA contained on pEcoB2.5 revealed the presence of 2 ORFs, one with similarity to a thioredoxin reductase, and the other to a chromosomal partitioning protein. Sequence analysis of the insert DNA of pEcoB4.5, revealed the presence of 4 ORFs, showing similarity to a glycosyltransferase involved in cell wall synthesis, two hypothetical proteins, and a transcriptional regulator. Since the sequences on recombinant plasmids pEcoB1.3, pEcoB1.7, pEcoB2.5, and pEcoB4.5 did not resemble any membrane located drug extrusion systems, they were not investigated further.

Sequence analysis of the ORFs contained on recombinant plasmids pEcoB4.13, pEcoB4.18, pEcoB4.19 and pEcoB4.23 revealed that they all showed sequence similarity to regions of the same ABC transporter system from *B. longum* NCC2705 (Table 3.2; Figure 3.2). In addition, pEcoB4.23 contained sequence with similarity to a transcriptional regulator upstream of the putative ABC transporter. Recombinant plasmid pEcoB4.21 shows sequence similarity to a solute binding of an ABC transporter system in *B. longum* NCC2705. Drug/metabolite uptake systems but not efflux systems possess an extracytoplasmic solute binding receptor [Saier & Paulsen, 2001]. No further work was therefore conducted on B4.21.

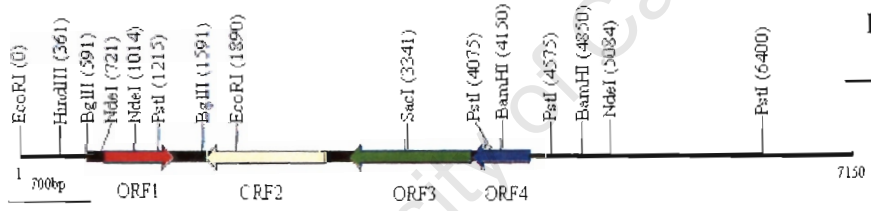
TABLE 3.2 Recombinant plasmids and the identity of the ORFs contained on the inserts as determined by sequence analysis.

Recombinant plasmid	DNA identity to genes lodged on NCBI database	Accession no. or reference	E value	EtBr MIC (μg/ml)
pEcoB1.3	• LPXTG-motif cell wall anchor domain protein	NP_977284	0.002	25
pEcoB1.5	• colicin uptake protein • metalloendopeptidase Gcp • acetyltransferase • putative molecular chaperone	ZP_00120427 NP_696616 NP_696615 ZP_00120471	2e-10 2e-50 2e-32 5e-28	25
pEcoB1.7	• aminopeptidase, PepN	NP_696360	4e-88	25
pEcoB2.5	• thioredoxin reductase • chromosomal partitioning protein, ParA	ZP_00151999 NP_695834	3e-07 2e-60	25
pEcoB4.5	• glycosyltransferase involved in cell wall synthesis • hypothetical protein • hypothetical protein • transcriptional regulator	NP_695422 ZP_00121655 NP_696762 NP_599277	8e-38 9e-14 2e-54 1e-07	25
pEcoB4.13	• 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase • ATP-binding protein of ABC transporter • ABC-type antimicrobial peptide transport component	NP_696169 NP_695373 NP_695374	6e-09 3e-28 8e-24	25
pEcoB4.18	• ATP-binding protein of ABC transporter • ABC-type antimicrobial peptide transport component	NP_695373 NP_695374	3e-24 4e-18	50
pEcoB4.19	• ATP-binding protein of ABC transporter • ABC-type antimicrobial peptide transport component	NP_695373 NP_695374	1e-21 8e-28	25
pEcoB4.21	• Solute binding protein of ABC transporter system	NP_696166	3e-16	25
pEcoB4.23	• Transcriptional regulator • ATP-binding protein of ABC transporter • ABC-type antimicrobial peptide transport component	NP_696075 NP_695373 NP_695374	2e-44 3e-25 2e-55	50
pMT104	• Vector control	Wehnert *et al.*, 1990		12.5

3.4.1.1 Recombinant plasmid pEcoB1.5

Further investigation was conducted on B1.5 to determine whether the membrane protein contained in ORF1 (Figure 3.1) was responsible for the elevated levels of ethidium bromide resistance. This was done through the deletion of the *BglII* fragment on which ORF1 occurs and the subcloning of the ORF into the high copy number plasmid pSK.

The MIC values presented in Figure 3.1 indicate that it is indeed the membrane protein that confers the elevated levels of resistance to *E. coli* KAM3. When ORF1 is more highly expressed, as in pSKTol$_f$, the level of ethidium bromide resistance increases compared to the original construct pEcoB1.5.



Recombinant Plasmid	EtBr MIC (µg/ml)
pEcoB1.5	25
pBgldel	12.5
pMT104 (vector control)	12.5
pSKTol$_f$	50
pSK (vector control)	12.5

FIGURE 3.1 Genetic organization of ORFs on recombinant plasmid pEcoB1.5. Transcriptional polarities are indicated by arrows. Thick and thin lines represent the insert and vector respectively. Plasmid pSKTol$_f$ contains a 1 kb *BglII* fragment from pEcoB1.5 subcloned into pSK, so that ORF1 is in same orientation as the *lacZ* promoter. The same 1 kb fragment was deleted to form pBgldel. The values presented are the ethidium bromide MIC values as determined by the broth dilution method. Plasmid pMT104 served as a negative control for pEcoR251-based plasmids, pEcoB1.5 and pBgldel. Plasmid pSK served as a negative control for pSK-based plasmid pSKTol$_f$.

Further sequence analysis of ORF1 revealed that it showed low sequence similarity to the porin TolA which is involved in colicin uptake in *E. coli* (Table 3.3). Loss of porins is generally considered to be a mechanism by which Gram-negative bacteria become resistant to a wide range of antimicrobials [Nikaido & Vaara, 1985]. It is, therefore, unexpected that ORF1 should confer ethidium bromide resistance to *E. coli* since the expression of porins should make the bacterial cell more susceptible to the influx of antimicrobial agents.

TABLE 3.3 Percent identity and similarity of the predicted ORFs in pEcoB1.5 to other proteins contained on the NCBI databases.

ORF	Homologous protein	Accession number	Identity (%)	Similarity (%)	E value
1	*B. longum*, COG3064, membrane protein involved in colicin uptake	ZP_00120427	30	48	2e-10
2	*B. longum*, COG0533, metalloendopeptidase Gcp	NP_696616	73	81	2e-50
3	*B. longum* COG1214, putative molecular chaperone	ZP_00120471	48	57	5e-28
4	*B. longum*, COG0456 putative acetyltransferase	NP_696615	58	69	2e-32

3.4.1.2 Recombinant plasmid pEcoB4.23

As mentioned previously, 4 of the clones isolated contained portions of the same ABC transport system. The sequences of all of the recombinant plasmids were compared and they were found to contain overlapping regions (Figure 3.2A). The overlapping regions all corresponded to ORF1 of pEcoB4.23, thus indicating that it was this ORF that conferred elevated levels of ethidium bromide resistance to the host *E. coli*. As shown in Figure 3.2B, all the recombinant plasmids contain sequence similarity to regions of the ATP-binding protein, BL0154, and to the permease/transport component, BL0155, of an ABC transporter in *B. longum* NCC2705.

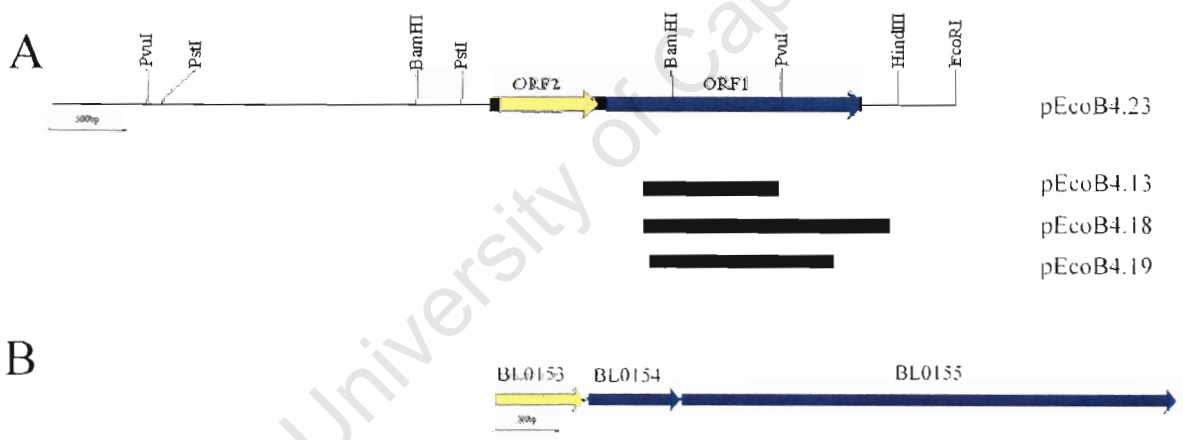


FIGURE 3.2 A: Genetic organization of ORFs on plasmid pEcoB4.23. Transcriptional polarities are indicated by arrows. Thick and thin lines represent the insert and vector respectively. Regions of recombinant plasmids pEcoB4.13, pEcoB4.18 and pEcoB4.19 with sequence similarity to ORF1 of pEcoB4.23 are shown. **B:** Organization of ABC transport system in *B. longum* NCC2705 with sequence similarity to recombinant plasmids. BL0153 encodes a transcriptional regulator (yellow arrow); BL0154 an ATP-binding protein of an ABC transporter; BL0155 the permease component of an ABC transporter (blue arrows). Transcriptional polarities are indicated by arrows.

Unfortunately, none of the 4 clones appeared to contain the entire ABC transporter in spite of the resistance phenotype shown. It was decided to continue with the study using the clone pEcoB4.23. This clone contained the largest portion of the same ABC transporter system (Figure 3.2A) and exhibited a high level of ethidium bromide resistance when compared to the other clones of the ABC transporter (Table 3.2). The ORFs contained on the insert DNA of pEcoB4.23 were further analyzed for sequence similarity to known genes (Table 3.4), especially those contained in *B. longum* NCC2705, since the genome sequence of *B. lactis* is not available on public databases.

TABLE 3.4 Percent identity and similarity of the predicted ORFs in pEcoB4.23 to other proteins contained on the NCBI database.

ORF	Homologous protein	Accession number	Identity (%)	Similarity (%)	E value
1	*B. longum,* COG1136: ATP binding protein of ABC transporter, **BL0154**	NP_695373	84	92	3e-25
	B. longum, COG0577, ABC-type antimicrobial peptide transport component, **BL0155**	NP_695374	34	47	2e-55
2	*B. longum,* COG2865: predicted regulator containing HTH domain and uncharacterized domain shared with mammalian protein Schlafen, **BL0898**	NP_696075	31	50	2e-44

Transporters belonging to the ABC superfamily consist of 4 domains, two transmembrane domains/proteins and two cytoplasmic domains/proteins, containing the ATP-binding domain. In many bacterial ABC transporters, the 4 domains are contained in separate proteins whereas in eukaryotes, the domains are contained on a single polypeptide [Kerr, 2002; Lage, 2003].

In bacteria, a membrane domain can be fused to a cytoplasmic domain to form a "half-transporter". This is shown in Figure 3.3. These proteins form an active unit by homo- or heterodimerization. LmrCD from *L. lactis* was the first heterodimeric ABC multidrug transporter to be characterized [Lubelski *et al.*, 2004].

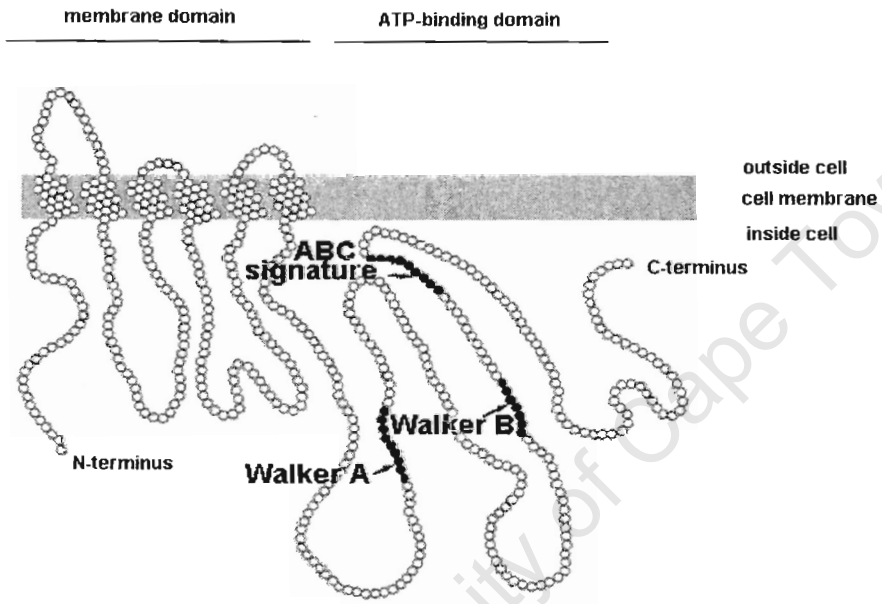


FIGURE 3.3 Membrane organization of a bacterial ABC multidrug transporter. The membrane/transport domain is fused to an ATP-binding cytoplasmic domain to form a "half-transporter". The protein forms an active unit by homo- or heterodimerization. Conserved regions are shown in black [adapted from Putman *et al.*, 2000].

ORF1 appears to be a single polypeptide. In *B. longum,* BL0154 and BL0155 are closely positioned but separate ORFs. (Figure 3.2B). The ATP-binding and permease domains of ORF1 were identified as well as the highly conserved Walker B motif and the ABC signature sequences of the ATP binding domain as (Figure 3.4).

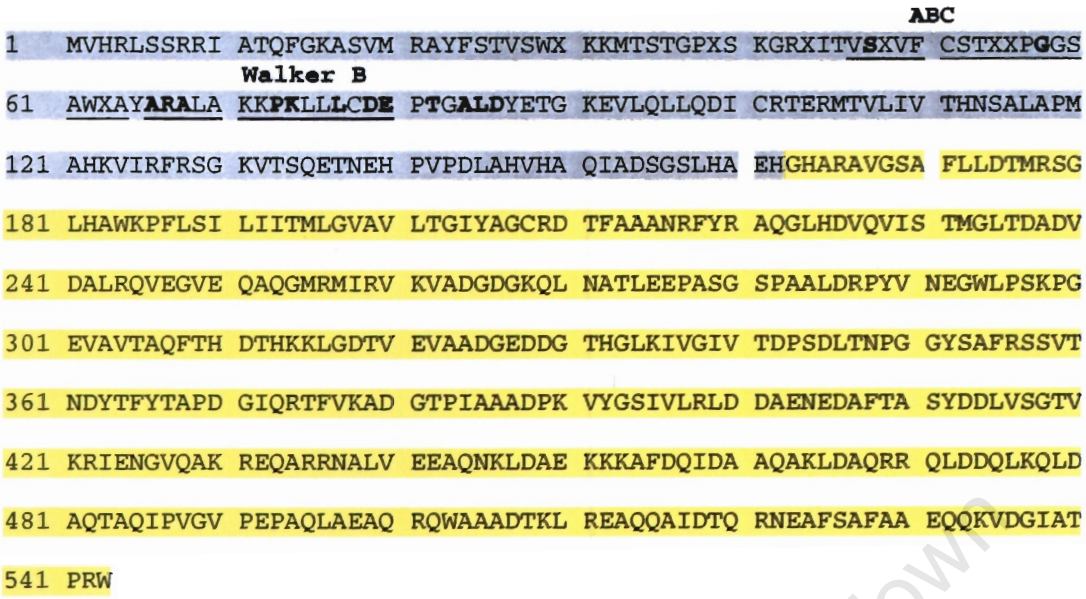


FIGURE 3.4 Derived amino acid sequence of ORF identified as an ABC transporter carried on pEcoB4.23. Conserved residues are indicated in bold. Conserved regions are underlined. The ATP-binding domain is shaded in grey and the permease component of the protein in yellow.

Analysis of ORF2 revealed that it belongs to the CRP family of cAMP regulatory proteins with a DNA-binding domain belonging to the catabolite activator protein family [Marchler-Bauer & Bryant, 2004]. As shown in Figure 3.2B, upstream of BL0154, is BL0153 identified as a putative transcriptional regulator. This regulator may be involved in controlling the expression of BL0154 and BL0155 in *B. longum* NCC2705. This regulator, however, only showed 12 % sequence similarity to ORF2. The regulator on pEcoB4.23 shows 31 % identity at the amino acid level to another bifidobacterial regulator, BL0898 (Table 3.4). This regulator is also upstream of a cluster of ABC transporter proteins on the chromosome of *B. longum* NCC2705 (Figure 3.5). ORF2 may therefore, encode a transcriptional regulator of the ABC transport system contained on ORF1.

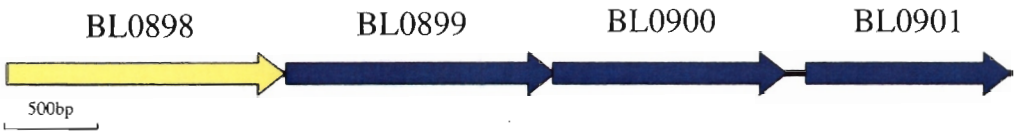


FIGURE 3.5 Position of the predicted ORFs BL00898, BL0899, BL0900 and BL0901 on the chromosome of *B. longum* NCC2705. BL0898 encodes a transcriptional regulator (yellow arrow); BL0899 and BL0900 permease components of an ABC transporter; and BL0901, the ATPase component of an ABC transporter (blue arrows). Transcriptional polarities are indicated by arrows

The components of the ABC transport system encoded by BL0899, BL0900 and BL0901 do not show high sequence similarity to ORF1 of pEcoB4.5 with 12.65 %, 15.58 % and 15.31 % respectively. The genes contained on the insert DNA of the recombinant plasmid and from *B. lactis* are, therefore, not identical to those on *B. longum*, and may be the result of the recruitment of a regulator form a different operon as discussed previously in Chapter 1, Section 1.7.3.

3.4.2 Characterization of antimicrobial agent resistance profile of B4.23

B4.23 was tested against a number of antimicrobial agents (Table 3.5). It conferred elevated levels of resistance to the cationic dye acriflavine orange, the detergents sodium glycocholate and SDS, as well as to the antibiotic erythromycin. B4.23 does not, however, confer resistance to tetracycline.

TABLE 3.5 MIC values of B4.23 against various antimicrobial agents with reference to the control vector pMT104.

Plasmid name	MIC values					
	ethidium bromide (μg/ml)	sodium glycocholate (%)	SDS (%)	acriflavine orange (μg/ml)	erythromycin (μg/ml)	tetracycline (μg/ml)
pMT104	12.5	0.25	0.005	6.25	1.6	0.5
pEcoB4.23	50	1	0.01	50	6.25	0.5

3.4.3 Transport assays

It was necessary to investigate whether the putative ABC transport system contained on B4.23 conferred resistance to *E. coli* KAM3 through the active extrusion of ethidium bromide. This was attempted by using ethidium bromide transport assays. Ethidium bromide influx into the cell is convenient to monitor since it becomes fluorescent upon intercalation with polynucleotides. For the same reasons, Hoescht 33342 is commonly used in the characterisation of multidrug transporters. Hoescht 33342 becomes fluorescent upon intercalation in the lipophilic membrane as well as upon binding to polynucleotides [Shapiro & Ling., 1995].

In these experiments, the cells appeared not to be loaded sufficiently as they continue to accumulate ethidium bromide without ever reaching a maximum value (Figure 3.6 A), irrespective of the ethidium bromide concentrations used in the range of 10 – 50 μM. A similar problem was encountered when Hoescht 3342 transport was monitored (Figure 3.6 C). It was, therefore, decided to preload the cells with ethidium bromide to determine whether the addition of glucose would cause a decrease in the amount of ethidium bromide associated with the cell. However, as shown in Figure 3.6 B, the addition of glucose did not cause the efflux of ethidium bromide.

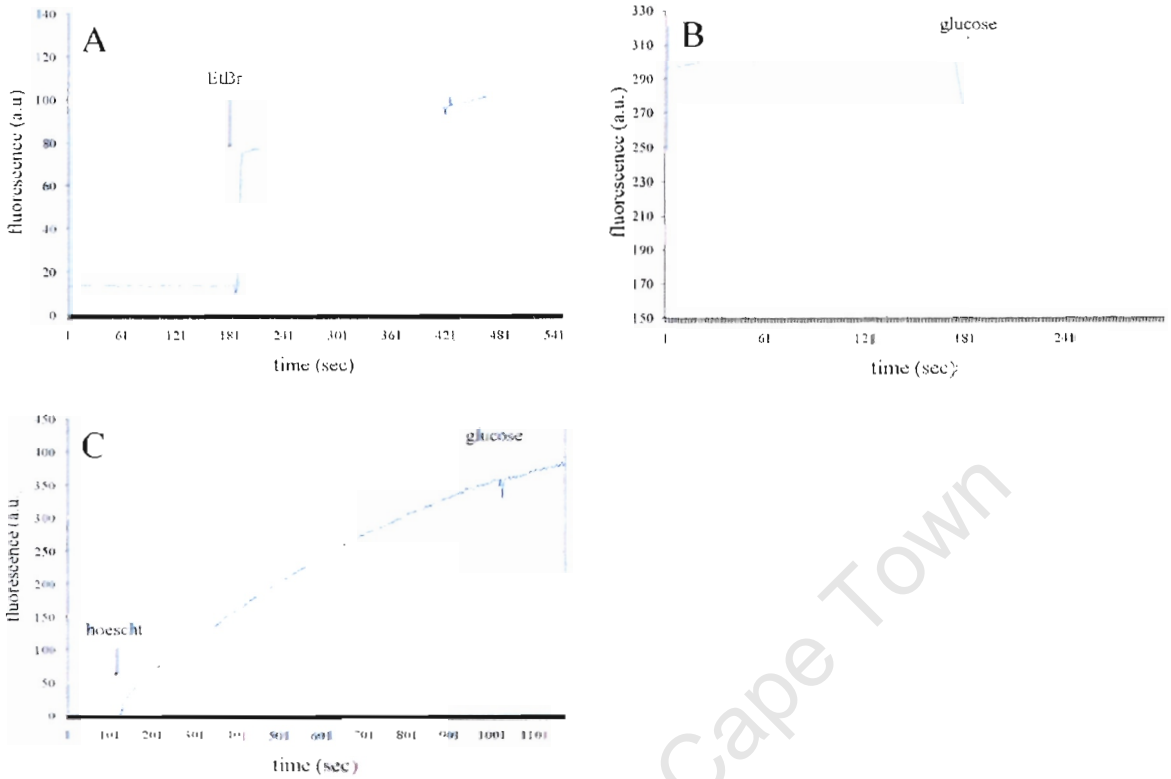


FIGURE 3.6 Measurement of ethidium bromide and Hoescht 3342 accumulation in de-energized cells of *E. coli* KAM3 harbouring the control plasmid pMT104 (pale blue line) and B4.23 (navy blue line). **A**, Ethidium bromide was added to washed cell suspension to a final concentration of 10 μM. Glucose was added to 10 mM at time points indicated. **B**, washed cell suspensions were pre-incubated in 20 μM ethidium bromide and glucose was added to 10 mM at time points indicated. **C**, Hoescht 3342 was added to washed cell suspension to a final concentration of 0.5 μM. Glucose was added to 10 mM at time points indicated. Fluorescence of ethidium bromide and Hoescht 3342 was monitored over time. *a.u.*, arbitrary units.

Higher concentrations of the ethidium bromide (up to 50 μM) and glucose (up to 50 mM) were used, but no change in the pattern was observed. An alternative protocol that could be used in future experiments, would be to preload the cells in the presence of the uncoupler CCCP, followed by washing of the cells [Lee *et al.*, 2003].

The rate at which the cells accumulated the compound did not change significantly whether the ABC transporter was present or not (Figure 3.6A and C). This could be due to the low level of

expression of the gene, or the fact that the entire ATP-binding and permease domains are not contained on ORF1. Plasmid pEcoR251 is a low copy number plasmid and although the level of expression may be sufficient to observe an increased level of resistance to ethidium bromide, it may not be sufficient to measure using these assay techniques. In addition, the cells did not appear to be sufficiently de-energized. Therefore the addition of glucose would have no effect on the rate of ethidium bromide or Hoescht 3342 influx. The use of 2-deoxyglucose to de-energize the cells may have produced the desired effect.

3.4.4 Expression of pNGABC in *Lactococcus lactis ΔlmrCD*

For the above reasons, the ORF containing the ABC transporter was subcloned into the *L. lactis* expression system pNG8048E. This allowed for gene expression under the control of the tightly regulated, inducible *nisA* promoter [de Ruyter *et al.*, 1996], and since *L. lactis* is a Gram-positive bacteria, the membrane and cell wall structure is more similar to bifidobacteria than the previously used heterologous host, *E. coli.*

The recombinant plasmid, pNGABC, contained the *B. lactis* ORF predicted to contain the ABC transporter under the control of the nisinA-inducible promoter contained on pNG8048E. The MIC values for pNGABC and pNG8048E were determined for cholate and ethidium bromide. However, no resistance was conferred on *L. lactis ΔlmrCD* by pNGABC. This could be due to the incorrect functioning of the ABC transporter in *L. lactis* because of the truncated membrane domain. Alternatively, while bifidobacteria have a high G+C content, lactococci do not. The gene may, therefore, not have been expressed in *L. lactis.*

3.5 CONCLUSIONS

Only one efflux system was identified in *B. lactis* through the screening of a genomic library on both ethidium bromide and tetracycline. According to sequence analysis using TransportDB [www.membranetransport.org; described in Ren *et al.,* 2004] there are at least 4 ABC multidrug transporters and 20 secondary multidrug transporters in *B. longum* (Table 3.6). This may be an indication of the number of multidrug transporters present in *B. lactis*. In *E. coli*, 37 putative multidrug transporters were identified through sequence analysis of the entire genome sequence [Nishino and Yamaguchi, 2001].

It is, therefore, reasonable to assume that the method used in this study was ineffective in identifying the vast number of multidrug transporters present in bifidobacteria. This could be due to a number of factors. Firstly, as already mentioned, the expression of genes from Gram-positive bacteria in a Gram-negative host is not ideal. This is especially true for membrane proteins. The difference in cell membrane structure could result in the incorrect functioning of an expressed gene. In addition, bifidobacterial DNA has a high G+C content which may lead to the genes contained on the recombinant plasmids not being expressed in *E. coli*. *E. coli* KAM3 has, however, been used to identify drug transporters in a number of organisms including *Arabidopsis thaliana* [Li *et al.,* 2002]. It was, therefore, a reasonable strategy.

Another reason for the failure to identify more multidrug transporters could be that other antimicrobials should have been used in the screening. These could include chloramphenicol and erythromycin. Bifidobacteria were able to adapt these antibiotics as shown in Section 2.4.3 of Chapter 2.

TABLE 3.6 Putative multidrug transporters in *B. longum* NCC2705 as predicted using TransportDB [www.membranetransport.org] with gene annotations as on the NCBI database.

ATP-dependent transporters		
The ABC superfamily		
	BL0162	probable permease protein of ABC transporter system
	BL0163	ABC-type multidrug transport system, ATPase and permease components
	BL0179	probable permease protein of ABC transporter system
	BL0180	probable permease protein of ABC transporter system
	BL1041	ATP binding protein of ABC transporter
	BL1766	ATP-binding protein of ABC transporter
	BL1767	ABC transporter, ATP-binding transmembrane protein
Secondary transporters		
SMR		
	BL0575	narrowly conserved hypothetical protein
	BL1406	possible integral membrane protein with duf6
	BL1444	widely conserved hypothetical transmembrane protein with duf6
	BL1566	conserved hypothetical transmembrane protein with duf6
MFS		
	BL0037	probable efflux-type transporter
	BL0251	widely conserved hypothetical transport protein
	BL0332	narrowly conserved hypothetical protein
	BL0475	hypothetical membrane protein with unknown function
	BL0681	conserved hypothetical transport protein
	BL0919	possible efflux transporter protein
	BL0920	possible efflux transporter protein
	BL1270	hypothetical transmembrane protein possibly involved in uptake or efflux
	BL1303	hypothetical transmembrane protein possibly involved in transport
	BL1699	hypothetical transmembrane protein possibly involved in efflux transport
	BL1703	hypothetical membrane protein in major facilitator transporter family
MATE		
	BL0432	hypothetical protein in upf0013
	BL0842	hypothetical protein
	BL0844	widely conserved hypothetical transmembrane protein with duf013
	BL1082	conserved hypothetical transmembrane protein in upf0013

For the above reasons, a more targeted approach may prove more successful in the identification and characterization of multidrug transporter genes in bifidobacteria. Although *B. longum* is not the predominant species of bifidobacteria used commercially, studies in this species could be applied to commercially used strains. A PCR approach to the cloning and substrate characterization of the putative transporters in *E. coli* KAM3 such as that used by Nishino and Yamaguchi (2001) may yield better results than those presented in this chapter. This approach would also circumvent the problem of the incomplete genes being cloned as occurred with recombinant plasmids pEcoB4.13, -B4.18, -B4.19 and -B4.23.

It is, however, important to note that the gene cluster on *B. longum* NCC2705 that showed sequence similarity to the ABC transporter cloned from *B. lactis*, namely BL0154 and BL0155, are not annotated as possible antibiotic extrusion systems. This illustrates that while genome analysis may identify many putative drug transporters, those with a novel signature sequence or membrane organization would be missed. Identification of drug efflux systems through functional complementation is therefore still a valid approach and more likely to identify novel drug transporters if they exist in *Bifidobacterium* spp.

CHAPTER 4

IDENTIFICATION AND CHARACTERIZATION OF CHOLATE TRANSPORTER

4.1 SUMMARY

The publication of the *Bifidobacterium longum* genome on public databases has allowed for the identification of putative efflux genes based on sequence similarity to known genes. Using this method, a putative sodium/bile symporter was identified in *B. longum*. The gene, BL1102, was isolated using a PCR amplification approach. BL1102 was subcloned and expressed in *Escherichia coli* KAM3, an efflux negative mutant. The construct was shown to confer greatly elevated levels of resistance to sodium glycocholate (16 fold). Energy-dependent efflux of the bile salt was demonstrated using radiolabelled cholate.

4.2 INTRODUCTION

For a bifidobacterial strain to be an effective probiotic it must be able to survive inhibitory host-produced substances such as bile salts [Margolles *et al.*, 2003].

The physiological property of bile resistance is a complex one. Enteric Gram-negative bacteria are inherently resistant to bile through the basic, asymmetric structure of their outer membranes which significantly reduces the diffusion of hydrophobic compounds, such as bile acids, across the outer membrane [Nikaido & Vaara, 1985]. Since the protection conferred by the outer membrane is only partial, the bacteria also express transport proteins that extrude bile acids. These have been identified in many Gram-negative bacteria such as *Escherichia coli* [Okusu *et al.*, 1996] and *Salmonella typhimurium* [Lacroix *et al.*, 1996].

Studies in *S. typhimurium* have revealed the presence of bile-salt-mediated induction of antimicrobial and bile resistance through the regulation of several different genes [Prouty *et al*, 2004]. DNA microarray analysis showed that the *marRAB* operon was bile-activated. In addition, experiments involving the fusion of the *acrAB* promoter to the *luc* gene, showed that the AcrAB efflux pump is also regulated by bile. The *marRAB* operon is involved in multidrug resistance in a number of bacteria including *E.* coli and *S. typhimurium* [reviewed in Alekshun & Levy, 1999]. MarR encodes a DNA-binding protein that functions as a repressor of the *mar* operon. MarA encodes a DNA-binding protein that is a positive global regulator, while MarB encodes a small protein of unknown function. The activation of *marAB* has been shown to increase levels of expression of the drug transport proteins AcrAB-TolC [reviewed in Alekshun & Levy, 1997]. A model was proposed by Prouty *et al.* (2004) linking the effect of bile on transcriptional regulation in *S. typhimurium.* In the model, bile salts enter the cell and deoxycholate interacts with MarR, inhibiting its binding to the *mar* operator. Although it was

initially hypothesized that this led to an increased the level of *acrAB* expression, and hence increased levels of bile resistance, this was found not to be the case. The regulation of the *mar* operon must, therefore, affect currently unknown genes that play a role in resisting the effects of bile. Concurrently, bile regulates *acrAB* independently of *marRAB*. The increased expression of *acrAB* would then allow the efficient removal of bile acids from the bacterial cell.

Less is known about the molecular basis for bile resistance in Gram-positive bacteria. It is likely that it is due to more than one physiological property of the bacterial cell. For example, in *Listeria monocytogenes,* the construction of a mutant bank allowed for the identification of 5 gene loci involved in bile resistance. These included a gene involved in capsule synthesis, a putative MFS transporter and a putative transporter of the glutamate decarboxylase acid resistance system [Begley *et al.,* 2002].

As mentioned in Chapter 1, Section 1.8.3, bile salt hydrolase (BSH) has been postulated to be involved in bile resistance in Gram-positive enteric bacteria. BSH activity has been detected in bifidobacteria and this genus possesses higher BSH activity than other bacterial groups [Kim *et al.,* 2004]. It is still unknown what benefit BSH activity affords the cell. A study by Grill *et al.* (2000) compared a *Lactobacillus amylovorus* strain with decreased conjugated bile salt hydrolase (CBSH) activity with the wild type strain. They found that the growth rate of the mutant in the presence of bile salts was affected more than the wild-type strain, but that bile was more toxic for the wild-type strain. De-energization experiments suggested that the chemical potential of protons (ZΔpH) was involved in the bile salt resistance of lactobacilli. They hypothesized that after deconjugation, the deconjugated bile salt may recapture a proton and be exported out of the cell. The transport would then decrease the ZΔpH and enhance the resistance to bile salts. In this way, a strain with a more active CBSH would survive better in the intestinal tract.

Moser and Savage tested the hypothesis that in lactobacilli, BSH protects the cells that produce it from the toxicity of conjugated bile salts [Moser & Savage, 2001]. They assayed 49 strains of a number of *Lactobacillus* spp. for the ability to express BSH activities and their ability to resist effects of the conjugated bile acid taurodeoxycholic acid (TDCA). They found that the capacities of lactobacilli to resist the toxicity of TDCA and the capacities of to express TDCA hydrolase and CA hydrolases were independent properties.

BSH isolated from bifidobacteria has been shown not to be induced by any extracellular factors including bile salts [Grill *et al.,* 1995]. However, in this study it was found that *B. longum* was able to withstand higher concentrations of bile salts following pre-exposure to the bile salts (Chapter 2, Figure 2.2). This suggested that *B. longum* may contain a transporter capable of extruding bile acids.

The publication of the *B. longum* NCC2705 genome on public databases [Schell *et al.,* 2002] has allowed for the identification of putative efflux genes based on sequence similarity to known genes. It was, therefore, decided to investigate whether any of the open reading frames (ORFs) identified in the genome encoded for a transport protein capable of extruding bile acids which might confer protection to bifidobacteria.

4.3 MATERIALS AND METHODS

4.3.1 Nucleotide sequence and analysis

Nucleotide sequences were determined by the fluorescent dideoxynucleotide chain termination method of Sanger *et al.* (1977) using the DYEnamic ET Dye Terminator Cycle sequencing Kit from MegaBACE (Molecular Dynamics). All reactions were performed according to the manufacturer's instructions and cycle sequenced on a GeneAmp PCR system 9700 (Perkin Almer, Applied Biosystems). The sequencing reaction products were analyzed on the MegaBACE 500 sequencer (AmershamPharmacia Biotech). The nucleotide sequences were analyzed using the MegaBACE 500 Sequence Analyser v2 and the DNAMAN software packages. Nucleotide and amino acid homology searches were performed, using the BLAST algorithm using the databases contained at NCBI [Altschul, *et al*, 1997].

4.3.2 Bacterial strains, Plasmids and Culture Conditions

Bifidobacterium longum NCIMB 702259^T were obtained from the NCIMB culture collection, UK. Bifidobacterial cultures were propagated anaerobically 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. Bifidobacteria were cultured in BYG medium (Appendix A.2).

E. coli KAM3 [Morita, *et al.,* 1998] served as the cloning and expression host for the introduction of recombinant plasmids. *E. coli* KAM3 was cultured in Luria-Bertani (LB) medium at 37°C. Ampicillin (100 µg/ml) was added to media for the growth of cells containing plasmid. Plasmid M13-derived Bluescript SK (pSK) (Stratagene, La Jolla, CA) was used for subcloning and sequencing.

4.3.3 DNA Extraction and General Recombinant DNA Procedures

Isolation of chromosomal DNA was performed as described by Trindade *et al.* (2003). Plasmid DNA was isolated by the alkali lysis method of Ish-Horowicz and Burke (1981) or with the QIAGEN Plasmid Midi Kit. All DNA modifications were performed according to standard procedures [Sambrook *et al.*, 1989]. All restriction and modifying enzymes were obtained from Roche Diagnostics and used according to general manufacturer's instructions. Competent *E. coli* KAM3 cells were prepared using the rubidium chloride method [Armitage *et al.*, 1988].

4.3.4 PCR of putative cholate transporter, BL1102

In order to isolate ORF BL1102 (*Bifidobacterium longum* NCC2705, Genbank: AE014295, NCBI database), primer ctrans-F was designed to 317 bp upstream of the putative start codon and ctrans-R to 116 bp downstream of the putative stop codon. Restriction enzyme sites were introduced where necessary to assist subcloning into pSK (Table 4.1). PCR amplification using bifidobacterial DNA as a template was performed with Taq Polymerase (0.25 U) (Supertherm) in a 50 µl reaction mixture containing: 50 ng chromosomal DNA; 0.5 mM of each of ctrans-F and ctrans-R; 0.5 mM $MgCl_2$; and 200 µM dNTP's. The amplification cycles were as follows: 96°C for 5 min; 25 cycles of 96°C for 1 min, 56°C for 45 s, and 72°C for 1 min, and finally 72°C for 5 min. PCR products were purified using the High Pure PCR Product Purification kit (Roche).

TABLE 4.1 Primers used for the isolation of BL1102. Base pair changes to introduce restriction sites are indicated in bold. Restriction sites included are underlined.

Primer name	Primer sequence
ctrans-F	**EcoRI** 5'-AGCTGAATTCGCGCAACAGG-3'
ctrans-R	**KpnI** 5'-ACGCCCG**G**TACCTCAATCG-3'

4.3.5 Determination of resistance to sodium glycocholate

Recombinant plasmid, pSKctrans, was expressed in *E. coli* KAM3. The MIC values for sodium glycocholate (Difco) were determined using the broth dilution method of Koneman *et al.* (1988).

4.3.6 Efflux of [^{14}C] cholate

The method used was based on that of Yokota *et al* (2000) using [*carboxyl*-^{14}C] cholic acid (New England Nuclear Corp). Exponentially growing *E. coli* KAM3 cells (culture volume of 100 ml) harbouring pSK or pSKctrans, were harvested and washed once with 50 mM potassium phosphate buffer containing 1 mM $MgSO_4$ pH 7.0 (KPi). The cells were resuspended to an OD_{600} of about 20 and de-energized by incubation in the presence of 10 mM 2-deoxyglucose [Sigma] at 37 °C for 30 min. The cells were then washed three times with KPi buffer and resuspended to an OD_{600} of about 4 in the same buffer. Aliquots (1.94 ml) of this cell suspension were placed at 37 °C. To preload the cells with cholate, 40 µl of 5.8 mM [^{14}C] cholate (16 mCi/mmol) was added so that the final cholate concentration was 0.116 mM. After incubation at 37 °C for 30 min, the aliquot was split in two, and glucose was added to one of the aliquots to a final concentration of 10 mM. At various time intervals, the amount of [^{14}C] cholate associated with the cells was determined using the rapid-filtration method. Briefly, 100 µl aliquots were removed and filtered over 0.45-µM-pore-size cellulose-acetate filters. The filters were washed twice with 2 ml of ice-cold 100 mM LiCl in 100 mM KPi, pH 7.0. The radioactivity retained on the filter was measured in a liquid scintillation counter after the addition of 2 ml of the scintillation cocktail (Emulsifier Scintillator Plus, Perkin Elmer). The background level of radioactivity associated with the filter (obtained from control incubations without cells) was subtracted from all readings. The amount of radioactivity associated with each aliquot was calculated for each experiment and used to calculate the counts per minute (cpm) per mM of

cholate. The results were expressed as nmol cholate/ µg protein. During each experiment, samples were taken in triplicate. The entire experiment was also performed in triplicate.

4.3.7 Protein quantitation

The cell suspension was boiled for 10 min in 1 N NaOH. The protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad) according to the manufacturer's instructions with BSA as the standard.

4.4 RESULTS AND DISCUSSION

4.4.1 Bioinformatic analysis of *Bifidobacterium longum* genome for candidate genes

Since the release of whole genome sequences onto public databases, function predictions based on sequence similarity to other genes of known function have been attempted. This has led to the identification of many putative transport genes. Using the annotation on the NCBI database, ORFs encoding putative transport genes were identified. From this group of genes BL1102 was identified as a possible candidate bile transport gene. ORF BL1102 is annotated as a probable sodium dependent transporter, possibly for ileal bile acids. Translation of the ORF yielded a predicted 353 aa protein. Using the blastp program, BL1102 was found to be similar to a number of sodium dependent (Na+) bile transporters as shown in Figure 4.1 and Table 4.2. Further analysis of BL1102 using the NCBI website revealed a number of conserved domains as shown in Table 4.3 [Marchler-Bauer & Bryant, 2004].

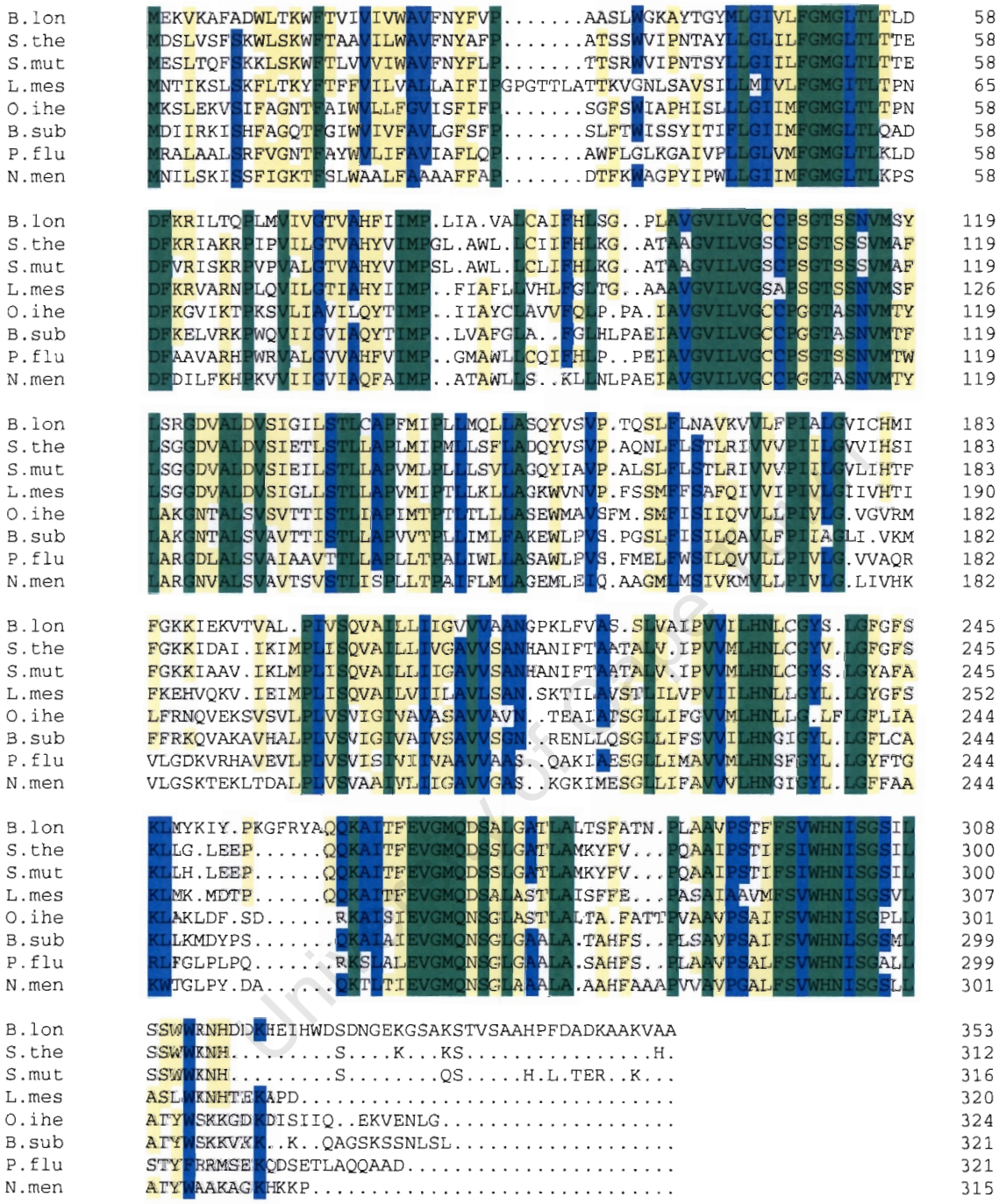


FIGURE 4.1 Multiple sequence alignment of the putative bile transporter BL1102 with other predicted Na^+ /bile transporters. Sequences included are from the following organisms: *B. longum* (B. lon), *Streptococcus thermophilus* (S. the), *Streptococcus mutans* (S. mut), *Leuconostoc mesenteroides* (L. mes), *Oceanobacillus iheyensis* (O. ihe), *Bacillus subtilis* (B. sub), *Pseudomonas fluorescens* (P. flu), and *Neisseria meningitides* (N. men). Sequence accession numbers are supplied in Table 4.2). Identical amino acids are shaded in green, and similar amino acids are shaded in blue and yellow.

TABLE 4.2 Analysis of genes with sequence similarity to BL1102

Accession no.	Species	Description	identity (%)	positive (%)	E value
YP_141686.1	*Streptococcus thermophilus* LMG 18311	bile acid:Na+ symporter (BASS) family protein	59	73	e-105
NP_721034.1	*Streptococcus mutans* UA159	putative sodium-dependent transporter	57	73	e-102
ZP_00063561.1	*Leuconostoc mesenteroides*	predicted Na+-dependent transporter	51	68	3e-85
NP_691915.1	*Oceanobacillus iheyensis* HTE831	sodium-dependent transporter	44	62	6e-70
CAB13827.1	*Bacillus subtilis* subsp. *subtilis* str. 168	probable sodium-dependent transporter yocS	44	61	7e-69
ZP_00262238.1	*Pseudomonas fluorescens* PfO-1	predicted Na+-dependent transporter	44	61	e-66
NP_273747.1	*Neisseria meningitidis* MC58	transporter	41	59	2e-65

TABLE 4.3 Analysis of conserved domains within BL1102

Accession no.	classification	description	alignment (aa residue)	score	E value
gnl|CDD|210259	COG0385	predicted sodium-dependent transporter	1-319	220	e-58
gnl|CDD|2288	pfam01758	Sodium bile acid symporter family (SBF)	43 – 199	104	2e-23
gnl|CDD|10666	COG0798	Arsenite efflux pump (ARC3); related permeases	1-292	62.9	6e-11
gnl|CDD|10548	COG0679	predicted permeases	41-154	47.2	3e-6
gnl|CDD|26102	pfam03547	auxin efflux carrier	22-147	44.5	2e-5

Proteins belonging to the sodium bile acid symporter family (SBF) are found in bacteria, yeast, plants and animals such as mice and humans, with over 50 members already identified [Hagenbuch & Dawson, 2004]. The size of ORF Bl1102 is consistent with other members of the family, for example the human gene *SLC10A1,* which is a 349 aa membrane protein. Analysis of the membrane topology revealed the presence of 9 TMS (Figure 4.2) [www.cbs.dtu.dk/services/TMHMM centre for biological sequence analysis, Technical University of Denmark, prediction of transmembrane helices]. Computer modeling of other members of the family has suggested between 7 and 9 TMS [Hagenbuch & Meier, 1994]. However, experimental data derived from translation/insertion scanning, alanine insertion and glucosylation site mutagenesis, supports a 9 TMS topology [Hallen *et al.*, 2002].

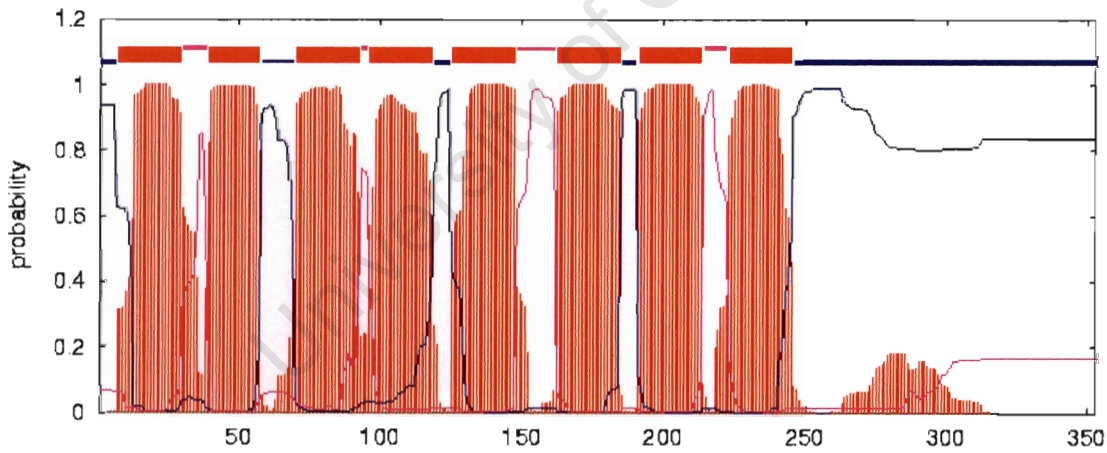


FIGURE 4.2 Membrane topology of BL1102. The transmembrane helices are represented by the red lines, while the orientation is marked by the blue (inside) and pink (outside) lines.

The position of BL1102 on the chromosome of *B. longum* was investigated to determine whether any genes in the flanking regions could also be involved in bile resistance (Figure 4.3). Analysis revealed that BL1102 is not part of an operon. Analysis of BL1101 did not result in any

significant sequence similarity to any known protein. Upstream of BL1102 is the tRNA-His sequence. It was, therefore, decided to isolate only BL1102 for further characterization.

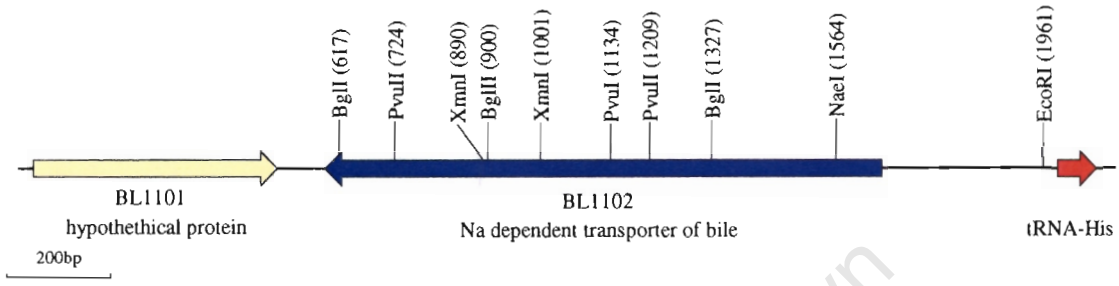


FIGURE 4.3 Genetic organization of BL1102 and flanking regions. Transcriptional polarities are indicated by arrows. Position 1 corresponds to 24 411 of the genome sequence.

4.4.5 PCR and subcloning of BL1102

PCR amplification of the *B. longum* chromosomal region coding for BL1102, using ctrans-F and ctrans-R, yielded the expected 1.38 kb fragment. The fragment was subcloned into pSK and sequencing revealed that the correct DNA fragment had been cloned. The resulting plasmid was named pSKctrans (cholate transporter).

4.4.6 MIC values of *E. coli* KAM3 (pSKctrans) to sodium glycocholate

E. coli KAM3 harbouring the plasmid pSK, had a MIC value of 0.25% to sodium glycocholate whereas pSKctrans increased this value to 8%. Taken together with the sequence analysis, this suggested that a bile transporter had indeed been isolated.

4.4.7 Efflux of [^{14}C] cholate

In order to investigate whether pSKctrans conferred resistance to bile through the active efflux of the compound, assays were performed using radiolabelled cholate. This would exclude the possibility of pSKctrans conferring bile resistance through the decreased influx of bile. The amount of cell-associated [^{14}C] cholate was monitored in *E. coli* KAM3 harbouring pSKctrans or the control vector pSK. De-energized washed cell suspensions were used with/without the addition of glucose as an energy source. The results are shown in Figure 4.4.

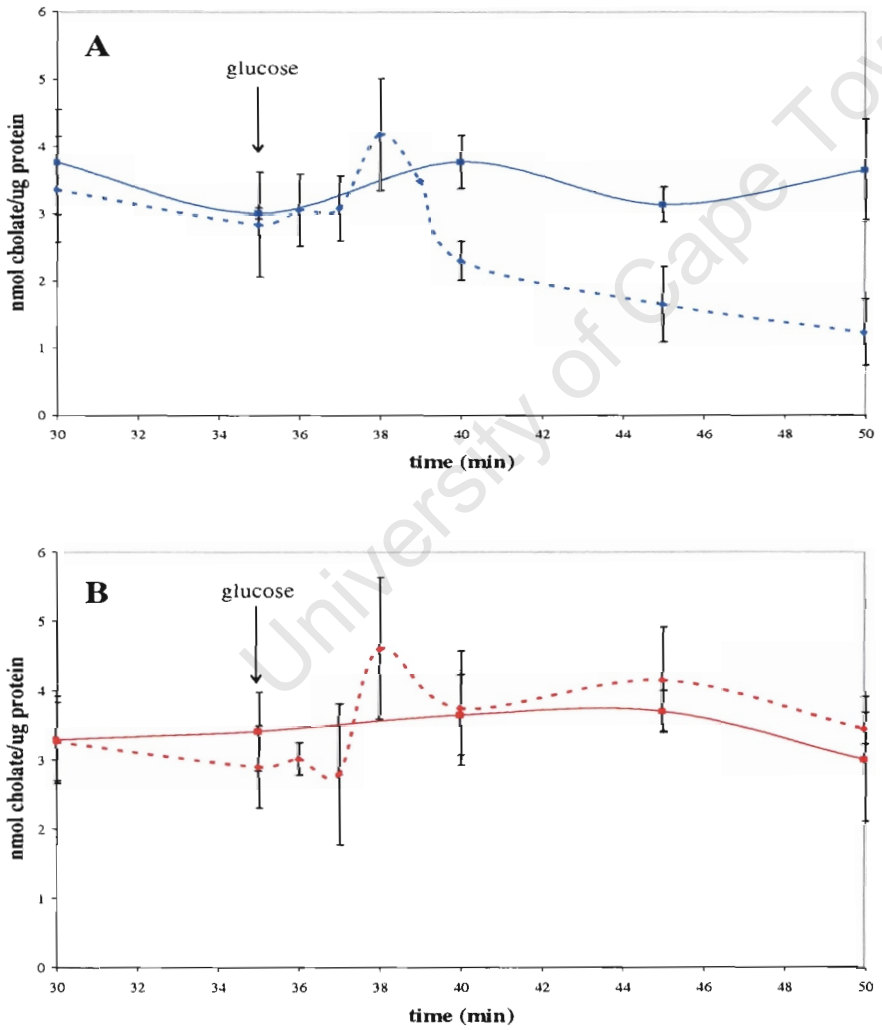


FIGURE 4.4 Energy-dependent extrusion of [^{14}C] cholate in *E. coli* KAM3. After the cells were loaded with cholate (solid lines), glucose was added to a final concentration of 10 mM at the time point indicated by the arrow. The amount of cell-associated cholate was subsequently measured over time (dotted lines). *E. coli* KAM3, (A) pSKctrans (blue), (B) pSK (red).

In the absence of glucose, an equilibrium of cholate between the inside and outside of the cells was obtained (Figure 4.4 A and B, solid lines). Upon addition of glucose cholate accumulated transiently. This is likely to be due to an increase in ΔpH (interior alkaline). Cholic acid is a weak acid, pKa of 6.4, which in its undissociated form, diffuses across the cell membrane. It then becomes trapped in the cytoplasm as cholate in a pH-dependent fashion [Yokota *et al.*, 2000]. The influx of cholate occurs whether or not ctrans is expressed. In the absence of pSKctrans a new equilibrium is reached, which is at a similar level to the de-energized cells (Figure 4.4 B). When pSKctrans is present, the initial accumulation of cholate is followed by a decrease in the level of cholic acid associated with the cell (Figure 4.4 A). Glucose metabolism results in a build up of ATP. The ATP is hydrolyzed so that the sodium gradient necessary to drive the cholate transporter is built up. The activity of ctrans is, therefore, strong enough to overcome the ΔpH-dependent passive influx of cholate.

4.5 CONCLUSIONS

The efflux negative mutant, *E. coli* KAM3, was used to express the putative cholate transporter. While the expression of genes from Gram-positive, G+C-rich bacteria in a heterologous Gram-negative host is not ideal, it was utilized in this study. This was done since there are currently no available Gram-positive efflux mutants that have been characterized in terms of expression of antibiotic resistance genes. In addition, as mentioned in Chapter 1, Section 1.2, there are no genetic methods by which specific genes can be inactivated in bifidobacteria. It is, therefore, not possible to ascertain whether BL1102 is essential for *B. longum* to survive in the presence of bile salts such as cholate.

The results reported in this chapter, show that when expressed in *E. coli,* pSKctrans mediates the energy-dependent efflux of cholate. This supports the notion that BL1102 codes for an energy-dependent cholate transporter. This is the first bile transporter described in bifidobacteria.

The active efflux of bile salts has been described in both Gram-negative and Gram-positive bacteria. However, Kurdi *et al* (2003) observed that cholic acid, the main free bile acid produced by BSH activity in the intestine, accumulated in bifidobacteria. This occurred as long as the bacteria were energized. They postulated that this may benefit the mammalian host since the entrapment of free bile acids may decrease the production of secondary bile acids which are considered cytotoxic and precarcinogenic. The enzyme responsible for this reaction has not been found in bifidobacteria. It has yet to be shown, however, what benefit this would afford the bacteria itself since it has not been shown that bifidobacteria can derive any energy from bile salts.

The study by Kurdi *et al,* (2003) was performed on 8 different bifidobacterial strains. However, neither *B. longum* NCC2705 nor *B. longum* NCIMB 702259^T were used. Therefore, BL1102 which was identified in strain NCC2705 and isolated from NCIMB 702259^T, may not have been present in the strains tested by Kurdi *et al.* Alternatively, BL1102 may not be constitutively expressed, or not expressed at high enough levels under the conditions tested to overcome the effect of the ΔpH-driven passive influx of cholic acid. Yokota *et al.* (2000) only showed the ATP-dependent efflux of cholate in *L. lactis* strain C41-2. This cholate-resistant strain was derived from the wild-type *L. lactis* strain MG1363 through growth on media containing stepwise-increasing cholate concentrations. No cholate efflux was observed in the wild-type strain. Using the protocol described in Section 4.3.6, they observed that in the wild-type strain, following the addition of glucose, cholate accumulated to a level at which the internal concentration was 7-fold higher that that of the extracellular medium [Yokota *et al*, 2000].

Future investigations into the role of BL1102 in conferring cholate resistance on bifidobacteria should include expression studies, such as northern hybridization. RNA isolation under conditions such as pre-exposure to low levels of cholic acid, would show whether the expression of BL1102 is bile-dependent. An alternative method to investigate the conditions under which BL1102 is expressed would be real-time PCR (RT-PCR). Using this method, RNA would be isolated under conditions such as growth in low concentrations of bile. This would be followed by cDNA synthesis and quantitative RT-PCR. This method has been used successfully in *Vibrio cholerae* [Chatterjee *et al.,* 2004] and *Pseudomonas aeruginosa* [Yoneda *et al.,* 2005] to study the expression of multidrug efflux pumps. Yoneda *et al.* (2005) found that this method was highly reproducible and allowed for the precise quantification of minute or substantial amounts of mRNA transcripts. The conditions under which BL1102 is expressed would also shed light onto the significance of the role that this gene plays in enabling *B. longum* to resist the effects of bile in the human GIT.

CHAPTER 5

GENERAL CONCLUSIONS

Safety concerns regarding the consumption of probiotic-containing food products, are important factors in the selection of suitable candidate strains for use as probiotics. Bifidobacteria are generally considered to be good probiotics candidates and are already used extensively in fermented food products, such as yoghurt. It is, therefore, important to develop a science-driven, evidence based overview of the safety of their use as probiotics [Borriello *et al*, 2003]. In particular, bifidobacteria have been shown to exhibit resistance to a wide range of antibiotics, but little is know about the molecular basis for this resistance. The aim of this project was, therefore, to investigate the molecular mechanisms responsible for the resistance against antibiotics and bile salts observed in bifidobacteria, and more specifically, to determine whether efflux systems contribute to this resistance.

In this study, a wide range of antimicrobial agents were tested against selected bifidobacteria, and adaptation to at least one of the antimicrobial agents tested was observed in every one of the bifidobacterial species under investigation. Bifidobacteria are resistant to a number of different antimicrobials with different targets and modes of action but this alone does not provide proof that multidrug efflux systems are responsible for the observed phenotypes. Antibiotic resistance in bacteria can be achieved via modification of the target of action, direct inactivation of the antibiotic, changes in the cell permeability and multidrug drug efflux pumps. The upregulation of multidrug efflux systems can provide cross-resistance to a number of antibiotics and/or antimicrobial agents [Levy, 2002], since the addition of the compounds exported by the multidrug transporters themselves actually enhances the expression levels of multidrug transporters

[Zheleznova *et al.*, 1999]. In order to examine whether this is the case in bifidobacteria, future experiments should include the investigation as to whether pre-exposure of cells to one compound results in the increased resistance to another/other antimicrobial(s).

In this study, genes conferring resistance to antimicrobials were identified using two approaches, namely the screening of a *Bifidobacterium lactis* genomic library in the drug hypersusceptible *Escherichia coli* KAM3, and the bioinformatic analysis of the *Bifidobacterium longum* genome sequence followed by functional complementation in *E. coli* KAM3.

Only one efflux system was identified in *B. lactis* through the screening of a genomic library in *E. coli* KAM3 plated on media containing ethidium bromide or tetracycline. However, according to sequence analysis using TransportDB [www.membranetransport.org; described in Ren *et al.*, 2004], there are at least 4 ABC multidrug transporters and 20 secondary multidrug transporters in *B. longum*. The method used in this study may therefore be ineffective in identifying the vast number of multidrug transporters likely to be present in *B. lactis*. The genes in the *B. longum* NCC2705 genome sequence that showed sequence similarity to the ABC transporter cloned from *B. lactis*, namely BL0154 and BL0155, are not, however, annotated as possible antibiotic extrusion systems. This illustrates that while genome analysis may identify many putative drug transporters, those with a novel signature sequence or membrane organization would be missed. Identification of drug efflux systems through functional complementation is, therefore, still a valid approach and more likely to identify novel drug transporters if they exist in *Bifidobacterium* spp.

With the advent of whole genome sequencing, complete genome sequences can be systematically analyzed and predicted antimicrobial efflux proteins can be identified. This targeted approach

was, therefore, used to identify the cholate transporter, ctrans. *E. coli* KAM3 was used to express the putative cholate transporter. While the expression of genes from Gram-positive, G+C-rich bacteria in a heterologous Gram-negative host is not ideal, it was utilized in this study because of the scarcity of suitable Gram-positive drug-susceptible hosts. Using [^{14}C]-cholate, it was shown that pSKctrans mediated the energy-dependent efflux of cholate when expressed in *E. coli*. This is the first bile transporter described in bifidobacteria.

Analysis of the expression of this gene in *B. longum* would shed light onto the significance of the role that this gene plays in enabling *B. longum* to resist the effects of bile in the human GIT. Future work could include the investigation into the conditions under which ctrans is expressed through the use of Northern hybridization or quantitative Real-Time PCR.

While resistance to antimicrobial agents may increase the survival rate of bifidobacteria in the human GIT, one of the aspects which should be studied is the safety and risks of acquisition of antimicrobial agents [Sullivan and Nord, 2002], especially if these bacteria are to be added in high numbers to food products. It is currently difficult to interpret studies of gene transfer *in vivo*, and further methods need to be developed, particularly for bifidobacteria. Also, the focus should be on possible gene transfer to pathogenic bacteria such as *Staphylococcus aureus*, rather than on homologous gene transfer [Borriello *et al*, 2003]. In this context, studies such as this make a significant contribution to the fundamental knowledge of bifidobacteria, which is important if the probiotic industry is to contribute to human health.

APPENDIX A

Preparation of Bifidobacterial Media

A.1 BHI (Brain Heart Infusion) Medium

In grams per litre:

Brain heart infusion (Difco)	37
Yeast extract (Difco)	5
Cystein HCl	0.5
Rezasurin	0.001
Agar (if required)	15

A.2 BYG (Basal+ Yeast + Glucose) Medium

In grams per litre:

Tryptone	10
Yeast extract (Difco)	5
Glucose	5
Tween 80	1
NaCl	4.5
$MgCl_2.6H_2O$	0.15
KH_2PO_4	0.4
K_2HPO_4	0.2
NH_4Cl	0.4

APPENDIX B

B.1 Bacterial Strains Used in this Study

Strain	Genotype and relevant characteristics	Reference
Bifidobacterium		
B. bifidum NCFB 2203	wild type	NCFB, UK
B. breve NCFB 2257	wild type	NCFB, UK
B.longum NCFB 2259	wild type	NCFB, UK
B. lactis DSM 10140	Industrial strain used in yoghurt	Darleon Distribution
B. bifidum W	Uncharacterized	Dr L. Brown, Food Technology, Cape Technikon
Escherichia coli		
DH5α	*supE*44 *Δlac* U169 (ϕ80*lacZ*Δm15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Sambrook *et al.*, 1985
JM109	*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi* *Δ*(*lac-proAB*)	Yanisch-Peron *et al.*, 1985
KAM3	***ΔacrAB*** Δ(lac-pro) *supE thi hsdΔ5/* F'*traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15	Morita *et al.*, 1998
Lactoccus lactis* subsp. *lactis NZ9000	wild type	NIZO Food Research, The Netherlands
ΔLmrCD	MG1363, deletion of LmrCD	J. Lubelski and R. van Merkerk, Department of Microbiology, University of Groningen

B.2 Plasmid Vectors Used in this Study

Plasmid vector	Antibiotic resistance genes and relevant characteristics	Reference
Escherichia coli		
pEcoR251	Ampicillin^R	Zabeau & Stanley, 1982
pMT104	Ampicillin^R, 0.15 kb *B. fragilis* fragment in pEcoR251	Wehnert *et al.,* 1990
pBluescript SK (pSK)	Ampicillin^R, *lacZ'*	Stratagene
Lactoccus lactis subsp. lactis		
pNG8048	Chloramphenicol^R, Erythromycin^R, NisinA-inducible promoter	NIZO Food Research, The Netherlands

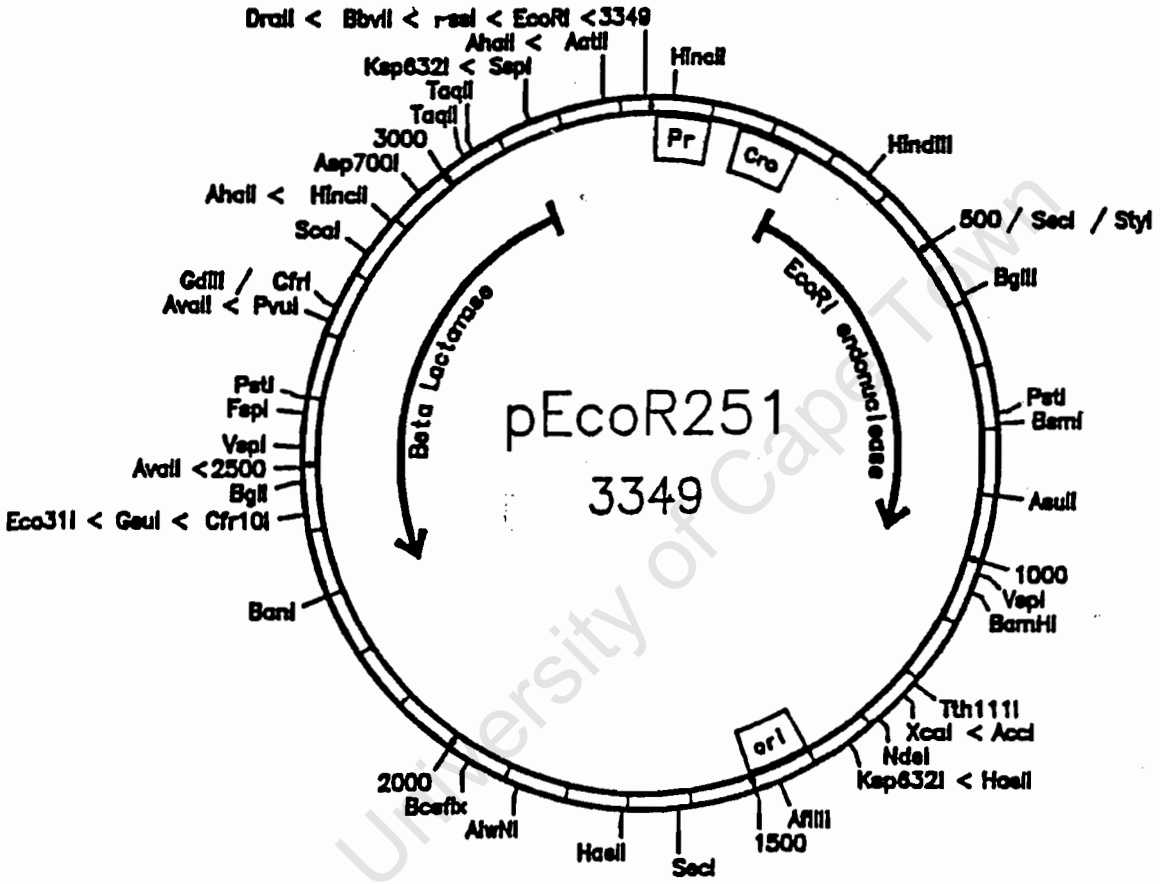


FIGURE B.1 Restriction map of pEcoR251 (Zabeau & Stanley, 1982)

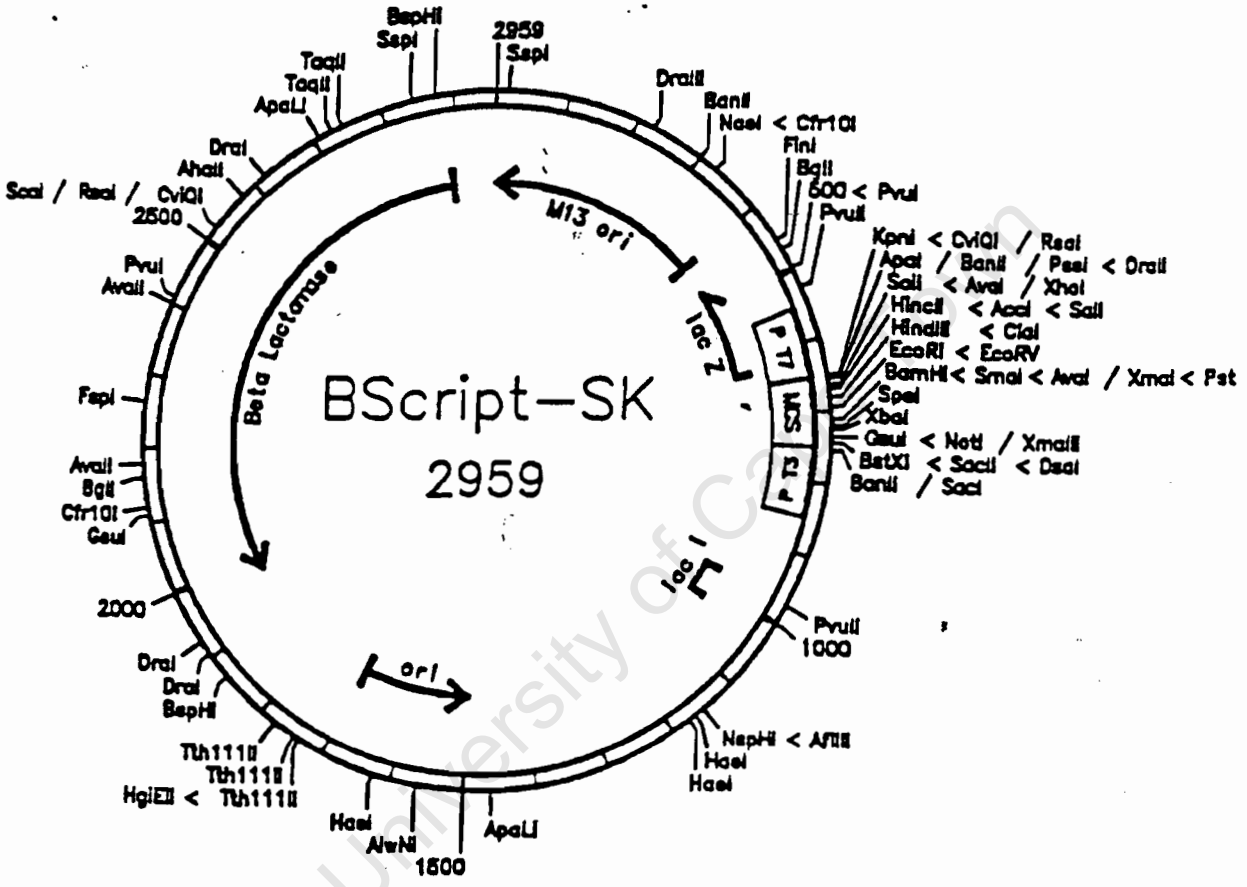


FIGURE B.2 Restriction map of pBluescript SK (Stratagene, La Jolla, CA)

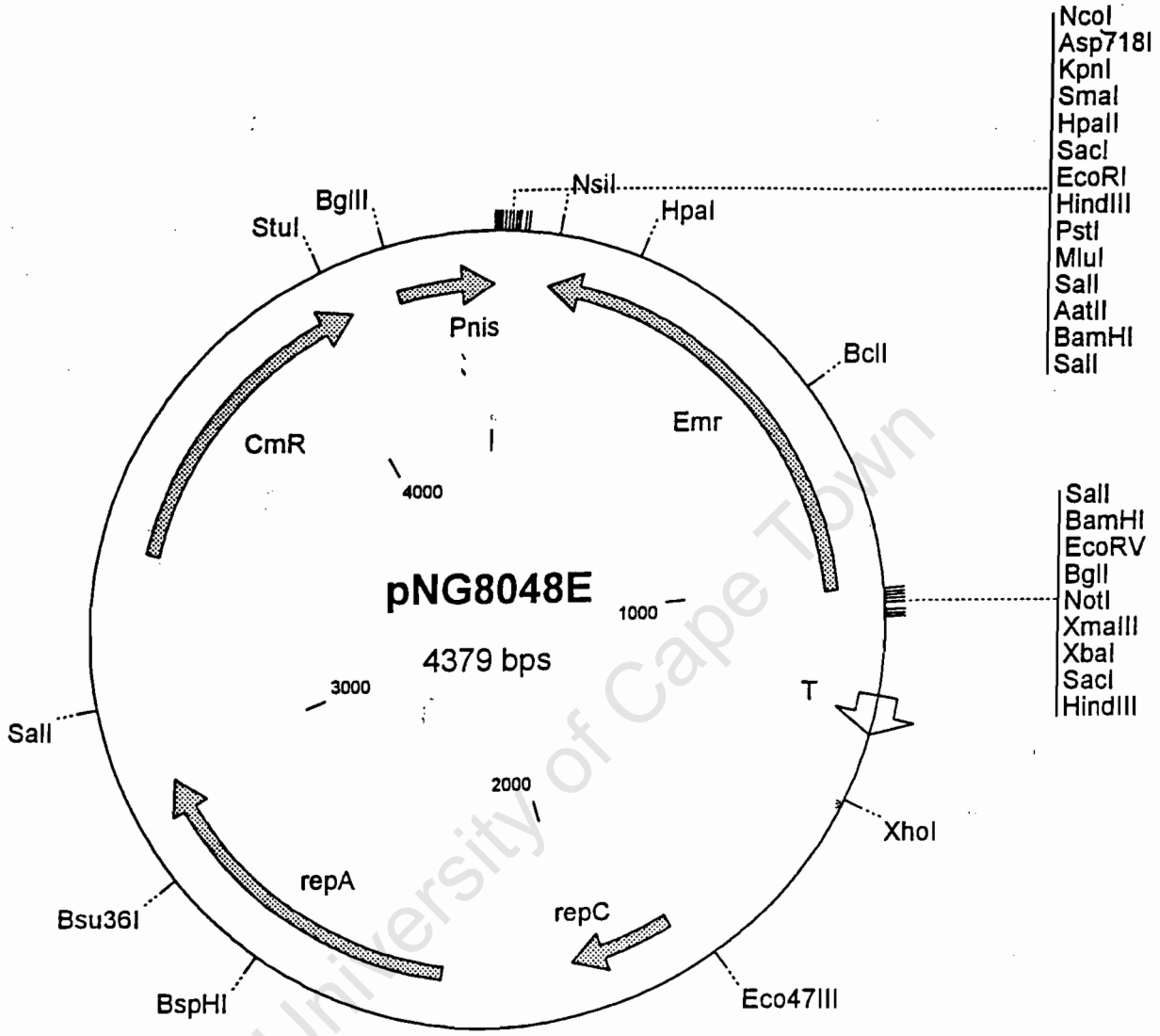


FIGURE B.3 Restriction map of pNG8048E (NIZO Food Reseach, The Netherlands)

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