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Coláiste na hOllscoile Corcaigh

Bifidobacterium breve Adaption to the Gut Environment



Ollscoil na hÉireann, Corcaigh

THE NATIONAL UNIVERSITY OF IRELAND, CORK

A Thesis presented to the National University of Ireland for the degree of Doctor of Philosophy by

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April 2018

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Declaration

I hereby declare that the research presented in this thesis is my own work and effort, and that it has not been submitted by me for any other degree either at University College Cork or elsewhere. Where contributions by others are involved, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

This research was completed under the guidance of Prof. Douwe van Sinderen at the APC Microbiome Institute & School of Microbiology, Biosciences Institute, University College Cork.

Signature: _____

Candidate

Date: _____

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“The more I learn, the more I realize how much I don't know.”

— Albert Einstein

List of Publications

Chapter II: Lanigan, N., Bottacini, F., Casey, P.G., O'Connell Motherway, M., and D. van Sinderen. (2017). Genome-Wide Search for Genes Required for Bifidobacterial Growth under Iron-Limitation. Front Microbiol; 8:964. doi: 10.3389/fmicb.2017.00964.

Christiaen, S.E., O'Connell Motherway, M., Bottacini, F., Lanigan, N., Casey, P.G., Huys, G., Nelis HJ, van Sinderen D, Coenye T (2014). Autoinducer-2 plays a crucial role in gut colonization and probiotic functionality of *Bifidobacterium breve* UCC2003. PLoS One 9:e98111. doi: 10.1371/journal.pone.0098111.

Ruiz, L., O'Connell Motherway, M., Lanigan, N., and D. van Sinderen D. (2013). Transposon mutagenesis in *Bifidobacterium breve*: construction and characterization of a Tn5 transposon mutant library for *Bifidobacterium breve* UCC2003. PLoS One 8:e64699. doi: 10.1371/journal.pone.0064699.

List of Abbreviations

ADP	Adenosine Diphosphate
Agl	Alpha glucosidase
Acka	Acetate kinase
Ald2	Alcohol dehydrogenase 2
Amp	Ampicillin
APC	Alimentary Pharmabiotic centre
ApuB	Amylopullunase
ATP	Adenosine Triphosphate
Bgl	Beta gluosidase
BLAST	Basic Local Alignment Search Tool
BSH	Bile Salt Hydrolase
cAMP	cyclic Adenosine Monophosphate
CCA	Carbon Catabolite Activation
CCC	Carbon Catabolite Control
CcpA	Catabolite control protein A
CCR	Carbon Catabolite Repression
CDM	Chemically Defined Medium
cDNA	complementary DNA
CFU	Colony Forming unit(s)
CldC	Beta glucosidase
Cm	Chloramphenicol
CoA	Coenzyme A
Cre	Catabolite Responsive Elements
Crp	cAMP receptor protein
DNA	Deoxyribonucleic Acid
DPS	DNA protecting proteins
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
Em	Erythromycin

EMSA	Electrophoretic Mobility Shift Assay
Eno	Enolase
EPS	Exopolysaccharide
F6P	Fructose 6-P
F6PPK	Fructose-6-Phospahte Phosphoketolase
Fe-S	Iron sulphur Cluster
Fe^{2+}	Ferrous Iron
Fe^{3+}	Ferric Iron
FMN	Flavin mononucleotide
fRCM	filtered Reinforced Clostridial Medium
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Guanine Cytosine
GEO	Gene Expression Omnibus
GH	Glycosyl Hydrolase
GIT	Gastrointestinal Tract
GlkA	Glucokinase
Gpi	Glucose- 6-p isomerase
Gpm	Phophoglycerate mutase
GusA	Beta-Glucoronidase
HCL	Hydrochloride
HPAEC-PAD	High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection
HPLC	High Performance Liquid Chromatography
HPr	Histidine-containing Protein
HTH	Helix-Turn-Helix
IPTG	Isopropyl-b-D-thiogalactopyranoside
Kan	Kanamycin
KCl	Potassium Chloride
kDA	kilo Daltons
LAB	Lactic Acid Bacteria
Ldh2	Lactate dehydrogenase 2

LNnT	lacto-N-neotetraose
MalQ	Glucanotransferase
Mb	Mega base
mMRS	modified de Man Rogosa and Sharpe (medium)
mRNA	messenger RNA
MRS	de Man Rogosa and Sharpe (medium)
MTC	Maximal Tolerable Concentration
MW	Molecular Weight
NAD+	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
NTPase	Nucleoside-Triphosphatase
OD	Optical Density
ORF	Open Reading Frame
ori+	Origin of Replication
P-Ser-Hpr	Seryl-Phosphorylated
PB	Porcine Bile
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEP-PTS	Phosphoenolpyruvate-dependent Phosphotransferase System
Pe	Primer Extension
Pfl	Pyruvate formate lyase
Pgk	Phosphoglycerate kinase
Pyk	Pyruvate kinase
RBS	Ribosome Binding Site
RCA	Reinforced Clostridial Agar
RCM	Reinforced Clostridial Medium
repA	replication Amplicon
Rk	Ribokinase
RNA	Ribonucleic acid

RT-PCR	Reverse Transcription-PCR
R5PI	Ribose-5-P isomerase
R5PR	Ribose-5-P reductase
S_2	Sulphur
SCFA	Short Chain Fatty Acid
SDS-PAG	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SFI	Science Foundation Ireland
Tal	Transaldolase
Tet	Tetracycline
TF	Transcription Factor
TFBS	Transcription Factor Binding Site
Tkt	Transketolase
Tn5	Transposon
Tris-HCL	Tris(hyroxymethyl)aminomethane- Hydrochloride
TSS	Transcriptional Start Site
UCC	University College Cork
UV	Ultra Violet
WT	Wild Type
wt/vol	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-D-galactopyranoside
X5P	Xylulose 5-P
XFPK	X5P/F6P Phosphoketolase

Thesis Abstract

Certain strains and species of the genus *Bifidobacterium* are considered probiotic organisms, whose presence in the gastrointestinal tract (GIT) may elicit one or more health benefits to the host. A number of factors impact on the ability of bifidobacteria to survive transit through the upper parts of the GIT, and to colonize and persist in the colon (where they are believed to exert their beneficial effects). They must for example be able to resist various environmental stresses, including oxidative stress and stresses imposed by low pH, bile exposure and nutrient starvation (such as iron or carbohydrate limitation). This thesis will take a focused view on the ability of the gut commensal *Bifidobacterium breve* UCC2003 to combat many of the rigours it faces upon gastrointestinal transit as a prototypical representative of its genus.

Chapter 2 of this thesis will investgate which genes are important for the survival of *B. breve* UCC2003 under iron-limiting conditions. Phenotypic screening of a Tn5-based random mutant library in *B. breve* UCC2003 and transcriptomic analysis of *B. breve* UCC2003 when exposed to iron-limiting conditions identified a number of genes, of diverse predicted cellular functions, that were implicated in the survival of the strain under iron restriction. Among the identified genes were two putative iron-uptake systems: (i) a presumed ferrous iron uptake system, designated here as *bfeUO*, and (ii) a predicted ferric iron/siderophore uptake system, designated *sifABCDE*. *In silico* analysis also illustrated that these two clusters are highly conserved across members of the genus *Bifidobacterium* and are invariably co-located. Murine colonization studies demonstrated that *B. breve* UCC2003-*bfeU* and *B. breve* UCC2003-*sifA* insertion mutants are able to colonize a healthy murine gut as efficiently as the wild type *B. breve* strain, indicating that while *bfeUO* and *sifABCDE* are important for *in vitro* growth under iron-limiting conditions, they are not crucial for gut survival or gut colonization of a healthy host.

Chapter 3 describes the *B. breve* UCC2003 global genome response to long-term iron starvation, which appears to be associated with an increased ability to resist bile stress. Analysis of the response of *B. breve* UCC20003 to chronic iron starvation was found to be distinct from the response of *B. breve* UCC2003 following exposure to iron-

limiting conditions as described Chapter 2. Chronic iron starvation caused in/decreased transcription of genes associated with carbon and nitrogen metabolism, genes predicted to be responsible for iron uptake, genes encoding putative DPS proteins (which are involved in iron storage/DNA protection) and Fe-S cluster-associated proteins, as well as a gene (*bshB*) encoding a bile salt hydrolase. Insertional mutagenesis and survival assays demonstrate that iron limitation imposed on *B. breve* UCC2003 evokes increased resistance to bile stress, being partly due to the iron-inducible transcription of the *bshB* gene. These findings therefore link bile salt hydrolase activity of *B. breve* UCC2003 to its ability to survive the adverse effects of bile exposure and suggests that this strain uses iron availability as a signal to adapt to the variable environment of the small intestine.

Being able to rapidly adapt to changing and/or adverse conditions is necessary for bifidobacteria to be able to survive and persist in the gut environment. Chapter 4 describes a novel mechanism by which bifidobacteria controls its central carbon metabolic pathway, again employing *B. breve* UCC2003 as the prototypical representative of its genus. Adaption to the gut environment requires rapid, energy-consuming adjustments in gene transcription, and *B. breve* UCC2003 is believed to achieve this through the utilisation of two predicted LacI-type transcription factors (TFs), designated BifR1 and BifR2. BifR1 and BifR2 were shown to be involved in regulating the central, carbohydrate-associated metabolic pathway, and thus, carbon flux of *B. breve* UCC2003. BifR1 and BifR2, though being encoded by two distinctly different genes, were found to be functionally very similar, due to their common control of genes within the central metabolic pathways, such as *tkt*, *tal*, *pyk*, *ldH*, *eno*, *pflA* and the *pflB*. Along with regulating the transcription of their own genes and each other, these TFs also appear to transcriptionally control additional genes. This complex network of BifR1/BifR2-mediated gene regulation provides novel insights into the decision-making process governing cell metabolism and physiology in bifidobacteria.

Chapter 5 describes the characterisation of four *B. breve* UCC2003-encoded LacI-type transcriptional regulators, namely MalR2, MalR3, MalR5 and MalR6. These transcription factors (TFs) have previously been proposed to be involved in the

utilisation of maltose, maltodextrins and related polysaccharides, such as starch, amylopectin, amylose, glycogen and pullulan. However, MalR2, MalR3 and MalR5 were found to have a more diverse role and were shown to participate in transcriptional regulation of a number of other carbon sources such as ribose and cellobiose. Interestingly, our *in vitro* data indicate that these regulators often cross-regulate the same carbohydrate utilization genes, while also regulating each other. This hierarchical regulatory system controls the transcription of genes involved in carbohydrate uptake, storage, breakdown and central metabolism. These four LacI-type regulators were shown to respond to differing carbohydrate effectors, such as turanose or galactose, thus indicating that each regulator is responsible for a different aspect of (α-glucoside-containing) carbohydrate metabolism. This complex network of gene regulation provides intriguing insights into the decision-making process of the cell with regards to carbohydrate utilisation, and into bifidobacterial metabolic adaption to and competitiveness in its environment

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Chapter 1 Literature Review

1.1 Microorganisms and the Gut Environment

The gastrointestinal environment harbours a complex assembly of microorganisms including bacteria, viruses, fungi, and protozoa that are believed to affect human metabolic and immune function. In order for microorganisms to colonise and survive in the gut they must first gain entry to this internal compartment, most likely by accidental or deliberate ingestion. Once internalised, microorganisms are faced with a diverse array of host defences or environmental factors that determine their chance of survival before they reach their optimal/preferred site for colonization/replication. Survival and persistence in the gut environment is a complex process with microorganisms being exposed to a number of environmental (stress) factors, which include low pH, oxygen, bile acids and nutritional limitations, such as, carbon or iron starvation.

The spatial distribution and concentration of bacteria present in the gut (Figure 1-1), can be correlated with a number of environmental factors. The low pH (pH 3) of the stomach -due to secretion of HCl- and high concentration of bile acids released into the duodenum are two important factors that dictate the concentration and diversity of bacteria in the upper gastrointestinal tract (stomach, duodenum, jejunum and ileum). From Figure 1-1 it is apparent that the stomach is one of the least populated areas of the gut with approximately 10^2 to 10^4 bacteria per ml. A substantial increase in bacterial population density is observed in the small intestine with an estimated 10^7 to 10^8 bacteria per ml. Finally, the colon contains up to 10^{11} bacterial cells/ml, including a diverse range of species such as members of the following genera: *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Dorea*, *Eubacterium*, *Faecalibacterium*, *Roseburia* and *Ruminococcus*, therefore making the colon the most densely populated area of the gastrointestinal tract from a bacterial perspective (1, 139).

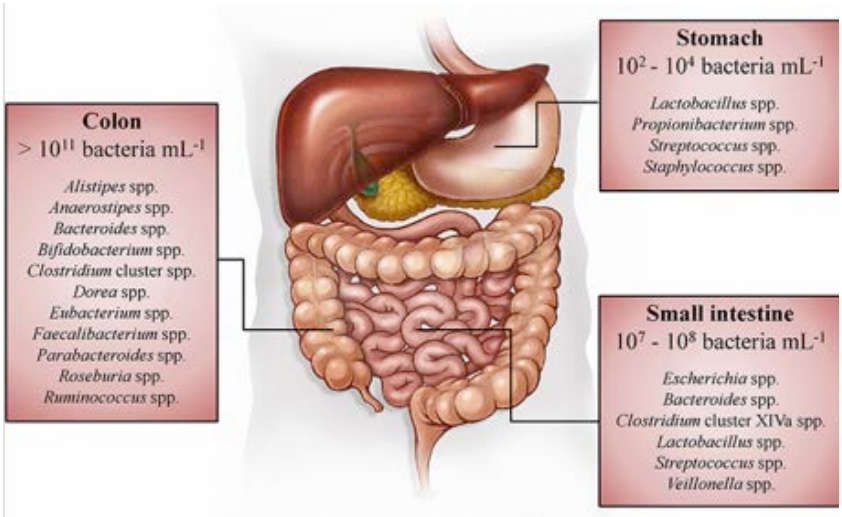


Figure 1-1 Spatial distribution/Concentrations of bacteria along the GIT.
The dominant genera in the stomach, small intestine, and colon are listed, based on 16S rRNA gene sequence studies. This image was obtained from Rivière *et al* 2016 (138).

1.2 General Features of the *Bifidobacterium* Genus

The *Bifidobacterium* genus belongs to the *Bifidobacteriaceae* family, being the sole member of the *Bifidobacteriales* order, which represents the deepest branch within the Actinobacteria phylum (2). The number of species within the *Bifidobacterium* genus is constantly increasing with 65 (sub)species identified to date (as of February 2018) (3).

Species of the genus *Bifidobacterium* represent Gram-positive, saccharolytic, bifid-shaped, strictly anaerobic bacteria (4). Bifidobacterial species characteristically possess a relatively high guanine plus cytosine content (>51 %) and harbour a genome that typically ranges in size from 2.0 to 2.8 Mb. Bifidobacteria are abundant and prevalent among the microorganisms that colonise the gut of mammals, birds and insects, in particular in species that raise their offspring by parental care (5).

Bifidobacteria are among the first colonisers of the gastrointestinal tract (GIT) of humans immediately following birth, although colonisation has been reported to occur at an even earlier stage of human development with vertical transmission from mother to infant via amniotic fluid/placenta, altough it is important to point out that the species of

Bifidobacterium identified in amniotic fluid/placenta was carrid out by DNA qualification (6). The gut of healthy full-term infants is colonised firstly by facultative anaerobes (e.g., Enterobacteriaceae such as *E. coli*), and growth of these microbes depletes any remaining oxygen in the GIT within days, thereby creating an anaerobic environment in which many obligate anaerobes can flourish. Such anaerobic bacteria are predominantly members of the genera *Bifidobacterium* and *Bacteroides* (7, 8). Bifidobacteria in particular have been found to dominate the microbiota of healthy, full-term, breast-fed infants (9-11), with one publication indicating that they represent approximately 80 % of the total infant gut microbiota (12). This may partially be due to the ability of certain bifidobacterial species to utilise human milk oligosaccharides (13-15). However, their abundance declines with age, with the adult microbiota comprising of 3-6 % bifidobacteria, and individuals over 65 years of age exhibiting a further reduction of bifidobacterial numbers (16-18).

1.3 Probiotic Effects

The positive impact of specific probiotic organisms, on human well-being has been recognized for a long time and was in particular put forward by the Russian zoologist Élie Metchnikoff who linked the enhanced longevity of Bulgarian rural people with their regular consumption of fermented dairy products such as yogurt (19). Not long after this report, the French paediatrician Henry Tissier reported that faeces of breast-fed infants was dominated by a bifid-shaped bacterium which was subsequently classified as a member of the *Bifidobacterium* genus (20).

Today various bifidobacterial strains are commercially exploited as probiotics, formally defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on their host” (21). Indeed, bifidobacteria have been included as bioactive ingredients in a number of functional foods, where they may be present as a single strain or combined with other (probiotic) microorganisms and/or prebiotic mixtures (in the latter case, this is called a synbiotic). Prebiotics are defined as selectively fermented ingredients that result in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thereby conferring health benefits(s) upon the host (22).

The reported health-promoting activities attributed to bifidobacteria are numerous and diverse, and include maturation and modulation of the immune system, preservation of

gut barrier function, vitamin production, inhibition of certain enteropathogens, such as *Clostridium difficile*, and amelioration of gastrointestinal diseases (23-28). Importantly, these beneficial activities are not necessarily elicited by every bifidobacterial strain or species, but are believed to be strain-specific.

In order for probiotics to exert their beneficial activities they must first be able to survive processing and storage at low temperature and suboptimal pH, as probiotics are commonly incorporated as functional ingredients in fermented dairy products. Such probiotic products characteristically have a low pH yet may also contain higher than optimal levels of molecular oxygen, which may negatively affect survival of many obligate anaerobes (29). Following this probiotic bacteria, like any gut commensal, will encounter various environmental challenges during their transit through the stomach, small intestine and colon, including variations in pH, nutritional limitations, fluctuations in oxygen concentration and exposure to bile salts, the latter being the main anti-microbial component of bile (30).

1.4 The Micronutrient Iron

Few elements are as widely used in nature as iron, a micronutrient which is essential for the growth of nearly all living organisms. Under physiological conditions iron can exist in two redox states, i.e. ferric iron (Fe^{3+}) or ferrous iron (Fe^{2+}) (31). Under aerobic and neutral pH conditions iron is present in its oxidized, but essentially insoluble trivalent, ferric form, while under anaerobic, neutral pH conditions iron is found in its reduced, divalent ferrous state, the latter being more biologically accessible due to its greater solubility (31). The redox potential of Fe^{2+}/Fe^{3+} makes iron an extremely versatile prosthetic component and as such can be incorporated into proteins as a catalytic centre or as an electron carrier (32).

Under reducing conditions Fe^{2+} can also complex with S^{2-} to form a so-called Fe-S cluster, which are utilised in many proteins to promote ligand exchange reactions and prevent oxidative degradation (33). These abilities of iron make it crucial for numerous biological processes, which include aerobic/anaerobic ATP biosynthesis, nitrogen fixation, photosynthesis, respiration, the tricarboxylic acid cycle, oxygen transport, gene regulation and DNA biosynthesis (34). The importance of iron in so many biological

processes is most likely due to the abundance of Fe^{2+} during the initial evolution of living organisms (34, 35).

1.5 Iron and the GIT Environment

The concentration of iron within the GIT can vary greatly based on age, gender, dietary habits (e.g., vegetarian, omnivore), sociodemographic factors and race (36-40). It is estimated that the average daily intake of dietary iron for an American child aged between 2 and 11 years is 11.5–13.7 mg/day, while this increases in adult men and women, who ingest between 16.3–18.2 mg/day and 12.6–13.5 mg/day, respectively (41). Remarkably, the portion of dietary iron which is absorbed in the human GIT is typically low and ranges from 5 to 35 % and is of course dependent on the circumstances and the type of iron present (42). This absorption occurs predominantly in the duodenum and upper jejunum (38), and the remaining unabsorbed dietary iron enters the colon, where, depending on its concentration, it has been found to cause microbiota alterations, specifically when iron is either limiting or in excess, as is the case during dietary iron supplementation (43-46). Increased iron concentration has been associated with increased abundance of (opportunistic) pathogenic (entero) bacteria and reduced numbers of bifidobacteria and lactobacilli (47).

Iron speciation, which relates to the changing concentration of varying forms of an ion as the pH of the solution changes, and iron availability are two important factors for iron availability within the gastrointestinal environment (Figure 1-2). Iron availability may vary greatly along the length of the GIT due to its tendency to complex with other molecules and because of its ability to exist in various oxidation states depending on its surrounding environment (34). The low pH in the stomach favours the solubility of both Fe^{2+} and Fe^{3+}, whereas the subsequent rise in pH upon entry into the small intestine results in a decrease in the solubility of Fe^{3+}.

Iron may then complex with food components or host/microbiota-derived compounds such as citrate, ascorbate, mucin, certain amino acids or lactate (32). Certain insoluble forms of Fe^{3+}, for example when complexed with phosphate, carbonate or oxides, cause this iron to be unavailable for the microbiota and may require reduction or removal from these complexes by siderophore activity (31). Soluble forms of Fe^{3+} may be reduced to

Fe^{2+} upon entry into the cytoplasm, while ferrous citrate or ferrous ascorbate may be taken up directly by (certain components of) the microbiota. Finally, lactate and short chain fatty acid (SCFA) production by the microbiota may also cause a modest, possibly localized drop in the pH in the colon resulting in an increase in iron solubility (48).

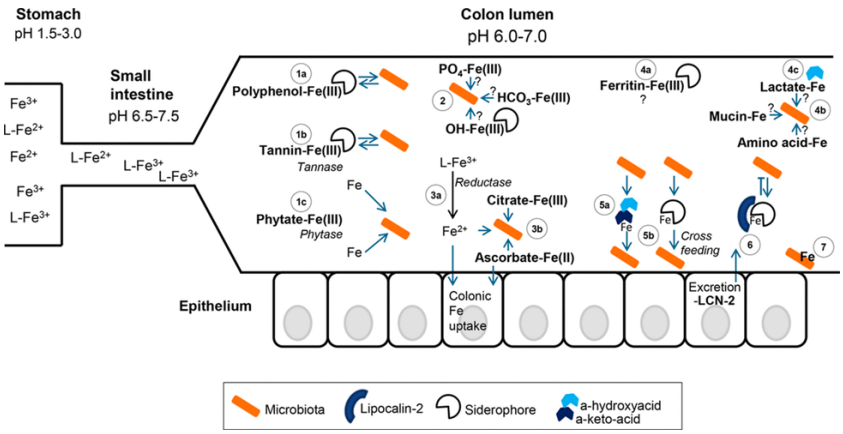


Figure 1-2. This Figure illustrates iron speciation and availability within the GIT
In the stomach (on the left), the low pH favours the solubility of both ferric and ferrous iron, not necessarily requiring a ligand (L) for solubility. When the pH increases in the small intestine, the solubility of mainly ferric iron decreases and more complexes with food components and host excretions will be formed. Within the colon, the pH drops slightly due to the production of, for example, lactate and SCFAs by the microbiota. The colonic part of the figure, where the microbiota depicted in orange represents both (beneficial) resident species and pathogenic species, shows the following aspects: (1) On the left, it is shown that iron bound to polyphenols (1a), for example tannins (1b) and phytate (1c), may be accessible via enzymatic degradation or via the removal of the iron by siderophores; (2) The rather insoluble forms like iron in a complex with phosphate, carbonate or oxides are likely not readily accessible for the microbiota, but they may be solubilised by bacterial reduction or siderophoric chelation; (3) Soluble forms of ferric iron may be reduced to ferrous iron after which it can be taken up by both the microbiota and the host (3a), and for instance, the soluble ferric citrate or ferrous ascorbate may be directly taken up by bacteria (3b). Notably, host iron uptake may be a mechanism to withdraw the iron from the colon lumen; (4) On the right, several forms of iron are depicted from which we also do not know very well how accessible they are. We envisage that ferritin–iron (dietary or from sloughed enterocytes) (4a) is difficult to access, but iron bound to mucin, amino acids or lactate (4b) may be relatively easy to access and that may hypothetically be promoted by the low-affinity siderophores α-hydroxyacids and α-keto-acids (4c); (5) These low-affinity siderophores (5a) may also play an important role in the phenomenon of iron cross-feeding by heterologous siderophores within the microbiota (5b); (6) The excretion of lipocalin-2 (LCN-2) in the colon lumen may scavenge iron bound to siderophores and hereby prevent uptake by (pathogenic) bacteria; (7) Finally, at the lower right, it is depicted that bacteria spp. may bind iron to their cell wall (as has been shown for *Bifidobacterium*), which likely prevents access to this iron by other (pathogenic) species. Relevant information can be found within the text above while further information is available from Kortman *et al* (31) from which this image was obtained.

Although essential for most organisms with the known exceptions of certain *Lactobacillus* and *Borrelia* species, which require very little if any of this metal (49, 50), iron can prove toxic to the cell due to its ability to induce the so-called Fenton reaction. This reaction results in the generation of free radicals which may damage

cellular components, such as protein and DNA, of both host and microbiota. Consequently, one mechanism by which probiotic bacteria can bestow a benefit upon their host is through the sequestration of iron, thereby reducing free radical production, which in turn is associated with inflammation and colorectal carcinoma via DNA damage induction within the GIT (51).

Another mechanism by which probiotic bacteria benefit their host is through their ability to effectively compete for micronutrients such as iron, consequently reducing proliferation of (opportunistic) pathogens (52-54). This phenomenon is known as nutritional immunity and was originally associated with higher organisms which were found to withhold trace elements such as iron thereby causing reduced proliferation of pathogens (54).

1.6 Iron Metabolism in Members of the Genus *Bifidobacterium*

Bifidobacteria are thought to be efficient scavengers of iron and are able to grow under low iron conditions. This notion is supported by the finding that certain bifidobacterial species are found in greater numbers under low luminal iron conditions as compared to normal or high luminal iron conditions (55). Previous research has found that iron is essential for growth of certain *Bifidobacterium* species (56, 57). As mentioned above, sequestration/withholding of iron is referred to as nutritional immunity and has long been known in both Gram-positive and Gram-negative bacteria as a defence mechanism, conferring a competitive advantage to certain commensals, such as bifidobacteria, which can thrive in low iron environments (58, 59). Indeed, a number of putative inorganic ion transporters and Fe-S cluster-associated proteins have recently been identified as essential for bifidobacterial growth and survival under iron-limiting conditions by screening a transposon-generated mutant library (56).

Bacteria have developed a wide range of mechanisms for iron sequestration, including a variety of systems for the uptake of ferrous iron, ferric iron, heme, hemephores (which scavenge heme from various hemoproteins), and siderophores (selective iron chelators which have a high affinity for ferric iron). Iron sequestration via siderophore-mediated and/or direct iron uptake has recently been reported for a number of bifidobacterial

species, with *B. breve* exhibiting the highest siderophore activity and *Bifidobacterium kashiwanohense* exhibiting the highest iron uptake (54, 60).

A number of bifidobacterial strains (e.g. *B. longum* DJ010A) have been found to inhibit growth of other bacteria, such as *Escherichia coli*, *Micrococcus luteus*, *Clostridium perfringens* and *Clostridium difficile*, through the production of siderophore complexes (61), although this characteristic seems strain specific among bifidobacteria (53, 62).

Bifidobacterial carbohydrate fermentation typically acidifies the surrounding environment, favouring the solubility of ferric and ferrous iron in a localized fashion (63). Interestingly, certain species of *Bifidobacterium* bind ferric iron to their cell wall and membranes, thereby limiting its availability to other bacteria (64). Bifidobacteria import iron (ions) across the cytoplasmic membrane by means of one or more ABC-type transporters (65). In the case of *B. breve*, this leads to a cytoplasmic iron concentration of about 100 to 200 µM, which is approximately 14/16-fold higher than the surrounding environment (66). While iron is essential for the survival of most living organisms, it may represent a double-edged sword because of its ability to generate hydroxyl radicals via the so-called Fenton reaction causing irreparable damage to DNA, lipids and proteins (67). Therefore, in order to utilise this essential, yet highly toxic ion (bifido)bacteria have evolved mechanisms for its storage, through the use of ferritin-like proteins. The primary role of these ferritin-like proteins is to store iron when present in adequate amounts, and to supply iron for cell function under limiting conditions. Taken together, the ability of (bifido)bacteria to withhold and sequester iron within the gut may result in a lower luminal iron level, which in turn may on the one hand, restrict radical-induced damage to intestinal tissue, while at the same time offer pathogen protection by means of nutritional immunity.

1.7 Bile

Bile is an aqueous secretion which is produced in the liver and is made up of a range of constituents including bile salts, bilirubin phospholipid, cholesterol, enzymes, porphyrins, steroids, amino acids, vitamins, and heavy metals (68). Bile salts are a major competent of bile (approximately 50 %) (69). In humans bile salts are mainly produced from the two primary bile acids cholic and chenodeoxycholic acid, which are generated

from cholesterol in the liver, and which, before secretion, are conjugated to either glycine or taurine moieties (via *N*-acyl amidation).

Conjugation of bile acids decreases their pKA, while it also increases their solubility (once conjugated bile acids are referred to as bile salts) (70-72). It is this attribute which makes bile salts amphipathic in nature and allows them to solubilise lipids, thereby forming mixed micelles.

Bile is excreted into the duodenum at a rate of approximately 750 ml per day, and concentrations of bile remain high along the length of the small intestine where it plays an important role in human nutrition, being required for food solubilisation and digestion, as well as absorption of lipids and lipid-soluble vitamins (73, 74), 95 % of the excreted bile salts are then re-absorbed into the blood-stream in the distal ileum (74, 75). The remaining 5 % bile salts pass on into the colon where they are believed to impose a strong selective pressure on the microbiota, either directly through their antimicrobial properties or indirectly via nuclear receptors. Bile has also been found to act as a growth stimulant for certain organisms, which have evolved to resist its bactericidal properties and apparently avail of its nutritional value (76-79).

1.8 Bacteria and Bile

Due to its strong lipophilic nature bile also acts as an antimicrobial compound by disorganising the bacterial cytoplasmic membrane (80). Several studies have demonstrated that the antimicrobial properties of bile prevent overgrowth of the commensal bacteria in the small intestine; such overgrowth is observed in individuals with reduced bile secretion output, which allows commensal bacteria to be present at high densities in the small intestine where they otherwise would not have been able to proliferate (68, 73, 74, 81).

Other characteristics of bile also allow it to play a major role in shaping the gut microbiota of mammals, such as its ability to denature proteins, chelate iron and calcium, cause oxidative damage to DNA, and control the expression of eukaryotic genes involved in host defence and immunity. Therefore, the ability of probiotic and commensal bacteria to

tolerate bile is presumed to be important for their survival and subsequent colonisation of and persistence in the GIT (82).

Bacteria employ a number of strategies to cope with bile-imposed stress, ranging from the production of exopolysaccharides, expression of efflux pumps for the cytoplasmic removal of (de-conjugated) bile salts, and the expression of bile salt hydrolases (BSHs; E.C.3.5.1.24) which de-conjugate bile salts (77, 83).

1.9 Bile Salt Hydrolases in *Bifidobacterium*

During transit through the GIT bile salts may undergo modification by the microbiota, resulting in a variety of so-called secondary bile acids. These modifications include 7-α-de-hydroxylation, de-conjugation and oxidation of the C-7 hydroxyl group. 7-α-de-hydroxylation is an activity that is restricted to members of the *Clostridium* genus, while de-conjugation can be carried out by various bacteria (74). De-conjugation is carried out by the enzymatic activity of a bile salt hydrolase (BSH) (chologlycine hydrolase; EC 3.5.1.24), which is a member of the chologlycine hydrolase family, and which has been identified in all major bacterial divisions and in two domains of life (*Bacteria* and *Archaea*). It is hypothesised that this enzyme has evolved as an adaption to bile-containing environments, due to its absence in microbes which do not encounter bile salts (84). The BSH enzyme catalyses the removal (or de-conjugation) of the amino acid taurine or glycine from the C-24 position of conjugated bile salts (74, 77). This ability to hydrolyse bile salts has been associated with a number of health benefits, including control of obesity, hypercholesterolemia, and metabolic syndrome (85). Furthermore, the ability to hydrolyse bile has been included in the EU criteria for probiotic strain selection (86).

Bifidobacteria display a varying ability to resist bile stress (63, 87, 88), with BSH activity being reported in a number of bifidobacterial strains (89-94). The BSH protein in *B. longum* SBT2928 has been characterised and found to have a preference for glycine-conjugated bile salts as opposed to taurine-conjugated bile salts, in line with the prevalence of these conjugated bile salts in the gut which is estimated to be 3:1 in the human gut (95). De-conjugation of bile salts has been shown to represent a means of acquiring carbon and/or nitrogen in *Bacteroides* species (96). This possible metabolic

function of BSH activity has also been suggested for *Bifidobacterium longum* where the *bsh* gene has been found to be co-transcribed alongside *glnE*, a putative glutamine synthetase adenylyltransferase-encoding gene (89).

GlnE is predicted to be involved in the nitrogen regulatory cascade in other bacteria and this may therefore explain the co-transcription of these genes with seemingly un-associated functions (89).

1.10 Carbohydrate Metabolism in *Bifidobacterium*

In humans and other mammals, carbohydrates are typically ingested as polysaccharides (e.g. starch, arabinoxylan or pectin), oligosaccharides (e.g. galacto-oligosaccharides and xylo-oligosaccharides) or disaccharides (e.g. sucrose and lactose) and must be broken down into monosaccharides before they can be transported across the epithelial barrier to generate energy in the body. Carbohydrate absorption usually occurs in the small intestine, however due to the relatively low number of glycosyl hydrolases (GHs) (seventeen) encoded by the human genome (97), a large proportion of ingested carbohydrates escape digestion/absorption and enter the large intestine where they are metabolised by the gut microbiota. Adaption of bifidobacteria to the carbon transience of the gut environment is exemplified by the large number of GHs it encodes, with the genome of *B. breve* UCC2003 for example encoding 55 (predicted and experimentally proven) GHs (98).

It has been estimated that between 10 - 60 g of dietary carbohydrates reach the colon every day (81), providing a constant supply of nutrients for the resident microbiota. These carbohydrates are typically plant or animal derived and are composed of monosaccharides which are connected through a variety of different glyosidic linkages. The variations seen in solubility, size and chemical structure result in a vast diversity of carbohydrates available for metabolism by the resident colonic microbiota. These microbial gut residents are in many cases equipped with a large collection of enzymes to allow hydrolysis (through the action of glycosyl hydrolases) and uptake (e.g. ABC-type or PEP-PTS transport systems) of these carbohydrates.

Competition within the gastrointestinal tract for these carbohydrate resources is fierce, and members of the *Bifidobacterium* genus typically dedicate a large proportion of their genome to encode activities involved in carbohydrate transport and metabolism, which on average within the genus represents about 13.7 % of their gene content (5, 99, 100). *B. breve* UCC2003, the strain which will be the focus of this thesis, dedicates approximately 10 % of its gene content to carbohydrate transport and metabolism (5, 14, 56, 101). To put this in context this is relative to the genome size nine times more than that encoded by *E. coli* (102).

Bifiobacteria are saccharolytic organisms and solely obtain energy in the form of ATP through fermentative metabolism (i.e. by substrate phosphorylation) of carbohydrates from their surrounding environment. Bifidobacterial species have been found to consume hexose- and pentose-containing sugars through the fructose-6-phosphate pathway (F6PK) pathway, which is also known as the 'bifid shunt'. The bifid shunt represents a unique metabolic pathway found in members of the *Bifidobacteriaceae* family, being distinct from the majority of gut microbiota members, many of which utilise the glycolytic pathway when metabolising (hexose) carbohydrates (103). Importantly, the identification of the bifid shunt was one of the factors which led to the reclassification of *Bifidobacterium* as a particular taxonomic group separate from the lactic acid bacteria (104), with the phosphoketolase called X5P/F6P phosphoketolase (XFPK) (EC 4.1.2.2) commonly being utilised as a taxonomic marker for the Bifidobacteriaceae family (105). XFPK is unique in exhibiting comparable affinities for both xylulose 5-P (X5P) and fructose 6-P (F6P) (106), and it directly converts X5P and F6P to acetyl phosphate (along with erythrose-4-phosphate and D-glyceraldehyde-3-phosphate) without using ATP (Fig. 1.3). This acetyl phosphate is subsequently converted into acetate to generate ATP. The success of bifidobacteria in the extremely competitive GIT environment may therefore be attributed to their ability to generate more ATP (2.5 molecules of ATP per molecule of glucose) via the XFPK pathway as compared to other oligosaccharide fermentative pathways such as glycolysis or the pentose phosphate pathway (107, 108).

The F6PK pathway furthermore results in a theoretical production of 1.5 Mol acetate and 1 Mol lactate per Mol of glucose consumed (99, 109). However, this ratio may vary depending on the carbon source, growth phase and other environmental conditions (99).

Some studies have found that different concentrations of metabolic end products impose varying physiological consequences on the bacterium. For example, increased ethanol production by members of the genera *Lactobacillus* and *Bifidobacterium* has been hypothesised to counter the oxidative stress effects of bile through the increased production of regenerated NAD^+ (110-113).

1.11 Regulation

Transcriptional control by LacI-type Regulators

LacI-type regulators were among the first transcription factors (TFs) to be discovered and were initially characterised in *E. coli* by Francois Jacob and Jacques Monod in 1961 (114). Today, the bacterial LacI family has >1000 members (115). LacI-type proteins typically modulate transcription through allosteric regulation and are composed of four domains: (i) the DNA-binding domain (represented by a helix-turn helix (HTH) structure), (ii) the regulatory/effector domain, (iii) the linker domain, and (iv) the tetramerization domain (Figure 1-3). LacI-type TFs bind to their recognition or operator sequence utilising their DNA binding domain, while the regulatory domain alters the shape of the protein and may either decrease (induction) or increase DNA-binding affinity (co-repression), thereby modulating transcription of associated genes (115) (Figure 1-3).

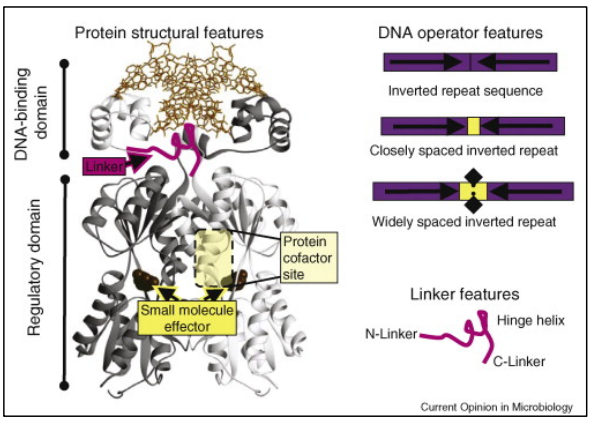


Figure 1-3. Common LacI features
Further information can be found at Swint-Kruse *et al.* (115), from which this image was obtained.

LacI-type TFs commonly regulate transcription of their regulons by means of repression, although some, for example CcpA TF (Carbon catabolite protein A) may act both as a transcriptional repressor as well as an activator (116-118). The majority of LacI-type TFs sense carbohydrate effectors and are involved in the transcriptional control of carbohydrate utilisation genes. Furthermore, 90 % of known LacI-type TFs are predicted to act at a local level, where they are responsible for transcriptional modulation of a small number of targets (<10) of a given carbohydrate metabolic pathway (119, 120).

One study which looked at LacI-type TFs across 344 genomes from 39 taxonomic groups reported that both the *Streptomycetaceae* and *Bifidobacteriaceae* families of the Actinobacteria phylum were found to encode the largest average number of LacI-type TFs relative to their genome size (ranging from 17 to 32 regulators), being in contrast to other bacteria which on average harbour less than 10 such regulator-encoding genes per genome (119). *B. breve* UCC2003 has 26 predicted LacI-type TFs, of which five have been characterised: MelR1 and MelR2, involved in melezitose utilisation, CldR involved in cellodextran utilisation, RbsR which is involved in ribose utilisation, and GalR involved in galactan metabolism (121-124).

Furthermore, a detailed *in-silico* analysis of the regulatory networks controlling carbohydrate metabolism in bifidobacteria has recently been carried out by Khoroshkin *et al.* (125). This study identified a number TFs which were predicted to control particular carbohydrate metabolic pathways at local or global level. These TFs included a number of LacI-type regulatory proteins (MalR1, MalR2a, MalR2b, MalR3 and MalR5) in *B. breve* UCC2003 which were predicted to be important in regulating maltose and maltodextrin utilisation pathways, along with a possible global regulatory network which was predicted to be regulated by a LacI-type regulatory protein denoted as AraQ.

Regulation of Central Carbon Metabolism in Various Bacteria

Carbon metabolism essentially provides living organisms a mechanism to both extract energy and synthesize biomolecules from their environmentally available substrates. Central metabolism is finely regulated in response to both intracellular and extracellular signals in order to balance catabolic and anabolic activities, and their associated energy consequences (i.e. ATP production and consumption).

Bacteria utilise multi-layered regulatory systems for the utilisation of carbon sources, and of particular interest in this context is a regulatory mechanism known as carbon catabolite control (CCC). CCC is a bacterial regulatory mechanism, which controls the metabolic pathways of carbon and energy sources, maximizing their efficiency while also regulating other metabolic processes (129). CCC is specific to carbon source-mediated regulation and encompasses both carbon catabolite repression (CCR), referring to the transcriptional repression of certain genes and operons when in the presence of a preferred carbon/energy source, and carbon catabolite activation (CCA), which involves increased transcription of particular genes and operons in the presence of a preferred carbon/energy source. CCC and the transcriptional regulation of central carbon metabolism has undergone extensive study in various bacterial prototypes (126-129).

The most thoroughly studied system for control of carbon flux/CCC is arguably that operating in the Gram-negative bacterium *Escherichia coli*. *E. coli* utilises two global transcription factors, Crp and Cra, to control central carbohydrate metabolism (130). Cra is a member of the LacI/GalR family of regulatory proteins and may act either as a repressor of a number of carbohydrate uptake systems (e.g. fructose) or as an activator of a number of genes involved in central metabolism (131).

Cra allosterically responds to two inducer molecules: D-fructose-1-phosphate and D-fructose-1,6-bisphosphate, while Crp (cAMP receptor protein) is a global regulatory protein, which in the presence of its effector molecule, cyclic adenosine monophosphate or cAMP, can either activate or repress transcription of a large number of genes involved in carbon/energy metabolism (132). In the absence of glucose cAMP is synthesised and can associate with two Crp monomers to form an active regulatory complex. Crp, which in *E. coli* is known to regulate more than 100 genes, is responsible for CCR, which prevents transcriptional activation of specific genes in the presence of glucose, the preferred carbon source of this metabolically versatile gut microbe (130). The most extensively studied system for CCC in Gram-positive bacteria is that of *Bacillus subtilis*, which utilises CcpA (Catabolite control protein A) and P-Ser-Hpr (seryl-phosphorylated form of the HPr (histidine-containing protein)) complex to bind to *cre* (catabolite responsive elements) sites in its genome, with CCC directly or indirectly regulating the expression of upwards of 300 genes in *B. subtilis* (129).

Very little work has been carried out on the regulatory mechanisms which control central carbon metabolism in other bacteria, including members of the genus *Bifidobacterium*. One study carried out in *B. longum* NCC2705 reported a lactose-over-glucose preference (133), which is manifested by lactose-mediated repression of glucose transport into this strain. The processes which control this proposed "reversed diauxie" still remain elusive. A number of factors set the *Bifidobacteriaceae* family apart from the regulatory systems which have been characterised in both *B. subtilis* and *E. coli.* These include the fact that many Gram-positive organisms including those in the *Bifidobacteriaceae* family and *B. subtilis* are not yet known to produce cyclic AMP which is utilised to control CCC in Gram-negative organisms such as *E. coli* (129).

Another factor which differentiates the regulatory mechanism of carbon metabolism in *Bifidobacterium* from other bacteria is that some members do not encode phosphoenolpyruvate-phosphotransferase systems, which play a crucial role in CCC in both *E. coli* and *B. subtilis*. In fact, bifidobacteria appear to prefer the use of ABC-type transport systems for the uptake of carbohydrate sources from their environment, although this may merely reflect carbohydrate availability in the gut environment (and in particular the colon, where free glucose or other monosaccharides are not expected to be present in substantial quantities). Furthermore, and as mentioned above, the *Bifidobacteriaceae* family possess a unique carbohydrate assimilatory pathway with metabolic intermediates that do not occur in other bacteria. Due to the above-mentioned factors it is likely that members of the genus *Bifidobacterium* have developed a distinct mechanism of CCC.

Research on regulation of central carbohydrate metabolism has also been carried out for certain members of the *Corynebacterium* genus, which, like *Bifidobacterium*, belongs to the phylum Actinobacterium. Such studies have shown that certain *Corynebacterium* species also contains the XFPK-encoding gene, and that this gene has likely been horizontally transferred between these two bacterial groups (104, 108, 134). *Corynebacterium glutamicum* is an industrial relevant species and is utilised for the production of amino acids, especially L-glutamic acid and L-lysine, which are important in human and animal nutrition (136).

These studies have found that *C. glutamicum* utilises two LacI type TFs, namely GntR1 and GntR2, to control the uptake and metabolism of various carbon sources (137). These TFs appear to have complementary functions and their corresponding genes are believed to have arisen from a duplication event (they share 78 % identity at amino acid level) (137). GntR1 and GntR2 were found to control central carbon metabolism through transcriptional repression of a number of genes involved in carbon uptake and metabolism (including transketolase and transaldolase), while also activating the expression of two PTS systems for the acquisition of sugars such as glucose and sucrose (137).

1.12 Conclusion

The understanding of how bifidobacteria interact with and respond to their surrounding environment is only now beginning to be uncovered. However, bifidobacterial iron metabolism remains a relatively poorly studied area, and many of the molecular mechanisms for iron uptake, storage and metabolism still remain to be identified and characterised. Also, bile resistance by means of de-conjugation of bile salts by BSH is not fully understood, and conflicting data appear to exist with respect to the role that BSH may play in bile tolerance/resistance in bifidobacteria. The regulatory mechanism responsible for the control of uptake and subsequent metabolism of energy sources is presumed to be crucial for members of the genus *Bifidobacterium* in order to effectively compete in an extremely densely populated gut environment. Such a regulatory control system is expected to enable *Bifidobacterium* species to balance its energy generating and biosynthetic demands, thereby allowing optimal growth and redox balance control, as based on environmental conditions. Understanding how bifidobacteria interact with and respond to the fluctuating environmental conditions within the gut is of critical importance for the development of effective probiotics.

1.13 Thesis Outline

Chapter 1 is a general introduction to this thesis which covers bifidobacterial (and in particular *B. breve* UCC2003) adaption to the gut environment with a focus on iron metabolism, bile resistance and regulation of carbon uptake and central metabolism.

Chapter 2 describes the identification of genetic loci involved in iron metabolism by *Bifidobacterium breve* UCC2003

Chapter 3 reports on how iron starvation increases bile resistance of *B. breve* UCC2003 through enhanced expression of a bile salt hydrolase.

Chapter 4 reports on the transcriptional control of the central carbon flux in *B. breve* UCC2003 by two distinct LacI-type regulators (BifR1 and BifR2).

Chapter 5 describes the roles played by various MalR TFs in controlling *B. breve* UCC2003 carbohydrate uptake and metabolism.

Chapter 6 contains a general discussion and the overall conclusions of this thesis.

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Chapter 2 Genome-Wide Search for Genes Required for Bifidobacterial Growth under Iron-Limitation

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2.1 Abstract

Phenotypic screening of a random mutant library combined with microarray analysis of the transcriptional response of *Bifidobacterium breve* UCC2003 to iron limitation, allowed the identification of a number of genes implicated in the survival of *B. breve* UCC2003 under iron-limiting conditions. Of the identified genes, two putative iron-uptake systems, were further characterised: (i) a presumed ferrous iron uptake system, designated here as *bfeUO*, and (ii) a predicted ferric iron/siderophore uptake system, designated *sifABCDE*. *In silico* analysis also illustrated that these two clusters are highly conserved across members of the genus *Bifidobacterium* and are invariably co-located. Murine colonization studies demonstrated that *B. breve* UCC2003-*bfeU* and *B. breve* UCC2003-*sifA* insertion mutants are able to colonize a healthy murine gut as efficiently as the wild type *B. breve* strain, indicating that these genes are not crucial for gut survival in or colonization of a healthy (murine) host.

2.2 Introduction

Iron is an essential nutrient for nearly all forms of life, having co-evolved with biological systems for billions of years; it is a key element in many redox reactions and is involved in various cellular processes such as DNA replication, nitrogen fixation, nucleotide biosynthesis and the synthesis of many metabolites (1). The prevalence and importance of iron in biological systems is most likely due to the abundance of Fe^{2+} during the initial evolution of living organisms (1, 2). The ionic forms of iron found in nature represent two interchangeable redox states: ferrous iron (Fe^{2+}) and its more oxidized form ferric iron (Fe^{3+}). These iron ions can also adopt different spin states, being either high or low, depending on its ligand environment. These attributes make iron a versatile prosthetic component in many proteins as a biocatalyst or electron carrier (3). Under aerobic, neutral pH conditions iron is present in its oxidized, but essentially insoluble trivalent, ferric form, while under anaerobic, neutral pH conditions iron is found in its reduced, divalent ferrous state, the latter being more biologically accessible due to its greater solubility (4).

Iron speciation, which relates to the changing concentration of varying forms of an ion as the pH of the solution changes, and iron availability are two factors which are important for the accessibility of iron within the gastrointestinal environment. Iron availability can vary greatly along the length of the gastrointestinal tract due to its tendency to complex with other molecules and because of its ability to exist in various oxidation states depending on its surrounding environment (1). The low pH in the stomach favours the solubility of both ferric and ferrous iron, whereas the subsequent rise in pH upon entry into the small intestine results in a decrease in the solubility of ferric iron. Iron may then complex with food components or host/microbiota-derived compounds such as citrate, ascorbate, mucin, certain amino acids or lactate (3). Certain insoluble forms of ferric iron, for example when complexed with phosphate, carbonate or oxides, are not readily available to the microbiota and may require reduction or removal from these complexes by siderophore activity (4). Soluble forms of ferric iron may be reduced to ferrous iron upon entry to the cytoplasm, while ferrous citrate or ferrous ascorbate may be taken up directly by (certain components of) the microbiota. Finally, lactate and short chain fatty acid (SCFA) production by the microbiota may also cause a modest, possibly localized drop in the pH in the colon resulting in an increase in iron solubility (5).

The human gut microbiota typically includes clostridia, eubacteria, and species of the genera *Bacteroides* and *Bifidobacterium* (6). Of particular interest, the genus *Bifidobacterium* belongs to the *Bifidobacteriaceae* family, which in turn belongs to the *Actinobacteria* phylum. Henri Tissier was the first to isolate a *Bifidobacterium* species in 1899 from the feces of a healthy, breast-fed infant, and due to his pioneering work and the subsequent work of many others, members of this genus are today considered to represent health-promoting or probiotic bacteria (7, 8).

Competitive sequestration/withholding of iron is referred to as nutritional immunity and has long been known in both Gram-positive and -negative bacteria as a defence mechanism, conferring a competitive advantage to certain commensals, such as bifidobacteria which thrive in low iron environments (9, 10). Bacteria have developed a wide range of mechanisms for iron sequestration, including a variety of systems for the uptake of ferrous iron, ferric iron, heme, hemephores (which scavenge heme from various hemoproteins), and siderophores (selective iron chelators which have a high affinity for ferric iron). Iron sequestration via siderophore-mediated and/or direct iron uptake has been reported recently for a number of bifidobacterial species, with *Bifidobacterium breve* exhibiting the highest siderophore activity and *Bifidobacterium kashiwanohense* exhibiting the highest iron uptake (11, 12). Furthermore, bifidobacterial carbohydrate fermentation typically acidifies the surrounding environment, favouring the solubility of ferric and ferrous iron in a localized fashion (13). Interestingly, certain species of *Bifidobacterium* have also been found to bind ferric iron to their cell wall and membranes, thereby limiting its availability to other bacteria (14). Bifidobacteria, like many other bacteria, are known to import iron (ions) across the cytoplasmic membrane by means of ABC-type transporters (15). In the case of *B. breve*, this leads to a cytoplasmic iron concentration (100/200 µM) which is about 14/16 fold higher than its surrounding environment (16).

Iron is also known to induce the generation of hydroxyl radicals through the Fenton reaction causing irreparable damage to DNA, lipids and proteins (17). Therefore, in order to utilise this essential, yet highly toxic ion (bifido)bacteria have evolved mechanisms for its storage, through the use of ferritin-like proteins.

The primary role of these ferritin-like proteins is to store iron when present in adequate amounts, and to supply iron for cell function under limiting conditions. Taken together, the ability of (bifido)bacteria to withhold and sequester iron within the gut may also result in a lower luminal iron level, which in turn may restrict radical-induced damage to intestinal tissue and offer pathogen protection by means of nutritional immunity.

The current work describes the identification of a number of genes that are involved in iron metabolism in the human gut commensal *B. breve* UCC2003. The identified genes are predicted to represent various iron-related functions, such as a ferrous iron uptake systems, a predicted ferric iron/siderophore uptake system and proteins involved in Fe-S cluster formation/repair/docking.

2.3 Materials and Methods

Bacterial Strains and Culture Conditions

Bacterial strains and plasmids used in this study are listed in Table 2.1. *B. breve* UCC2003 was routinely grown at 37°C in either de Man Rogosa and Sharpe medium (MRS medium; Difco, BD, Le Pont de Claix, France), modified de Man Rogosa and Sharpe (mMRS) medium made from first principles (18), or reinforced clostridial medium (RCM; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) supplemented with 0.05 % cysteine-HCl. For iron-limitation experiments, filtered reinforced clostridial medium (RCM; Oxoid Ltd.) was prepared in de-mineralised water. Bifidobacterial cultures were incubated anaerobically in a modular, atmosphere-controlled system (Davidson and Hardy, Belfast, Ireland). Where appropriate growth medium was supplemented with tetracycline (Tet; 10 µg ml^{-1}), chloramphenicol (Cm; 5 µg ml^{-1} for *L. lactis* and *E. coli*, 2.5 µg ml^{-1} for *B. breve*), ampicillin (Amp; 100 µg ml^{-1}), erythromycin (Em; 100 µg ml^{-1}) or kanamycin (Kan; 50 µg ml^{-1}) for plasmid selection and maintenance.

Phenotypic Screening and Monitoring of Dipyridyl-Sensitive Growth

Phenotypic screening and determination of transposon insertion sites was carried out as described previously (19) with the following adjustments: *B. breve* UCC2003 transposon mutants were sub-cultured twice in RCM supplemented with tetracycline and spotted onto Q-Trays (Molecular Devices, Berkshire, United Kingdom) containing RCA or RCA supplemented with the iron chelator dipyridyl at a concentration of either 250 µM or 275 µM. Transposon mutants which grew on RCA, but failed to grow or showed poor growth on RCA supplemented with (either concentration of) dipyridyl were then selected for further analysis. The phenotype of such dipyridyl-sensitive mutants was confirmed by monitoring growth in filtered RCM broth and filtered RCM broth containing dipyridyl (at a final concentration of 250 µM or 275 µM). Such mutants were incubated anaerobically at 37°C, with OD_{600nm} readings taken at 6, 8, 10 and 24 hours following inoculation.

Nucleotide Sequence Analysis

Sequence data were obtained from the Artemis-mediated (20) genome annotations of the *B. breve* UCC2003 genome sequence (21). Data base searches were carried out using non-redundant sequences accessible at the National Centre for Biotechnology Information internet site (http://www.ncbi.mlm.nih.gov) utilising the basic alignment search tool (Blast). Sequence analysis was performed using the Seqbuilder and Seqman programs of the DNASTAR software package (DNASTAR, Madison, WI). Protein functions were assigned with the use of the basic protein alignment search tool BlastP and Homology detection and structure prediction by HMM-HMM comparison; HHpred (22, 23). Rho-independent terminators were identified utilising ARNold (24).

Table 2-1. Bacterial strains and plasmids used in this study.

Strains and plasmids	Relevant features	Reference or source
Strains		
B. breve		
UCC2003	Isolate from nursling stool	(69)
UCC2003-*bfeU*	pORI19-*tetW-bfeU* insertion mutant of UCC2003	(68)
UCC2003-*sifA*	pORI19-*tetW-sifA* insertion mutant of UCC2003	(68)
UCC2003-PK1	UCC2003 harbouring pPKCM	(21)
E. coli		
xl1 BLUE	supE44 hsdR17 recA1 gyrA96 thi relA1 lac F= [proAB laclq lacZ M15 Tn10(Tet^r)]	Stratagene
EC101	Cloning host; *repA*$^+$ *km*^{r}	(69)
L. lactis NZ9000	MG1363, pepN::nisRK, nisin-inducible overexpression host	(70)
Plasmids		
pORI19	Em^r, repA$^-$, ori$^+$, cloning vector	(69)
pORI19-*bfeU*	pOR19 harbouring internal fragment of *bfeU* (*Bbr_0221)*	(68)
pORI19-*bfeU*-tet	pORI19 harbouring internal fragment of *Bbr_0221* + Tet^r	(68)
pORI19-*sifA*	pOR19 harbouring internal fragment of *bfeB* (*Bbr_0223)*	(68)
pORI19-*sifA*-tet	pORI19 harbouring internal fragment of *bfeB* + Tet^r	(68)
pBC1.2	pBC1-pSC101-Cm^r	(71)
pBC1.2-bfeU-IR	pBC1.2 harbouring *bfeU* promoter	This study
pBC1.2-*sifA*-IR	pBC1.2 harbouring *sifA* promoter	This study
pNZ272	Cm^r, pSH71 derivative containing promoterless glucuronidase gene for promoter screening	(33)
pNZ272-*bfeU*-IR	pNZ272 derivative carrying the *bfeU* promoter	This study
pNZ272-*sifA*-IR	pNZ272 derivative carrying the *sifA* promoter	This study

DNA Manipulations

DNA manipulations were carried out as previously reported (25). Restriction enzymes and T4 DNA ligase were obtained from Roche Diagnostics (Basel, Switzerland), and were used according to the manufacturer's instructions. PCRs were performed using Extensor Long Range PCR Enzyme master mix (Thermo Scientific, Glouchester, UK). Synthetic oligonucleotides were synthesized by Eurofins (Ebersberg, Germany) and are listed in Table 2-2. PCR products were purified by the use of a High-Pure PCR product purification kit (Roche, Basel, Switzerland). Plasmid DNA was introduced into *E. coli* and *B. breve* by electroporation and large-scale preparation of chromosomal DNA from *B. breve* was performed as described previously (26). Plasmid DNA was obtained from *B. breve* and *E. coli* using the Roche High Pure plasmid isolation kit (Roche Diagnostics, Basel, Switzerland). An initial lysis step was performed using 30 mg ml^{-1} of lysozyme for 30 min at 37°C as part of the plasmid purification protocol for *B. breve*.

Transcriptome Analyses

B. breve UCC2003's transcriptome response to iron limitation was tested by subjecting exponentially growing cells to a high concentration of dipyridyl for 30 minutes. Cells were prepared as follows; an overnight culture of *B. breve* UCC2003 in RCM was inoculated at 1 % into filtered RCM broth and incubated until an OD_{600nm} of 0.5 was reached. Cells were then exposed to 700 µM dipyridyl for 30 minutes. *B. breve* UCC20003 controls were treated in the same manner except for the addition of dipyridyl. Following dipyridyl exposure as outlined above (or in the absence of dipyridyl for controls), cells were harvested by centrifugation at 10,000 rpm for 2 min at room temperature and immediately frozen at -80°C prior to RNA isolation. DNA microarrays containing oligonucleotide primers representing each of the 1,864 annotated genes on the genome of *B. breve* UCC2003 were designed by and obtained from Agilent Technologies (Palo Alto, CA, USA). Cell disruption, RNA isolation, RNA quality control, and cDNA synthesis and labelling were performed as described previously (27). The labelled cDNA was hybridized using the Agilent Gene Expression hybridization kit (part number 5188-5242) as described in the Agilent Two-ColorMicroarrayBased Gene Expression Analysis v4.0 manual (G4140-90050). Following hybridization, the microarrays were washed in accordance with Agilent's standard procedures and scanned using an Agilent DNA microarray scanner (model G2565A).

The generated scans were converted to data files with Agilent's Feature Extraction software (v9.5). The DNA microarray data sets were processed as previously described (28-30). Differential expression tests were performed with the Cyber-T implementation of a variant of the t-test (31). A gene was considered to exhibit a significantly different expression level relative to the control when $p < 0.001$ and an expression ratio of > 2 or < 0.25. The microarray data obtained in this study have been deposited in NCBI's Gene Expression Omnibus database and are accessible through GEO series accession number GSE92758.

Real Time-PCR

Primers were designed to amplify upstream regions of all genes from Bbr_0220-Bbr_0227 (Table 2-1). *B. breve* UCC2003 was prepared for RNA extraction as follows: cells were incubated until an OD_{600nm} of 0.5 was achieved and were then exposed to 700 µM or 900 µM dipyridyl for 30 minutes, cells were harvested by centrifugation at 10,000 rpm for 2 minutes at room temperature and immediately frozen at -80°C. Cell disruption, RNA isolation and cDNA synthesis were performed as previously described (32). Two micrograms of RNA was treated with 2 units of DNase, RNase free (Roche, Basel, Switzerland) for 1 hour at 37°C. cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen). Absence of chromosomal DNA contamination was checked by real-time PCR. RT-PCR experiments were carried out using the cDNA-containing sample as a template and employing primers as above and Extensor Hi-Fidelity PCR Master Mix (Thermo Scientific, Glouchester, UK).

Beta-Glucoronidase Assay

The potential promoter-containing regions of *bfeU* and *sifA* were amplified by PCR using primer combinations *bfeU*-GUS-F, *bfeU*-GUS-R and *sifA*-GUS-F, *sifA*-GUS-R (Table 2-2. Oligonucleotide primers used in this study.), which contain EcoRI and BamH1 restriction sites at their 5′ ends. Amplicons were digested with EcoRI and BamHI, and cloned upstream of the promoterless *gusA* gene present in the similarly restricted pNZ272 reporter vector (33). Ligation mixtures were introduced by electroporation in *Lactococcus lactis* NZ9000 competent cells. The resulting plasmids; pNZ272-bfeU-IR and pNZ272-sifA-IR, once verified by restriction and sequence analysis, were then transformed into *B. breve* UCC2003 by electroporation (Table 2-1).

GusA activity assays in *B. breve* UCC2003 were carried out in triplicate by independent assay as previously described (34), with the following modifications: cells were grown in filtered RCM to an OD_{600nm} of approximately 0.3 to 0.4, at which transcription was induced by the addition of 700 µM dipyridyl for 20 minutes. 0.2 ml of cell culture were used in the assay. GusA activity was expressed in Miller Units and calculated using the following equation: $1000*((OD_{420nm}-(1.75*OD_{550nm}))/(t \times v \times OD_{600nm}))$, where t is reaction time (min), v is cell volume and OD_{420nm}, OD_{550nm} and OD_{600nm} are absorbance values at wave lengths of 420 nm, 550 nm and 600 nm, respectively.

Table 2-2. Oligonucleotide primers used in this study.

Purpose	Primer	Sequence
Cloning *bfeU* promotor in pNZ272	*bfeU*-GUS-F	atagctggatccgagatctgtccgttggcgctg
	bfeU-GUS-R	atagctgaattcgaaggaatcggcaacgtg
Cloning *feB* promotor in pNZ272	*sifA*-GUS-F	atagctggatcctcgacaactgggactacacc
	sifA-GUS-R	atagctgaattccatcacgctcaggcacatcac
Cloning *bfeU* promoter in pBC1.2	*bfeU*-PE-F	atagctctgcaggagatctgtccgttggcgctg
	bfeU-PE-R	cctgactctagagatggccttggacacgtc
Cloning *feB* promoter in pBC1.2	*sifA*-PE-F	atagctctgcagtcgacaactgggactacacc
	sifA-PE-R	cctgactctagaagcaccggatagttgacgaa
RT-PCR Bbr_0220-Bbr_0228	Bbr_0220-RT-1	aagtgcgtgccatgatgatc
	bfeU-RT-2	caacgtgcgtatcgtgttct
	bfeU-RT-3	ctccatggctgtttcgatgg
	bfeO-RT-4	ccatcgaaacagccatggag
	bfeO-RT-5	tcgacaactgggactacacc
	sifA-RT-6	tgccacgaattgttcaagca
	sifA-RT-7	cgattccgttcccgtacaag
	sifB-RT-8	catacggtaagcgcgatgag
	sifB-RT-9	gattctgaacctcaagcccg
	sifC-RT-10	catcctgaagaacatgccgg
	sifC-RT-11	cgctccatcggattcaactg
	sifD-RT-12	cggtcagattgaggtcgtct
	sifD-RT-13	gcgagaagtggaatgacgag
	sifE-RT-14	gctcttcctgcgatttctgg
	sifE-RT-15	ggtctttggtggcgtatgagg
	Bbr_0228-RT-16	ccgttcaccaagatttccaagg
Sequencing primers, Tn5 random mutant library	pMOD-fw-seq	gccaacgactacgcactagcc
	pMOD-rev-seq	gagccaatatgcgagaacacc
Inverse PCR primers, Tn5 random mutant library	i-PCR-fw	gcataccgtactgatctg
	i-PCR-rev	caatcataccggcttcc
IRD700 primers, primer extension products	*bfeU*ifR	tcaatgcgaacaggaacacgac
	*bfeO*ifF	gacgaaccgcaagcagcc
	*bfeO*ifR	cttcttggcggtgtcggag
	*sifA*ifF	ctcaggcacatcaccagtaa
	*sifA*ifR	gccgatgagacgccaatg

Primer Extensions

The Upstream (intergenic) regions of *bfeU* and *sifA* were PCR amplified using *B. breve* UCC2003 DNA as the template and oligonucleotide primer combinations listed in Table 2-2. These PCR products were then individually ligated into pBC1.2, using PstI and XbaI restriction sites and transformed into *E. coli* XL1 BLUE cells by electroporation. *E. coli* XL1 BLUE transformants containing the pBC1.2 constructs were selected for on LB agar with appropriate antibiotics. The integrity of the constructs were then confirmed by restriction and sequence analyses, and plasmid preparations of resulting recombinant plasmids, designated pBC1.2-*bfeU*-IR and pBC1.2-*sifA*-IR (names correspond to the gene downstream of the promoter in the UCC2003 genome, see Table 2-1), were introduced by electroporation into *B. breve* UCC2003 with selection on RCA supplemented with the appropriate antibiotic.

B. breve UCC2003-pBC1.2-bfeU-IR and *B. breve* UCC2003-pBC1.2-sifA-IR were prepared as follows for RNA extraction; Cells were incubated until an OD_{600nm} of 0.5 was achieved and were then exposed to 700 µM or 900 µM dipyridyl for 30 minutes, cells were harvested by centrifugation at 10,000 rpm for 2 minutes at room temperature and immediately frozen at -80°C. RNA extraction was carried out as previously described (27). The 5′ ends of the RNA transcripts were determined by annealing 1 pmol of an Ird-700-labeled synthetic oligonucleotide to 20 µg of RNA, as previously described (35).

The following Ird-700 labelled primer pairs were used: *bfeU*ifF and *bfeU*ifR, *sifA*ifF and *sifA*ifR (Table 2-2). Corresponding sequence ladders of the promoter regions of *bfeU* and *sifA* were produced using the same primer as in the primer extension reaction and employing a DNA cycle-sequencing kit (Jena Bioscience, Germany) and were run alongside the primer extension products to allow precise alignment of the transcriptional start site with the corresponding DNA sequence. Separation was achieved on a 6.5 % Li-Cor Matrix KB Plus acrylamide gel. Signal detection and image capture were performed with a Li-Cor sequencing instrument (Li-Cor Biosciences).

Murine Colonization Experiments

Experiments with mice were approved by the University College Cork Animal Experimentation Ethics Committee and experimental procedures were conducted under license from the Irish Government (license number B100/3729). Seven-week-old female, BALB/c mice were housed in individually vented cages (Animal Care Systems) under a strict 12 hour light cycle. Mice (n=7 per group) were fed a standard polysaccharide-rich mouse chow diet and water *ad libitum*.

Mice were inoculated by oral gavage (10^9 cfu of *B. breve* UCC2003PK1, *B. breve* UCC2003-*bfeU*, *B. breve* UCC2003-*sifA*, or a mixture of either *B. breve* UCC2003PK1 and *B. breve* UCC2003-*bfeU*, or *B. breve* UCC2003PK1 and *B. breve* UCC2003-*sifA* in 100 µl of PBS) (see Table 2-1 for descriptions of strains). Fecal pellets were collected at intervals for 18 days to enumerate bacteria. Eighteen days after inoculation, mice were sacrificed and their intestinal tracts immediately dissected. The small intestine, cecum and large intestine were harvested for determination of colony forming units (cfu) (serial dilution plating on RCA agar plates with appropriate antibiotics).

2.4 Results

B. breve UCC2003 Transposon Mutants Sensitive to Iron Limitation

Screening of a Tn5-mediated mutant library containing ~20,000 *B. breve* UCC2003 derivatives was performed in order to identify mutants with increased sensitivity to the iron chelator dipyridyl. The dipyridyl-sensitive phenotype was then validated for nearly 250 mutants by sub-cultivation in RCM broth supplemented with dipyridyl, resulting in a final collection of 29 verified, dipyridyl-sensitive mutants. Dipyridyl-sensitive mutants, which had been identified in the screening of the random mutant library, were also analysed by growth profile analysis for their sensitivity to two other iron chelators, ciclopirox olamine and phenanthroline. These results also support the notion that the obtained mutants were specifically impaired due to iron limitation rather than due to an aspecific effect of the dipyridyl. The transposon insertion site of validated dipyridyl-sensitive mutants was then determined for these 33 mutants using an approach that was described previously (19). A list of these mutants and the location of their corresponding transposon insertion site can be found in Table 2-3 and Figure 2-1, respectively.

In a number of mutants, the link between the disrupted gene and the corresponding dipyridyl-sensitive phenotype was obvious. For example, we found a mutant with a transposon insertion in a gene (Bbr_0427, designated here as *mntH*, after its homolog in *Escherichia coli*) encoding a divalent cation transporter of the NRAMP family. Studies on *mntH* in *E. coli* have found that this divalent cation transporter has affinity for more than one cation and can have a broad range of specificity including Fe^{2+} and Mn^{2+} (36). Furthermore, transposon-mediated disruption of Bbr_1816 (designated here as *oppC*) caused impaired growth under dipyridyl-induced, iron-limiting conditions. Based on BlastP and HHpred analysis, it appears that the deduced product of *oppC* is part of an ABC transport system involved in the transport of cations and iron-carrying compounds.

In addition, our transposon screening identified eleven distinct mutants, carrying Tn5 insertions in a gene cluster encompassing Bbr_0221–Bbr_0227, and displaying a severely impaired growth phenotype when cultivated in the presence of dipyridyl (Table 2-3). The products of Bbr_0221-Bbr_0222 (previously called *bfeUO*) are predicted to represent a high affinity ferrous iron uptake system which is homologous to (two genes of) the *efeUOB* gene cluster found in *E. coli* (37, 38), while Bbr_0223-Bbr_0227 gene cluster,

designated here as *sifABCDE*, are predicted to represent a ferric iron/siderophore uptake system, based on its protein similarity to previously characterised ferric iron uptake/aerobactin siderophore uptake systems in *E. coli* 1520, plasmid pIP1206 and *Bacillis subtilis* 168 (39).

Interestingly, mutations in two genes involved in iron-sulphur (Fe-S) cluster formation, repair and docking were identified as causing growth impairment under iron limitation. Fe-S clusters act as cofactors for many cellular proteins which have prominent roles in many cellular processes including but not limited to respiration, central metabolism, gene regulation, DNA repair and replication (40-42). The first, Bbr_1825 (designated here as *apbE*), the gene product is similar to *apbE* of *Salmonella enterica* which is known to play a role in the assembly and repair of Fe-S clusters in this pathogen (43). The second, Bbr_0669 (designated as *apbC*), encodes a protein with similarity to a P loop NTPase which binds and transfers Fe-S clusters to cytosolic apo-proteins (44, 45). Studies in *Salmonella enterica* have found that mutations in *apbC* and *apbE* result in cellular deficiencies which could be reversed by the addition of ferric chloride (46). This study concluded that the addition of iron may compensate for the absence of *apbC* and *apbE* by increasing repair of oxygen-labile Fe-S clusters. In the case of *B. breve* UCC2003 this may mean that under dipyridyl-induced, iron-limiting conditions the loss of *apbC* and *apbE* results in *B. breve* UCC2003 not being able to construct/repair Fe-S clusters or to insert these Fe-S clusters into one or more essential apo-proteins.

The remaining dipyridyl-sensitive mutants which were selected from the Tn5 library screening efforts, whose involvement in iron uptake or requirement for iron is not immediately clear from similarity searches, are listed in Table 2-3.

Table 2-3. Growth profiles of iron sensitive Tn5 transposon mutants.

Gene	Predicted function	No chelator	250 μM dipyridyl[a]	275 μM dipyridyl[a]	100 μM ciclopirox olamine[b]	80 μM phenanthroline[b]	Upregulation transcriptome analysis[c]
TRANSPORT ASSOCIATED							
UCC2003	*B. breve* UCC2003 WT strain	+++	+++	+++	+++	+++	N/A
Bbr_0221	*bfeU*, high affinity Fe^{2+} periplasmic transporter	+++	–	–	–	–	Yes
Bbr_0222	*bfeO*, high affinity Fe^{2+} periplasmic transporter	+++	–	–	–	–	Yes
Bbr_0223	*sifA*, possible siderophore binding protein	+++	–	–	+++	–	Yes
Bbr_0224[d]	*sifB*, permease protein, ferric iron/siderophore uptake	+++	–	–	+++	–	Yes
Bbr_0225[d]	*sifC*, permease protein, ferric iron/siderophore uptake	+++	–	–	+++	–	Yes
Bbr_0226[e]	*sifD*, heme ATP binding domain, heme ABC transporter	+++	–	–	+++	–	No
Bbr_0227	*sifE*, possible ferric iron reductase, FMN binding domain	+++	–	–	+++	+	No
Bbr_0427	*mntH*, Mn^{2+}/Fe^{2+} transporter, NRAMP family	+++	+	–	+++	++	No
Bbr_1656	Sugar ABC transporter, permease protein	+++	++	++	++	++	No
Bbr_1657	Binding-protein-dependent transport system	+++	++	++	++	++	No
Bbr_1669	Hypothetical membrane spanning protein	++	+	–	++	–	No
Bbr_1816	*oppC*, permease protein iron siderophore uptake	+++	+++	++	+++	++	Yes
Fe-S CLUSTER ASSEMBLY ASSOCIATED							
Bbr_0669	*apbC*, Iron-sulfur cluster binding protein	++	+	–	–	–	No
Bbr_1825	*apbE*, Iron-sulfur cluster formation/repair	+++	+	+	+++	–	No
CELL ENVELOPE ASSOCIATED							
Bbr_0246	*murE*, UDP-N-acetylmuramoylalanyl-D-glutamate–2,6-diaminopimelate ligase	+++	++	++	+	–	No
Bbr_1592	Putative lipid kinase	+++	++	++	++	–	No
Bbr_1916	Hypothetical secreted protein, in operon with peptidoglycan lipid II flipase	+++	+	++	+	–	No
DNA REPAIR/REPLICATION							
Bbr_0511	Alanine aminopeptidase	+++	++	++	++	++	No
Bbr_0514	DNA polymerase III, epsilon subunit or related 3′-5′ exonuclease	+++	++	++	+++	++	No
Bbr_1224	*scpA*, segregation and condensation protein	++	++	+	++	+	No
Bbr_1370	Hypothetical protein, in operon with Uracil-DNA glycosylase	+++	++	+	+++	+	No
Bbr_1603	*radA*, DNA repair protein	+++	++	++	++	–	No
REGULATORY PROTEINS							
Bbr_0466	Hypothetical regulatory protein	+++	++	++	+++	++	No
Bbr_0589	Hypothetical regulatory protein, tetR family	+++	++	++	+++	+	No
REQUIRE METAL ION AS COFACTOR							
Bbr_0702	Haloacid dehalogenase-like hydrolase	+++	++	++	+++	++	No
Bbr_1743	Short chain dehydrogenase	+++	++	++	+++	++	No
OTHER							
Bbr_1644[d]	BD-type quinol oxidase subunit II; oxidoreductase	+++	++	++	+++	+	No
Bbr_1647	*glgX*, glycogen operon protein	+++	+++	+++	++	+	No

Growth profiles of transposon mutants which were sensitive to iron chelation, selected for in RCM supplemented with 250 and 275 μM of 2′2-dipyridyl. A minus sign (−) indicates that final OD_{600} < 0.5; (+) indicates OD_{600} between 0.5 and 1.5, (++) indicates OD_{600} of 1.5 to 2.5 and (+++) indicates OD_{600} > 2.5.

[a]MTC for WT was found to be 275 μM of dipyridyl, two concentrations were used in the screening process in order to reduce the number of false positives obtained

[b]MTC for WT was found to be 100 μM of ciclopirox olamine and 80 μM of phenanthroline.

[c]Genes which were differentially regulated in transcriptomic analysis of B. breve UCC2003 under iron limiting conditions.

[d]Two transposon insertional mutants were identified in this gene.

[e]Three transposon insertional mutants were identified in this gene.

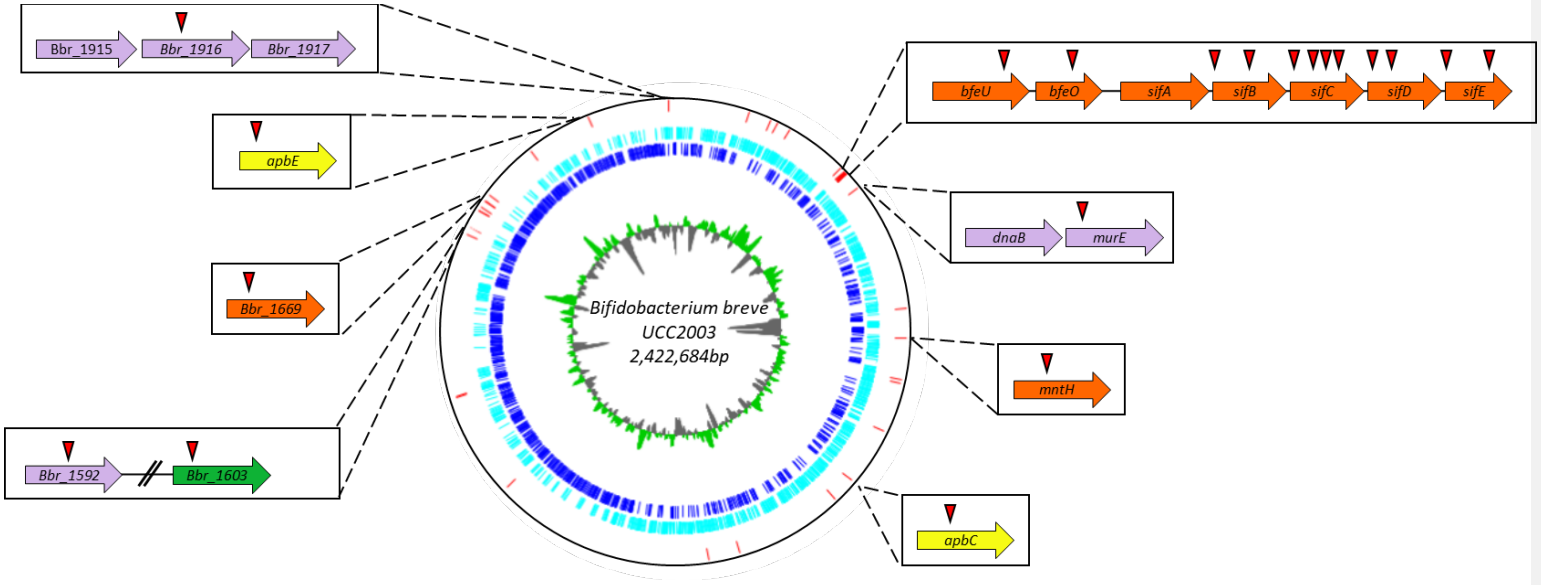


Figure 2-1. Circular genome map of *B. breve* UCC2003.
The innermost circle illustrates GC skew, shown in green on the forward strand and in gray on the reverse strand. The dark blue circle displays the ORFs on the forward strand, while the light blue circle representing all the ORFs on the reverse strand. The red vertical bars indicate the transposon insertion sites of dipyridyl-sensitive mutants identified in the screening of the random mutant library. Genes in which transposon mutations were shown to cause complete growth impairment in the presence of either dipyridyl, phenanthroline or ciplopirox olamine are indicated in boxes together with their names or locus tags with the position(s) of the transposon insertion indicated by a red triangle.

Transcriptomic Response of *B. breve* UCC2003 to Iron Limitation.

Microarray analysis was carried out so as to complement the results obtained from the phenotypic screening of the *B. breve* UCC2003 Tn5 random mutant library under iron-limiting conditions. The transcriptomic response of *B. breve* UCC2003 upon iron limitation was assessed by subjecting exponentially grown cells to a high concentration of dipyridyl for 30 minutes (Results are summarised in Table 2-4 and Table 2-5). Genes with a significantly different transcription level relative to the control when grown under iron limiting conditions, were identified in this manner ($p < 0.001$ and an expression ratio of >1.7 or < 0.25). Based on these criteria a total of 41 genes were found to be differentially regulated under iron-limiting conditions.

One noteworthy feature of the *B. breve* UCC2003 transcriptome response to iron limitation was the transcriptional activation of most genes of the *bfeUO* and the *sifABCDE* clusters, which encode a putative high affinity ferrous iron uptake system and a predicted ferric iron /siderophore uptake system, respectively. These clusters were also identified in the Tn5 transposon screening (see above).

Other putative metal uptake systems were shown to be transcriptionally up-regulated under the imposed iron-limiting conditions, including a solute binding protein of an ABC-type transport system (Bbr_0579). Based on BlastP and HHpred analysis this protein displays similarity to solute binding proteins for metal cations found in many species including *E. coli* and *Streptococcus pneumoniae* (47, 48). The gene associated with locus tag Bbr_0268 (and designated here as *silP*) displayed a 6.7-fold transcriptional increase under iron-limiting conditions. The *B. breve* UCC2003-encoded *silP* contains an ATPase domain and is predicted to be involved in the transport of iron-carrying compounds and copper ions based on BlastP analysis. Bbr_0269 designated here as *csoR*, was also up-regulated 2.1-fold under microarray analysis this gene encodes a predicted copper-sensitive operon repressor based on BlastP analysis.

Also, of interest was the up-regulation of Bbr_1850, a NADPH-dependent FMN reductase. Studies in *Pseudomonas putida* have found that these reductases have ferric iron reductase activity and are attributed to playing a role in NADH-dependent ferric/flavin reduction under iron stress conditions (49). In short, it is possible that *B. breve* UCC2003 may be utilising this reductase to convert cytosolic ferric iron or ferric

iron bound to siderophore complexes into its more biologically available ferrous form for further use within the cell.

Table 2-4. Transcripomic response (up-regulation) of *B. breve* UCC2003 to iron limitation.

Locus tag	Up	Predicted Function	P value
Bbr_0221	2.4*	*bfeU*, High-affinity Fe^{2+} permease	1.65E-04
Bbr_0222	3.7	*bfeO*, High affinity Fe^{2+} periplasmic transporter	4.43E-05
Bbr_0223	1.8	*sifA*, hypothetical protein, heme uptake	4.45E-05
Bbr_0224	1.7	*sifB*, Permease protein ABC transporter heme uptake	7.10E-04
Bbr_0225	1.7	*sifC*, Permease protein ABC transporter heme uptake	7.10E-04
Bbr_0268	6.7	*silP*, Cation transport ATPase	1.32E-05
Bbr_0269	2.1	Transcriptional regulator CsoR (copper-sensitive operon repressor)	1.39E-04
Bbr_0573	1.7	*fur*, Ferric uptake regulation protein	3.76E-02
Bbr_0579	7.8	Solute binding protein of ABC transporter system, iron siderophore, metallic cations	4.49E-03
Bbr_0750	1.8	ATP-binding protein of ABC transporter system for metals	1.19E-01
Bbr_0826	3.3	SAM-dependent methyltransferase	2.45E-01
Bbr_0827	1.8	Hypothetical protein, containing cupin domain	2.53E-01
Bbr_1815	1.7	*oppD*, Cation/iron containing molecules transport ATP-binding protein	6.69E-08
Bbr_1817	1.9	*oppB*, Cation/iron containing molecules transporter system permease protein	1.50E-07
Bbr_1850	2.4	NADPH-dependent FMN reductase/Oxygen-insensitive NADPH nitroreductase	1.56E-07

*Fold up-regulation of logarithmically growing *B. breve* UCC2003 cells when exposed to iron limitation induced by exposure to 700µM dipyridyl for 30 minutes.

Table 2-5. Transcripomic response (down-regulation) of *B. breve* UCC2003 to iron limitation

Locus tag	Down	Predicted Function	P value
Bbr_0329	2.5	*atpD*, ATP synthase beta chain	2.20E-09
Bbr_0843	2.1	Conserved hypothetical secreted protein with excalibur domain	3.79E-14
Bbr_0888	1.9	*rpsD*, SSU ribosomal protein S4P	2.77E-09
Bbr_0899	2.1	Endonuclease involved in recombination	1.17E-11
Bbr_0925	2	Permease MFS superfamily	4.61E-09
Bbr_0973	1.9	*pyrB*, Aspartate carbamoyltransferase	3.71E-11
Bbr_0974	1.9	*pyrI*, Aspartate carbamoyltransferase	6.58E-12
Bbr_1014	1.9	*ruvB*, Holliday junction DNA helicase	1.34E-10
Bbr_1104	3.4	*tsf*, Protein Translation Elongation Factor Ts (EF-Ts)	9.71E-10
Bbr_1446	2.4	*nrdG*, Anaerobic ribonucleoside-triphosphate reductase activating protein	4.44E-16
Bbr_1581	2.1	Narrowly conserved hypothetical membrane spanning protein	9.72E-13
Bbr_1582	2.6	Narrowly conserved hypothetical membrane spanning protein	2.01E-14
Bbr_1583	2.1	Histidine kinase sensor of two component system	6.21E-10
Bbr_1622	2.3	*rplO*, 50S ribosomal protein L15	5.33E-15
Bbr_1623	2.2	*rpmD*, 50S ribosomal protein L30	1.01E-12
Bbr_1624	2.2	*rpsE*, 30S ribosomal protein S5	3.20E-12
Bbr_1626	2.2	*rplF*, 50S ribosomal protein L6	3.11E-11
Bbr_1627	2.3	*rpsH*, 30S ribosomal protein S8	9.26E-12
Bbr_1628	2.2	*rpsN*, 30S ribosomal protein S14-1	9.95E-11
Bbr_1632	2.1	*rpsQ*, 30S ribosomal protein	1.11E-11
Bbr_1638	1.9	*rplB*, 50S ribosomal protein	1.48E-11
Bbr_1675	2.1	*rplL*, LSU ribosomal protein L12P (L7/L12)	4.51E-11
Bbr_1726	2.3	*rlpA*, LSU ribosomal protein L1P	2.12E-12
Bbr_1898	4.8	*nrdF*, Ribonucleoside-diphosphate reductase beta chain	0.00E+00
Bbr_1899	4.2	*nrdE*, Ribonucleoside-diphosphate reductase alpha chain	2.22E-16
Bbr_1900	1.9	*nrdI*, NrdI protein	1.69E-08

Fold down-regulation of logarithmically growing *B. breve* UCC2003 cells when exposed to iron limitation induced by exposure to 700µM dipyridyl for 30 minutes.

In-silico Analysis of the Predicted *bfeUO and sifABCDE* Gene Clusters

Phenotypic screening of the random mutant library in *B. breve* UCC2003 and microarray analysis showed that the *bfeUO* and *sifABCDE* clusters are essential for their ability to survive during iron-limitation. As the aforementioned gene clusters were unique in being identified by both of these different approaches we decided to characterise their products in more detail. BfeU encodes a conserved hypothetical membrane spanning protein with an iron permease FTR1 family domain.

BfeU contains 9 transmembrane domains based on TMpred analysis and has a high degree of similarity to a high affinity ferrous iron permease. BfeU is similar to the EfeU a membrane spanning protein of a ferrous iron uptake system found in *E. coli* and *Pseudomonas syringae*, based on BlastP and HHpred analysis. BfeO encodes a conserved hypothetical protein, which has a hydrophobic N-terminus suggesting it is a membrane associated protein and is predicted to function in metal ion binding. BfeO also has homology to a human lactoferrin transporter of *Treponema pallidum* based on HHpred analysis. SifA is a conserved hypothetical membrane-spanning protein which contains 8 transmembrane helices based on TMpred analysis, it displays similarity to the cytochrome B_C1 complex, and these proteins are a part of a family of oxidoreductases. SifB and SifC are ABC transporter permeases with FtsX-like family domains, while SifD is an ATP-binding subunit protein with homology to a ferric ion import ATP-binding subunit. SifE contains a hydrophobic N-terminus indicating that it is membrane associated. The N-terminus region of SifE from amino acid 3 to 33 also displays homology to ABC-type transport solute binding proteins, while the C-terminal domain contains a FMN-binding domain which is homologous to NADH reductases.

Cronin *et al.* previously noted sequence similarity between the putative BfeUO ferrous iron-uptake system in *B. breve* UCC2003 and the EfeUOB iron-uptake machinery in *E. coli*, and between the SifCD proteins encoded by *B. breve* UCC2003 and a siderophore uptake system in *Bacillus subtilis* (38). In the current study we were interested in identifying whether the corresponding *bfeUO* and *sifABCDE* gene clusters are conserved throughout the *Bifidobacterium* genus. *In silico* analysis utilising blastP was carried out by comparing the degree of protein similarity of the BfeUO/SifABCDE proteins found in *B. breve* UCC2003 with the deduced protein complement of representative bifidobacterial species. The results are displayed in Table 2-6 as a heat map, which illustrates protein similarity, as well as the genetic organisation and presence/absence of BfeUO- and SifABCDE-encoding genes across a number of bifidobacterial species. From Table 2-6 it is obvious that the *bfeUO/sifABCDE* gene cluster is highly conserved across the bifidobacterial genus, and that *bfeUO*/*sifABCDE* gene cluster is present in the genomes of 35 of the 47 bifidobacterial species assessed. The 12 Bifidobacterial species which do not contain these gene clusters are members of three distinct branch points of a phylogenetic tree constructed by Milani *et al*, 2014 (50).

Members of these branch points are furthest away from the root of the evolutionary phylogenetic tree, which indicates that they represent the most evolved from the common bifidobacterial ancestor (50). Furthermore, the five genes of the *sifABCDE* cluster are, when present, always co-located on the bifidobacterial genomes. The only exception to this was SifE, which encodes a predicted ferric reductase protein, and which appears to be absent from the genomes of *Bifidobacterium bohemicum* and *Bifidobacterium bombi*, bifidobacterial species that originate from the hind-gut of a bumblebee.

Comparative analysis of the *bfeUO*/*sifABCDE* gene cluster as compared to the corresponding loci in various bacteria and yeast shows that both clusters were highly conserved in the *Bifidobacteriaceae* family, for example in *Bifidobacterium longum* NCC2705 and in *Gardinella vaginalis*. The *bfeUO/sifABCDE* gene cluster was also present although at a low level of similarity on pIP1206 and pRSB107 plasmids from *E. coli* (51, 52)

Table 2-6. Heat map of BfeUO and SifABCDE homologs across the *Bifidobacterium* genus.

Bifidobacterium genomes	Origin	locus_tag	*bfeU*	*bfeO*	*sifA*	*sifB*	*sifC*	*sifD*	*sifE*
Bifidobacterium actinocoloniiforme	Bumblebee digestive tract	BACT							
Bifidobacterium adolescentis	Adult faeces	BAD_0097-0103	63	67	69	66	70	90	67
Bifidobacterium angulatum	Adult faeces	BIANG_1097-1091	63	67	68	76	70	89	73
Bifidobacterium animalis subsp. animalis	Sewage	BASA							
Bifidobacterium animalis subsp. lactis	Fermented milk	Bl12							
Bifidobacterium asteroides	Bee intestine	BAST_1624-1618	54	66	63	68	62	78	47
Bifidobacterium biavatii	Tamarind faeces	BBIA_1594-1587	82	81	84	87	91	93	67
Bifidobacterium bifidum	Infant faeces	BBPR_1727-1721	79	84	78	88	83	90	73
Bifidobacterium bohemicum	Bumblebee digestive tract	BBOH_0687-0682	53	54	60	70	64	80	
Bifidobacterium bombi	Bumblebee digestive tract	BBOMB_0050-0055	55	56	58	68	56	83	
Bifidobacterium boum	Bovine rumen	BBOU_1030-1024	59	78	69	72	71	85	53
Bifidobacterium breve	Infant faeces	Bbr_0221-0227	100	100	100	100	100	100	100
Bifidobacterium callitrichos	Marmoset faeces	BCAL_1913-1919	77	78	77	87	79	90	74
Bifidobacterium catenulatum	Adult faeces	BIFCAT_0140-0147	62	63	69	76	70	91	70
Bifidobacterium choerinum	Piglet faeces	BCHO							
Bifidobacterium coryneforme	Bee intestine	BCOR_1391-1385	56	67	65	69	67	81	49
Bifidobacterium crudilactis	Raw milk cheese	BCRU_1683-1689	56	62	64	67	66	81	58
Bifidobacterium cuniculi	Rabbit faeces	BCUN							
Bifidobacterium dentium	Oral cavity	BDP_0163-0169	65	67	70	76	71	91	67
Bifidobacterium gallicum	Human faeces	BIFGAL							
Bifidobacterium gallinarum	Chicken caecum	BIGA_1581-1587	58	57	54	64	59	87	59
Bifidobacterium indicum	Bee intestine	BINDI_1328-1322	56	66	64	69	67	81	48
Bifidobacterium kashiwanohense	Infant faeces	BKAS_0947-0954	62	64	70	77	70	91	69
Bifidobacterium longum subsp. infantis	Infant faeces	Blon_0196-0202	83	93	94	95	93	99	89
Bifidobacterium longum subsp. longum	Adult faeces	BL_0455-0449	75	85	87	95	92	99	88
Bifidobacterium longum subsp. suis	Piglet faeces	BLSS_0743-0737	83	94	94	94	93	98	89
Bifidobacterium magnum	Rabbit faeces	BMAGN							
Bifidobacterium merycicum	Bovine rumen	BMERY_0538-0544	64	67	68	77	68	89	73
Bifidobacterium minimum	Sewage	BMIN_1327-1334	55	76	72	72	66	78	62
Bifidobacterium mongoliense	Fermented milk	BMON							
Bifidobacterium pseudocatenulatum	Infant faeces	BIFPSEUDO_4117-4125	62	63	69	76	70	91	68
Bifidobacterium pseudolongum subsp. globosum	Bovine rumen	BPSG							
Bifidobacterium pseudolongum subsp. pseudolongum	Pig faeces	BPSP							
Bifidobacterium psychraerophilum	Porcine caecum	BPSY_0711-0717	55	62	69	69	67	80	56
Bifidobacterium pullorum	Chicken faeces	BPULL_1164-1158	58	57	54	64	59	87	59
Bifidobacterium reuteri	Marmoset faeces	BREU_0405-0412	80	82	83	76	86	94	77
Bifidobacterium ruminantium	Bovine rumen	BRUM_0487-0493	63	68	71	70	70	90	66
Bifidobacterium saeculare	Rabbit faeces	BSAE_1410-1404	58	57	54	64	59	87	61
Bifidobacterium sanguini	Tamarind faeces	BISA_0498-0491	81	84	82	77	87	94	84
Bifidobacterium scardovii	Human sources	BSCA_0091-0085	88	91	91	89	89	93	77

Table 2-6. Heat map continued for BfeUO and SifABCDE homologs across the *Bifidobacterium* genus.

Bifidobacterium genomes	Origin	locus_tag	*bfeU*	*bfeO*	*sifA*	*sifB*	*sifC*	*sifD*	*sifE*
Bifidobacterium scardovii	Human sources	BSCA_0091-0085	88	91	91	89	89	93	77
Bifidobacterium stellenboschense	Tamarind faeces	BSTEL_1997-2003	79	81	78	86	80	94	79
Bifidobacterium stercoris	Adult faeces	BSTER_0114-0120	63	67	69	66	69	90	69
Bifidobacterium subtile	Sewage	BISU_833-834-836-838	58	46				79	47
Bifidobacterium thermacidophilum subsp. porcinum	Piglet faeces	BPORC							
Bifidobacterium thermacidophilum subsp. thermoacidophilum	Anaerobic digester	THER5							
Bifidobacterium thermophilum	Piglet faeces	BTHER_1257-1263	59	78	69	71	71	85	52
Bifidobacterium tsurumiense	Hamster dental plaque	BITS_0255-0249	57	74	65	72	66	88	54

Heat map of the percentage protein identity of BfeUO and SifABCDE iron uptake systems from *B. breve* UCC2003 as compared with bifidobacterial representative species, Dark green shading represents > that 70% protein identity, green shading represents between 60 and 69% protein identity, light green shading below 60% identity and white shading indicates the absence of a gene.

Transcriptional Analysis of the *bfeUO and sifABCDEF* Gene Clusters

In order to assess if the *bfeUO/sifABCDE* gene cluster of *B. breve* UCC2003 represents one or more transcriptional units we investigated its transcription product(s) by means of RT-PCR. For this purpose, total RNA was extracted from logarithmically growing *B. breve* UCC2003 cultured in filtered RCM supplemented with dipyridyl (i.e. growth under iron-limiting conditions so as to maximize transcription of the *bfe/sif* gene cluster), and then used to generate cDNA by reverse transcription (see Materials and Methods). The resulting cDNA was used as a template for the amplification of the intergenic regions of *bfeUO* and *sifABCDE* using various primer sets listed in Table 2-1. RT-PCR analysis indicated that the *bfe/sif* gene cluster encompasses two transcriptional units represented by the *bfeUO* operon and the *sifABCDE* operon (Figure 2-2). Consistent with this analysis was the identification of a potential rho-independent transcriptional terminator sequence located downstream of *bfeO*, while the *sifABCDE* operon is followed by a gene, Bbr_0228, which is oriented in the opposite direction (Figure 2-2).

The RT-PCR results indicate that transcription of the *bfeUO* and *sifABCDE* operons initiates upstream of the *bfeU* and *sifA* genes, respectively. In order to validate this notion and to investigate these presumed promoters in more detail, plasmids pNZ-*bfeU* and pNZ-*sifA* were constructed, in which the upstream regions of *bfeU* and *sifA* were cloned in front of the promoter-less *gusA* gene in the promoter-probe vector pNZ272 (see Materials and Methods). Plasmids pNZ-*bfeU* and pNZ-*sifA*, as well as pNZ272 (negative control) were individually introduced into *B. breve* UCC2003, logarithmically growing cells of these three strains that either had or had not been subjected to iron limitation by means of dipyridyl addition were then utilised for β-glucuronidase (the product of *gusA*) activity assays. The obtained results clearly demonstrate that each of the cloned fragments in pNZ-*bfeU* and pNZ-*sifA* elicit promoter activity and that these promoters are inducible by iron limitation (Figure 2-2), which is consistent with previously reported findings for the *bfeU* promoter region by Cronin *et al* (2012) (38).

In order to identify transcriptional initiation sites of the *bfeUO* and *sifABCDE* promoters, primer extension analysis was carried out, which identified a single transcriptional start site upstream of *bfeU* and *sifA*.The transcriptional start site (TSS) of the *bfeUO* operon was shown to be located 48 bp upstream of the predicted *bfeU* start codon, and a putative

promoter sequence was identified nine base pairs upstream of this TSS with putative -10 (CATAGT) and -35 (TTGAAG) elements which are similar to vegetative promoter sequences found in *B. breve* (53-56). The transcriptional start site identified for *sifABCDE* operon is located 93 bp upstream of *sifA* start codon, a possible putative promoter sequence is located eight base pairs upstream of this TSS with putative -10 (AACAGT) while no obvious -35 elements could be identified (Figure 2-2). However, this possible -10 deviates from the recently identified typical vegetative promoter consensus for *B. breve* UCC2003 which was found to be particularaly conserved in genes which were highly/medium expressed genes. (-10; TATAAT, -35; TTGACA). The deviance from typical promoter consensus of *B. breve* UCC2003 may indicate that this gene utilises an alternative sigma factor for transcription initiation; however this would require further investigations. Previous studies carried out by Cronin *et al*. identified a putative regulatory motif (AAAATCAAGACTGTTGTT), upstream of *bfeUO* which is also present upstream of a number of genes involved in iron utilisation. The location of this binding operator sequence in relation to the *bfeUO* promoter region suggests that this as yet unidentified transcription factor may act as a repressor (Figure 2-2) (57).

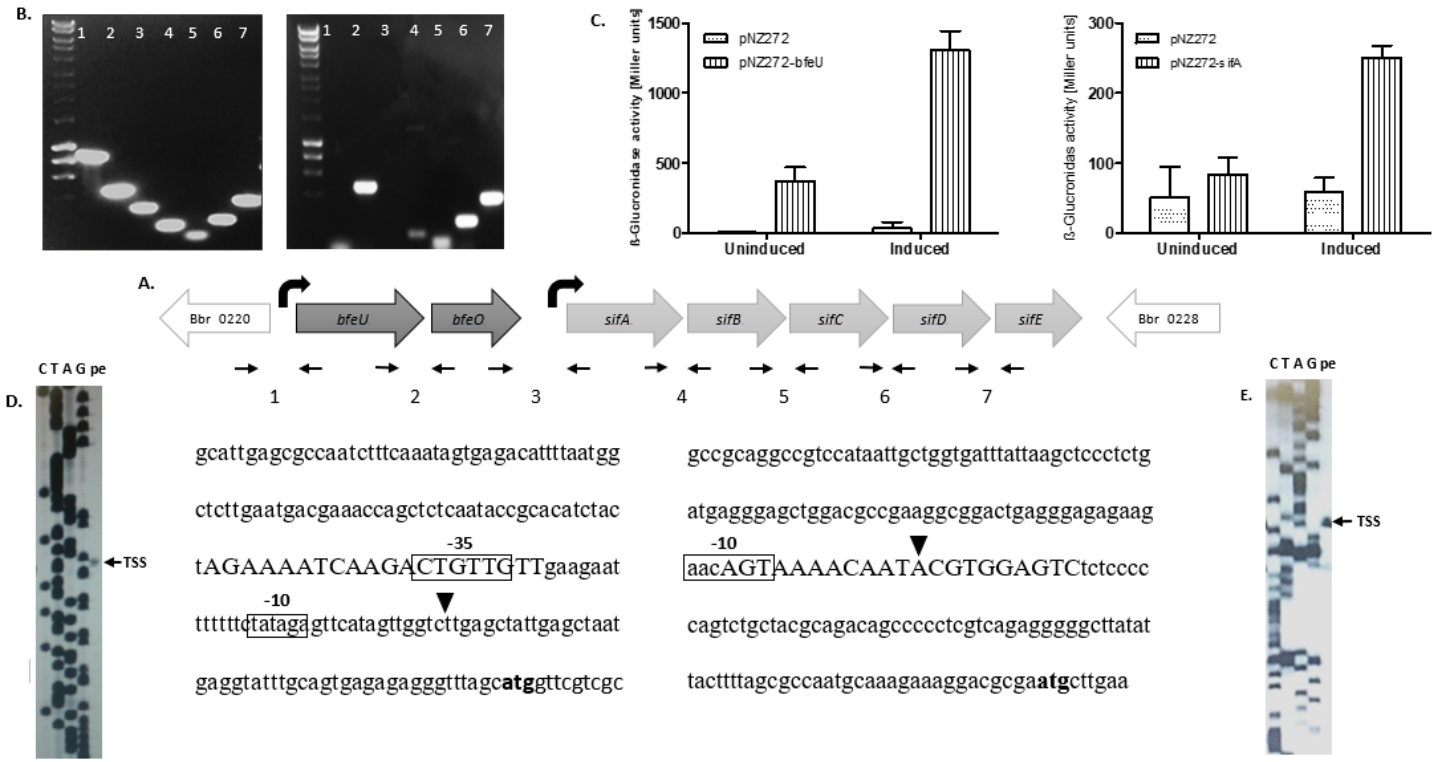


Figure 2-2. Transcriptional analysis of *bfeUO*/*sifABCDE* gene cluster
(A) is a schematic representation of the *bfeUO/sifABCDE* gene cluster, curved black arrows represent the predicted transcriptional start sites present, straight small arrows indicate positions of primers used. Intergenic region between genes *bfeU* to *sifE*, are labeled as follows; 1 (Bbr_0220/*bfeU*), 2 (*bfeU*/*bfeO*), 3 (*bfeO*/*sifA*), 4 (*sifA*/*sifB*), 5 (*sifB*/*sifC*), 6 (*sifC*/*sifD*), and 7 (*sifD*/*sifE*). (B) RT-PCR of the intergenic regions from Bbr_0220 to Bbr_0228, intergenic regions are labeled as in (A). (C) β-glucuronidase assay of pNZ272+*bfeU*, pNZ272+*sifA* and pNZ272 (negative control). β-glucuronidase activity is expressed in Miller units. (D) Primer extension (PE) analysis of the transcriptional start site of *bfeU*. (E) PE analysis of transcriptional start site of *sifA*. In both (D,E) the start codon (ATG) is indicated in bold, the transcriptional start site (TSS) is indicated by a black triangle, the proposed −10 and −35 motifs are boxed and a possible operator sequence is displayed in uppercase.

Murine Colonization Experiments

Murine colonization experiments were carried out to assess if *bfeUO*, a high affinity ferrous iron uptake cluster, or *sifABCDE*, a predicted ferric iron/siderophore uptake system, play a role in gut colonization. In order to analyse this the gut colonization capacity of *B. breve* UCC2003, *B. breve* UCC2003-*bfeU*, *B. breve* UCC2003-*sifA* was tested in BALB/c mice. In conventional BALB/c mice with a resident microbiota (i.e. in a competitive environment), WT *B. breve* UCC2003, *bfeU* and the *sifA* insertion mutants were all able to colonize the gastrointestinal tract, as was shown by plating of faecal samples (1×10^5 CFU/g faeces retrieved 15 days after last administration; Figure 2-3). Viable count determinations of the contents of the small intestine, large intestine and cecum of individual mice confirmed these findings. Therefore, the functionality of *bfeUO* and *sifABCDE* did not have any obvious impact on the ability of *B. breve* UCC2003 to colonize the gut of healthy BALB/c mice with a resident microbiota. These results are in agreement with similar work carried out by Christiaen *et al.*, who found that *B. breve* UCC2003-*bfeU* and *B. breve* UCC2003-*sifA* insertion mutant were able to colonize the gastrointestinal tract of nematodes as efficiently as the wild type strain.

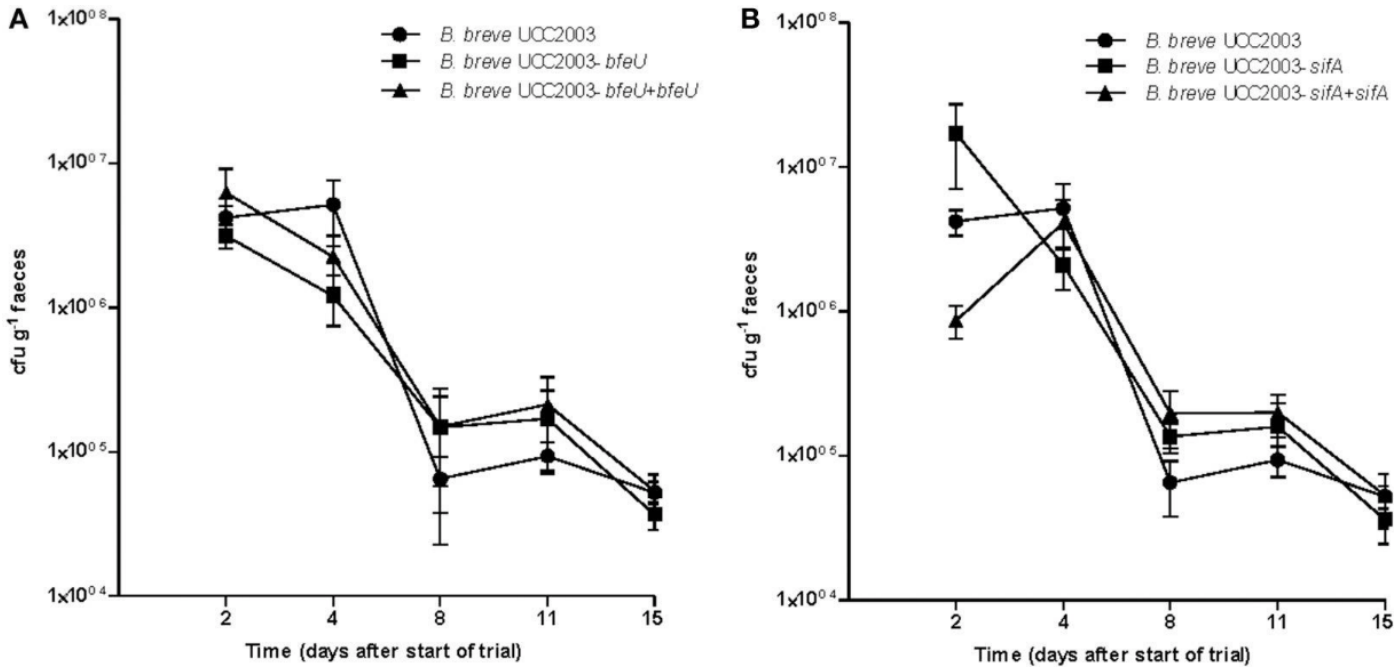


Figure 2-3 Murine colonisation trial:
(A) Illustrates the cfu g^{-1} faeces of *B. breve* UCC2003 (circle) and *B. breve* UCC2003 -*bfeU* (square) and *B. breve* UCC2003 -*bfeU*+pBC1.2+*bfeU* (triangle), while (B) illustrates cfu g^{-1} faeces of *B. breve* UCC2003 (circle), *B. breve* UCC2003-sifA (square), and *B. breve* UCC2003-sifA+pBC1.2+sifA (triangle) administered individually, administration started at day 0 and was continued for 3 consecutive days. Data shown are mean ± SEM. (n = 6).

2.5 Discussion

Bifidobacteria are believed to be able to propagate under low iron conditions and are thought to be efficient scavengers of iron, a notion being supported by the finding that certain bifidobacterial species are found in greater numbers under low luminal iron conditions as compared with normal or high luminal iron conditions (58). Much of the relevant literature to date focuses on the ability of bifidobacteria to internalise and sequester iron, and to proliferate in either high or low concentrations of iron (16, 59-61). The current study was aimed at uncovering the molecular mechanisms and systems responsible for iron uptake and metabolism in *B. breve* UCC2003, as a prototypical representative of its genus.

Severe iron limitation has a profound effect on *B. breve* UCC2003, as illustrated by the plethora of genes whose transcription is altered under such iron restrictive conditions and by the number of genes which were identified in the screening of the random mutant library under iron limiting conditions. This response includes the up-regulation of Bbr_1850, a gene encoding a predicted NADPH-dependent FMN reductase. A similar reductase was identified to be involved in iron metabolism in *Pseudomonas putida*, and it has been speculated that such reductases may be needed in higher amounts to reduce ferric iron during iron limitation (49, 62). Among the *B. breve* UCC2003 genes that were shown to be essential for growth under iron limiting conditions, we identified *apbC* and *apbE*, which are predicted to encode proteins required for Fe-S cluster formation, repair and docking. Mutations in homologs of *apbC* and *apbE* in *Salmonella enterica* result in cellular deficiencies which are reversed by the addition of ferric chloride, which suggests that iron addition compensates for such mutation by increasing repair of oxygen-labile Fe-S clusters (46). Therefore, in the case of *B. breve* UCC2003 it is possible that under iron limiting conditions the loss of *apbC* and *apbE* causes an inability for the cell to construct, repair or to load these Fe-S clusters into one or more essential apo-proteins. This phenomenon, although not studied in full in this paper, would be of interesting for future study.

Screening of the random mutant library and transcriptome analysis led to identification of several genes encoding factors for the transport of cations and siderophore complexes.

From the current study it therefore appears that there are two key uptake systems for bifidobacterial survival under the imposed iron-limiting conditions: BfeUO, a predicted high affinity Fe^{2+} iron uptake system, and SifABCDE, a predicted iron/siderophore uptake system, which potentially binds ferric iron. BfeUO is similar to EfeUOB, an Fe^{2+} uptake system identified in *E. coli*, and the Fet3P-Ftr1P system in *Saccharomyces cerevisiae*. EfeUOB and the Fet3P-Ftr1P iron uptake systems both transport ferrous iron (63, 64). A similar system is present in *Bacillus subtilis*, being responsible for ferrous and ferric iron transport depending on the extracellular conditions and the oxidant supply (65). Homologs of both of these iron uptake clusters are also present on plasmids pIP1206 and pRSB107 from *E. coli* (51, 52).

SifABCDE represents a possible ferric iron/siderophore uptake system, which was found to be important for *B. breve* UCC2003 survival under iron limiting conditions. A relatively small number of siderophore uptake systems have been characterised in Gram-positive bacteria, however, in those characterised there are a number of common features, including a siderophore binding protein (possible SifA), ABC transporter permeases (SifB/C), ATPase (SifD) and a ferric iron reductase (SifE) (66, 67). The identified dipyridyl-sensitive mutants within the *bfeUO* and *sifABCDE* gene clusters exhibited impaired growth in the presence of dipyridyl. Transcriptional analysis of *bfe*UO and *sifABCDE* via RT-PCR, and primer extension analysis found that the gene cluster is organised into two transcriptional units, which are subject to transcriptional induction upon Fe^{2+} iron limitation.

Previously *Bifidobacterium* species have been found to operate a LuxS-mediated system for gut colonization and pathogen protection, which is linked to iron acquisition (68). Similar to the findings of the murine colonization experiments carried out in this study, Christiaen *et al*. found that *B. breve* UCC2003-*bfeU* and *B. breve* UCC2003-*sifA* insertion mutants are able to colonize the GIT of nematodes as efficiently as *B. breve* UCC2003 WT strain. In addition, these authors found that the *B. breve* UCC2003-*bfeU* and *B. breve* UCC2003-*sifA* mutants exhibit a significantly decreased ability to confer protection to *Salmonella*-infected nematodes as compared to the WT *B. breve* UCC2003 in the nematode model (68).

The fact that insertion mutations within these uptake systems (*bfeUO* and *sifABCDE*) did not affect the colonisation efficiency of *B. breve* UCC2003 in the healthy nematode or murine gut leads us to believe that iron availability in a healthy gut is sufficient for colonization of *B. breve* UCC2003, *B. breve* UCC2003-*bfeU* or *B. breve* UCC2003-*sifA*, even though the latter two mutants exhibit an *in vitro* growth deficiency under iron limiting conditions. Therefore, we postulate that either iron is available in the gut in a form which is not taken up by the *bfeUO* or *sifABCDE* or that these systems for iron acquisition may be important for *B. breve* UCC2003 survival during times in which iron is more limiting, for example during GIT infection.

This study has identified a high affinity ferrous iron uptake system, a predicted ferric iron/siderophore uptake system and a number of other genes which are important for *B. breve* UCC2003 survival under iron-limiting conditions. The identification and characterisation of a ferrous iron uptake system and ferric iron/siderophore uptake system in *B. breve* UCC2003 demonstrates that *Bifidobacterium* can utilise both ferrous and ferric iron depending upon its availability with the gastrointestinal tract. This study has also helped to broaden our knowledge regarding *Bifidobacterium* transcriptomic response to iron limitation, with the identification of a number of presumed iron uptake systems, of reductases which may be involved in ferric iron reduction, and of *apbC*/*apbE* which may be involved in Fe-S cluster formation/repair/docking. Taken together, this versatile response of bifidobacteria to iron limitation may be one of the factors that afford such gut commensals a competitive edge among the many microbes within the gastrointestinal tract.

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Chapter 3 Iron starvation increases bile resistance of *Bifidobacterium breve* UCC2003 through enhanced expression of a bile salt hydrolase

3.1 Abstract

Several members of the genus *Bifidobacterium* are considered to be beneficial for human health and have therefore been exploited as functional ingredients in probiotic foods. Probiotic bacteria must be able to resist various environmental stress factors in order to reach and survive in the gastrointestinal environment (where they are believed to exert their beneficial role). The current study was aimed at investigating the transcriptome of *Bifidobacterium breve* UCC2003 global genome response when this strain was grown under iron limitation. The observed transcriptomic changes involved the in/decreased transcription of genes associated with carbon and nitrogen metabolism, genes predicted to be responsible for iron uptake, genes encoding DPS proteins (which are predicted to be involved in iron storage/DNA protection) and Fe-S cluster associated proteins, as well as a gene (*bshB*) encoding bile salt hydrolase. Insertional mutagenesis and survival assays demonstrate that iron limitation imposed on *B. breve* UCC2003 evokes increased resistance to bile stress, being partly due to the iron-inducible transcription of *bshB*. This study links bile salt hydrolase activity of *B. breve* UCC2003 to its ability to survive the adverse effects of bile salt and suggests that this strain uses iron availability as a signal to adapt to the gut environment.

3.2 Introduction

Species of the genus *Bifidobacterium* represent Gram-positive, bifid-shaped, strictly anaerobic bacteria, which are commonly found among the intestinal microbiota of mammals, including humans (1). Certain bifidobacterial strains have been commercially exploited as probiotics, defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on their host (2). Indeed, bifidobacteria have been included as bioactive ingredients in a number of functional foods, where they have been incorporated as a single strain or in combination with other probiotic microorganisms and/or prebiotic mixtures. The purported health-promoting activities are diverse and include, among others, maturation and modulation of the immune system, vitamin production, inhibition of certain enteropathogens, such as *Clostridium difficile*, and amelioration of gastrointestinal diseases (3-7). These gut commensals have to cope with various environmental challenges during their transit through the stomach, small intestine and colon, such as variations in pH, nutritional limitations (including micronutrients such as iron) and exposure to bile salts, the latter being the main component of bile (8).

Bile is produced in the liver and secreted into the duodenum, at a rate of approximately 750 ml per day, and elicits powerful antimicrobial activity against many bacteria, including *Bifidobacterium* (9). Bile plays an important role in lipid solubilisation and emulsification, and due to its strong lipophilic nature acts as an antimicrobial compound by disorganising the cellular membrane (10). Therefore, the ability of probiotic and commensal bacteria to tolerate bile is presumed to be important for their survival and subsequent colonization of and persistence in the gastrointestinal tract (11).

Bacteria employ a number of strategies to cope with bile stress, ranging from the production of exopolysaccharides, expression of efflux pumps for the cytoplasmic removal of bile salts, and the expression of bile salt hydrolases (BSHs; E.C.3.5.1.24) which deconjugate bile salts (12, 13). BSHs belong to the cholloglycine hydrolase family, have been identified in all major bacterial divisions and in two domains of life (*Bacteria* and *Archaea*), and are thought to have evolved as an adaption to bile-containing environments, consequently being absent in microbes which do not encounter bile salts (14). The BSH enzyme catalyses the removal (or de-conjugation) of the amino acid

taurine or glycine from the C-24 position of conjugated bile salts (13, 15). This ability to hydrolyse bile salts has been associated with a number of health benefits, including control of obesity, hypercholesterolemia, and metabolic syndrome (16). Furthermore, the ability to hydrolyse bile has been included in the criteria for probiotic strain selection (17). Bifidobacteria display a varying ability to resist bile stress (18-20), while BSH activity has been reported for a number of bifidobacterial strains (21-26). One spontaneous mutant and two UV-induced, BSH-negative mutants, all derived from *Bifidobacterium longum* SBT2928, have previously been isolated (21), although, to the best of our knowledge no site-directed mutants in a BSH-encoding gene have been created in any bifidobacterial strain. Furthermore, no in-depth characterisation was carried out for these *B. longum* SBT2928-derived BSH-negative mutants, leaving distinct knowledge gaps regarding the precise role of BSH in resisting bile stress, and regarding the environmental factors that control BSH expression.

Iron is essential for almost all living organisms; it is involved in a wide variety of metabolic processes due to its ability to act as a prosthetic component for a number of essential enzymes as a biocatalyst and/or electron carrier. The importance of iron for particular species of *Bifidobacterium* has been investigated in a number of publications (27-30). Recently, we identified genes, which are essential for growth and survival under iron-limiting conditions, and which include a number of uptake systems, Fe-S cluster associated proteins, and other genes of diverse function (31).

In the current study, transcriptional analysis of the prototypical human gut commensal *Bifidobacterium breve* UCC2003 was explored when grown under ferrous (Fe^{2+}) and/or ferric (Fe^{3+}) iron starvation. Various genes exhibit up/down-regulation under such conditions, in particular the *bshB* gene. Our results suggest that transcription of the *bshB* gene in *B. breve* UCC2003 is responsive to iron limitation and show that iron starvation results in an increased resistance to bile stress due, in part, to iron-inducible transcription of the *bshB* gene. These findings are highly interesting as it links *bshB* to bile resistance, while it also correlates the bifidobacterial response to iron limitation to bile stress/resistance. This may have practical relevance with respect to the preparation, manufacture, delivery and storage of bifidobacteria.

3.3 Materials and Methods

Bacterial Strains and Culture Conditions

Bacterial strains and plasmids used in this study are listed in Table 3-1. *B. breve* UCC2003 was routinely grown at 37°C in either de Man Rogosa and Sharpe medium (MRS medium; Difco, BD, Le Pont de Claix, France), modified de Man Rogosa and Sharpe (mMRS) medium made from first principles (32), or reinforced clostridial medium (RCM; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) supplemented with 0.05 % cysteine-HCl. For iron-limitation experiments, filtered RCM (fRCM) was prepared in de-mineralised water, supplemented with the maximal tolerable concentration (MTC) of iron chelators 2,2-dipyridyl, ciclopirox olamine or phenanthroline, as determined previously (33).

Table 3-1. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant features	Reference or source
Strains		
B. breve		
UCC2003	Isolate from nursling stool	(83)
UCC2003-*bsh*	pORI19-*tetW*-*bsh* insertion mutant of UCC2003; Tet^r.	This study
E. coli		
EC101	Cloning host for pORI19 for insertional mutagenesis; $repA^+$ Km^r	(82)
Plasmids		
pORI19	Em^r, $repA^-$, ori^+, cloning vector	(82)
pORI19- *bsh*	pOR19 harbouring internal fragment of *bsh* (*Bbr_0971)*	This study

Em^r, Km^r and Tet^rresistance to erythromycin, kanamycin and tetracycline respectively.

Nucleotide Sequence Analysis

Sequence data were obtained from Artemis-mediated (35) genome annotations of *B. breve* UCC2003 (36). Data base searches were carried out using non-redundant sequences accessible at the National Centre for Biotechnology Information internet site (http://www.ncbi.mlm.nih.gov) using BLAST (37, 38). The Universal Protein Resource (UniProt) is a comprehensive resource for protein sequence and annotation data (39) and was used to acquire functional information on the proteins of interest.

DNA Manipulations

DNA manipulations were carried out as previously reported (40). Restriction enzymes and T4 DNA ligase were obtained from Roche Diagnostics, and were used according to the manufacturer's instructions. PCRs were performed using Extensor Long Range PCR Enzyme master mix (Thermo Scientific, Glouchester, UK). Synthetic oligonucleotides were synthesized by Eurofins (Ebersberg, Germany) and are listed in Table 3-2. PCR products were purified by the use of a High-Pure PCR product purification kit (Roche, Basel, Switzerland). Plasmid DNA was introduced into *Escherichia coli, Lactococcus lactis* and *B. breve* by electroporation and large-scale preparation of chromosomal DNA from *B. breve* was performed as described previously (41). Plasmid DNA was obtained from *B. breve, L. lactis* and *E. coli* using the Roche High Pure plasmid isolation kit (Roche Diagnostics, Basel, Switzerland). An initial lysis step was performed using 30 mg ml^{-1} of lysozyme for 30 min at 37°C as part of the plasmid purification protocol for *B. breve*.

Construction of *B. breve* UCC2003 insertion mutant in the *bshB* gene

An internal fragment of the *bshB* gene, corresponding to locus tag Bbr_0971 (fragment encompasses 387 bp, representing codon numbers 67 to 196 of the 317 codons of this gene), was amplified by PCR using *B. breve* UCC2003 chromosomal DNA as a template and primers pair Bbr_0971F and Bbr_0971R (Table 3-2). The insertion mutant was constructed as described previously (42). Site specific recombination of tetracycline-resistant mutants was confirmed by colony PCR using primer combinations tetW_F and tetW_R to verify the integration of the tetracycline cassette, and bshB_confirm, a primer located upstream of the selected internal fragments of Bbr_0971 in combination with primer tetW_F to confirm that homology-mediated integration had occurred at the expected chromosomal location. One of the thus identified *bshB* mutants, designated here as *B. breve* UCC2003-*bshB*, was selected for further analysis.

Table 3-2 Oligonucleotide primers used in this study

Purpose	Primer	Sequence
Cloning of internal fragment of *bshB* in pORI19	*bshB*_IM_F	tagcattctagacgactgcgccaatgaacatg
	*bshB*_IM_R	tagcataagcttggttcagccatctcgttgc
Confirmation of site specific homologous recombination	*bshB*_confirm	ctggcgcaatacggaaccg
	*tetW*_F	tcagctgtcgactgctcatgtacggtaag
	*tetW*_R	gcgacggtcgaccattaccttctgaaacat
qPCR primer	*bshB*_PL_14_F	ggtggcacgtaatttcgact
	*bshB*_PL_14_R	ccagtgcagcagagactcct
	*bshB*_PL_18_F	ggtggcacgtaatttcgact
	*bshB*_PL_18_R	gatgaaccagtgcagcagag
	*glnE*_PL_90_F	tcctggatgagcggagtaat
	*glnE*_PL_90_R	cgtcgtacacgtcggtcat
	*glnE*_PL_10_F	accgaatccggctacctc
	*glnE*_PL_10_R	aatcgcgagttggacagg

Transcriptome Analyses

Global gene transcription of *B. breve* UCC2003 in response to iron starvation was determined by means of microarray analysis as follows: an overnight culture of *B. breve* UCC2003 cultured in RCM was inoculated at 1 % into filtered RCM supplemented with either 275 µM dipyridyl, 85 µM ciclopirox olamine or 100 µM phenanthroline, and incubated until an OD_{600nm} of 0.6 was achieved. Cells were then harvested by centrifugation at 6000 rpm for 5 min at room temperature and immediately frozen at -80°C prior to RNA isolation. DNA microarrays containing oligonucleotide primers representing each of the 1,864 annotated genes on the genome of *B. breve* UCC2003 were designed by and obtained from Agilent Technologies (Palo Alto, CA, USA). Cell disruption, RNA isolation, RNA quality control, and cDNA synthesis and labelling were performed as described previously (43). The labelled cDNA was hybridized using the Agilent Gene Expression hybridization kit (part number 5188-5242) as described in the Agilent Two-ColorMicroarrayBased Gene Expression Analysis v4.0 manual (G4140-90050). Following hybridization, the microarrays were washed in accordance with Agilent's standard procedures and scanned using an Agilent DNA microarray scanner (model G2565A). The generated scans were converted to data files with Agilent's Feature Extraction software (v9.5). The DNA microarray data sets were processed as previously described (44-46). Differential expression tests were performed with the Cyber-T implementation of a variant of the student t-test (47). A gene was considered to exhibit a significantly different expression level relative to the control when $p < 0.001$ and an expression ratio of > 2.5 or < 0.25. The microarray data obtained in this study have been

deposited in NCBI's Gene Expression Omnibus database and are accessible through GEO series accession number GSE110080.

Q-PCR Analyses

Quantitative RT-PCR was used to assess the expression levels of *bshB* and *glnE* under iron-limiting conditions utilising one of three different iron chelators; ciclopirox olamine, phenanthroline and dipyridyl. Primers for qPCR were designed utilising the Universal ProbeLibrary (Roche); these primer pairs, listed in Table 3-2, were then used for qPCR with the LightCycler 480 System (Roche, Basel, Switzerland). Cells were cultured and prepared for RNA isolations as above for microarray analysis. RNA was treated with DNA-*free* (Ambion), cDNA was synthesized using Transcriptor reverse transcriptase (Roche, Basel, Switzerland). Q-PCR was then carried out utilising 2x Sensi fast probe No-Rox mix (Bioline), Protector RNase inhibitor (Roche, Basel, Switzerland) was also utilised to inhibit RNase activity in the reactions. The *2-ΔΔC* method was used to calculate relative changes in gene expression; the *2-ΔΔC* method reflects the difference in threshold for each target gene relative to *rpnA.* The *rpnA* gene was selected as a housekeeping gene as its transcription is constitutive (43, 48). *P* values of <0.05 were considered significant.

Phenotypic Assays

Bile salt hydrolase activity-plate assay

Qualitative bile salt hydrolase activity tests – by means of plate assays - were carried out as previously described (49) with some minor modifications. Briefly, *B. breve* UCC2003 and its isogenic *bshB* mutant derivative (*B. bre*ve UCC2003-*bshB*) were cultivated o/n in RCM broth supplemented with the appropriate antibiotics. The following morning 10 µl of culture was spot plated on mMRS medium supplemented with 1 % glucose and either 0 %, 0.1 % or 0.2 % oxgal (Sigma). Plates were then incubated anaerobically at 37°C for 48 hours. Plates were then inspected for the presence or absence of a halo indicating bile salt hydrolase activity.

Iron limitation and Bile Salt Survival Assay

The Miles and Mersa method was employed to assess survival of *B. breve* UCC2003 and *B. bre*ve UCC2003-*bshB*, which were grown under normal or iron-limiting conditions prior to bile salt exposure (50). *B. breve* UCC2003 and *B. breve* UCC2003-*bshB* mutant were first cultivated overnight (o/n) in RCM broth supplemented with the appropriate antibiotics.These o/n cultures were then subcultured in fRCM broth supplemented with varying concentrations of the iron chelator dipyridyl (0 µM, 25 µM, 50 µM and 100 µM) and incubated o/n under anaerobic conditions at 37°C. Cells contained in 1 ml of each culture were then collected by centrifugation and re-suspended in fRCM supplemented with increasing concentration of porcine bile (PB) (0, 0.5, 1 and 2 % v/v) for 20 minutes, after which cells were serially diluted in PBS buffer to 10^{-7} and spot plated on RCA. Plates were incubated anaerobically at 37°C for 48 hours. CFU/ml was calculated using the following formula: CFU/ml = (no. of colonies x dilution factor) / volume of culture plated.

3.4 Results

Transcriptome Response of *B. breve* UCC2003 to Iron Starvation

Previous studies have investigated the transcriptome response of *B. breve* UCC2003 to acute and severe iron limitation by exposing exponentially growing cells to a high (i.e. growth-arresting; 700 μM) concentration of the ferrous iron chelator dipyridyl (28, 31). In the current study, we wanted to investigate the global transcriptional response of *B. breve* UCC2003 when grown in the presence of a non-lethal level of ferrous and/or ferric iron chelators. For this purpose, *B. breve* UCC2003 was grown to an OD_{600nm} of 0.6 in the presence of the Maximal Tolerable Concentration (MTC) of either dipyridyl which specifically binds Fe^{2+}, ciclopirox olamine, which is a Fe^{3+}-specific chelator, or phenanthroline, an Fe^{2+}/Fe^{3+} chelator. MTC values had previously been determined (33). The transcriptome response of *B. breve* UCC2003 to these Fe^{2+}/Fe^{3+} starvation conditions is summarised in the heat map displayed in Figure 3-1. The chronic iron starvation conditions caused enhanced transcription of the *bfeUO* operon, which encodes a predicted high affinity ferrous iron uptake system, and of *sifABCDE*, which encodes a predicted ferrous iron/siderophore uptake system. Transcription of both of these operons had previously been found to be important for *B. breve* UCC2003 survival when actively growing cells were exposed to an acute, growth-arresting level of iron limitation (31).

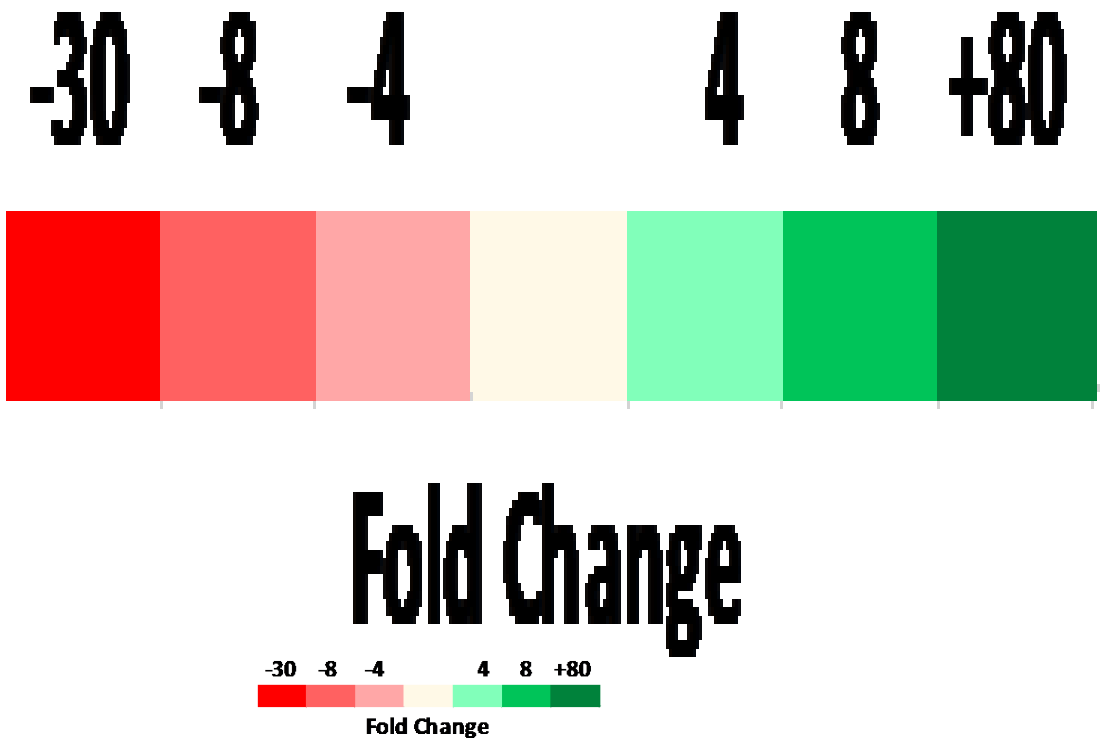


Figure 3-1. Heat map summarising global genome response to iron starvation
B. breve UCC2003 global genome response iron starvation with dipyridyl, ciclopirox olamine and phenanthroline which act as chelating compounds for Fe^{2+} / Fe^{3+}, Fe^{2+} and Fe^{3+} iron respectively. Colour intensity is derived from mean relative expression fold changes of dye swap results, green for up-regulated, red for down-regulated and cream for no change.

This analysis also found that the tracription of two homologous genes predicted to encode DNA protecting proteins (DPS) (corresponding to locus tags Bbr_0016 and Bbr_0032) were increased when *B. breve* UCC2003 was grown under iron starvation conditions. Furthermore, 14 gene clusters associated with carbohydrate uptake/utilisation (and corresponding regulatory proteins), were differentially regulated under all tested iron limitation reactions, as well as a number of genes associated with cysteine/methionine metabolism and nucleotide biosynthesis and repair were found to be differentially transcribed (Figure 3-1).

Growth under the applied iron-limiting conditions also led to the increased transcription of two Fe-S clusters related proteins; Bbr_0669 (designated here as *apbC*) and Bbr_1825 (designated here as *apbE*). Transcription of *apbC* was increased 12.6 fold under Fe^{2+}/Fe^{3+} limitation and 5.4-fold under Fe^{2+} limtiation conditions, while *apbE* was up-regulated 4.7-fold under Fe^{2+}/Fe^{3+} limitation. The *apbC* gene encodes a protein with similarity to a P-loop NTPase, which binds and transfers Fe-S clusters to cytosolic apo-proteins (51, 52). The *apbE* gene is homologous to *apbE* of *Salmonella enterica*, and the product of this latter gene is known to play a role in the assembly and repair of Fe-S clusters (53, 54). Both *apbC* and *apbE* have previously been identified as important for growth/survival of *B. breve* UCC2003 under iron limiting conditions by means of a random mutagensis approach (31).

A number of genes were differentially expressed in the presence of the Fe^{3+} chelator, yet not in the presence of the Fe^{2+} chelator. These include three out the four genes encoding MarR-type transcription factors (TFs) (corresponding to locus tags Bbr_0199, Bbr_1435 and Bbr_1826), which are encoded on the genome of *B. breve* UCC2003. MarR-type TFs have been characterised in *E. coli*, and are believed to be involved in the regulation of multiple efflux pumps, being responsive to the presence or absence of cations (55). For example, transcription of the gene corresponding to locus tag Bbr_0199 was up-regulated 7.3-fold when grown under Fe^{3+} limiting conditions and was slightly but not significantly down-regulated (1.5-fold) under Fe^{2+} limiting conditions. Bbr_0048 and Bbr_0049 were also found to be up-regulated under Fe^{2+} limiting conditions, these genes encode a predicted cation transport system and a predicted diguanylate cyclase, respectively. Transcription of the latter two genes was only significantly up-regulated under Fe^{3+}

limiting conditions, yet not during Fe^{2+} limitation. A number of genes without any predicted function (e.g. those corresponding to locus tags Bbr_0264, Bbr_1785 and Bbr_1905) also exhibited differential transcription under particular iron-limiting conditions (Fe^{2+}, Fe^{3+} or Fe^{2+} /Fe^{3+} limitation; see Figure 3-1)

Our current analysis furthermore found that transcription of one particular gene, designated here as *bshB* (Bbr_0971) and representing a homolog of the previously characterised *bsh* gene from *B. longum* SBT2928 (21), was increased when *B. breve* UCC2003 was grown under chronic, but non-growth arresting ferric and/or ferrous iron starvation conditions (see Figure 3-1). This transcriptional induction of *bshB* was verified by q-PCR analysis (which revealed induction levels of 4, 7, 16-fold for Fe^{2+}, Fe^{3+} and Fe^{2+}/Fe^{3+} iron-limiting conditions, respectively) (Figure 3-2).

Presumably co-transcribed and (thus) up-regulated with the *bshB* gene under iron starvation is a gene adjacent to *bshB*, designated here as *glnE* (Bbr_0972). The *glnE* is predicted to encode a nitrogen cascade regulatory protein, which in *Mycobacterium tuberculosis* has been found to regulate the activity of the glutamine synthethase (GS), an enzyme which is responsible for the conversion of L-glutamate to L-glutamine (56). This analysis is in line with findings in other bacteria, as both GSs (Bbr_0670 and Bbr_1270) in *B. breve* UCC2003 were down-regulated when *glnE* levels are high (57-59). Coinciding with this was the up-regulation of a possible glutamine uptake system (Bbr_0817-Bbr_0814). The heat map in Figure 3-1 illustrates the profound, yet perhaps indirect effect of iron starvation on nitrogen metabolism in *B. breve* UCC2003 with as many as seven genes involved in nitrogen uptake and metabolism being differentially transcribed.

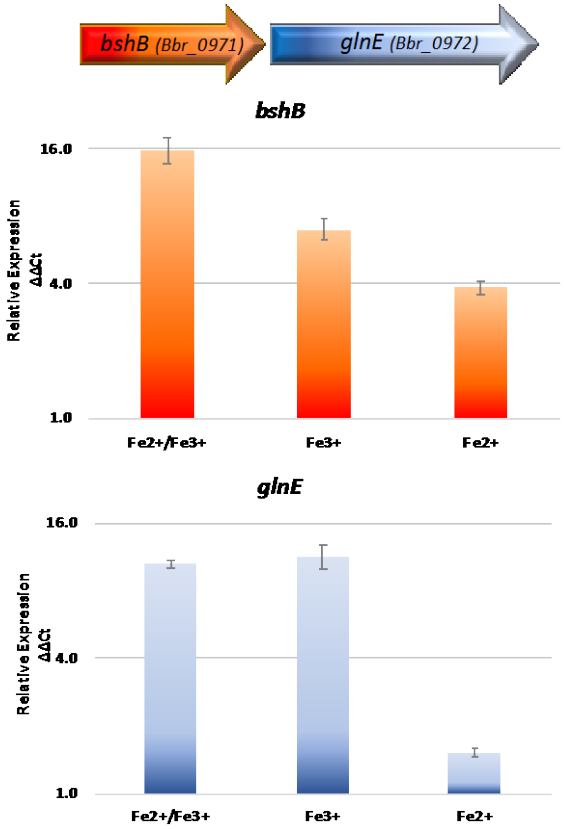


Figure 3-2 Q-PCR analysis of *bshB* and *glnE* to ferrous and/or ferric iron starvation:
Q-PCR analysis of *bshB* and *glnE* to ferrous and/or ferric iron starvation: Relative gene expression of *bshB* and *glnE* when *B. breve* UCC2003 is exposed to Fe^{2+}/Fe^{3}, Fe^{2+} and Fe^{3+} iron starvation. The Y-axis represents the $\log^2$ of fold changes which were calculated by the ΔΔCt method in which the Ct values of each gene were normalized to the level of a housekeeping gene (*rpnA*) in control RNA. Each value is the mean ± SD of three independent experiments.

Disruption of *bshB* gene and BSH activity in *B. breve* UCC2003

In order to investigate if disruption of the *bshB* gene would affect the ability of *B. breve* UCC2003 to survive bile stress, an insertional mutant in the *bshB* gene of *B. breve* UCC2003 was constructed (designated here as *B. breve* UCC2003-*bshB*, see Materials and Methods section). A qualitative BSH activity assessment of *B. breve* UCC2003 and *B. breve* UCC2003-*bshB* was then carried out by a bile salt plate assay, where BSH activity is visually observed as an opaque halo of precipitated de-conjugated bile salt around a colony (61). Figure 3-3 illustrates that growth of *B. breve* UCC2003 on mMRS supplemented with bile salt caused the formation of a halo of bile acid precipitate while

growth on mMRS medium alone did not. In contrast, *B. breve* UCC2003-*bshB* was unable to produce such a halo of bile acid precipitate, thus indicative of its inability to de-conjugate bile acids.

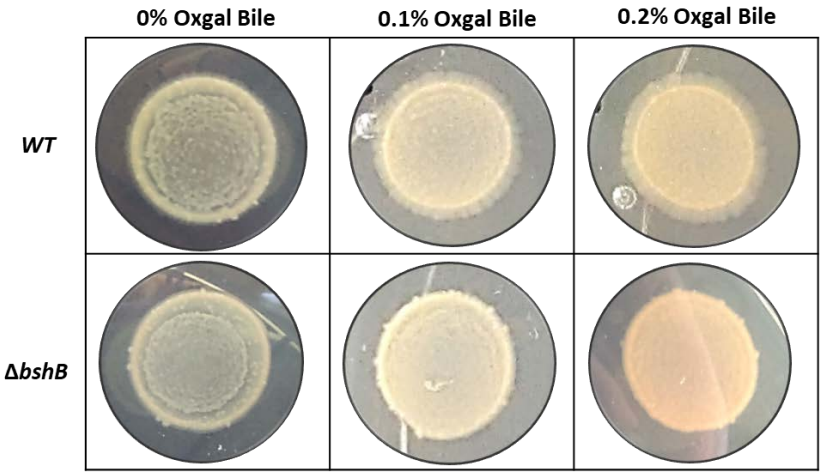


Figure 3-3 Bile salt hydrolase Assay
Bile salt hydrolase activity of *B. breve* UCC2003 WT and the *B. breve* UCC2003-*bsh* mutant on dMRS agar supplemented with increasing concentration of ox-gall (0, 0.1 and 0.2 %). The precipitation or the formation of an opaque halo around the colonies is indicative of bile salt hydrolase activity.

Iron Starvation and its Effect on Bile Salt Survival

A bile salt survival assay was performed in order to investigate the differences in bile salt resistance/sensitivity between WT and *B. breve* UCC2003-*bshB* mutant following exposure to iron starvation. Briefly, o/n cultures were inoculated (1 %) into fRCM broth containing increasing concentrations of the iron chelator dipyridyl (0, 25, 50 and 100 µM), which was used to impose no or different levels of iron starvation (and also to cause induced transcription of the *bshB* gene). These cultures were then collected by centrifugation and re-suspended in fRCM containing increasing concentrations of porcine bile (0, 0.2, 0.5 and 1 %) for 20 minutes and subsequently spot plated on RCA. Cell survival was measured by viable cell count. In this assay (Figure 3-4), *B. breve* UCC2003 exhibits increased resistance to bile stress when previously exposed to iron starvation. The *B. breve* UCC2003-*bshB* mutant also exhibits slight increase in its survival under iron limitation, yet had decreased survival at all concentrations of bile tested as compared with the WT. This is most apparent at 2 % porcine bile where the WT strain was recovered at 8.83 x 10^6 CFU/ml, while no growth/survival was observed for the *B. breve* UCC2003-*bshB* mutant.

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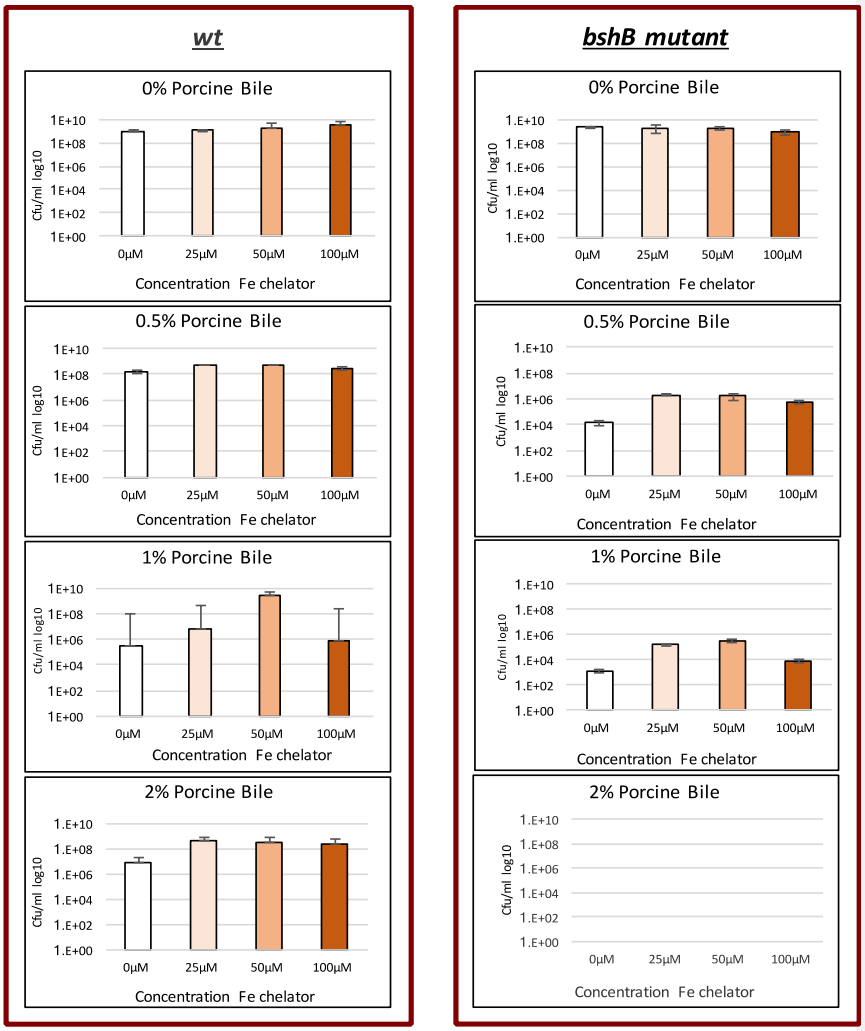


Figure 3-4 Iron starvation and its effect on survival when exposed to bile stress
B. breve UCC2003 *WT* and *B. breve* UCC2003-*bshB* grown o/n in fRCM broth supplemented with increasing concentration of iron chelator dipyridyl (0, 25, 50 and 100 µM), followed by exposure to 0 %, 0.5 %, 1 % or 2 % porcine bile for 20 minutes. Cell survival was measured by viable cell count and is expressed as CFU/ml on a $\log_{10}$ scale. Each value is the mean ± SD of two independent experiments.

3.5 Discussion

Transcriptomic analyses carried out in this study demonstrate that the transcriptomic patterns of *B. breve* UCC2003 obtained during growth under chronic, yet non-growth arresting iron starvation conditions is distinct from those obtained when exponentially growing cells were exposed to acute and growth-arresting iron limitation. In the latter case *B. breve* UCC2003 responds by increased transcription of a range of predicted cation uptake systems, while in the former *B. breve* UCC2003 induced transcription of just two (predicted) iron uptake systems, *bfeUO* and *sifABCDE*, possibly indicating their importance for the long-term survival of *B. breve* UCC2003 to iron starvation. Growth under chronic iron starvation conditions was also shown to induce transcription of two genes encoding predicted DPS proteins, which in other bacteria are known to be crucial in coping with oxidative stress due to its dual biochemical functions, DNA binding and ferroxidase activity (62). This dual function allows DPS proteins to both non-specifically bind DNA, thereby blocking it from damage and to counter the toxic effects of both iron and hydrogen peroxide through their ferroxidase activity. Structural studies in *E. coli* and *Listeria* have also found that each ferritin-like DPS protein had maximum iron binding capacity of 500 iron atoms making it an important factor in maintaining a sufficient intracellular iron pool (63-65). It has also been shown for *Listeria monocytogenes* that the expression of a DPS-like protein is inducible under iron-limiting conditions (66).

Previous studies have suggested that bile salt hydrolase activity is involved in resisting the toxic bactericidal effects exerted by bile stress (61, 67). *In vitro* experiments in a number of bifidobacterial strains, including *B. longum*, *B. animalis* and *B. breve*, suggest that the corresponding bile salt hydrolase-encoding gene is constitutively transcribed, rather than being induced by the presence of bile salts (68-71). However, a study carried out on the proteome of *B. longum* in the GIT of rabbits, found that transcription of the *bsh* gene was increased under *in vivo* conditions, with the authors suggesting that BSH expression in this strain may be responding to factors other than bile (72). The environmental signals which induce transcription of the bifidobacterial BSH-encoding gene are currently unknown. Our findings reveal that subjecting *B. breve* UCC2003 to chronic, non-lethal iron starvation induces *bshB* transcription, and that this induction is in turn associated with an enhanced ability of this strain to cope with bile stress.

This phenomenon may be explained in a number of ways, the first possibility is that the transcription factor(s) controlling the expression of the *bshB* gene may be directly/indirectly responding to iron limitation and consequently activating or de-repressing *bshB* transcription. Another possibility is that the pre-exposure of *B. breve* UCC2003 to iron limitation may have allowed the strain to gain enhanced protection to bile stress in a phenomenon known as cross-adaption (13, 73).

Within the *Bifidobacterium* genus the *bsh* gene has been found to be co-located with a *glnE* gene encoding a putative glutamate:ammonia-ligase adenylyltransferase. The protein encoded by *glnE* homologs in other bacteria is known to control the activity of glutamine synthetase by adenylylating the latter protein, which in turn modulates its glutamine producing activity (74), and thus plays a crucial role in nitrogen metabolism. The co-location and transcription of *bshB* and *glnE* may indicate that the *bsh* gene is involved in or responsive to nitrogen metabolism/starvation (60). However, our findings suggest that the co-location perhaps may be due to their mutual requirement for iron limitation as a signal for transcriptional induction. Supporting this possibility is the fact that glutamine synthases are metalloproteins which require cations such as Fe^{2+} as a metal co-factor (75).

A previous study carried out in *E. coli* has found that the presence of bile salts causes increased transcription of a number of genes associated with iron scavenging and metabolism (76). These findings indicate that *E. coli* utilises bile as an environmental signal to adapt to the specific conditions found in (different parts of) the GIT including, amoung others, iron limitation. We demonstrate here that *B. breve* UCC2003's ability to express the *bshB* is very important for its survival under bile stress conditions. What's more, *B. breve* UCC2003 induces *bshB* transcription in response to iron starvation and the latter may act as an environmental signal for *B. breve* UCC2003 to respond to bile stress. Increasing BSH activity under iron chelation conditions seems to result in enhanced survival. However, this phenomenon seems to be concentration dependent, as higher levels of iron chelation elimante this protective effect and this is reflected in the results, where exposure to 100µM iron chelator effects growth/survival negatively. Iron chelation was also found to increase the survival of the *B. breve* UCC2003-*bshB* mutant altough to a lesser degree. Consequently, we must conclude that this increased survival

phenotype identified under iron chelation conditions may also be a results of the increased expression of other protection systems (for example the DPS proteins identified in this study which may also endow *B. breve* UCC2003 with increased survival to the toxic effects of bile salts).

A number of publications have found that bile has an inherent ability to acts as an iron chelator and suggest that bile salts, and in particular cholate, taurocholate, glycocholate, chenodeoxycholate, and deoxycholate, are able to bind ferrous iron (77-79). Furthermore, it has been shown that both taurocholate and glycocholic acid can form soluble Fe^{2+}-bile salt complexes, leading to increased Fe^{2+} uptake in the host (78, 80, 81). Bacteria may be taking advantage of this characteristic of bile through the use of the BSH protein which de-conjugates bile salts, thereby releasing taurine and glycine, and perhaps any Fe^{2+} that may be complexed with the bile salt.

In conclusion, this study has revealed the profound impact of prolonged iron starvation on the transcriptional profile of *B. breve* UCC2003, including the increased expression of iron uptake systems, DPS proteins, Fe-S cluster associated proteins, activation of a nitrogen cascade regulatory protein *glnE* and increased expression of the *bshB* gene, which appears to be crucial to cope with bile stress. Notably, the expression of *bshB* in *B. breve* UCC2003 is vital for the survival of this organism under bile stress conditions. Interestingly, *B. breve* UCC2003 appears to utilise iron limitation as a signal to adapt to the changing conditions of the GIT (such as bile stress). To the best of our knowledge this represents an environmental signalling system that has not been reported for other bacteria, although we currently do not know how this iron starvation signal is perceived or how it causes increased transcription of the *bsh* gene (and other regulated genes).

3.6 References

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Chapter 4 Transcriptional control of central carbon flux in *Bifidobacterium breve* UCC2003 by two functionally similar, yet distinct LacI-type regulators

Noreen Lanigan and Emer Kelly contributed equally to this work. Noreen Lanigan carried out all work with respect to the BifR1 protein/mutant, while Emer Kelly carried out all work with respect to the BifR2 protein/mutant. Analysis of results and writing of this chapter was also jointly contributed to by Noreen Lanigan and Emer Kelly.

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4.1 Abstract

Bifidobacteria resident in the gastrointestinal tract (GIT) are subject to constantly changing and stressful environmental conditions, such as bile exposure and nutrient starvation. Adaptation to these environmental conditions requires rapid adjustments in gene expression. Here, we show that two predicted LacI-type transcription factors (TFs), designated BifR1 and BifR2, are involved in regulating the central, carbohydrate-associated metabolic pathway (the so-called phosphoketolase pathway or bifid shunt) of *Bifidobacterium breve* UCC2003. These TFs not only control the transcription of genes involved in the bifid shunt and each other, but also appear to commonly and directly affect transcription of other transcriptional regulator-encoding genes, as well as genes related to uptake and metabolism of various carbohydrates. This complex and interactive network of BifR1/BifR2-mediated gene regulation provides novel insights into the governance of overall cell metabolism in bifidobacteria.

4.2 Introduction

Humans and other mammals harbour complex microbial consortia within their gastrointestinal tract (GIT), commonly referred to as the gut microbiota. The bacterial communities within the GIT have enjoyed intense scientific scrutiny and are believed to be predominantly made up of members of the phyla Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria (1, 2). Of particular interest to the current study is the genus *Bifidobacterium*, a member of the Actinobacteria phylum. *Bifidobacterium* members represent Gram-positive, non-motile, non-spore forming, saccharoclastic anaerobes that commonly colonize the gut of a wide variety of animals, including birds, insects and mammals. Bifidobacteria have consequently been isolated from a number of sources including human and animal faeces and/or intestinal tracts, reproductive tract, oral cavity, sewage and fermented foods. Several bifidobacterial strains/species are called probiotic, which means that when such bacteria are administered in adequate and viable numbers, they may confer a health benefit to their (human) host (3). These health benefits include, among others, modulation of the host immune response (4), mitigation of lactose intolerance, the ability to lower serum cholesterol levels in humans (5-7), pathogen inhibition/exclusion (8) and propholaxis of tumour growth in certain cancers (e.g. colorectal cancer) (9-11). Bifidobacteria are one of the first and predominant colonisers of the (healthy, full-term and breast-fed) infant GIT and they often persist throughout adulthood, although their relative abundance is lower (compared to the infant gut), with a further reduction in the elderly gut (12-14).

It is estimated that approximately 8–15 % of the bifidobacterial coding capacity is dedicated to carbohydrate uptake and metabolism, were the observed variability is dependent on the species and the functional assignment of the genes included in the analysis (15, 16). These estimates are consistent with the well-documented ability of bifidobacteria to utilise a diverse array of carbohydrate sources (15). This remarkable genetic dedication to a particular metabolic behaviour probably reflects the importance of carbohydrate utilisation for bifidobacterial gut colonization and persistence. Just as important is their ability to control expression of the genes involved in the associated metabolic pathways. Being able to regulate which genes are expressed allows (bifido)bacteria to only express those genes that are relevant in order to prevent wasting energy and resources on something which is unnecessary. As a classical example, the *lac*

operon of *Escherichia coli* is only switched on if the substrate lactose is present and if a better (i.e. more energy generating) substrate such as glucose is absent (17).

Additionally, regulation of carbon metabolism enables bifidobacteria to balance their energy and biosynthetic demands, thereby allowing optimal growth and redox balance control, as based on environmental conditions. This control is extremely important for bifidobacteria, as carbohydrate metabolism is their sole mechanism to produce ATP by substrate phosphorylation (18), while metabolic intermediates of carbon metabolism (such as pyruvate) are used as precursors for a range of biosynthetic processes (e.g. biosynthesis of fatty acids, amino acids, and nucleotides).

Carbohydrate metabolism of bifidobacteria is performed by a rather unique pathway, the so-called phosphoketolase pathway or bifid shunt, which allows this taxonomic group to produce more energy in the form of ATP from carbohydrates than fermentative pathways employed by other bacteria (19). From 1 mol of glucose the bifid shunt theoretically yields 2.5 mol ATP, along with 1.5 mol of acetate and 1 mol of lactate (20), whereas for example homofermentative lactic acid bacteria (LAB) produce 2 mol of ATP and 2 mol of lactic acid per mol of glucose (21). This superior ATP production ability is due to the presence of a phosphoketolase called X5P/F6P phosphoketolase (XFPK) which is unique in exhibiting comparable affinities for both xylulose 5-P (X5P) and fructose 6-P (F6P) (22). It directly converts X5P and F6P to acetyl phosphate (along with erythrose-4-phosphate and D-glyceraldehyde-3-phosphate) without using ATP (Figure 4-3). Acetyl phosphate is subsequently converted into acetate to generate ATP. It was the identification of this phosphoketolase pathway that led to the reclassification of bifidobacteria as a separate taxonomic group, being distinct from LAB (23). All members of the *Bifidobacteriaceae* family are said to have homologs of the *xfpK* gene, and thus employ the bifid shunt (24). Phylogenetic and protein modelling studies have recently suggested that the XFPK from bifidobacteria is specifically related to that found in members of the Coriobacteriales order and that the XFPK gene was horizontally transferred between (an ancestor of) these two groups (25-27). Another interesting difference between bifidobacteria (and now possibly Coriobacteriales) and other bacteria is that they lack a number of enzymes, such as phosphofructokinase, which are crucial for control of central carbohydrate metabolic pathways in other bacteria (28).

Carbon/carbohydrate metabolism in bifidobacteria has been the focus of substantial research efforts, and a number of specific carbohydrate utilisation pathways, sometimes including their regulation, have been characterised (32-40). Several carbohydrate metabolism-related LacI-type regulators are known in *B. breve* UCC2003 (35, 36, 38, 64), and are typically involved in local (i.e. the gene encoding the LacI-type regulator is located in the vicinity of the genes it regulates) transcriptional regulation of genes that are responsible for the metabolism of a particular carbohydrate. These LacI-type transcription factors (TFs) typically block (and are then called repressors), yet may sometimes also enhance (then designated as activators) access of the RNA polymerase to the promoter region of the regulated gene. This ability to act as a repressor or activator is dependent on a number of factors such as the location of the DNA binding motif of the TF with respect to the -10/-35 RNA polymerase recognition sites and the presence or absence of effector molecules, which may bind to the TF to alter its conformation and consequently its DNA-binding ability. The binding state of the TF must be in accord with the external and/or cytoplasmic environment, and, as mentioned, bacteria achieve this through the use of effector molecules. In the case of carbohydrate utilisation effector molecules are typically a carbohydrate or a metabolic intermediate.

It had previously been predicted that the *Bifidobacterium breve* UCC2003-encoded LacI-type regulators MalR1, MalR2, MalR3, MalR5 and MalR6 are involved in the metabolism of starch-like carbohydrates, while BifR1 (referred to as AraQ in that publication) was envisaged to be responsible for transcriptional control of genes encoding a number of bifid shunt enzymes (41). Chapter 5 of this thesis will focus on MalR2, MalR3, MalR5 and MalR6, demonstrating that all of these MalR TFs are indeed involved in the transcriptional regulation of genes and gene clusters required for the metabolism of starch-like carbohydrates. Central metabolism and its regulation have not been experimentally characterised in *B. breve* UCC2003 or other members of the extended *Bifidobacteriaceae* family. The bifid shunt has been investigated in terms of its enzymatic capabilities in animal-derived species, such as *Bifidobacterium globosum* (65, 66). One such study has indeed established that the bifid shunt is the sole pathway to satisfy all energy demands in *Bifidobacterium animalis* subsp. *lactis* Bb-12 (as a representative species of its genus) when grown on either glucose or lactose (22). Employing *B. breve* UCC2003 as a prototype for other bifidobacteria, we show here that two of the previously

identified LacI-type TFs appear to be directly involved in transcriptionally regulating genetic components of the bifid shunt, as well as genes involved in a range of specific carbohydrate metabolic pathways, thereby revealing a heretofore undiscovered global carbohydrate control network.

4.3 Methods

Bacterial Strains and Culture Conditions

Bacterial strains and plasmids are listed in Table 4-1. B. breve strains were routinely grown at 37°C in either de Man Rogosa and Sharpe medium (MRS medium; Difco, BD, Le Pont de Claix, France), modified de Man Rogosa and Sharpe (mMRS) medium made from first principles (42), or reinforced clostridial medium (RCM; Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with 0.05 % cysteine-HCl. Bifidobacteria were incubated anaerobically in a modular, atmosphere-controlled system (Davidson and Hardy, Belfast, Ireland). Where appropriate growth medium was supplemented with tetracycline (Tet; 10 µg ml-1), chloramphenicol (Cm; 5 µg ml-1 for *L. lactis* and *E. coli*, 2.5 µg ml-1 for *B. breve*), ampicillin (Amp; 100 µg ml−1), erythromycin (Em; 100 µg ml-1) or kanamycin (Kan; 50 µg ml-1) for plasmid/strain selection and/or maintenance.

Nucleotide Sequence Analysis

Sequence data were obtained from the Artemis-mediated (43) genome annotations of the *B. breve* UCC2003 genome sequence (44). Data base searches were carried out using non-redundant sequences accessible at the National Centre for Biotechnology Information internet site (http://www.ncbi.mlm.nih.gov) utilising the basic local alignment search tool (Blast). Sequence analysis was performed by the use of Seqbuilder and Seqman programs of the DNASTAR software package (DNASTAR, Madison, WI). Protein functions were assigned with the use of the basic protein local alignment search tool for proteins (BlastP), and Homology detection and structure prediction by HMM-HMM comparison and HHpred (45, 46).

Table 4-1. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant features	Reference or source
Strains		
B. breve		
UCC2003	Isolate from nursling stool	(77)
UCC2003-*bifR2*	pORI19-*tetW-bifR2* insertion mutant of UCC2003	This study
UCC2003-*bifR1*	pORI19-*tetW*-bifR1 insertion mutant of UCC2003	This study
E. coli		
XL1-BLUE	Host for pQE60 plasmids; supE44 hsdR17 recA1 gyrA96 thi relA1 lac F= [proAB laclq lacZ M15 Tn10(Tet^r)]	Stratagene
XL1-BLUE + pQE60	pQE60 *E. coli* expression vector, Amp^r	This study
EC101 + PQE60_*bifR2*	pQE60+*bifR2*	This study
XL1-BLUE + pQE60_*bifR1*	pQE60+*bifR1*	This study
EC101	Cloning host for pORI19 for insertional mutagenesis; *repA*$^+$ *Km*^{r} *and* pQE60 *E. coli* expression vector	(78)
Plasmids		
pORI19	Em^r, repA$^-$, ori$^+$, cloning vector	(78)
pORI19- *bifR2*	pOR19 harbouring internal fragment of *bifR2* (*Bbr_1846)*	This study
pORI19- *bifR1*	pOR19 harbouring internal fragment of *bifR1* (*Bbr_0411)*	This study
pORI19- *bifR2*-tet	pOR19 harbouring internal fragment of *bifR2* (*Bbr_1846)* + Tet^r	This study
pORI19- *bifR1*-tet	pOR19 harbouring internal fragment of *bifR1* (*Bbr_0411)* + Tet^r	This study
pQE60	*E. coli* expression vector, Amp^r	Qiagen
pQE60+bifR2	pQE60 harbouring *bifR2*	This study
pQE60+bifR1	pQE60 harbouring *bifR1*	This study

Em^r, Km^r, Tet^r and Amp^r: resistance to erythromycin, kanamycin, tetracycline and ampicillin, respectively.

DNA Manipulations

DNA manipulations were carried out as previously reported (47). Restriction enzymes and T4 DNA ligase were obtained from Roche Diagnostics (Basel, Switzerland), and were used according to the manufacturer's instructions. PCRs were performed using either Q5® High-Fidelity DNA polymerase (New England Biolabs, Hertfordshire, UK) or Extensor Long Range PCR Enzyme master mix (Thermo Scientific, Glouchester, UK). Synthetic oligonucleotides were synthesized by Eurofins (Ebersberg, Germany) and are listed in Table 4-2. Ird-labelled synthetic oligonucleotides were provided by IDT (Integrated DNA technologies, Dresden, Germany) and are listed in Table 4-3. PCR

products were purified by the use of a High-Pure PCR product purification kit (Roche, Basel, Switzerland). Plasmid DNA was introduced into *E. coli* and *B. breve* by electroporation, and large-scale preparation of chromosomal DNA from *B. breve* was performed as described previously (48). Plasmid DNA was obtained from *B. breve* and *E. coli* using the Roche High Pure plasmid isolation kit (Roche Diagnostics, Basel, Switzerland). An initial lysis step was performed using 30 mg ml^{-1} of lysozyme for 30 min at 37°C as part of the plasmid purification protocol for *B. breve*.

Purpose	Primer	Sequence
Cloning of internal fragment of *bifR2* in pORI19	*bifR2_IM_F*	ttgctaaagcttgaaatcaggccgatcactgcg
	bifR2_IM_R	ttgctatctagacaatatcgcggtggccaagg
Cloning of internal fragment of *bifR1* in pORI19	*bifR1_IM_F*	atagctaagcttgattcgccggatgtgtctg
	bifR1_IM_R	atagcttctagagtacagggcggacttatggc
Confirmation of site specific homologous recombination	*bifR2_Con*	gccatcgccgacgaactc
	bifR1_Con	gtgtgcaggccgccattg
	tetW_F	tcagctgtcgactgctcatgtacggtaag
	tetW_R	gcgacggtcgaccattaccttctgaaacat
Cloning of *bifR2* in pQE60	*bifR2_F*	ttgctaccatggatgaccacaagtatccaagatgtcgcc
	bifR2_R	ttgctaggatccgcggatcttggcggtagagg
Cloning of *bifR1* in pNZ44	*bifR1_F*	atagcttctagaatggaggttcgggcagtatggtg
	bifR1_R_hist	atagctaagcttttacatcaccatcaccatcaccat caccatcacgaacggcactcagcacagc

Table 4-2. Oligonucleotide primers used in this study.

Construction of *B. breve* UCC2003 Insertion Mutants

Internal fragments of Bbr_0411 (designated here as *bifR1*; fragment used was 402 bp in length, representing codons 74 through to 208 of the 371 codons of this gene) and Bbr_1846 (designated here as *bifR2*; fragment used was 504 bp in length, representing codons 102 through to 267 of the 338 codons of this gene) were amplified by PCR using *B. breve* UCC2003 chromosomal DNA as a template (primers employed are listed in Table 4-2). Insertional mutagenesis was carried out as previously described (49). The presence of the tetracycline resistant cassette was confirmed by colony PCR with TetF and TetR primers, while site-specific recombination of potential Tet-resistant mutants was confirmed by colony PCR using a combination of the TetR primer and a primer located upstream of the recombination site in *bifR1* and *bifR2* in the chromosome of *B. breve* UCC2003 (see Table 4-2 for primer details). The confirmed insertional mutants within *bifR1* and *bifR2* were designated here as *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2*, respectively.

Microarray Analysis

The transcriptome of *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2* was compared to the global gene expression patterns of *B. breve* UCC2003 (WT). Insertional mutants and the WT strain were cultivated in mMRS medium supplemented with 0.5 % ribose until an OD_{600nm} of ~0.6 was achieved. Cells were harvested by centrifugation at 10,000 rpm for 2 min at room temperature and immediately frozen at -80°C prior to RNA isolation. DNA microarrays containing oligonucleotide primers representing each of the annotated genes on the genome of *B. breve* UCC2003 were designed by and obtained from Agilent Technologies (Palo Alto, CA, USA). Cell disruption, RNA isolation, RNA quality control, and cDNA synthesis and labelling were performed as described previously (50). The labelled cDNA was hybridized using the Agilent Gene Expression hybridization kit (part number 5188-5242) as described in the Agilent Two-ColorMicroarrayBased Gene Expression Analysis v4.0 manual (G4140-90050).

Following hybridization, the microarrays were washed in accordance with Agilent's standard procedures and scanned using an Agilent DNA microarray scanner (model G2565A). The generated scans were converted to data files with Agilent's Feature Extraction software (v9.5). The DNA microarray data were processed as previously described (51-53). Differential expression tests were performed with the Cyber-T implementation of a variant of the t-test (54). A gene was considered to exhibit a significantly different expression level relative to the control when $p < 0.001$ and an expression ratio of > 2 or < 0.25. The microarray data obtained in this study have been deposited in NCBI's Gene Expression Omnibus database and is accessible through GEO series accession number GSE108949.

Plasmid Constructions

For the construction of plasmids pQE60+bifR1 and pQE60+bifR2 DNA fragments encompassing bifR1 (Bbr_0411) and bifR2 (Bbr_1846) were generated by PCR amplification employing chromosomal DNA of *B. breve* UCC2003 as a template, Q5 high-fidelity DNA polymerase, and primers bifR1_F and bifR1_R, and bifR2_F and bifR2_R, respectively (Table 4-2). An in-frame His10-encoding sequence is contained within the 3' end of the pQE60 construct to facilitate downstream protein purification.

The bifR1-encompassing PCR product was digested with NcoI and BglII, while the bifR2-encompassing PCR product was digested with NcoI and BamHI. The digested PCR products were ligated into a similarly digested pQE60, an IPTG-inducible translational fusion plasmid. The ligation mixtures were introduced into *E. coli* XL1-Blue or *E. coli* EC101 by electro-transformation, and transformants were then selected on the basis of Amp resistance (AmpR). The plasmid contents of a number of AmpR transformants were screened by restriction analysis, and the integrity of positively identified clones was verified by sequencing. One verified clone of plasmid pQE60+bifR1 and pQE60+bifR2 (i.e. plasmid pQE60 in which either bifR1 or bifR2, respectively, was cloned) (Table 4-1) was then selected for protein expression and purification purposes.

Protein (Over)Expression and Purification

E. coli XL1-BLUE was utilised as the host for the heterologous expression of BifR1 and BifR2. *E. coli* XL1-BLUE strains containing either pQE60+*bifR1* or pQE60+*bifR2* were inoculated at 2 % in LB medium until an OD_{600nm} of ~0.5 was reached, at which point protein expression was induced by the addition of IPTG (65). Following incubation for a further two hours cells were harvested by centrifugation and re-suspended in EMSA buffer (see below). Bacterial cells were disrupted by bead beating in a mini-bead beater (BioSpec Products, Bartlesville, OK, USA) using glass beads. Cellular debris was then removed by centrifugation to produce a crude cell extract.

Recombinant BifR1 and BifR2 proteins, each tagged with an incorporated C-terminal His_{10} sequence, were purified from a crude cell extract using a nickel-nitrilotriacetic acid column (Qiagen, Hilden, Germany) according to the manufacturer's instructions (QIAexpressionist, June 2003). Lysis, wash and elution buffers were supplemented with 10 % glycerol; the addition of 10 % glycerol in the above buffers during the purification process considerably improved the (binding) activity of BifR1 and BifR2 proteins. Elution fractions were analysed by SDS-polyacrylamide gel electrophoresis, as described previously (79), on a 12.5 % polyacrylamide gel. Following electrophoresis, gels were fixed and stained with Coomassie brilliant blue to identify fractions containing the purified protein. Colour Prestained Protein Standard, Broad Range (11–245 kDa) (New England BioLabs, Hertfordshire, UK) was used to estimate the molecular weights of the purified proteins. Protein was concentrated using Amicon® Ultra Filters from Merck

Millipore and dialysed into EMSA binding buffer (80 mM Tris-HCl [pH 8.0], 20 mM $MgCl_2$, 2 mM dithiothreitol [DTT], 4 mM EDTA, 400 mM KCl and 40 % glycerol). Concentration of the purified BifR1/BifR2 protein and subsequent dialysis into EMSA binding buffer also improved the binding ability of these two LacI-type proteins. Protein concentrations were determined using the Qubit® fluorometer as per manufacturer's instructions (Thermofisher scientific, Glouchester, UK). Purified protein was aliqoted and stored at -80°C for subsequent use in EMSAs.

Electrophoretic Mobility Shift Assay (EMSA)

The promoter regions of genes of interest were amplified by PCR utilising individual primer pairs, of which one or both were 5' Ird-700-labelled (provided by IDT, Dresden, Germany) as listed in Table 4-3. Electrophoretic mobility shift assays (EMSAs) were performed as described previously (55). All binding reactions were carried out with poly(dI-dC) (0.05 µg/µl), DNA probe (0.5 nmol), BSA (0.2 µg/µl), binding buffer (20 mM Tris-HCl, 5 mM MgCl2, 0.5 mM dithiothreitol (DTT), 1 mM EDTA, 50 mM KCl, and 2.5 % glycerol at pH 7.0) and water to a final volume of 20 µl. Binding reactions were carried out in the presence of various amounts (0, 10, 50, 100, 150 and 250 nM) of the purified BifR1 or BifR2 protein. Binding reactions were then incubated for 20 min at 37 °C and were loaded onto a 6 % non-denaturing polyacrylamide (PAA) gel prepared in TAE buffer (40 mM Tris-acetate [pH 8.0], 2 mM EDTA) and bound/unbound DNA fragments were then separated by electrophoresis on a 0.5X to 2.0X gradient of TAE at 100 V for 90 min in an Atto Mini PAGE system (Atto Bioscience and Biotechnology, Tokyo, Japan).

Signals and percentage binding inferred from the Integrated Intensity (II) were detected/calculated using an Odyssey infrared imaging system (Li-Cor Biosciences, United Kingdom, Ltd., Cambridge, United Kingdom), and images were captured by the use of Odyssey software v3.0. The ability of BifR1 and BifR2 to bind DNA fragments was graded utilising a non-linear scale which spanned from no affinity, low, medium to a high affinity (-, +, ++, +++, respectively). This non-linear scale was calculated based on the percentage of DNA which was bound as compared to unbound DNA within a given reaction with – representing no DNA bound, + represents up to 15 % DNA bound, ++ represents 15 % - 50 % DNA bound and +++ represents 50 – 100 % DNA bound. Once

a DNA fragment had shown to possess binding affinity, the binding site was in some cases more precisely determined by generating sub-fragments by PCR, followed by EMSAs as described above utilising 150 nM of either BifR2 or BifR1. To identify possible effectors for BifR1 or BifR2, 10 mM of a particular compound (Table S4-9) was added to the binding reaction mixture.

Motif Searches

EMSA analysis and previous prediction by Khoroshkin *et al* (41) identified a number of DNA promoter regions with which BifR1/BifR2 had affinity, DNA regions with which BifR1/BifR2 had a medium affinity (++) and high affinity (+++) (based on the non-linear scale, see EMSA protocol above for further information) were utilised for input into the MEME software tool (81) in order to search for overrepresented nucleotide sequences. A graphical representation of the identified motif was obtained using WebLOGO software (82). WebLOGO software expresses the motif scores as a "bit score", the bit score is a measure of sequence similarity and is independent of query sequence length and database size and is normalized based on the raw pairwise alignment score.

Table 4-3 Oligonucleotide primers used in EMSA analysis.

Locus tag*	Primer name#	Sequence
Bbr_0023	*malR2*_Ird	gctcgcttttgccatggc
Bbr_0023	*malR2*	ccgaaaccagccgttgcac
Bbr_0027	*malFG 2*_Ird	ccatgccggtctcctttgc
Bbr_0027	*malFG 2*	gccagcctactttctcctcc
Bbr_0032/Bbr_0033	*malR5/malE2*_Ird	cgccatgatgtctcctttcgc
Bbr_0032/Bbr_0033	*malR5/malE2*	gctcatgtgatacgtgcctcc
Bbr_0060	*glgP 1*_Ird	cattctgactttccttccgggg
Bbr_0060	*glgP 1*	gcgttctatccttgcactgagcc
Bbr_0105	*cldR*_Ird	gctgcgcgctatgttctcct
Bbr_0105	*cldR*	ccacgtcacgaatagttgcc
Bbr_0106	*cldE*_Ird	gcgacgatgacgaatccg
Bbr_0106	*cldE*	gcacttgggcgctcattg
Bbr_0111	*agl3*_Ird	gttcattcagcccgacgc
Bbr_0111	*agl3*_Ird	gtcattgaggttggcg
Bbr_0112/Bbr_0113	*malR6/*Bbr_0113_Ird	cgcttgaatgcttgcttttgc
Bbr_0112/Bbr_0113	*malR6/*Bbr_0113	ccggaacttctcgctatcatcatg
Bbr_0116	*malQ 2*_Ird	cctgttctgctcttaccgtagctc
Bbr_0116	*malQ 2*	gagctacggtaagagcagaacagg
Bbr_0117/Bbr_0118	*agl4/malFG*_Ird	cggttcctacgccaagtaatc
Bbr_0117/Bbr_0118	*agl4/malFG*	aagtgctgctgctgtcatcg
Bbr_0122/Bbr_0123	*malR3/apuB*_Ird	ggtagatgtctgccttgccc
Bbr_0122/Bbr_0123	*malR3/apuB*_Ird	gcaaggcgtttggcgagcg
Bbr_0411	*bifR1*_Ird	cgcacttcctggcatttg
Bbr_0411	*bifR1*	gaacttcaggccgccagc
Bbr_0725	*eno*_Ird	caaggaagtcgccgacaatc
Bbr_0725	*eno*	ggttgccacgagaatccag
Bbr_0747	*carD*_Ird	ggcacattgatgaccagacc
Bbr_0747	*carD*_Ird	gccgttgtattcgtgtgtca
Bbr_0757	*pyk*_Ird	cgctgagaaggcctgaaatc
Bbr_0757	*pyk*_Ird	ggttgtcgtaatcctcggtg
Bbr_0787	*pflBA*_Ird	gccgatagaacagcgtatgg
Bbr_0787	*pflBA*	cttggcgtcgagctcctcttg
Bbr_0845	*glgp 2*_Ird	cgcacctccttccacgctg
Bbr_0845	*glgp 2*	ccgttagattggagattgtccg
Bbr_1002-1003	*tkt – tal*_Ird	gctcggtctccttgaattcg
Bbr_1002-1003	*tkt – tal*	ggctcttgcgaacgaatg
Bbr_1233	*gap*_Ird	gcattgcctcaggtaagcc
Bbr_1233	*gap*	gaccaatgcgaccgaagc
Bbr_1273	*ldh*_Ird	ggatgtcgattcgcacttgg
Bbr_1273	*ldh*_Ird	agcttgctgttattggtgcc
Bbr_1419	*rbsA1*_Ird	gctcaatagtccttcgccgcc
Bbr_1419	*rbsA1*	catacgcctctcgctttcgtc
Bbr_1420	*lacI*_Ird	gatcatgctcagatgcggcg
Bbr_1420	*lacI*	cgaatgccatatccgtctcc
Bbr_1595	*pgma*_Ird	ccatactttcattctgccacg
Bbr_1595	*pgma*	gcgacatctcttactccattcc
Bbr_1658	Bbr_1658_Ird	ggtcaagctcatcgtgcg
Bbr_1658	Bbr_1658	ctggtcagcatagccgcac
Bbr_1659	*LacI*_Ird	caacgtgcgcagcctatgg
Bbr_1659	*LacI*	cttcgccacgtcatacacc
Bbr_1841	Bbr_1841_Ird	cgacagcttccttgccatgc
Bbr_1841	Bbr_1841	gcgtgcgatgtccctgatg
Bbr_1845/Bbr_1846	*malFG/bifR2*_Ird	cataacagccccttgcc
Bbr_1845/Bbr_1846	*malFG/bifR2*	catgactttcctcctccttgag

Bbr_1847	*malE*_Ird	ggaatgcctgagctgagccg
Bbr_1847	*malE*	cgaacctttctctttcatcgtcg
Bbr_1891	*gntR*_Ird	gatgagtgcgcgtgagaag
Bbr_1891	*gntR*	cacgctggcgaagattgtc
Bbr_1894	*PTS*_Ird	gatatgcgcgaggattgg
Bbr_1894	*PTS*	gatcgacatacagcatgccg
Bbr_1901	*nrdH* & *nrdI* & *nrdE_Ird*	gtctcgaacggcacacca
Bbr_1901	*nrdH* & *nrdI* & *nrdE*	tggacatccggtcaggcc
Generation of DNA fragments for EMSA Fragmentations		
Bbr_0725	*eno*_1_Ird	caaggaagtcgccgacaatc
Bbr_0725	*eno*_2	ggtgtgccgcgtgattgc
Bbr_0725	*eno*_3	ccaaaatttgatgtgagcgctc
Bbr_0725	*eno*_4	ggttgccacgagaatccag
Bbr_0757	*pyk*_1_Ird	cgctgagaaggcctgaaatc
Bbr_0757	*pyk*_2	gcgccgattgggttttgag
Bbr_0757	*pyk*_3_Ird	ccggtgtgttgtgagcgc
Bbr_0757	*pyk*_4_Ird	ggttgtcgtaatcctcggtg
Bbr_0032/Bbr_0033	*malR5/malE2*_1_Ird	cgccatgatgtctcctttcgc
Bbr_0032/Bbr_0033	*malR5/malE2*_2	caacgcgcacatcgtggtac
Bbr_0032/Bbr_0033	*malR5/malE2*_3_Ird	gtaccacgatgtgcgcgttg
Bbr_0032/Bbr_0033	*malR5/malE2*_4	cacaccgtcaaccgccgc
Bbr_0032/Bbr_0033	*malR5/malE2*_5	gcggcggttgacggtgtg
Bbr_0032/Bbr_0033	*malR5/malE2*_6	gctcatgtgatacgtgcctcc
Bbr_0032/Bbr_0033	*malR5/malE2*_7_Ird	gcagccggcatccgatcc
Bbr_0112/Bbr_0113	*malR6/agl3*_1_Ird	cgcttgaatgcttgcttttgc
Bbr_0112/Bbr_0113	*malR6/agl3*_2	gacaatcgaaacgcacacacc
Bbr_0112/Bbr_0113	*malR6/agl3*_3	ccaccgggcatgatacc
Bbr_0112/Bbr_0113	*malR6/agl3*_4	ccatgtcggcgaatttcctc
Bbr_0112/Bbr_0113	*malR6/agl3*_5	ccatgctattatgcaaacgatgtcag
Bbr_0112/Bbr_0113	*malR6/agl3*_6_Ird	ccggaacttctcgctatcatcatg
Bbr_0122/Bbr_0123	*malR3/apuB*_1_Ird	ggtagatgtctgccttgccc
Bbr_0122/Bbr_0123	*malR3/apuB*_2	gattacacatcgtggatggcgc
Bbr_0122/Bbr_0123	*malR3/apuB*_3_Ird	gcgccatccacgatgtgtaatc
Bbr_0122/Bbr_0123	*malR3/apuB*_4	gggaagtgttgcttggtgtgg
Bbr_0122/Bbr_0123	*malR3/apuB*_5	ccacaccaagcaacacttccc
Bbr_0122/Bbr_0123	*malR3/apuB*_6	cggcatgcagcacagttgac
Bbr_0122/Bbr_0123	*malR3/apuB*_7_Ird	gtcaactgtgctgcatgccg
Bbr_0122/Bbr_0123	*malR3/apuB*_8	ctgaccgtgcgatagggg
Bbr_0122/Bbr_0123	*malR3/apuB*_9	cccctatcgcacggtcag
Bbr_0122/Bbr_0123	*malR3/apuB*_10_Ird	gcaaggcgtttggcgagcg

*The locus tag number refers to the gene upstream of which a presumed promoter region is present that was amplified by the primer pair. If two locus tags are indiciated, it means that the promoter region is located in between two corresponding and divergently oriented genes (i.e., representing an intergenic region). #Primers used in the EMSA experiments, _Ird represents primers which were labelled with an iridescent probe at the 5' end.

Evaluation of Growth of *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2* on a Range of Carbohydrate Sources

B. breve UCC2003 and its isogenic derivatives *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2* were inoculated at 1 % (v/v) from stock into RCM broth and were cultured overnight under anaerobic conditions at 37°C. The above strains were then inoculated at 1 % (v/v) into mMRS medium containing 1 % carbohydrate (glucose, maltose, ribose or starch) along with the addition of 0.5 % (v/v) L-cysteine HCl. mMRS without the addition of a carbohydrate source served as a negative control. Bacterial strains were evaluated for carbohydrate-dependent growth using a Life Sciences UV/VIS spectrophotometer DU5300 (Beckman Coulter). Optical density (OD_{600nm}) readings were taken at 24 hr post-inoculation. All samples were assessed as biologically independent triplicates.

Growth of *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2* in the presence of bile

B. breve UCC2003 and its isogenic derivatives *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2* were inoculated at 1 % (v/v) from stock into mMRS medium supplemented with 1 % maltose and 0.5 % (v/v) L-cysteine HCl and were cultured overnight under anaerobic conditions at 37°C. The above strains were then inoculated at 1 % (v/v) into mMRS medium containing 1 % maltose, 0.5 % (v/v) L-cysteine HCl along with increasing concentration of porcine bile at 0, 1 and 2 % (v/v). mMRS without the addition of a carbohydrate source served as a negative control. Bacteria were evaluated for their ability to grow in the presence of bile with Optical density (OD_{600nm}) readings taken at 10 and 24 hr post-inoculation. All samples were assessed as biologically independent triplicates. Statistical analysis was carried out using the Microsoft Excel Data Analysis ToolPak. Statistical significance was determined for each strain, comparing growth without the presence of bile against growth in the presence of bile at both 1 % and 2 %, separately.

Evaluation of *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2* survival upon bile exposure

B. breve UCC2003 wild type (*wt*) and its isogenic derivatives *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2* mutants were inoculated at 1 % (v/v) from stock into mMRS medium supplemented with 1 % maltose and 0.5 % (v/v) L-cysteine HCl and were cultured overnight under anaerobic conditions at 37 °C. The cells from 1 ml of the above cultures were then collected by centrifugation and re-suspended in mMRS medium containing 1 % maltose, 0.5 % (v/v) L-cysteine HCl along with increasing concentration of porcine bile at 0, 1 and 2 % (v/v) and incubated under anaerobic conditions at 37 °C for 20 minutes, after which cells were serially diluted in PBS buffer to 10^{-7} and spot plated on RCA. Plates were incubated anaerobically at 37 °C for 48 hours. Cfu/ml was calculated using the following formula: cfu/ml = (no. of colonies x dilution factor) / volume of culture plated. All samples were assessed as biologically independent triplicates. Statistical analysis was carried out using the Microsoft Excel Data Analysis ToolPak. The statistical significance was determined for the two mutants, comparing the drop in cell numbers when exposed to bile against the drop in cell numbers for the wild type strain when similarly exposed to bile.

Metabolite analysis by High Performance Liquid Chromatography (HPLC)

HPLC analysis was used to quantify the concentration of produced metabolites following growth of *B. breve* UCC2003 or its isogenic derivatives *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2* on mMRS supplemented with 1 % glucose as the sole carbohydrate source. The collected samples, taken at 8 and 24 hours post inoculation, were then prepared for HPLC analysis by centrifugation at 5000 rpm for 5 min, the resulting supernatants were filter sterilized (0.45 µm filter, Costar Spin-X Column) and stored at −20 °C prior to analysis. An Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) with a refractive index detector was used to quantify the production of a given metabolite. Metabolite peaks and concentrations were identified and calculated based on retention times and concentrations of lactic acid, acetic acid and ethanol, which were used as standards. Non-fermented mMRS medium preparations containing individual carbohydrates served as controls. A REXEX 8 µ 8 % H organic acid column (300 mm × 7.8 mm, Phenomenex, Torrance, CA, USA) was utilized and

maintained at 65 °C. Elution was performed for 25 min using a 0.01 M H_2SO_4 solution at a constant flow rate of 0.6 ml/min.

4.4 Results

Identification and genetic analysis of *bifR1 and bifR2* on the *B. breve* UCC2003 genome

This study was initially aimed at the characterization of six previously described LacI-type transcription factors (TFs), named AraQ, MalR1, MalR2, MalR3, MalR5 and MalR6, and their proposed regulation of central metabolic pathways and malto-oligosaccharide utilisation pathways in *B. breve* UCC2003 (41). In the course of our experiments we determined that MalR1 was in fact functionally more similar to AraQ and for this reason our experimental scope was narrowed to specifically characterise AraQ and MalR1. The AraQ and MalR1 proteins, encoded by the genes Bbr_0411 and Bbr_1846, were renamed here as BifR1 and BifR2, respectively, to better reflect, as will be outlined below, their conserved role as global regulators of central metabolism in *B. breve* UCC2003. The encoded products of *bifR1* and *bifR2* represent proteins of 371 and 338 amino acids, respectively, which exhibit 26 % overall identity to each other. Their respective DNA binding domains (encompassing residues 11-74 for BifR1 and residues 3-66 for BifR2) exhibit a higher level of identity at 48 %, perhaps a reflection of the conserved nature of the DNA-binding helix-turn-helix regions of LacI-type proteins in general (83), coupled to the finding that, as will be outlined below, BifR1 and BifR2 recognize near identical operator sequences.

***In vitro* characterisation of the regulons of BifR1 & BifR2 by EMSA assays**

In order to assess BifR1 and BifR2 interaction with their possible operator sequences, EMSA assays were carried out using DNA fragments encompassing upstream regions of genes corresponding to the predicted regulons as identified by Khoroshkin *et al.* (41), and to a number of (differentially transcribed) genes that were identified in the microarray analyses (discussed below).

Obtaining purified and active BifR1 or BifR2 protein from *B. breve* proved to be troublesome, in line with similar issues observed for other bifidobacterial LacI-type proteins (35, 36, 38, 64, 80). The current work adapted previous protocols and developed a method which allows for the purification of active (i.e. DNA-binding competent) LacI-type proteins from *B. breve* UCC2003 (see Materials and Methods section for a more detailed description). The main changes which were found to enhance the ability of

purified BifR1/BifR2 to bind to its DNA targets include (i) the addition of 10 % glycerol to all buffers utilised during the purification process, (ii) the utilisation of Amicon® Ultra Filters from Merck Millipore for the concentration and dialysis of BifR1/BifR2, and (iii) the storage of the purified protein in EMSA buffer as opposed to Tris-HCl (pH 8) buffer. In order to determine the most suitable protein concentration for EMSA analysis (concentration at which the TF binding was deemed specific to their target transcription factor binding sites (TFBS)), varying concentrations of purified BifR1 and BifR2 were tested for their ability to bind to DNA sequences encompassing the BifR1 predicted TFBS (41). The chosen concentration for both proteins was 150 nM when incubated with 0.5 nM Ird-labelled DNA encompassing the predicted TFBS (Figure 4-1).

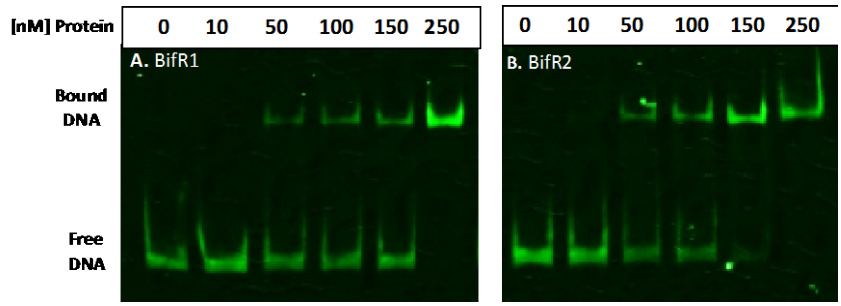


Figure 4-1. EMSA increasing concentration BifR1/BifR2.
EMSA analysis carried out with increasing concentrations (0, 10, 50, 100, 150, 250 nM) of purified BifR1 (panel A) or BifR2 (panel B) incubated with 0.5 nM Ird-labelled DNA fragment encompassing the Bbr_1233 (*gap*) promoter region.

The ability of BifR1 and BifR2 to bind DNA fragments was graded utilising a non-linear, arbitrary scale. This non-linear scale was based on the percentage of DNA which was bound as compared to unbound DNA within a given reaction with – representing no DNA bound, + represents up to 15 % DNA bound, ++ represents 15 % - 50 % DNA bound and +++ represents 50 – 100 % DNA bound. Example EMSAs illustrating this non-linear scale can be found in Figure S4-7 to S4-10 while Table 4-4 summarises the EMSA experiments carried out in this study. EMSA analysis revealed that BifR1 and BifR2 bind, though to varying degrees, to upstream, presumed promoter-containing, regions of 23 and 20 genes, respectively. Of the total of 33 promoter regions tested via EMSA analysis, BifR1 and BifR2 were unable to bind to 10 and 13, respectively, under the conditions tested. A number of these regions contain LacI-type motifs and were considered to act as good negative controls for non-specific binding. Furthermore, some of the promoter

regions to which BifR1/BifR2 did not appear to bind, are associated with genes that had shown differentially transcription in the microarray analysis of the corresponding *bifR1*/*bifR2* mutants, indicating this maybe caused by an indirect effect.

Six of the genes/gene clusters whose promoter regions were shown to be bound by BifR1 and BifR2 encode enzymes that are part of the bifid shunt (Figure 4-3), several of which have recently been shown to be essential for growth on glucose (63) (Table 4-4), while they are also highly transcribed during logarithmic growth on glucose as a sole carbon source (87). The ability of BifR1 to bind to the promoter regions of these six genes/gene clusters involved in central metabolism as well as those of certain genes involved in the metabolism of starch-like/-derived carbohydrates is consistent with previous *in silico* predictions (41). Our EMSA analyses furthermore demonstrate that, similar to BifR1, BifR2 binds to promoter regions of genes/gene clusters involved in central carbon metabolism along with genes involved in the metabolism of starch-like/derived carbohydrates (and that the deduced BifR1 and BifR2 regulons therefore largely overlap). Though the ability of BifR2 to transcriptionally control genes that are required for the metabolism of starch-like/-derived carbohydrates is consistent with previous *in silico* predictions by Khoroshkin *et al* (41), its presumed ability to control genes involved in central carbon metabolism was an unexpected additional function.

Based on their binding behaviour BifR1 and BifR2 appear to have a similar affinity for the six promoter regions of the central metabolism genes (Table 4-4). However, some divergence in binding specificity between these two TFs appears to exist as they exhibit differential binding abilities to certain promoter regions. This divergent binding behaviour is exemplified by the ability of BifR1 (and not BifR2) to bind to the promoter region of a gene encoding a putative solute binding protein for a sugar ABC transport system, located adjacent to the *bifR2* gene and also a promoter region of a gene encoding a putative permease protein involved in sucro-oligosaccharide uptake (57). Additionally, BifR2 (and not BifR1) was shown to bind to the promoter regions of two gene clusters involved in the uptake of cellobiose and ribose (35, 36). Other examples of differential binding behaviour between BifR1 and BifR2 are listed in Table 4-4.

Finally, BifR1 and BifR2 were both found to be capable of binding their own and each other's presumed promoter regions. This finding suggests that these genes are subject to interactive autoregulation, in which they not only control the transcription of their own gene, but also that of the other. Furthermore, as based on their binding behaviour, BifR1 and BifR2 appear to control transcription of the LacI-type-encoding genes MalR2, MalR3 MalR5 and MalR6, as well as a CarD-like TF (Table 4-4).

Table 4-4. EMSA Analysis

Gene	Locus tag; Bbr	Function (predicted or experimentally validated)	BifR1	BifR2	High level of transcription on glucose (H) and essential (E)	Repression (R) or Activation (A) by BifR1 & BifR2
		Predicted BifR2 Regulon				
**malG2 (F2)*	0027	Permease protein of sugar ABC transporter	++	-	-	R
**malR5*	0032	LacI Transcriptional regulator	+	+	-	-
**malE2*	0033	Solute binding protein for sugar ABC transport system	+	+	-	-
glgP1	0060	Glycogen/Amylose phosphorylase	++	-	H	R
agl3	0111	α-glucosidase	-	-	-	-
**malR6*	0112	LacI Transcriptional regulator	-	++	-	-
Bbr_0113	0113	Sortase	++	++	-	-
malQ	0116	Glucanotransferase	+	-	-	R
**agl4/ malE1*	0117/0118	α-glucosidase-like protein/ Solute binding protein for sugar ABC transport system	++	++	-	R
**malR3*	0122	LacI Transcriptional regulator	+	+	-	-
**apuB*	0123	Amylopullulanase	+	+	-	-
**malF / bifR2*	1845/1846	Permease protein of sugar ABC transporter/ LacI Transcriptional regulator	++	++	-	R
malE	1847	Solute binding protein for sugar ABC transport system	++	-	-	A
		Predicted Global BifR1 Regulon				
bifR1	0411	LacI Transcriptional regulator	+++	+++	-	-
eno	0725	Enolase	+++	+++	H, E	A
carD	0747	CarD-like transcriptional regulator	+++	++	H, E	A
pyk	0757	Pyruvate Kinase	+++	+++	H	A
pflB - pflA	0787	Formate acetyl transferase - Pyruvate formate-lyase activating	++	+++	H	A
tkt - tal	1002	Transketolase - Transaldolase	++	++	H, E	A
gap	1233	Glyceraldehyde Phosphatase	+++	+++	H, E	A
ldh2	1273	Lactate dehydrogenase	+++	+++	H,E	A
		Additional Genes Identified by Microarray Analysis				
malR2	0023	LacI Transcriptional regulator	++	-	-	R
cldR	0105	Cellodextrin LacI Transcriptional regulator	-	-	-	-
cldE	0106	Cellodextrin permease	-	++	-	R#
glgP2	0845	Glycogen phosphorylase (Non functional)	-	-	-	-
rbsA1	1419	Ribose transport ATP binding	-	++	-	R
Bbr_1420	1420	LacI Transcriptional regulator (Ribose utilisation)	-	+	-	-
Bbr_1658	1658	Sugar binding protein of abc transport system	-	-	-	-
Bbr_1659	1659	LacI Transcriptional regulator	-	-	-	-
Bbr_1841	1841	ATP binding protein of ABC transport system	+	-	-	-
gntR	1891	GntR transcriptional regulator	-	-	-	-
Bbr_1894	1894	PTS uptake System	++	+	H	-
nrdH	1901	Glutaredoxin	++	-	E	-

EMSA analyses were carried out with 150 nM protein (BifR1 or BifR2) incubated with 0.5nM Ird-labelled DNA fragments encompassing the promoter region of the specified gene. Binding affinity was calculated based on the total percentage DNA bound, – is representative of no binding, + is representative of an ability to bind up to 15 % of the total DNA, ++ is representative of an ability to bind 15 % - 50 % of the total DNA present and +++ is representative of an ability to bind 50 – 100 % of the DNA present in the reaction. * represents divergently orientated genes. # represents genes which are located at divergently orientated gene/gene clusters in which the transcriptional start site has been experimentally validated. The Table has been colour coded, genes highlighted in orange are genes which were predicted to be part of the BifR2-controlled regulon by Khoroshkin *et al.* (41), genes in green were predicted to be part of the BifR1-controlled regulon by Khoroshkin *et al.* (41), while genes highlighted in blue were selected based on their differential expression found in the transcriptomic analysis of either *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2* as compared with the *B. breve* UCC2003 WT strain conducted in this study.

Determination of the operator sequences recognized by BifR1 and BifR2

As shown above, EMSA analyses employing BifR1 and BifR2 clearly demonstrate their *in vitro* affinity for various promoter regions, including those that correspond to several central metabolic genes. In order to identify the operator sequences or TFBSs that are recognized by either BifR1 or BifR2, fragmentation analysis was carried out on the promoter regions of two key metabolic pathway genes (both bound by BifR1 and BifR2). This analysis entailed incubating DNA fragments encompassing various sections of the promoter region upstream of the *eno* (encoding enolase and corresponding to locus tag Bbr_0725) and *pyk* (encoding pyruvate kinase and corresponding to locus tag Bbr_0757) with purified BifR1 or BifR2. EMSA analyses of these *eno* and *pyk* promoter regions revealed that a 47 and a 48-bp DNA segment, respectively, contained the operator site for both BifR1 and BifR2 (fragmentation of the *pyk* promoter region is illustrated in Fig. 4.2). The 47-bp segment of DNA located upstream of the *eno* gene contains a 20 bp imperfect inverted repeat 5'-TGATGTGAGC><GCTCACAATG-3' (with "><" indicating the centre of the inverted repeat), while the 48-bp DNA segment located upstream of the *pyk* gene contains a very similar 20 bp inverted repeat 5'-TGTTGTGAGC><GCTCACACTG-3' (NB. Both sequences have a 14-bp central, perfect repeat in common). The features of these BifR1/BifR2 operator sequences are consistent with those typical of Lac-I type regulators, being an even palindrome of 16-22 nucleotides in length with a consensus CG at the centre and a highly conserved A and T at nucleotides 8 and 13 respectively of the 20 nucleotide consensus motif (84).

A screen for similar sequences in the BifR1/BifR2-bound promoter regions generated a consensus BifR1/BifR2-recognition sequence RNTGNKARC><GCTMACRNN for central metabolic genes (Figure 4-2, Panel 3 (A)). This consensus sequence (although individual nucleotides were not weighted for importance) was then utilised to identify any further genes which may be regulated by BifR1 and BifR2, resulting in the identification of three additional genes/gene clusters that appear to contain this TFBS in their predicted promoter region (though this will require experimental verification). These three promoter regions are associated with locus tags Bbr_0037/0038, Bbr_1650, Bbr_1723/1724, and are predicted to encode a carbonic anhydrase/alkyl hydroperoxide reductase, a glucanotransferase and a BirA family transcriptional regulator containing a biotin (acetyl-coA carboxylase) ligase domain, respectively.

As mentioned above, some divergence in the DNA binding specificity of the two TFs for the promoter regions of certain genes (not involved in central carbon metabolism) was observed (Table 4-4; NB the affinity for some of these differentially bound fragments is low). In order to identify the consensus sequence for each TF, DNA regions upstream of those genes for both BifR1 and BifR2 was analysed. From Figure 4-2, Panel 3 it is evident that the deduced TFBS for the genes associated with central metabolism is highly conserved, with 13 of the 20 nucleotides analysed in the TFBS being highly conserved. On inclusion of deduced TFBSs from bound fragments associated with non-bifid shunt genes, a gradual resemblance to the generic LacI consensus motif is observed, although 8 of the 20 nucleotides still remain highly conserved (Figure 4-2, Panels 3B and 3C display the deduced TFBS for BifR1 and BifR2, respectively). Of note, a large number of hits were returned when these consensus sequences were employed to search for additional BifR1/BifR2-binding sites on the *B. breve* UCC2003 genome.

Interestingly, the deduced TFBSs of BifR1 and BifR2 are located upstream of recently assigned -10/-35 promoter sites of six bifid shunt-associated genes/gene clusters (Table 4-4 and Table S4-10) (22). This TFBS location relative to the -10/-35 sites is therefore typical of a transcriptional activator and in this context it should be noted that all genes encoding the bifid shunt enzymes exhibit a high level of transcription when grown on glucose (22). Further analysis showed that the TFBS of BifR1/BiR2 is not located at a particular distance from these promoter sequences: Table S4-10 illustrates that the BifR1/BifR2 TFBS is located 16 and 15 bp upstream of the -35 site in the promoter regions of *eno* and *pyk*, and 33, 34 and 35 bp upstream of the -35 site of *pfl*, *icfA* and *gap*, respectively. Therefore, the position of the BifR1/BifR2 TFBS relative to the -35 sites implies that BifR1/BifR2 bind in multiples of one helical turn plus one half (i.e. 1.5, 3.5 or 6.5 helical turns) away from the promoter region. The mechanistic implications of such an apparently phased binding pattern will be covered below in the discussion.

Analysis of the transcriptional start sites with respect to the (deduced) location of the TFBS of BifR1/BifR2 upstream of *carD*, *glgP* and *cldE* revealed that the TFBS is overlapping with predicted -35/-10 regions, indicating that BifR1/BifR2 in these cases act as repressors, being typical LacI-type TFs (85).

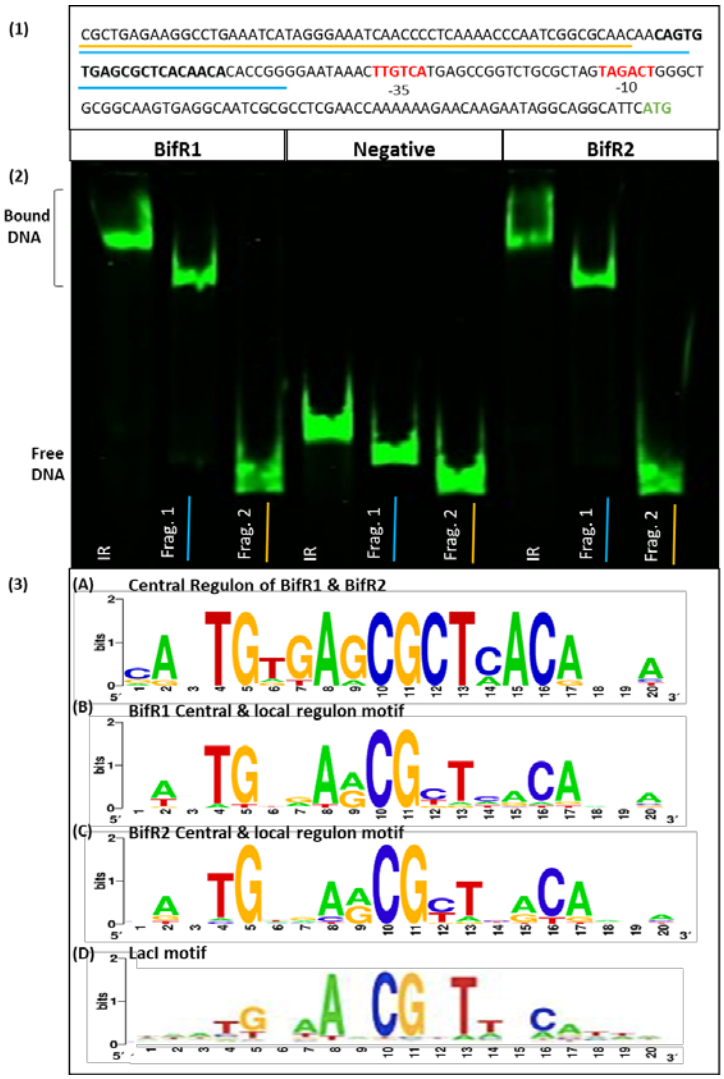


Figure 4-2. Fragmentation analysis and BifR1/BifR2 binding.
Panel (1) depicts the promoter region of *pyk* (Bbr_0757) used for fragmentation analysis. -35 and -10 sites are indicated in red and the ATG start codon is indicated in green. The TFBS is indicated in bold. The fragment including the TFBS (Frag. 1) is underlined in blue, the fragment excluding the TFBS (Frag. 2) is underlined in yellow, while the full fragment is referred to as IR.
Panel (2) EMSA to investigate BifR1 and BifR2 abilities to bind to Bbr_0757 promoter region fragments (IR, Frag 1 and Frag 2). All reactions contain 0.5 nM Ird labelled DNA and 150 nM of either BifR1 or BifR2 protein, while negative reactions contain 0 nM protein.
Panel (3), (A) Illustrates consensus BifR1/BifR2 binding motif for genes involved in central metabolism. (B) Depicts the binding motif of BifR1 central and peripheral regulons. (C) Displays the binding motif of BifR2 central and peripheral regulons. (D) Illustrates the logo for the alignment 370 LacI family members (BS sequences from RegTransBase (46)). Motif scores are expressed in bits (see materials and methods).

Attempts to identify effector molecules for BifR1 and BifR2

Microarray and *in silico* analyses, coupled to EMSA analyses allowed the deduction of the BifR1/BifR2 regulons and associated operator sequences. We next wanted to gain further insight into the nature of the environmental/metabolic signals that impact on the recognition ability and/or binding affinity of BifR1 and BifR2 to their target DNA sequences. For this purpose, EMSA analyses were performed whereby BifR1 or BifR2 was incubated with one of its binding targets, in this case the *bifR1* DNA promoter region, in the presence of each of a variety of possible (commercially available/relevant) effectors (Table S4-9). These effectors were selected for two main reasons: (i) because they occupy key positions within the bifid shunt (e.g. Phosphoenolpyruvate, D-erythrose-4-Phosphate and Glyceraldehyde 3-phosphate), which may allow them to act as cues to provide information on the metabolic status of the cell, and (ii) because they are known to act as effector molecules in other organisms (58, 59). The results obtained indicate that none of the effector molecules tested in this study consistently decreased/increased BifR1 or BifR2 ability to bind to DNA fragment encompassing the *bifR1* promoter region (example effector EMSA can be found in Figure S4-11). It is possible that the *in vitro* experimental set up did not allow for the identification of the effector molecule, or perhaps that the molecule is the product of another metabolic pathway outside of central carbon metabolism.

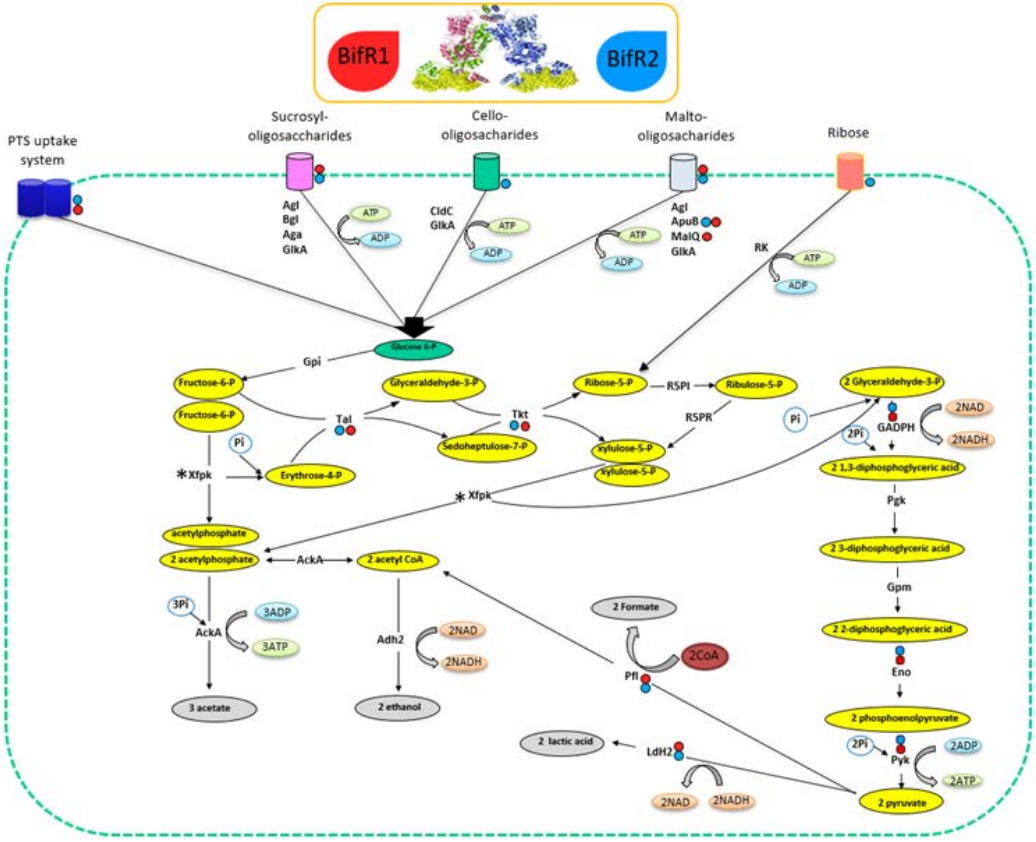


Figure 4-3. Schematic of BifR1 and BiR2 effects on Central metabolism.
Schematic of predicted central metabolism steps for carbohydrate metabolism in *B. breve* UCC2003. The XFPK enzymatic steps are indicated by an asterisk. As determined by EMSA analysis in Table 4-4, enzymatic steps which are under the regulation of BifR1 and BifR2 are indicated by a red or blue dot, respectively. Abbreviation: Agl, alpha glucosidase; Bgl, beta gluosidase; GlkA, glucokinase; CldC, beta glucosidase; ApuB, amylopullunase; MalQ, glucanotransferase; Rk, ribokinase; Gpi, glucose- 6-p isomerase; Tal, transaldolase; Tkt, transketolase; R5PI, ribose-5-P isomerase; R5PR, ribose-5-P reductase; GADPH, glyceraldehyde-3-phosphate dehydrogenase; Xfpk, Xylose-5-P/Fructose-6-P phosphoketolase; Acka, acetate kinase; Ald2, alcohol dehydrogenase 2; Pgk, phosphoglycerate kinase; Gpm, phophoglycerate mutase; Eno, enolase; Pyk, pyruvate kinase; Pfl, pyruvate formate lyase; ldh2, lactate dehydrogenase 2.

Microarray analysis - Transcriptome of *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2*

In order to investigate the role of *bifR1* and *bifR2* in controlling central carbon metabolism, insertional mutants were created in each of these genes in *B. breve* UCC2003, resulting in strains *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2* (respectively; see Materials and Methods). These two mutants were then employed to assess transcriptional changes due to either of these mutations using microarray analysis. It was hypothesized that if *bifR1* and *bifR2* encode repressors, which is the most commonly encountered activity of LacI-type transcription factors (TFs), mutation of either *bifR1* or *bifR2* would cause increased transcription of the genes they control, even in the absence of the inducing carbohydrate or condition as had been previously shown for other (LacI-type) regulators in *B. breve* UCC2003 (35-38).

The transciptome of *B. breve* UCC2003-*bifR1*, when compared to that of *B. breve* UCC2003 (Table S4-5 and Table S4-6), revealed that 32 genes were transcriptionally up-regulated and 45 were down-regulated above a fold change of 2.0, p-value < 0.001. Microarray data furthermore revealed that compared to *B. breve* UCC2003, several genes which are predicted or had previously been shown to be involved in malto-oligosaccharide metabolism, such as *malQ* and *apuB* (56), were significantly up-regulated in *B. breve* UCC2003-*bifR1* (>2.5-fold change; P < 0.001), along with a number of other genes which are predicted to encode carbohydrate transport systems. The latter observation is consistent with the notion that *bifR1* encodes a transcriptional repressor, though as mentioned above various genes were also found to be down-regulated in the transcriptomic analysis. Rather surprisingly, none of the differentially regulated genes are members of the BifR1 regulon as predicted by Khoroshkin *et al.* (41) (Table S4-5 and Table S4-6). A number of genes involved in exopolysaccharide production (EPS) (Bbr_0442 – Bbr_0449 inclusive) were substantially down-regulated in the *bifR1* mutant microarray along with a number of other genes which are listed in Table S4-6. The genes which were transcriptionally down-regulated were not predicted to be under BifR1 control, and their reduced transcription in *B. breve* UCC2003-*bifR1* may therefore be due to an indirect effect of the *bifR1* mutation since BifR1 appears to regulate a number of other transcription factors (see EMSA results below).

The transcriptome of *B. breve* UCC2003-*bifR2* revealed, when compared to that of the *B. breve* UCC2003 control (Table S4-7 and Table S4-8), just a single gene, *malE*, to be transcriptionally up-regulated above a fold change of 2.0, while no genes were found to be significantly (fold change of >2.0) down-regulated in the transcriptomic analysis of the *B. breve* UCC2003-*bifR2* mutant. Two genes, *apuB* and *malC*, that form part of the predicted *bifR2*-governed regulon (41), were up-regulated to a lower, yet significant degree (1.8 and 1.3-fold change, respectively; $P < 0.001$). Notably, some of the central metabolic genes were found to be up-regulated at a rather modest level. These results were found to be statistically significant with a p-value of less than 0.004, these were *xfp*, *eno*, *tkt*, *pfl*, *ldh* (1.5, 1.3, 1.3, 1.2 and 1.2-fold change, respectively). BifR2 was previously predicted to specifically control the expression of a number of genes involved in the utilisation of starch-like carbohydrates (41). Consequently, the (modest) increased transcription of several genes of the bifid shunt in the *B. breve* UCC2003-*bifR2* mutant (as compared to the wild type UCC2003 strain) was unexpected.

Phenotypic analysis of *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2*

Analysis of B. breve UCC2003-bifR1 and B. breve UCC2003-bifR2 growth on varying carbon sources.

In order to gain a better understanding of the phenotypic implications of inactivation of *bifR1* or *bifR2*, strains *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2* were tested for their ability to grow on a number of carbon sources (glucose, maltose, starch and ribose) with OD_{600nm} readings taken at 24 hours post-inoculation (Figure 4-4). These carbohydrates were selected as they were perceived to be the most relevant carbon sources based on the regulons of BifR1 and BifR2 (based on transcriptomic and EMSA analysis). This analysis found that all strains displayed similar growth abilities on all sugars, although *B. breve* UCC2003-*bifR2* was shown to exhibit somewhat (but not statistically significant) reduced growth on starch as compared to *B. breve* UCC2003.

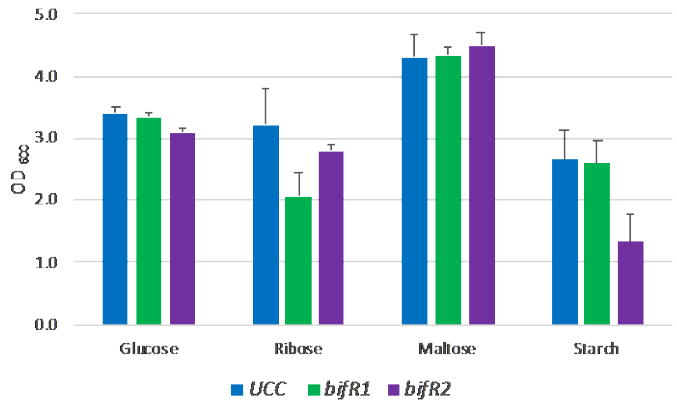


Figure 4-4 Analysis of the growth
B. breve UCC2003 *wt (UCC)*, *B. breve* UCC2003-*bifR1 (bifR1)*, and *B. breve* UCC2003-*bifR2* (bifR2) were analysed for their ability to grow on varying carbon sources (starch, ribose, glucose and maltose) at 24 hours post inoculation. Error bars for each represent the standard deviation calculated from three replicates.

Survival of B. breve UCC2003-bifR1 and B. breve UCC2003-bifR2 when exposed to porcine bile or when grown in the presence of bile salts

Several publications have reported that the (in)ability to survive bile stress is linked with shifts in the expression of genes involved in central metabolism and with the concentration of its resulting end products in various *Bifidobacterium* species (52, 74-76). For this reason, survival assays were carried out on *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2* mutants, along with the wild type control strain *B. breve* UCC2003, to test their ability to survive exposure to porcine bile (0, 1 and 2 %) for a time period of 20 minutes. Following the imposition of bile stress, cultures were assessed for survival by spread plating at serial dilutions. The resulting colony counts after 2 days of growth are displayed in Figure 4-5 Panel (A). Firstly, this analysis revealed that *B. breve* UCC2003 recovers very efficiently post bile stress, with no dramatic drop in viable counts following exposure to either 1 % or 2 % porcine bile. In contrast, *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2* exhibited a reduced ability to withstand short-term bile stress, although this reduction did not reach statistical significance (*p*-value of 0.07). We next investigated if these two mutants were able to resist long-term exposure to porcine bile. *B. breve* UCC2003 wt, *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2* mutants were analysed for their ability to grow in the absence (control) as compared to the presence of 1 % porcine bile with OD_{600nm} readings taken at 24 hours post inoculation. Figure 4-5 Panel (B) displays the fold reduction in OD_{600nm} following 24

hours growth in the presence of 1 % porcine bile as compared to the control. As expected, there was a significant fold reduction in OD_{600nm} readings for all three strains when grown in the presence of porcine bile, as compared to OD_{600nm} readings without porcine bile. However, the fold reduction in growth of *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2* mutants was more pronounced: in the presence of 1 % porcine bile, the *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2* mutants demonstrated a fold reduction of 11.1 (p-value < 0.010) and 6.4 (p-value < 0.010) in OD_{600nm} readings, respectively, whereas the fold reduction for the control strain *B. breve* UCC2003 was 3.5.

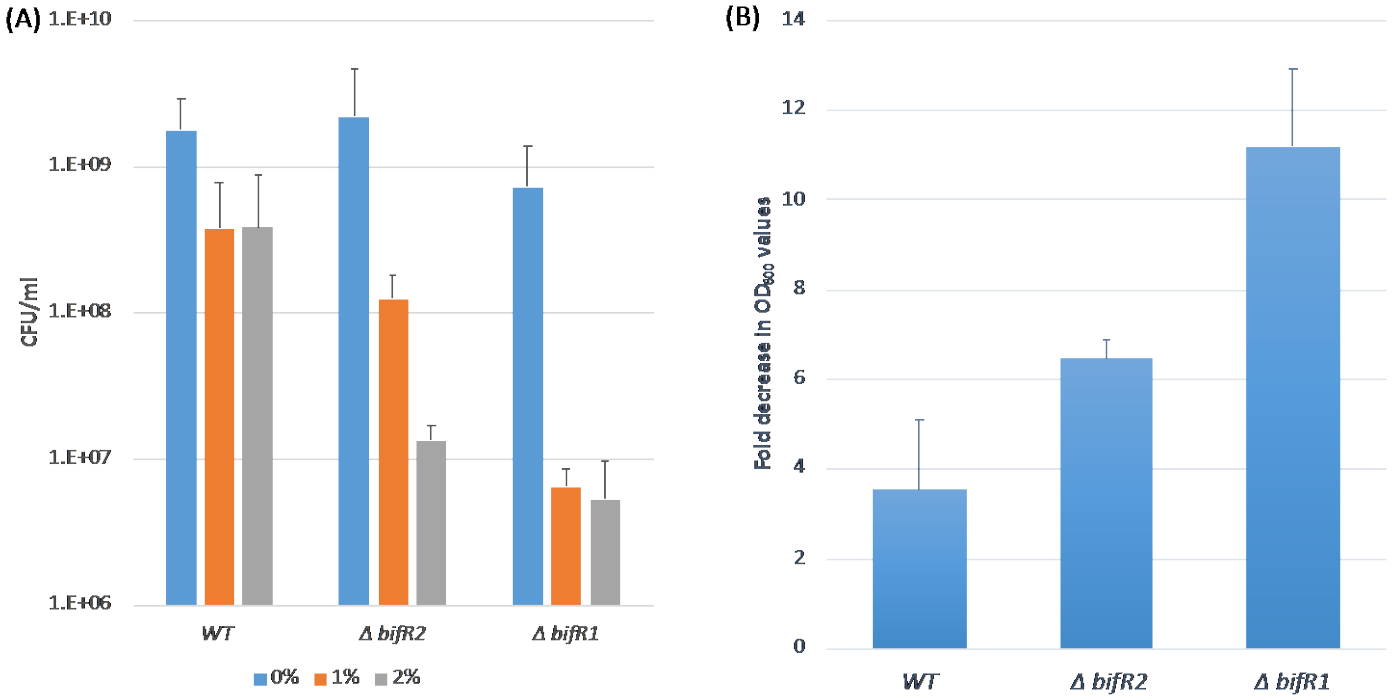
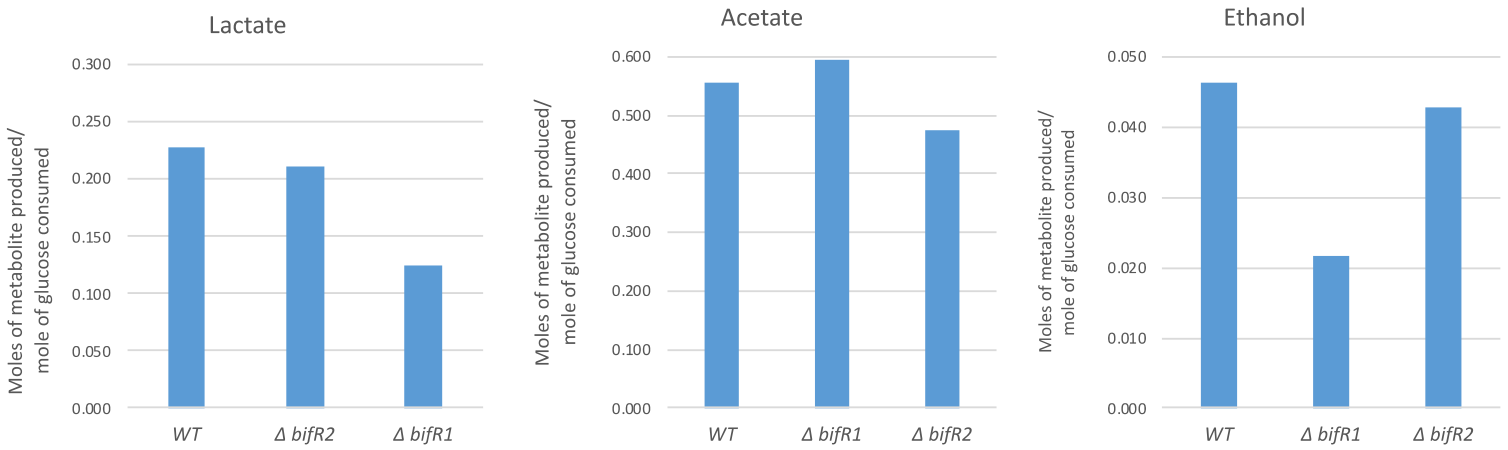


Figure 4-5 Bile survival Assays:
Part A: Survival assay of *B. breve* UCC2003 WT (*WT*), *B. breve* UCC2003-*bifR1* (Δ*bifR1*) and *B. breve* UCC2003-*bifR2* (Δ*bifR2*) when exposed to increasing concentrations of porcine bile (0, 1 and 2 % (v/v) for 20 minutes, survival is expressed in CFU/ml).
Part B: Analysis of the growth of *B. breve* UCC2003 WT (WT), *B. breve* UCC2003-*bifR1* (Δ*bifR1*) and *B. breve* UCC2003-*bifR2* (Δ*bifR2*) when grown in the presence of 1 % porcine bile. The values are expressed as fold decrease in OD_{600nm} values between growth on 0 and 1 % bile. Error bars for each represent the standard deviation calculated from three replicates.

Metabolic Flux Analysis by HPLC

Phenotypic analysis of *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2* revealed that insertional mutagenesis of the *bifR1* and *bifR2* genes resulted in a reduced ability to withstand bile stress. In order to investigate if these mutations have any impact on the generation of metabolic end products, HPLC analysis was carried out of spent medium following growth of the control strain *B. breve* UCC2003 and the two strains that carry a mutated *bifR1* or *bifR2* gene (Figure 4-6). In short, *B. breve* UCC2003, *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2* were grown on mMRS medium supplemented with glucose, with samples taken at 8 hours post-inoculation for analysis. This analysis found that *B. breve* UCC2003-*bifR1* consumed more glucose at 8 hours post inoculation as compared to the control strain and *B. breve* UCC2003-*bifR2*. The resulting lactate:acetate ratio was 1:2.43 in the WT, which is rather different from that reported by O'Loughlin *et al* (60), who found the ratio of lactate:acetate of *B. breve* UCC2003 grown on glucose as a sole carbon source to be 1:4.989. However, the exact growth phase at which this ratio was recorded is unknown, as previously mentioned the theoretical ratio of lactate:acetate produced by the bifid shunt pathway is 1:1.5 (20), but lactate:acetate ratios are rarely reproducible during bifidobacterial carbohydrate fermentation, due to the production of formate, acetate, and ethanol from pyruvate instead of lactate, depending on the available energy source and its consumption rate (61). In comparison to the metabolite production in the *B. breve* UCC2003 WT strain (1:2.43), *B. breve* UCC2003-*bifR1* and *B. breve* UCC2003-*bifR2* both exhibited a higher lactate:acetate ratio than the WT with ratio of 1:2.82 (however this did not reach significance) and 1:3.80 being recorded, respectively, at 8 hours growth (Figure 4-6). However further analysis of this phenomenon will be necessary to validate the biological significance of these findings.



	Lactate : Acetate Ratios
WT	1 : 2.43
Δ bifR1	1 : 2.82
Δ bifR2	1 : 3.80

Figure 4-6 Metabolic end product analysis
HPLC of *B. breve* UCC2003 (*WT*), *B. breve* UCC2003-*bifR1 (Δ bifR1)*, and *B. breve* UCC2003-*bifR2 (Δ bifR2).* All strains were grown on glucose as the sole carbon source, and samples were taken after 8 hours. Concentration of lactate, acetate and ethanol are expressed as moles of the metabolite produced per mole of glucose consumed.

4.5 Discussion

Several bifidobacterial colonisation factors have been identified in recent years (62), often using *B. breve* UCC2003 as a model organism (63). One particular property of interest with respect to gut colonisation is the metabolic flexibility of *B. breve* UCC2003. The distal gut does not supply bacteria with reliable carbon sources; available carbohydrates are dependent on many factors such as host diet and other (competing) bacteria present in the GIT. This carbohydrate transience deems it necessary for bifidobacteria to be capable of utilising a multitude of carbon sources, and importantly to be capable of doing so swiftly and efficiently, making sure energy generation and biosynthetic demands are in balance, so as to (out)compete (with) other GIT resident bacteria. As for most living organisms, bifidobacteria conserve energy by only transcribing (metabolic) genes when necessary.

Previous studies have reported difficulties in retaining binding activity of bifidobacterial LacI-type proteins during purification. Consequently, many of these studies utilised crude cell extracts in their EMSA assays (35, 36, 38, 64, 80). The addition of glycerol and the resuspension of the purified protein in EMSA buffer allowed for the purification of active BifR1 and BifR2 proteins, although it is unlikely that all purified BifR1/BifR2 protein was obtained in a stable, binding-competent state. As with all *in vitro* assays, EMSA and transcriptomic analyses have their limitations and it is important to consider these factors on interpretation of the obtained results. The same can be said for *in silico* analysis, as exemplified by the publication by Khoroshkin *et al.* (41), in which BifR2 (previously designated MalR1) was predicted to regulate the transcription of a number of genes involved in the metabolism of starch-like/-derived carbohydrates. Our findings clearly indicate that BifR2, along with BifR1, plays a role in the regulation of central metabolism of *B. breve* UCC2003. Khoroshkin *et al.* (41) found that both *bifR1* and *bifR2* are conserved across nine out of the ten assessed *Bifidobacterium* genomes, while genes encoding other LacI-type TFs, such as MalR2/MalR5, are not. This suggests that BifR1/BifR2-mediated global regulation of central metabolism is operational in many, if not all, bifidobacteria.

From the current study it appears that BifR1 and BifR2 together impose global shifts in gene transcription in *B. breve* UCC2003 through their ability to regulate transcription of

other TF's (i.e. CarD , MalR2, MalR3 and MalR5; see Table 4-4), thus effecting the expression of their respective regulons while also controlling the transcription of various bifid shunt genes in *B. breve* UCC2003 (Table 4-4; , Figure 4-1 and Figure 4-2). The presumed dual ability of two TFs to either enhance or repress transcription of their target genes is reminiscent of a similar phenomenon reported in *Corynebacterium glutamicum* (67). Where BifR1 and BifR2 are deduced to repress transcription of genes related to sugar uptake and to activate transcription of genes related to central carbon metabolism, the opposite was seen for two LacI-type TFs, GntR1 and GntR2, in *C. glutamicum* (68). Furthermore, BifR1 and BifR2 appear to regulate transcription of their own genes (auto-regulation), while also regulating each other's expression. Assuming that both BifR1 and BifR2 act as repressors of each other transcription (-10/-35 sites for bifR1 and bifR2 remain to be determined), a decrease in the concentration of either TF would consequently result in the increase of the other, exemplifying just how complex and intertwined this regulatory system is in *B. breve* UCC2003.

Initially, we believed that the functional redundancy of the *bifR2* gene was the product of a gene duplication event, as was predicted to be the case for two functionally equivalent TFs, encoded by *gntR1* and *gntR2*, in *C. glutamicum* (68). However, GntR1 and GntR2 exhibit 78 % amino acid similarity to each other, while BifR1 and BifR2 display just 26 % similarity, and a higher level of similarity would be more in line with a (recent) gene duplication event. A number of attempts were made to "knockdown" transcription of both *bifR1* and *bifR2*, in order to determine the combined effect on global transcription. Unfortunately, these attempts were not successful, and we believe that reducing expression of both TFs at the same time causes serious growth issues due to its combined negative impact on central carbon metabolism.

The location of BifR1 and BifR2 TFBS in relation to the cognate -10/-35 sites of their regulated genes revealed that BifR1 and BifR2 both act as activators of a number of genes involved in central carbon metabolism including *eno*, *pyk*, *tkt*, *gap*, *ldh2*, and a number of other genes outside of central metabolism including *Bbr_1723* and *malE2* (a carbohydrate uptake system), while probably acting a repressor of a number of other carbohydrate related uptake and utilisation genes (listed in Table S4-9).

Analysis of the transcriptional start sites with respect to the location of the TFBS of BifR1/BiR2 (on genes which BifR1/BifR2 were deemed to act as activators) revealed that, although there is no fixed distance upstream at which they bind (relative to the predicted -35 site), they bind at a distance of a multiple of one turn plus one half (i.e. 1.5, 3.5 or 6.5 helical turns) away from the promoter region. This is consistent with findings for another LacI family member, CcpA. Grundy *et al.* reported that CcpA acts both as a repressor and an activator (85), and that this TF binds 14 bp upstream of the -35 site when identified as an activator. These authors suggested that CcpA binds to the same side of the helix face as the position of the -35 site. The BifR1/BifR2 TFBS was found (in some cases) to be located at a similar distance upstream of the -35 site implying that BifR1/BifR2 may also bind on the same side of the helix face as the position of the -35 site. One important feature of LacI-type TFs is that they can alter the local DNA structure (85), which in this case may stimulate the docking of the RNA polymerase holoenzyme to its cognate -35/-10 sites.

It has been reported that the metabolic redirection towards acetate production in the bifid shunt allows for additional ATP generation, yet reduces the ability to equilibrate the redox balance (52), which may consequently make the strain more susceptible to bile stress. It has previously been shown in *Lactobacillus* and *Bifidobacterium* that increased ethanol production results in an increased level of regenerated NAD+ which in turn helps to combat the oxidative stress effects of bile. Therefore, the reduced production of ethanol, particularly in the *bifR1* mutated strain, may have caused increased bile sensitivity (73-76).

Where there is a lack of information relating to the transcriptional regulation of central metabolism in bifidobacteria, there is less so in the area of bifidobacterial bile stress response. In order for bifidobacteria to transiently colonise the GIT, they have to respond and adapt to the potentially lethal effects of bile. A number of publications have reported a change in the central carbon flux in bifidobacteria upon exposure to bile (69-72). In one such publication (69), the authors reported that the expression of certain key metabolic enzymes was up-regulated in the presence of bile, some of which form part of the BifR1 and BifR2 regulon, such as the transketolase, the lactate dehydrogenase, the glyceraldehyde-3-phosphate dehydrogenase and the pyruvate kinase. It appears that the effect of a *bifR1* mutation has a more deleterious effect on growth in the presence of bile

as compared to a *bifR2* mutation. The cause of this increased sensitivity to bile is most likely due to an inability of the corresponding *B. breve* UCC2003 mutant to control its central metabolism in a way that could optimize survival under stress conditions, as has previously been reported (70). Optimal bifid shunt performance as governed by BifR1 and BifR2 may be expected to lead to increased ATP production, which in turn can be employed to combat bile stress. Mutating *bifR2* altered this strains metabolic end product profile, such as a changed lactate:acetate ratio and ethanol production, which may be a reflection of the inability of such mutants to adjust central carbon metabolism, perhaps also contributing to the increased bile sensitivity.

The observed phenotypes point towards the important role of BifR1 and BifR2 in *B. breve* UCC2003 in regulating the metabolic flux of the bifid shunt, thereby contributing to metabolic agility and associated ability to adapt to stressful conditions. In conclusion, this work has shed light on a novel and apparently unique regulatory mechanism employed by *B. breve* UCC2003 and probably many bifidobacterial species to regulate the central carbon flux. This regulatory system appears to be crucial for this organism in order to provide adaptability to survive in the constantly changing and highly competitive environment of the gastrointestinal tract.

4.6 Supplemental Tables

Table S4-5. Transcriptomic analysis of *B. breve* UCC2003-*bifR1* mutant (Up-regulation)
Global transcriptomic analysis of *B. breve* UCC2003-bifR1 mutant as compared with *B. breve* UCC2003 when grown on mMRS supplemented with ribose (up-regulated gene above a 2-fold threshold are indicated)

Locus tag	Up	Gene name and/or predicted Function	P value
Bbr_0026	2.2	Permease protein of ABC transporter system for sugars	4.50E-01
Bbr_0027	2.2	Permease protein of ABC transporter system for sugars	4.53E-01
Bbr_0030	2.7	Conserved hypothetical protein	3.67E-01
Bbr_0060	2.6	*glgP1*, Glycogen phosphorylase	3.78E-01
Bbr_0110	6.5	*ilvC2*, Ketol-acid reductoisomerase/2-dehydropantoate 2-reductase	1.54E-01
Bbr_0116	2.5	*malQ*, 1 4-alpha-glucanotransferase	3.98E-01
Bbr_0117	2.8	*agl3*, Alpha-glucosidase	3.59E-01
Bbr_0118	4.4	*malE*, Maltose/maltodextrin-binding protein	2.25E-01
Bbr_0119	4.4	*malC*, Maltodextrin transport system permease protein	2.28E-01
Bbr_0120	4.5	*malG*, Maltose transport system permease protein	2.21E-01
Bbr_0121	3.9	Conserved hypothetical membrane spanning protein	2.59E-01
Bbr_0122	3.7	*malR3*, Transcriptional regulator, LacI family	2.73E-01
Bbr_0123	4.3	*apuB*, Amylopullulanase	2.34E-01
Bbr_0164	4.3	*oppA1* Oligopeptide-binding protein	2.35E-01
Bbr_0165	4.6	*oppB1*, Oligopeptide transport system permease protein	2.18E-01
Bbr_0171	2.2	Sialidase A	4.60E-01
Bbr_0284	2.5	Sugar/Sodium symporter	4.04E-01
Bbr_0285	2.3	*lacZ2*, Beta-galactosidase	4.34E-01
Bbr_0538	2.2	*cysK*, cysteine synthase	4.63E-01
Bbr_1416	2.0	*rbsD1*, Ribose transport system permease protein	4.95E-01
Bbr_1429	2.1	*cbiO2*, Cobalt transport ATP-binding protein	4.70E-01
Bbr_1430	2.1	*cbiQ*, Cobalt transport protein	4.81E-01
Bbr_1530	2.8	Conserved hypothetical protein with CHAP and transglycosylase SLT	3.62E-01
Bbr_1644	2.3	Narrowly conserved hypothetical membrane spanning protein	4.35E-01
Bbr_1742	2.3	L-fucose permease	4.37E-01
Bbr_1743	2.3	Short chain dehydrogenase	4.38E-01
Bbr_1842	2.2	*aap6*, Amino acid permease	4.64E-01
Bbr_1845	2.4	Permease protein of ABC transporter system for sugars	4.24E-01
Bbr_1889	5.1	Cell surface protein with gram positive anchor domain	1.95E-01
Bbr_1890	6.2	ATP-binding protein of ABC transporter system for sugars	1.62E-01
Bbr_1891	7.6	Transcriptional regulator, GntR family	1.32E-01
Bbr_1892	8.6	PTS system, IIC component	1.16E-01

The level of expression is shown as a fold-value of increase in expression, with a cut-off of a minimum >2-fold increase in expression.

Table S4-6. Transcriptomic analysis of *B. breve* UCC2003-*bifR1* mutant (Down-regulation)
Global transcriptomic analysis of *B. breve* UCC2003-bifR1 mutant as compared with *B. breve* UCC2003 when grown on mMRS supplemented with ribose (down regulated gene above a 2-fold threshold are indicated)

Locus tag	Down	Gene name and/or predicted Function	P value
Bbr_0104	4.0	*ilvC1*, Ketol-acid reductoisomerase/2-dehydropantoate 2-reductase	0.00E+00
Bbr_0113	3.6	Cell surface protein precursor with Cna protein B-type domain and Gram-positive cocci surface proteins LPxTG motif profile	1.43E-08
Bbr_0114	2.3	Cell surface protein with Gram positive anchor and Cna protein B-type domain	0.00E+00
Bbr_0115	2.4	Sortase	1.48E-05
Bbr_0267	2.1	*glpF*, Glycerol uptake facilitator protein	7.11E-15
Bbr_0366	7.3	Narrowly conserved hypothetical secreted protein with Gram	0.00E+00
Bbr_0368	2.8	Conserved hypothetical protein	1.10E-08
Bbr_0441	14.1	Capsular polysaccharide biosynthesis protein	1.17E-11
Bbr_0442	11.7	Capsular polysaccharide biosynthesis protein	3.77E-15
Bbr_0443	6.3	Glycosyltransferase	1.66E-12
Bbr_0444	41.2	Membrane spanning polysaccharide biosynthesis protein	0.00E+00
Bbr_0445	8.7	Glycosyltransferase	0.00E+00
Bbr_0446	14.9	Acetyltransferase (cell wall biosynthesis)	2.33E-12
Bbr_0447	6.5	Conserved hypothetical protein	1.22E-15
Bbr_0448	4.3	Glycosyltransferase	4.05E-07
Bbr_0449	5.2	Hypothetical membrane spanning protein	5.71E-14
Bbr_0450	2.7	Membrane spanning protein involved in polysaccharide biosynthesis	1.44E-08
Bbr_0532	6.1	Transcriptional regulator, homologs of Bvg accessory factor	4.44E-16
Bbr_0533	3.3	Solute-binding protein of ABC transporter system for peptides	0.00E+00
Bbr_0534	2.7	Permease protein of ABC transporter system for peptides	0.00E+00
Bbr_0535	2.6	Permease protein of ABC transporter system for peptides	0.00E+00
Bbr_0536	3.0	ATP-binding protein of ABC transporter system for peptides	3.38E-12
Bbr_0602	4.0	Low specificity-threonine aldolase	1.19E-08
Bbr_0610	2.1	Conserved hypothetical membrane spanning protein	7.10E-08
Bbr_0611	2.4	Narrowly conserved hypothetical protein	3.37E-07
Bbr_0612	2.1	*crcB*, family protein	1.98E-07
Bbr_0674	2.4	Peptidase family M20A protein	7.44E-15
Bbr_0675	2.6	Permease protein of ABC transporter system	6.63E-11
Bbr_0889	2.3	Glutamine amidotransferase	1.48E-13
Bbr_0924	2.6	pntB NAD(P) transhydrogenase subunit beta	0.00E+00
Bbr_1078	2.1	Lantibiotic transport ATP-binding protein	3.23E-04
Bbr_1327	6.7	dTDP-rhamnosyl transferase	0.00E+00
Bbr_1328	4.2	Conserved hypothetical membrane spanning protein	0.00E+00
Bbr_1364	2.3	*groEL*, 60 kDa chaperonin	0.00E+00
Bbr_1474	3.0	Sua5/YciO/YrdC/YwlC family protein	0.00E+00
Bbr_1475	2.0	*livF*, Branched-chain amino acid transport ATP-binding protein	8.88E-16
Bbr_1476	2.1	*livG*, Branched-chain amino acid transport ATP-binding protein	2.47E-12

Bbr_1477	2.0	*livM*, Branched-chain amino acid transport system permease protein	3.12E-11
Bbr_1478	2.6	*livH* , Branched-chain amino acid transport system permease protein	5.95E-14
Bbr_1642	2.2	*rpsJ*, 30S ribosomal protein	1.58E-12
Bbr_1718	2.1	Hypothetical protein	5.55E-16
Bbr_1719	2.3	*fas*, Type I multifunctional fatty acid synthase	0.00E+00
Bbr_1720	2.1	*accD*, Acetyl-/propionyl-CoA carboxylase beta chain	2.22E-16
Bbr_1884	2.3	*galT2*, Galactose-1-phosphate uridylyltransferase	4.19E-06
Bbr_1886	2.1	Narrowly conserved hypothetical secreted protein	9.42E-12

The level of expression is shown as a fold-value of increase in expression, with a cut-off of a minimum >2-fold increase in expression.

Table S4-7. Transcriptomic analysis of *B. breve* UCC2003-*bifR2* mutant (Up-regulation)
Global transcriptomic analysis of *B. breve* UCC2003-bifR2 mutant as compared with *B. breve* UCC2003 when grown on mMRS supplemented with ribose (up-regulated gene above a 1.2-fold threshold are indicated)

Locus tag	Up	Gene name and/or predicted Function	P value
Bbr_0118	2	*malE*, Maltose/maltodextrin-binding protein	3.61E-13
Bbr_0119	1.3	*malC*, Maltodextrin transport system permease protein	2.46E-09
Bbr_0123	1.8	*apuB*, Amylopullulanase	6.34E-12
Bbr_0299	1.2	Holin	2.45E-07
Bbr_0391	1.2	*ilvB*, Acetolactate synthase large subunit	3.24E-07
Bbr_0558	1.3	Transcriptional regulator, LacI family	4.66E-12
Bbr_0607	1.4	*Tuf*, Protein Translation Elongation Factor Tu (EF-TU)	3.61E-03
Bbr_0725	1.3	*eno*, Enolase	2.93E-04
Bbr_0776	1.5	Xylulose-5-phosphate/Fructose-6-phosphate phosphoketolase	4.61E-06
Bbr_0787	1.2	*pfl*, Formate acetyltransferase	1.36E-01
Bbr_0847	1.3	*nagB2*, Glucosamine-6-phosphate isomerase	3.99E-10
Bbr_0848	1.3	Sugar kinase, ROK family	4.91E-06
Bbr_0921	1.3	*fadD2*, Long-chain-fatty-acid--CoA ligase	4.65E-09
Bbr_0969	1.3	*metE*, 5-methyltetrahydropteroyltriglutamate—homocysteine methyltransferase	6.99E-11
Bbr_0970	1.3	*metF*, Methylenetetrahydrofolate reductase	1.28E-12
Bbr_0973	1.3	*pyrB*, Aspartate carbamoyltransferase	5.89E-11
Bbr_1003	1.3	*tkt*, Transketolase	5.39E-04
Bbr_1079	1.3	Two-component response regulator	7.20E-11
Bbr_1233	1.3	Glyceraldehyde 3-phosphate dehydrogenase	4.01E-03
Bbr_1273	1.2	*ldh2*, L-lactate dehydrogenase	3.43E-04
Bbr_1367	1.3	*ung*, Uracil-DNA glycosylase	3.31E-11
Bbr_1505	1.2	*fucO*, Lactaldehyde reductase	2.05E-05
Bbr_1537	1.3	Hypothetical protein	2.53E-11
Bbr_1635	1.3	rpsC, 30S ribosomal protein	9.91E-03
Bbr_1649	1.3	*rplM*, 50S ribosomal protein L13	2.09E-02
Bbr_1719	1.2	*fas*, Type I multifunctional fatty acid synthase	6.48E-08
Bbr_1909	1.3	Conserved hypothetical protein	3.96E-08

The level of expression is shown as a fold-value of increase in expression, with a cut-off of a minimum >1.2-fold increase in expression.

Table S4-8 Transcriptomic analysis of *B. breve* UCC2003-*bifR2* mutant (Down-regulation)
Global transcriptomic analysis of *B. breve* UCC2003-bifR2 mutant as compared with *B. breve* UCC2003 when grown on mMRS supplemented with ribose (down-regulated gene above a 1.2-fold threshold are indicated)

Locus tag	Down	Gene name and/or predicted Function	P value
Bbr_0112	1.8	*malR6*, Transcriptional regulator, LacI family	7.65E-07
Bbr_0030	1.3	Conserved hypothetical proetin	1.63E-01
Bbr_0044	1.3	*pelF*, Glycosyl transferase (Polysacharide biosynthesis protein)	1.65E-08
Bbr_0045	1.2	Conserved hypothetical membrane spanning protein	4.09E-09
Bbr_0107	1.2	*cebF*, Cellobiose/cellotriose transport system permease protein	8.37E-11
Bbr_0468	1.2	Hypothetical protein	3.17E-10
Bbr_0535	1.3	Permease protein of ABC transporter system for peptides	5.61E-11
Bbr_1299	1.2	i*spA*, Lipoprotein signal peptidase	3.42E-07
Bbr_1482	1.3	*rpmE2*, LSU ribosomal protein L31P	2.97E-06
Bbr_1597	1.3	*rrf2*, family protein	3.53E-09
Bbr_1598	1.4	Pyridine nucleotide-disulphide oxidoreductase family protein	2.38E-14
Bbr_1658	1.2	Sugar-binding protein of ABC transporter system	2.32E-08
Bbr_1667	1.5	*rpmG*, LSU ribosomal protein L33P	3.67E-06
Bbr_1668	1.4	*Hsp10*, 10 kDa chaperonin GROES	1.24E-08
Bbr_1669	1.3	Narrowly conserved hypothetical membrane spanning protein	2.92E-10
Bbr_1731	1.3	*aspC*, Aspartate aminotransferase	3.75E-10
Bbr_1774	1.7	Transposase	3.19E-09
Bbr_1828	1.2	Transcriptional regulator, MarR family	4.07E-10
Bbr_1843	1.2	Narrowly conserved hypothetical membrane spanning protein	1.76E-07
Bbr_1844	1.3	Permease protein of ABC transporter system for sugars	2.67E-07
Bbr_1845	1.4	Permease protein of ABC transporter system for sugars	2.95E-08
Bbr_1854	1.3	*tdcB*, Threonine dehydratase	2.88E-10
Bbr_1873	1.3	Phospholipase/carboxylesterase	1.74E-11
Bbr_1890	1.2	ATP-binding protein of ABC transporter system for sugars	5.35E-04
Bbr_1905	1.3	Narrowly conserved hypothetical protein	7.05E-08
Bbr_1914	1.3	*pcnA*, tRNA nucleotidyl transferase	4.98E-09
Bbr_1918	1.2	*trxB2*, Thioredoxin reductase	3.41E-08
Bbr_1920	1.3	*parA*, Chromosome partitioning protein	7.06E-11
Bbr_1926	1.3	*rpmH*, LSU Ribosomal protein	6.81E-03

The level of expression is shown as a fold-value of increase in expression, with a cut-off of a minimum >1.2-fold increase in expression.

Table S4-9 Effector molecules tested

Effector Molecule
Maltose
Maltotriose
Maltulose
Isomaltose
Lactose
Glucose
Galactose
Sucrose
Trehalose
Glucose 6-phosphate
Glucose 1-phosphate
Pyruvic acid
Sodium Acetate
Fructose 6-phosphate
Cellobiose
Palatinose
Turanose
DL-Glyceraldehyde 3-phosphate
Acetyl coenzyme A sodium salt
Phosphoenolpyruvate
Acetyl Phosphate
D-Sedoheptulose-7-phosphate
D-Ribose 5-phosphate disodium salt
D-(-)-3-Phosphoglyceric acid disodium salt
Butyrate
Lactate
Propioante
Acetate
Acetyl Aldehyde
1,2 Propanediol
D-erythrose-4-Phosphate
Oxaloacetic acid
Cyclic-AMP
Succinic acid
D-Ribulose 5-phosphate disodium salt

Table S4-10 Location of the BifR1/BifR2 TFBS in relation to the -10/-35 sites.
The distance in base pairs [bp] is measured fromt the 3' -end of the TFBS to the 5' -end of the predicted -35 sequence.

Locus Tag Bbr_	**Gene Name**	**Activator/ Repressor/ Unknown**	**Distance between TFBS and predicted -35 [bp]**
0725	*eno*	Activator	16
0747	*carD*	Possible Repressor	-1 (overlaps with -35)
0757	*pyk*	Activator	15
0787	*pfl*	Activator	33
1002	*tkt*	Activator	6
1233	*gap*	Activator	35
1273	*ldh2*	Activator	65
1847	*malE3*	Activator	92
1723	-	Activator	41
0037	*icfA*	Activator	34
0038	*ahpC*	Unknown	84
0117	*agl4*	Repressor	-9 (overlaps with -35)
0060	*glgP*	Repressor	-13 (overlaps with -10)
0106	*cldE*	Repressor	-5 (overlaps with -10/-35)

BifR1

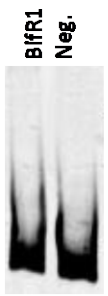
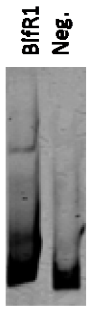
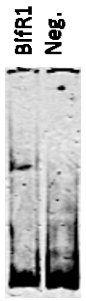
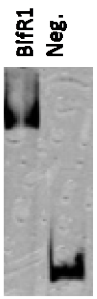
Gene Name				*malQ*	*pfl*	*pyk*	*ldh*	*eno*
Locus tag	Bbr_1891	Bbr_1420	Bbr_1841	Bbr_0116	Bbr_0787	Bbr_0757	Bbr_1273	Bbr_0725
EMSA Image								
Level Binding	–	–	+	+	++	++	+++	+++

Figure S4-7 BifR1 Example EMSA
All EMSA analysis carried out in the above figure were carried out with 150 nM protein (BifR1) or 0nM protein (Neg.) incubated with 0.5nM Ird labelled DNA fragments encompassing the promoter region of the specified gene. The level of binding was calculated based on the total percentage DNA bound, – is representative of no binding, + is representative of an ability to bind 5 – 15 % of the total DNA, ++ is representative of an ability to bind 15 % - 50 % of the total DNA present and +++ is representative of an ability to bind 50 – 100 % of the DNA present in the reaction.

BifR1

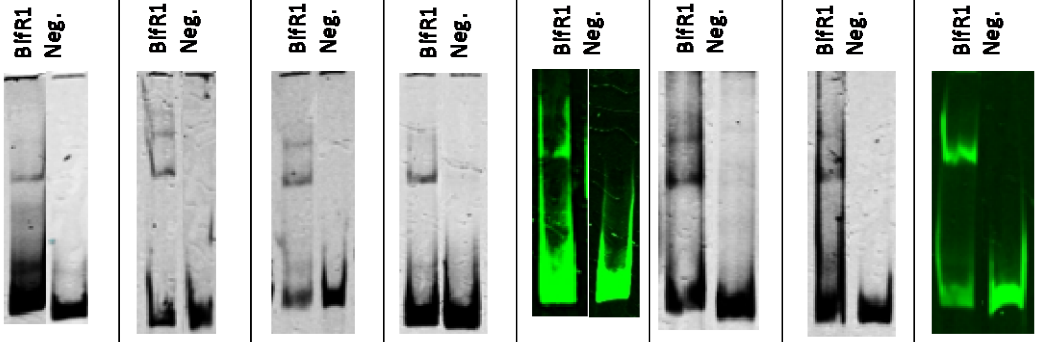
Gene Name		*glgp*	*malG2*	*malR2*	*malR5*	*nrdH*	*bifR2*	*bifR1*
Locus tag	Bbr_1841	Bbr_0060	Bbr_0027	Bbr_0023	Bbr_0032	Bbr_1901	Bbr_1846	Bbr_0411
EMSA Image								
Level Binding	+	++	++	++	++	++	++	+++

Figure S4-8 BifR1 Example EMSA
All EMSA analysis carried out in the above figure were carried out with 150 nM protein (BifR1) or 0nM protein (Neg.) incubated with 0.5 nM Ird labelled DNA fragments encompassing the promoter region of the specified gene. The level of binding was calculated based on the total percentage DNA bound, – is representative of no binding, + is representative of an ability to bind 5 – 15 % of the total DNA, ++ is representative of an ability to bind 15 % - 50 % of the total DNA present and +++ is representative of an ability to bind 50 – 100 % of the DNA present in the reaction.

BifR2

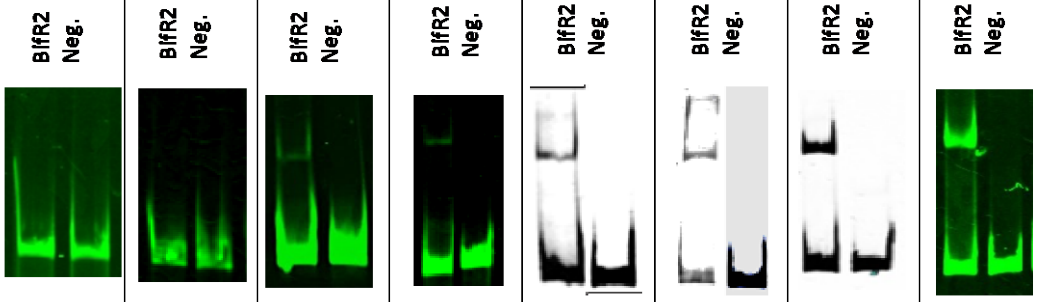
Gene Name	*malE*	*malG2*	*lacI*	*apuB/malR3*	*malR1*	*cldE*	*ldh*	*bifR1*
Locus tag	Bbr_1847	Bbr_0027	Bbr_1420	Bbr_0122/ 0123	Bbr_1846	Bbr_0106	Bbr_1273	Bbr_0411
EMSA Image								
Level Binding	–	–	+	+	++	++	+++	+++

Figure S4-9 BifR2 Example EMSA
All EMSA analysis carried out in the above figure were carried out with 150 nM protein (BifR2) or 0nM protein (Neg.) incubated with 0.5 nM Ird labelled DNA fragments encompassing the promoter region of the specified gene. The level of binding was calculated based on the total percentage DNA bound, – is representative of no binding, + is representative of an ability to bind 5 – 15 % of the total DNA, ++ is representative of an ability to bind 15 % - 50 % of the total DNA present and +++ is representative of an ability to bind 50 – 100 % of the DNA present in the reaction.

BifR2

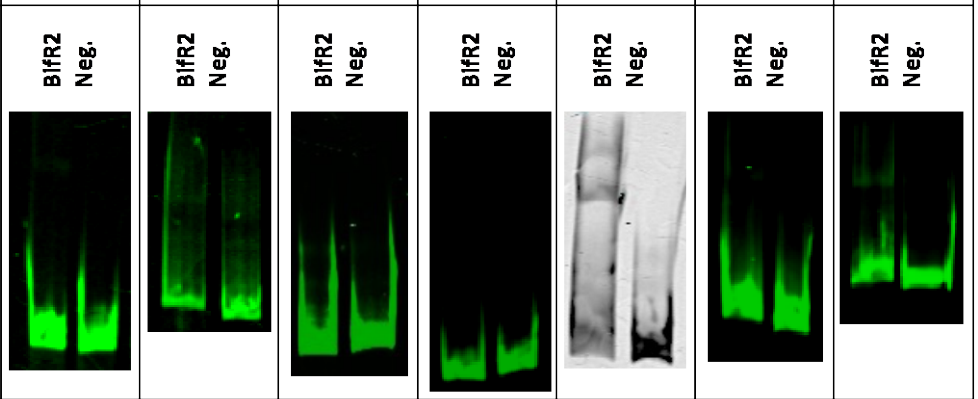
Gene Name	*glgP*	*malR6*	*malQ*	*malR2*	*rbsA1*		
Locus tag	Bbr_0060	Bbr_0112	Bbr_0116	Bbr_0023	Bbr_1419	Bbr_1841	Bbr_1901
EMSA Image							
Level Binding	–	–	-	-	++	-	-

Figure S4-10 BifR2 Example EMSA
All EMSA analysis carried out in the above figure were carried out with 150 nM protein (BifR2) or 0nM protein (Neg.) incubated with 0.5 nM Ird labelled DNA fragments encompassing the promoter region of the specified gene. The level of binding was calculated based on the total percentage DNA bound, – is representative of no binding, + is representative of an ability to bind 5 – 15 % of the total DNA, ++ is representative of an ability to bind 15 % - 50 % of the total DNA present and +++ is representative of an ability to bind 50 – 100 % of the DNA present in the reaction.

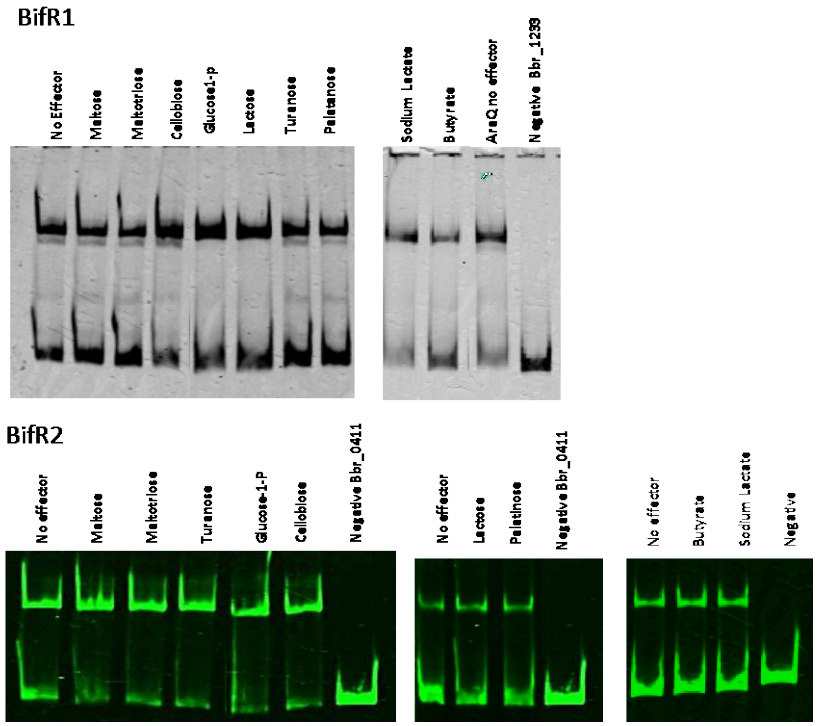


Figure S4-11 Example EMSA of BifR1 and BifR2 Effector Assays
All EMSA analysis were carried out with 150 nM protein (BifR1 or BifR2) or 0nM protein (Neg.), 10mM Effector Listed above and with 0.5 nM Ird labelled DNA fragments encompassing the promoter region of the specified gene

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Chapter 5 Regulation of carbohydrate metabolism by LacI-type MalR regulators in *Bifidobacterium breve* UCC2003

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*Both authors contributed equally to this work.

Noreen Lanigan carried out all work with respect to MalR2/MalR5 while Emer Kelly carried out all work with respect to MalR3/MalR6 while the analysis of results and writing of this chapter was jointly contributed to by Noreen Lanigan and Emer Kelly.

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5.1 Abstract

Bifidobacteria resident in the gastrointestinal tract are subject to many stresses such as bile exposure, osmotic shifts and nutritional starvation. Adaptation to these stressful conditions requires energy and an ability to rapidly adjust gene transcription. Four *Bifidobacterium breve* UCC2003-encoded LacI-type transcription factors (TFs), termed MalR regulators, had previously been proposed to be involved in the utilisation of maltose, maltodextrins and related polysaccharides, such as starch, amylopectin, amylose, glycogen and pullulan. Our current work indicates that these regulators are also involved in the utilisation of other carbon sources such as ribose and cellobiose. Interestingly, our *in vitro* data show that these regulators may cross-regulate the same carbohydrate utilization genes, while they also appear to regulate the expression of each other. These four TFs were shown to respond to distinct carbohydrate effectors, such as turanose or galactose, thus indicating that each regulator is responsible for a different aspect of carbon metabolism. This complex network of MalR-mediated gene regulation provides intriguing insights into the decision-making process of the cell with regards to carbohydrate utilisation, and into metabolic adaption of bifidobacteria to their environment.

5.2 Introduction

Members of the genus *Bifidobacterium* are Gram-positive, non-motile, non-spore forming, anaerobic, irregular rod-shaped bacteria, possessing a genome with a relatively high (>51 %) G+C content. Bifidobacteria have been isolated from a number of environments including human and animal and/or intestinal tracts, reproductive tract, the oral cavity and fermented foods. Various bifidobacterial strains and species are considered to elicit beneficial or probiotic activities (1-3). The health benefits with which *Bifidobacterium* are associated range from serum cholesterol level reduction in humans (4-6), modulation of host immune function (3), mitigation of lactose intolerance, pathogen inhibition/exclusion (7), and prevention or treatment of certain cancers such a colorectal cancer (8-10).

The genus *Bifidobacterium* consists of microorganisms with a chemoorganotrophic fermentative metabolism, being capable of degrading of variety of carbon sources including many mono/di-saccharides and more complex carbohydrates of plant origin. Carbohydrate fermentation in *Bifidobacterium* occurs via a rather unique metabolic pathway, the so-called bifid shunt, which results in a theoretical production of 1.5 mol acetate and 1 mol of lactate per mol of glucose utilised (11, 12). However, this ratio can vary dependent on growth rate and phase, cultivation conditions and carbohydrate substrate utilised. Consistent with their ability to utilise a diverse array of carbon sources is the fact that approximately 8 - 15 % of the coding capacity of an average bifidobacterial genome is involved in carbohydrate metabolism, the observed variability being due to different species and the functional assignment of the genes included in the respective analysis (12, 62). Furthermore, the (predicted) number of sugar uptake systems encoded by a bifidobacterial genome exceeds that of uptake systems for other compounds, such as amino acids, peptides and metals (63). This genetic dedication to carbohydrate metabolism indicates how important (flexible and diverse) carbohydrate utilisation is to bifidobacteria, particularly in the context of their ability to compete with other microbial inhabitants in the gastrointestinal tract (GIT).

Concurrent with the extensive array of carbohydrate uptake systems/utilisation genes is the presence of a large number of bifidobacterial Transcription Factors (TFs) (predicted to be) involved in controlling the uptake and utilisation of carbohydrates (13). Regulatory

networks are likely to play a vital role in the ability of bifidobacteria to respond to fluctuations in the type and quantity of nutrients available in their particular environmental niche. Carbohydrate utilisation pathways are generally governed by TFs, which bind to specific DNA sequences (referred to as operators or Transcription Factor Binding Sites (TFBSs)) in order to negatively or positively regulate transcription of a given gene by either preventing (TF is a repressor) or encouraging (TF is an activator) the RNA polymerase to gain access to its corresponding promoter, respectively. The activity of a TF may be modulated by an effector molecule, which may bind to an allosteric site on the TF, change its conformation, and either increase or reduce its affinity to its DNA target (14). In the case of carbohydrate utilisation such an effector molecule is typically a carbohydrate or a corresponding metabolic intermediate (15, 16).

Carbohydrate metabolism in bifidobacteria has been the focus of a substantial amount of research, and has resulted in the elucidation and characterisation of a number of specific carbohydrate utilisation pathways and corresponding regulons (17-25), along with a detailed *in silico* analysis investigating the regulatory networks controlling carbohydrate metabolism in bifidobacteria (13). The latter publication investigated the regulatory networks in ten bifidobacterial genomes and identified a number of TFs, termed MalR regulators, predicted to be involved in the utilisation of maltose and maltodextrins, as well as of larger α-glucosidic linkage-containing polysaccharides, such as starch, amylopectin, amylose, glycogen and pullulan. MalR1 (which will be referred to here as BifR2), MalR2 and MalR3 were predicted to recognize a common DNA motif and it was hypothesised that these TFs are involved in an interactive carbohydrate-dependent regulatory network. Two other TFs, MalR2b (designated here as MalR6) and MalR5 were proposed to encode maltose-specific regulators which act at a local level. The reconstructed BifR2, MalR2, and MalR3 regulons were conserved in nine out of the ten assessed *Bifidobacterium* genomes and were found to be overlapping with the AraQ (here denoted as BifR1) regulon in *B. breve* and *B. longum*. BifR1 was predicted to be a global regulatory protein involved in the regulation of central carbon metabolism in *Bifidobacterium*. From the results presented in Chapter 4 it became clear that BifR1 and BifR2 essentially recognize the same operator sites and it is currently believed that together they are responsible for the transcriptional regulation of several genes involved in the central carbon metabolic pathway in *Bifidobacterium breve* UCC2003. The

observed BifR1 and BifR2 binding abilities also indicate that these two TFs control not only their own but also each other's transcription, while they were also appear to control the transcription of the genes encoding MalR2, MalR3 and MalR5, whose regulons in turn are predicted to be involved in carbohydrate internalisation and processing before their entry into the bifid shunt.

The current study will take a focused view on the DNA binding abilities of MalR2, MalR3, MalR5 and MalR6, employing *B. breve* UCC2003 as a model organism to experimentally validate/explore the regulons governed by these TFs.

5.3 Materials and Methods

Bacterial Strains and Culture Conditions

Bacterial strains and plasmids used in this study are listed in Table 5-1. *B. breve* UCC2003 was routinely grown at 37°C in either de Man Rogosa and Sharpe medium (MRS medium; Difco, BD, Le Pont de Claix, France), modified de Man Rogosa and Sharpe (mMRS) medium made from first principles (26), or reinforced clostridial medium (RCM; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) supplemented with 0.05 % cysteine-HCl. Bifidobacterial cultures were incubated anaerobically in a modular, atmosphere-controlled system (Davidson and Hardy, Belfast, Ireland). Where appropriate growth medium was supplemented with tetracycline (Tet; 10 µg ml^{-1}), chloramphenicol (Cm; 5 µg ml^{-1} for *L. lactis* and *E. coli*, 2.5 µg ml^{-1} for *B. breve*), ampicillin (Amp; 100 µg ml^{-1}), erythromycin (Em; 100 µg ml^{-1}) or kanamycin (Kan; 50 µg ml^{-1}) for plasmid selection and maintenance.

Nucleotide Sequence Analysis

Sequence data were obtained from the Artemis-mediated (27) genome annotations of the *B. breve* UCC2003 genome sequence (28). Data base searches were carried out using non-redundant sequences accessible at the National Centre for Biotechnology Information internet site (http://www.ncbi.mlm.nih.gov) utilising the basic local alignment search tool (Blast). Sequence analysis was performed by using the Seqbuilder and Seqman programs of the DNASTAR software package (DNASTAR, Madison, WI). Protein functions were assigned with the use of the basic local alignment search tool for proteins (BlastP), and Homology detection and structure prediction by HMM-HMM comparison, and HHpred (29, 30).

Table 5-1. Bacterial strains and plasmids used in this study.

Strains and plasmids	Relevant features	Reference or source
Strains		
B. breve		
UCC2003	Isolate from nursling stool	(58)
UCC2003-*malR2*	pORI19-*tetW-malR2* insertion mutant of UCC2003	This study
UCC2003-*malR6*	pORI19-*tetW-malR6* insertion mutant of UCC2003	This study
UCC2003-*malR3*	pORI19-*tetW-malR3* insertion mutant of UCC2003	This study
UCC2003-*malR5*	pORI19-*tetW-malR5* insertion mutant of UCC2003	This study
E. coli		
XL1 BLUE	Host for pQE60 plasmids; supE44 hsdR17 recA1 gyrA96 thi relA1 lac F= [proAB laclq lacZ M15 Tn10(Tet^r)]	Stratagene
XL1 BLUE+pQE60	pQE60 *E. coli* expression vector, Amp^r	This study
XL1 BLUE+malR2	pQE60+*malR2*	This study
XL1 BLUE+malR5	pQE60+*malR5*	This study
EC101	Cloning host for pORI19 for insertional mutagenesis; $repA^+$ Km^r	(59)
L. lactis		
NZ9000	MG1363, pepN::nisRK, nisin-inducible overexpression host	(60)
NZ9000+pNZ8150	pNZ8150; Cm^r, nisin inducible translational fusion vector	This study
NZ9000+malR6	pNZ8150+*malR6*	This study
NZ9000+malR3	pNZ8150+*malR3*	This study
Plasmids		
pORI19	Em^r, $repA^-$, ori^+, cloning vector	(59)
pORI19-*malR2*	pOR19 harbouring internal fragment of *malR2* (*Bbr_0023)*	This study
pORI19-*malR6*	pOR19 harbouring internal fragment of *malR6* (*Bbr_0112)*	This study
pORI19-*malR3*	pOR19 harbouring internal fragment of *malR3* (*Bbr_0122)*	This study
pORI19-*malR5*	pOR19 harbouring internal fragment of *malR5* (*Bbr_0032)*	This study
pORI19-*malR2*-tet	pOR19 harbouring internal fragment of *malR2* (*Bbr_0023)* + Tet^r	This study
pORI19-*malR6*-tet	pOR19 harbouring internal fragment of *malR6* (*Bbr_0112)* + Tet^r	This study
pORI19-*malR3*-tet	pOR19 harbouring internal fragment of *malR3* (*Bbr_0122)* + Tet^r	This study
pORI19-*malR5*-tet	pOR19 harbouring internal fragment of *malR5* (*Bbr_0032)* + Tet^r	This study
pQE60	*E. coli* expression vector, Amp^r	Qiagen
pQE60+*malR2*	pQE60 harbouring *malR2*	This study
pQE60+*malR5*	pQE60 harbouring *malR5*	This study
pNZ8150	*L. lactis* expression vector, P*nisA*, Cm^r, ScaI site	(61)
pNZ8150+*malR3*	pNZ8150 harbouring *malR3*	This study
pNZ8150+*malR6*	pNZ8150 harbouring *malR6*	This study

Cm^r, Em^r, Km^r, Tet^r and Amp^r resistance to chloramphenicol, erythromycin, kanamycin, tetracycline and ampicillin, respectively.

DNA Manipulations

DNA manipulations were carried out as previously reported (31). Restriction enzymes and T4 DNA ligase were obtained from Roche Diagnostics (Basel, Switzerland), and were used according to the manufacturer's instructions. PCRs were performed using either Q5® High-Fidelity DNA polymerase (New England Biolabs, Hertfordshire, UK) or Extensor Long Range PCR Enzyme master mix (Thermo Scientific, Glouchester, UK). Synthetic oligonucleotides were synthesized by Eurofins (Ebersberg, Germany) and are

listed in Table 5-2. Ird-labelled synthetic oligonucleotides were supplied by IDT (Integrated DNA Technologies, Dresden, Germany) and are listed in Table 5-3. PCR products were purified by the use of a High-Pure PCR product purification kit (Roche, Basel, Switzerland). Plasmid DNA was introduced into *E. coli* and *B. breve* by electroporation and large-scale preparation of chromosomal DNA from *B. breve* was performed as described previously (32). Plasmid DNA was obtained from *B. breve* and *E. coli* using the Roche High Pure plasmid isolation kit (Roche Diagnostics, Basel, Switzerland). An initial lysis step was performed using 30 mg ml^{-1} of lysozyme for 30 min at 37°C as part of the plasmid purification protocol for *B. breve*.

Construction of *B. breve* UCC2003 insertion mutants.

Internal fragments were amplified by PCR using *B. breve* UCC2003 chromosomal DNA as a template; for the *malR2* gene (Bbr_0023) the internal fragment used was 382 bp in length, representing codons 54 through to 181 of the 338 codons of this gene; for the *malR5* gene (Bbr_0032) the internal fragment used was 387 bp in length, representing codons 60 through to 189 of the 345 codons of this gene; for the *malR3* gene (Bbr_0122) the internal fragment used was 380 bp in length, representing codons 36 through to 162 of the 342 codons of this gene; and finally for the *malR6* gene (Bbr_0112) the internal fragment used was 415 bp in length, representing codons 48 through to 186 of the 344 codons of this gene. Corresponding primers are listed in Table 5-2. Insertional mutagenesis was carried out as previously described (33). The presence of the tetracycline resistance cassette was confirmed by colony PCR with TetF and TetR primers, while site-specific recombination of Tet-resistant mutants was confirmed by colony PCR using a combination of the TetR primer and a primer located upstream of the recombination site in the chromosome of *B. breve* UCC2003 (primers listed in Table 5-2). Insertional mutants created in *malR2*, *malR3*, *malR5* and *malR6* genes of *B. breve* UCC2003 were thus obtained and verified, and were designated *B. breve* UCC2003-*malR2*, *B. breve* UCC2003-*malR3*, *B. breve* UCC2003-*malR5* and *B. breve* UCC2003-*malR6*, respectively.

Table 5-2 Oligonucleotide primers used in this study for cloning and site-mutagenesis

Purpose	Primer	Sequence
Clone *malR2* fragment in pORI19	*malR2_IM_F*	ttgctaaagcttcgcgttgaaatccggacaatc
	malR2_IM_R	ttgctatctagagttgcgatggccgagcgaaatc
Clone *malR6* fragment in pORI19	*malR6_IM_F*	ttgctaaagcttcatctcccgatcggccg
	malR6_IM_R	ttgctatctagaggcatcgcattcgttggc
Clone *malR3* fragment in pORI19	*malR3_IM_F*	ttgctaaagcttcaaggtgctggatgtggc
	malR3_IM_R	ttgctatctagacggaagacggcgtattgatg
Clone *malR5* fragment in pORI19	*malR5_IM_F*	ttgctaaagcttgcgcgtcgtcatgctgttc
	malR5_IM_R	ttgctatctagaggtttcggatgtgcgacg
Confirmation of site specific homologous recombination	*malR2_Con*	cgagcattcaagcggtagcg
	malR6_Con	ctcgtgacaaagtaatggccg
	malR3_Con	cggaactcgtctcggcgaag
	malR5_Con	catgaatagcgtccggccgt
	tetW_F	tcagctgtcgactgctcatgtacggtaag
	tetW_R	gcgacggtcgaccattaccttctgaaacat
Cloning of *malR2* in pQE60	*malR2_F*	ttgctaccatggatggcaaaagcgagcattcaagc
	malR2_R	ttgctaggatcccgcggcgatggccgtggtgtc
Cloning of *malR6* in pNZ8150	*malR6_F*	ttgctagatatcatggcaaaagcaagcattcaagcgg
	malR6_R	ttgctatctagatcagtgatggtgatggtgatggtgatggt gatgcccctgtgcgggcacgg
Cloning of *malR3* in pNZ8150	*malR3_F*	ttgctagatatcatgggcaaggcagacatctaccaag
	malR3_R	ttgctatctagattagtgatggtgatggtgatggtgatggt gatgcgccatttccccgaatg
Cloning of *malR5* in pQE60	*malR5_F*	ttgctaggatccatggcgacaatccacgacgtgg
	malR5_R	ttgctaagatctcggaagggatggtgcggc

Microarray Analysis

The transcriptome of each of the generated insertion mutants (*B. breve* UCC2003-*malR2*, *B. breve* UCC2003-*malR3*, *B. breve* UCC2003-*malR5* and *B. breve* UCC2003-*malR6*, which represent isogenic *B. breve* UCC2003 derivatives carrying an insertional mutation in the *malR2*, *malR3*, *malR5* or *malR6* gene, respectively; see previous paragraph) was compared to that of *B. breve* UCC2003 (WT). The insertion mutants and the WT strain were cultivated in mMRS medium supplemented with 0.5 % ribose as a sole carbon source until an OD_{600nm} of ~0.6 was reached. Cells were then harvested by centrifugation at 10,000 rpm for 2 min at room temperature and immediately frozen at -80°C prior to RNA isolation. DNA microarrays containing oligonucleotide primers representing each of the annotated genes on the genome of *B. breve* UCC2003 were designed by and obtained from Agilent Technologies (Palo Alto, CA, USA). Cell disruption, RNA isolation, RNA quality control, and cDNA synthesis and labelling were performed as described previously (34). The labelled cDNA was hybridized using the Agilent Gene

Expression hybridization kit (part number 5188-5242) as described in the Agilent Two-ColorMicroarrayBased Gene Expression Analysis v4.0 manual (G4140-90050). Following hybridization, the microarrays were washed in accordance with Agilent's standard procedures and scanned using an Agilent DNA microarray scanner (model G2565A). The generated scans were converted to data files with Agilent's Feature Extraction software (v9.5). The DNA microarray data were processed as previously described (35-37). Differential expression tests were performed with the Cyber-T implementation of a variant of the student t-test (38). A gene was considered to exhibit a significantly different expression level relative to the control when $p < 0.001$ and an expression ratio of > 2 or < 0.25. The microarray data obtained in this study have been deposited in NCBI's Gene Expression Omnibus database and is accessible through GEO series accession number GSE108950.

Plasmid construction

For the construction of plasmids pQE60+*malR2*, pQE60+*malR5*, pNZ8150+*malR6* and pNZ8150+*malR3* DNA fragments encompassing the full coding sequence of *malR2* (locus tag Bbr_0023), *malR5* (locus tag Bbr_0032), *malR6* (locus tag Bbr_0112) and *malR3* (locus tag Bbr_0122) were generated by PCR amplification of chromosomal DNA of *B. breve* UCC2003 with *Q5 high-fidelity* DNA polymerase and primers listed in Table 5-2. Cloning attempts in the case of *malR2* or *malR5* were unsuccessful in *Lactococcus lactis* and for this reason these two genes were cloned and their encoded products expressed in *E. coli*. An in-frame His_{10}-encoding sequence is incorporated into the 3' end of the pQE60 and pNZ8150 constructs to facilitate downstream protein purification. The amplicons generated for *malR2*, *malR5*, *malR6* and *malR3* were digested with NcoI/BamHI, BglII/BamHI, EcoRV/XbaI and EcoRV/XbaI, respectively. The restricted amplicons were then ligated into the NcoI and BamHI sites of pQE60 for *malR2* cloning, into the BglII and BamHI sites of pQE60 for *malR5* cloning, and into the ScaI and XbaI sites of pNZ8150 for cloning of *malR3* and *malR6*. pQE60 is an IPTG-inducible translational fusion plasmid and pNZ8150 is a nisin-inducible translational fusion plasmid. The ligation mixtures were introduced into *E. coli* XL1 Blue or *L. lactis* NZ9000 by electrotransformation. Transformants were then selected on the basis of Amp or Cm resistance. A number of transformants for each cloning were screened by restriction analysis, and the integrity of positively identified clones was verified by

sequencing, resulting in recombinant plasmids pQE60+*malR2*, pQE60+*malR5*, pNZ8150+*malR6* and pNZ8150+*malR3* (where the first part of the name refers to the original cloning and expression vector and the second part to the cloned gene).

Protein expression and purification

Proteins were overexpressed in 25 ml of M17 supplemented with 0.5 % (wt/vol) glucose or LB depending on the expression host (i.e. *L. lactis* or *E. coli*, respectively). Growth medium was then supplemented with a 2 % inoculum of a culture that had grown overnight in the same medium for 16 h; strains harbouring the empty cloning vector pNZ8150 or pQE60 were used as negative controls. Cultures were grown until an OD_{600nm} of ~0.5 was reached, at which point protein expression was induced by the addition of the cell-free supernatant of a nisin-producing strain for *L. lactis* strains or by the addition of IPTG for *E. coli* strains (65). Following incubation for a further two hours cells were harvested by centrifugation and re-suspended in electrophoretic mobility shift assay (EMSA) binding buffer (20 mM Tris-HCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol [DTT], 1 mM EDTA, 50 mM KCl, and 10 % glycerol at pH 7.0). Bacterial cells were disrupted by bead beating in a mini-bead beater (BioSpec Products, Bartlesville, OK) using glass beads. Cellular debris was then removed by centrifugation to produce a crude cell extract which was stored in aliquots at -20°C.

Recombinant MalR2, MalR3, MalR5 and MalR6 proteins, each with an incorporated C-terminal His10-tag, were purified from a crude cell extract using a nickel nitrilotriacetic acid column (Qiagen GmbH) according to the manufacturer's instructions (QIAexpressionist, June 2003). Lysis Buffer, wash buffer and Elution buffer were supplemented with 10 % glycerol in order to increase the stability/activity of each of these LacI-type proteins. Elution fractions were analysed by SDS-polyacrylamide gel electrophoresis, as described previously (66), on a 12.5 % polyacrylamide gel. Following electrophoresis, gels were fixed and stained with Coomassie brilliant blue to identify fractions containing the purified protein. Colour Pre-stained Protein Standard, Broad Range (11–245 kDa) (New England BioLabs, Hertfordshire, UK) was used to estimate the molecular weights of the purified proteins. Proteins were concentrated using Amicon® Ultra Filters from Merck Millipore and dialysed into EMSA binding buffer (80 mM Tris-HCl [pH 8.0], 20 mM $MgCl_2$, 2 mM dithiothreitol [DTT], 4 mM EDTA, 400

mM KCl and 40 % glycerol). Protein purification yields were calculated as a percentage of total protein present in the purified fraction over total protein present in crude cell extract. Protein concentrations were determined using the Qubit® fluorometer as per manufacturer's instructions (Thermofisher scientific, Glouchester, UK). Purified protein was aliquoted and stored at -20°C for further use.

Electrophoretic mobility shift assays (EMSAs)

The (deduced) promoter regions of genes of interest were amplified by PCR utilising either one (while the other was non-labelled) or two 5' IRD-700-labelled (supplied by Integrated DNA Technologies, Dresden, Germany) primers as listed in Table 5-3, Electrophoretic mobility shift assays (EMSAs) were performed as described previously (39). All binding reactions were carried out with poly(dI-dC) (0.05 µg/µl), DNA probe (0.5 nmol), BSA (0.2 µg/µl), binding buffer (20 mM Tris-HCl, 5 mM MgCl2, 0.5 mM dithiothreitol (DTT), 1 mM EDTA, 50 mM KCl, and 10 % glycerol at pH 7.0) and water to a final volume of 20 µl. Binding reactions were carried out utilising a 150 nM quantity of one of the purified His-tagged regulatory protein (i.e. MalR2, MalR3, MalR5 or MalR6) preparations. Binding reactions were then incubated for 20 min at 37°C and were loaded onto a 6 % non-denaturing polyacrylamide (PAA) gel prepared in TAE buffer (40 mM Tris-acetate [pH 8.0], 2 mM EDTA) and run in a 0.5× to 2.0× gradient of TAE at 100 V for 90 min in an Atto Mini PAGE system (Atto Bioscience and Biotechnology, Tokyo, Japan). Signals and percentage binding inferred from the Integrated Intensity (II) were detected/calculated using an Odyssey infrared imaging system (Li-Cor Biosciences, United Kingdom, Ltd., Cambridge, United Kingdom), and images were captured by the use of Odyssey software v3.0. To identify the effector for each regulator 10 mM of each one of the effectors listed in Table S5-14 was added to the binding reaction mixture prior to the binding reaction and corresponding EMSA. Any effector molecules which were found to reduce binding at the 10 mM concentration were then tested again, this time utilising decreasing concentration of the effector molecule as follows; 10, 5, 2.5, and 1 mM including a control without effector addition.

Table 5-3 Oligonucleotide primers used in EMSA analysis.

Locus Tag	Primer name	Sequence
Bbr_0023	*malR2*_Ird	gctcgcttttgccatggc
Bbr_0023	*malR2*	ccgaaaccagccgttgcac
Bbr_0027	*malFG 2*_Ird	ccatgccggtctcctttgc
Bbr_0027	*malFG 2*	gccagcctactttctcctcc
Bbr_0032-0033	*malR5/malE2*_Ird	cgccatgatgtctcctttcgc
Bbr_0032-0033	*malR5/malE2*	gctcatgtgatacgtgcctcc
Bbr_0060	*glgP 1*_Ird	cattctgactttccttccggg
Bbr_0060	*glgP 1*	gcgttctatccttgcactgagcc
Bbr_0105	*cldR*_Ird	gctgcgcgctatgttctcct
Bbr_0105	*cldR*	ccacgtcacgaatagttgcc
Bbr_0106	*cldE*_Ird	gcgacgatgacgaatccg
Bbr_0106	*cldE*	gcacttgggcgctcattg
Bbr_0111	*agl3*_Ird	gttcattcagcccgacgc
Bbr_0111	*agl3*_Ird	gtcattgaggttggcg
Bbr_0112-0113	*malR6/*Bbr_0113_Ird	cgcttgaatgcttgcttttgc
Bbr_0112-0113	*malR6/* Bbr_0113	ccggaacttctcgctatcatcatg
Bbr_0116	*malQ 2_Ird*	cctgttctgctcttaccgtagctc
Bbr_0116	*malQ 2*	gagctacggtaagagcagaacagg
Bbr_0117-0118	*agl4/malFG*_Ird	cggttcctacgccaagtaatc
Bbr_0117-0118	*agl4/malFG*	aagtgctgctgctgtcatcg
Bbr_0122-0123	*malR3/apuB*_Ird	ggtagatgtctgccttgccc
Bbr_0122-0123	*malR3/apuB*_Ird	gcaaggcgtttggcgagcg
Bbr_0411	*bifR1*_Ird	cgcacttcctggcatttg
Bbr_0411	*bifR1*	gaacttcaggccgccagc
Bbr_0725	*eno*_Ird	caaggaagtcgccgacaatc
Bbr_0725	*eno*	ggttgccacgagaatccag
Bbr_0746	*glgB*_Ird	ggcacattgatgaccagacc
Bbr_0746	*glgB*_Ird	gccgttgtattcgtgtgtca
Bbr_0757	*pyk*_Ird	cgctgagaaggcctgaaatc
Bbr_0757	*pyk*_Ird	ggttgtcgtaatcctcggtg
Bbr_0787	*pflBA*_Ird	gccgatagaacagcgtatgg
Bbr_0787	*pflBA*	cttggcgtcgagctcctcttg
Bbr_0845	*glgp 2*_Ird	cgcacctccttccacgctg
Bbr_0845	*glgp 2*	ccgttagattggagattgtccg
Bbr_1002-1003	*tkt – tal*_Ird	gctcggtctccttgaattcg
Bbr_1002-1003	*tkt - tal*	ggctcttgcgaacgaatg
Bbr_1233	*gap*_Ird	gcattgcctcaggtaagcc
Bbr_1233	*gap*	gaccaatgcgaccgaagc
Bbr_1273	*ldh*_Ird	ggatgtcgattcgcacttgg
Bbr_1273	*ldh*_Ird	agcttgctgttattggtgcc
Bbr_1419	*rbsA1*_Ird	gctcaatagtccttcgccgcc
Bbr_1419	*rbsA1*	catacgcctctcgctttcgtc
Bbr_1420	*lacI*_Ird	gatcatgctcagatgcggcg
Bbr_1420	*lacI*	cgaatgccatatccgtctcc
Bbr_1595	*Pgma*_Ird	ccatactttcattctgccacg
Bbr_1595	*Pgma*	gcgacatctcttactccattcc
Bbr_1658	Bbr_1658_Ird	ggtcaagctcatcgtgcg
Bbr_1658	Bbr_1658	ctggtcagcatagccgcac
Bbr_1659	*LacI*_Ird	caacgtgcgcagcctatgg
Bbr_1659	*LacI*	cttcgccacgtcatacacc
Bbr_1841	Bbr_1841_Ird	cgacagcttccttgccatgc
Bbr_1841	Bbr_1841	gcgtgcgatgtccctgatg
Bbr_1845-1846	*malFG/bifR2*_Ird	cataacagccccttttgcc
Bbr_1845-1846	*malFG/bifR2*	catgactttcctcctccttgag
Bbr_1847	*malE*_Ird	ggaatgcctgagctgagccg
Bbr_1847	*malE*	cgaacctttctctttcatcgtcg
Bbr_1891	*gntR*_Ird	gatgagtgcgcgtgagaag
Bbr_1891	*gntR*	cacgctggcgaagattgtc

Bbr_1894	*PTS*_Ird	gatatgcgcgaggattgg
Bbr_1894	*PTS*	gatcgacatacagcatgccg
Bbr_1901	*nrdH* & *nrdI* & *nrdE*_Ird	gtctcgaacggcacacca
Bbr_1901	*nrdH* & *nrdI* & *nrdE*	tggacatccggtcaggcc
Generation of DNA fragments for EMSA Fragmentations		
Bbr_0032/Bbr_0033	*malR5/malE2*_1_Ird	cgccatgatgtctcctttcgc
Bbr_0032/Bbr_0033	*malR5/malE2*_2	caacgcgcacatcgtggtac
Bbr_0032/Bbr_0033	*malR5/malE2*_3_Ird	gtaccacgatgtgcgcgttg
Bbr_0032/Bbr_0033	*malR5/malE2*_4	cacaccgtcaaccgccgc
Bbr_0032/Bbr_0033	*malR5/malE2*_5	gcggcggttgacggtgtg
Bbr_0032/Bbr_0033	*malR5/malE2*_6	gctcatgtgatacgtgcctcc
Bbr_0032/Bbr_0033	*malR5/malE2*_7_Ird	gcagccggcatccgatcc
Bbr_0112/Bbr_0113	*malR6/agl3*_1_Ird	cgcttgaatgcttgcttttgc
Bbr_0112/Bbr_0113	*malR6/agl3*_2	gacaatcgaaacgcacacacc
Bbr_0112/Bbr_0113	*malR6/agl3*_3	ccaccgggcatgatacc
Bbr_0112/Bbr_0113	*malR6/agl3*_4	ccatgtcggcgaatttcctc
Bbr_0112/Bbr_0113	*malR6/agl3*_5	ccatgctattatgcaaacgatgtcag
Bbr_0112/Bbr_0113	*malR6/agl3*_6_Ird	ccggaacttctcgctatcatcatg
Bbr_0122/Bbr_0123	*malR3/apuB*_1_Ird	ggtagatgtctgccttgccc
Bbr_0122/Bbr_0123	*malR3/apuB*_2	gattacacatcgtggatggcgc
Bbr_0122/Bbr_0123	*malR3/apuB*_3_Ird	gcgccatccacgatgtgtaatc
Bbr_0122/Bbr_0123	*malR3/apuB*_4	gggaagtgttgcttggtgtgg
Bbr_0122/Bbr_0123	*malR3/apuB*_5	ccacaccaagcaacacttccc
Bbr_0122/Bbr_0123	*malR3/apuB*_6	cggcatgcagcacagttgac
Bbr_0122/Bbr_0123	*malR3/apuB*_7_Ird	gtcaactgtgctgcatgccg
Bbr_0122/Bbr_0123	*malR3/apuB*_8	ctgaccgtgcgatagggg
Bbr_0122/Bbr_0123	*malR3/apuB*_9	cccctatcgcacggtcag
Bbr_0122/Bbr_0123	*malR3/apuB*_10_Ird	gcaaggcgtttggcgagcg
Bbr_0032/Bbr_0033	*malR5/malE2*_1_Ird	cgccatgatgtctcctttcgc
Bbr_0032/Bbr_0033	*malR5/malE2*_2	caacgcgcacatcgtggtac
Bbr_0032/Bbr_0033	*malR5/malE2*_3_Ird	gtaccacgatgtgcgcgttg
Bbr_0032/Bbr_0033	*malR5/malE2*_4	cacaccgtcaaccgccgc
Bbr_0032/Bbr_0033	*malR5/malE2*_5	gcggcggttgacggtgtg
Bbr_0032/Bbr_0033	*malR5/malE2*_6	gctcatgtgatacgtgcctcc
Bbr_0032/Bbr_0033	*malR5/malE2*_7_Ird	gcagccggcatccgatcc
Bbr_0112/Bbr_0113	*malR6/agl3*_1_Ird	cgcttgaatgcttgcttttgc
Bbr_0112/Bbr_0113	*malR6/agl3*_2	gacaatcgaaacgcacacacc
Bbr_0112/Bbr_0113	*malR6/agl3*_3	ccaccgggcatgatacc
Bbr_0112/Bbr_0113	*malR6/agl3*_4	ccatgtcggcgaatttcctc

Primers used in the EMSA experiments, the suffix _Ird represents primers which were labelled with an iridescent probe attached at their 5' end.

Phenotypic analysis of *malR* mutations

Evaluation of growth of malR mutants on a range of carbohydrate sources.

B. breve UCC2003 and its derived, isogenic *B. breve* UCC2003-*malR2, B. breve* UCC2003-*malR3, B. breve* UCC2003-*malR5* and *B. breve* UCC2003-*malR6* insertional mutants were inoculated from stock into RCM medium and were cultured overnight under anaerobic conditions at 37°C. The strains were then inoculated at 1 % (v/v) into mMRS medium containing 1 % (w/v) carbohydrate (glucose, maltose, cellobiose or starch) along with the addition of 0.5 % (v/v) L-cysteine HCl. mMRS without the addition of a carbohydrate source served as a negative control. Bacterial strains were evaluated for carbohydrate-dependent growth using a Life Sciences UV/VIS spectrophotometer

DU5300 (Beckman Coulter, California,US). Optical density (OD_{600nm}) readings were taken at 8 and 24 hours post-inoculation. All samples were assessed as biologically independent duplicates.

Evaluation of growth of malR mutants in the presence of bile.

B. breve UCC2003 and its derivatives *B. breve* UCC2003-*malR2, B. breve* UCC2003-*malR3, B. breve* UCC2003-*malR5* and *B. breve* UCC2003-*malR6* were inoculated at 1 % (v/v) from stock into mMRS medium supplemented with 1 % maltose and 0.5 % (v/v) L-cysteine HCl and were cultured overnight under anaerobic conditions at 37 C. The above strains were serially diluted in PBS buffer to 10^{-7} and spot plated on RCA supplemented with increasing concentration of ox-gall bile (0, 0.0625, 0.125 or 0.25 % v/v). Plates were incubated anaerobically at 37°C for 48 hours. CFU/ml was calculated using the following formula CFU/ml = (no. of colonies x dilution factor) / volume of culture plated. All samples were assessed as biologically independent duplicates.

Analysis of metabolic end products generated by malR mutants as compared with B. breve UCC2003

HPLC analysis was used to quantify the concentration of metabolites produced (lactate, acetate and ethanol) when *B. breve* UCC2003 or its isogenic derivatives were grown on glucose as a sole carbohydrate source. All strains were cultivated on mMRS medium supplemented with 1 % glucose, samples were taken at 8 and 24 hours post inoculation. The collected samples were then prepared for HPLC analysis by centrifugation at 5000 rpm for 5 min, the resulting supernatants were filter sterilized (0.45 μm filter, Costar Spin-X Column) and stored at −20°C prior to analysis. An Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) with a refractive index detector was used to quantify the production of lactate, acetate and ethanol as a result of carbohydrate fermentation. Metabolite peaks and concentrations were identified and calculated based on lactic acid, acetic acid and ethanol retention times and known concentrations of corresponding standards. Non-fermented mMRS medium containing carbohydrates served as controls. A REXEX 8 μ 8 % H organic acid column (300 mm × 7.8 mm, Phenomenex, Torrance, CA, USA) was utilized and maintained at 65 °C. Elution was performed for 25 min using a 0.01 M H_2SO_4 solution at a constant flow rate of 0.6 mL/min.

Preferential carbohydrate utilisation by *B. breve* UCC2003 and its isogenic derivatives carrying a mutation in *malR2, malR3, malR5,* or *malR6.*

B. breve UCC2003 and its isogenic derivatives *B. breve* UCC2003-*malR2, B. breve* UCC2003-*malR3, B. breve* UCC2003-*malR5* and *B. breve* UCC2003-*malR6* were inoculated into RCM medium from stock and cultured overnight anaerobically at 37°C. Strains were then inoculated into mMRS medium containing a mix of six sugars (glucose, maltose, maltotriose, lacto-N-neotetraose (LNnT), ribose or cellobiose) with a final concentration of 0.1 % of each sugar. Bacterial strains were evaluated for carbohydrate-dependent growth using a Life Sciences UV/VIS spectrophotometer DU5300 (Beckman Coulter). Optical density (OD_{600nm}) readings and HPAEC-PAD samples were taken over a 24 hour period at times 0, 4, 6, and 8 hrs post-inoculation. HPAEC-PAD samples were then prepared for analysis by centrifugation at 5000 rpm for 2 minutes and subsequent membrane filtration using Filtropure S filters (pore size, 0.45 µm; Sarstedt, Nümbrecht, Germany) and stored at 4 °C.

HPAEC-PAD analysis was conducted utilising a Dionex (Sunnyvale, CA, USA) ICS-3000 system. Samples (25 µl aliquots) were separated on a CarboPac PA1 analytical-exchange column (dimensions, 250 mm by 4 mm) with a CarboPac PA1 guard column (dimensions, 50 mm by 4 mm) and a pulsed electrochemical detector (ED40) in the PAD mode (all from Dionex). Elution was performed at a constant flowrate of 1.0 ml min^{-1} at 30 °C using the following eluents for the analysis: eluent A, 200 mM NaOH; eluent B, 100 mM NaOH plus 550 mM Na acetate; eluent C, Milli-Q water. The following linear gradient of sodium acetate was used with 100 mM NaOH: from 0 to 50 min, 0 mM; from 50 to 51 min, 16 mM; from 51 to 56 min, 100 mM; from 56 to 61 min, 0 mM. Standard curves were generated from chromatographic profiles of known quantities (0.005, 0.025, 0.05, 0.1 and 0.125 mg/ml) of each carbohydrate (glucose, maltose, maltotriose, LNnT, ribose and cellobiose). Chromeleon software (version 6.70; Dionex Corporation) was used for the integration and evaluation of the chromatograms obtained.

5.4 Results

EMSA assays

Previously, a number of transcription factors (TFs) had been identified, that were proposed to be involved in controlling the ability of bifidobacteria to utilize carbohydrates (13). In particular, this *in silico* work described a number of LacI-type regulatory proteins (designated here as BifR2, MalR2, MalR3, MalR5 and MalR6) in *B. breve* UCC2003 which were believed to be important in regulating starch-like/-derived (such as amylose, glycogen, maltose and maltodextrin) utilisation pathways along with a number of uncharacterised predicted carbohydrate uptake systems. In order to experimentally validate the predicted role of the implicated transcription factors (TFs; i.e. MalR2, MalR3, MalR5 and MalR6), these proteins were overexpressed in *E. coli* or *L. lactis* and consequently purified. As mentioned in the Materials and Methods section, cloning of *malR2* and *malR5* was unsuccessful in *L. lactis* NZ9000, and for this reason they were instead cloned into pQE60 in *E. coli* Xl1blue. The purified MalR proteins were then utilised to perform EMSAs in order to assess their (previously predicted) ability to bind to possible operator-containing DNA fragments (see Materials and Methods).

The rational for selecting (predicted) promoter regions for EMSA analysis was based on genes which can be divided into three categories: (i) genes which had previously been predicted to be part of a given MalR regulon (13), (ii) genes predicted to be in the BifR1 regulon (13) (which is now also the regulon of BifR2 as discussed in chapter 4), and (iii) selected genes that exhibited differential transcription in a given malR mutant (*B. breve* UCC2003-*malR2, B. breve* UCC2003-*malR3, B. breve* UCC2003-*malR5* and *B. breve* UCC2003-*malR6*; these data will be discussed below). The ability of MalR2, MalR3, MalR5 or MalR6 to bind a particular DNA fragment was graded as either negative, or from low, to medium, to high (-, +, ++, +++, respectively, calculated based on the proportion of bound DNA relative to the overall amount of DNA). Example EMSA experiments illustrating this arbitrary scale can be found in Figure S5-8 to S5-11, while Table 5-4 summarises all EMSAs carried out in this chapter.

EMSA analysis revealed that the regulons of MalR2 and MalR3 are overlapping; these two TFs are shown to bind the promoter regions of various genes that (are predicted to) specify various carbohydrate uptake systems (corresponding to locus tags Bbr_0118-

Bbr_0120, Bbr_1845, and Bbr_0026-Bbr_0027), several glycosyl hydrolases involved in malto-oligosaccharide utilisation (*apuB*, *agl4*, *agl3*), regulatory proteins (*malR3*, *malR2*, *bifR2*), and *glgP* an enzyme which is predicted to be involved in catalysing the intracellular breakdown of a glycogen-like polymer.

MalR5 was found to bind to the promoter regions of 5 genes/gene clusters with the following (putative functions): (i) a solute binding protein of an ABC transport system (Bbr_0033), (ii) an uncharacterised ABC uptake system (Bbr_0118-Bbr_0121), (iii) a cellodextrin ABC type uptake system (Bbr_0106-Bbr_0109), (iv) a 4-α-glucotransferase (MalQ; Bbr_0116), (v) MalR5 (Bbr_0032), suggestive of transcriptional autoregulation. Finally, the binding targets of MalR6 were found to be confined; this TF was shown to bind to only two of the promoter regions tested in this study, representing the presumed promoter regions of *malR6* itself and *agl3*, encoding an α-glucosidase (Bbr_0111) (42).

MalR2, MalR3, MalR5 and MalR6 had no apparent affinity for the promoter regions of genes associated with central metabolic pathways (results not shown), thus confirming the prediction that these TF do not appear to (directly) affect central carbon metabolism, while also showing that these four TFs are functionally distinct from BifR1 and BifR2 (the latter had previously been referred to as MalR1, and predicted to function as a MalR type TF, as discussed in Chapter 4). Finally, the results of the EMSA analysis are consistent with the previously predicted regulons for MalR2, MalR3, MalR5 and MalR6 (these genes are highlighted in orange in Table 5-4) (13).

Table 5-4. MalR EMSA Analysis.

Gene	Bbr_	Predicted MalR regulon	MalR2	MalR3	MalR5	MalR6
malR2	0023	LacI Transcriptional regulator	++	++	-	-
malG2	0027	Permease protein of sugar ABC transporter	+++	++	-	-
malR5	0032	LacI Transcriptional regulator	-	-	+++	-
malE2	0033	Solute binding protein for sugar ABC transport	-	-	+++	-
glgP1	0060	Glycogen/Amylose phosphorylase	++	++	-	-
agl3	0111	Alphaglucosidase	+++	-	-	+++
malR6	0112	LacI Transcriptional regulator *malR6*	+++	+++	-	+++
malQ	0116	Glucanotransferase	+	-	++	-
agl4	0117	α-glucosidase-like protein	+	-	+++	-
malE1	0118	Solute binding protein for sugar ABC transport	+	+++	+++	-
malR3	0122	LacI Transcriptional regulator	-	++	-	-
apuB	0123	Amylopullulanase	++	++	-	-
malF -	1845	Permease protein of sugar ABC transporter	++	+	-	-
bifR2	1846	LacI Transcriptional regulator	+	+	-	-
malE	1847	Solute binding protein for sugar ABC transport	-	+++	-	-
		Global BifR1/BifR2 regulon	**MalR2**	**MalR3**	**MalR5**	**MalR6**
bifR1	0411	LacI Transcriptional regulator	-	-	-	-
eno	0725	Enolase	-	-	-	-
carD	0746	carD-like Transcriptional regulator	-	-	-	-
pyk	0757	Pyruvate Kinase	-	-	-	-
pflB - pflA	0787	Formate acetyl transferase- Pyruvate formate-	-	-	-	-
tkt - tal	1002	Transketolase/Transaldolase	-	-	-	-
gap	1233	Glyceraldehyde Phosphatase	-	-	-	-
ldh	1273	Lactate dehydrogenase	-	-	-	-
		Additional Genes Identified by Microarray Analysis	**MalR2**	**MalR3**	**MalR5**	**MalR6**
cldR	0105	Cellodextrin related LacI Transcriptional		-	-	-
cldE	0106	Cellodextrin permease	+++	-	++	-
glgP2	0845	Glycogen phosphorylase (Non-functional)	-	-	-	-
rbsA1	1419	Ribose transport ATP binding	-	-	-	-
Bbr_1420	1420	Ribose related LacI Transcriptional regulator	-	-	-	-
Bbr_1658	1658	Sugar binding protein of abc transport system	-	-	-	-
Bbr_1659	1659	LacI Transcriptional regulator	-	-	-	-
Bbr_1841	1841	Permease of sugar uptake system	-	-	-	-
gntR	1891	Gntr transcriptional regulator	-	-	-	-
Bbr_1894	1894	PTS uptake System	-	-	-	-
nrdH	1901	Glutaredoxin	-	-	-	-

All EMSA analyses carried out in the above table were carried out with 150 nM protein (MalrR2, MalR3, MalR5 and MalR6) incubated with 0.5 nM Ird labelled DNA fragments encompassing the promoter region of the specified gene. Binding affinity was calculated based on the total percentage DNA bound – is representative of no binding, + is representative of an ability to bind 5 %-15 % of the total DNA, ++ is representative of an ability to bind 15 %-40 % of the total DNA present and +++ is representative of an ability to bind greater than 40 % of the DNA present in the reaction. Table has been colour coded, genes highlighted in orange are genes which were predicted to be part of the MalR-controlled regulon by Khoroshkin *et al.* (13), genes in green are members of central carbon metabolism and have been determined in Chapter 4 to be part of the BifR1/BifR2 controlled regulons, while genes highlighted in blue were selected based on their differential expression found in the transcriptomic analysis of the MalR mutants as compared with the *B. breve* UCC2003 WT strain conducted in this study

Microarray analysis

As mentioned above, EMSA analyses confirmed previous predictions regarding the regulons of MalR2, MalR3, MalR5 and MalR6 (13). Following on from this, insertional mutants were created in the four TFs (*malR2*, *malR3*, *malR5* and *malR6*) to generate *B. breve* UCC2003-*malR2*, *B. breve* UCC2003-*malR3*, *B. breve* UCC2003-*malR5* and *B. breve* UCC2003-*malR6*, respectively (see Materials and Methods), in order to substantiate our EMSA results and perhaps identify additional genes that could form part of their respective regulons. Henceforth, the insertional mutants created for each TF-encoding gene mentioned above, will collectively be referred to as *malR* mutant strains. The transcriptional profiles of these mutants were then investigated employing microarray analysis when cultivated in the presence of ribose as a sole carbon source until an OD_{600nm} of 0.6 was achieved and compared to that of *B. breve* UCC2003 grown under similar conditions.

The global transcriptional response of *B. breve* UCC2003 to the inactivation of each of these four TF-encoding genes resulted in complex and extensive outputs, summarised in Table 5-5, while a comprehensive microarray data set display can be found in additional Tables (Table S5-6 to S5-13). Transcriptome analysis revealed that the expression of 19 predicted carbohydrate uptake systems (highlighted in yellow in Table 5-5) was altered as compared to the *B. breve* UCC2003 WT strain. The majority of these putative uptake systems represent ABC type transport systems previously shown or predicted to be involved in the uptake of a diverse array of carbohydrates including maltose/maltooligosacharides (28), cellobiose/cellodextrins (21), ribose (20) sucrose-related oligosaccharides (40), fucose (28), sialic acid (24) and glucose (28), while the remainder await characterisation. The transcription of two PTS-type carbohydrate uptake systems was also altered in the microarray analysis of *B. breve* UCC2003-*malR5* and *B. breve* UCC2003-*malR2*. One of these systems is homologous to a glucose uptake system, based on BLASTp analysis (29). A number of genes involved in carbohydrate catabolism were also identified (highlighted in grey in Table 5-5). These include but are not limited to two α-glucosidase-encoding genes (*agl3* and *agl4*), three β-glucosidase-encoding genes (*bgl1*, *bgl2* and *bgl3*), a gene encoding a α-galactosidase (*lacZ1*), *apuB*, which encodes an extracellular amylopullulanase, *malQ*, which encodes a 4-α-glucanotransferase, *rbsK3*, which specifies a ribokinase, Bbr_1416, which encodes a

fructokinase, *glgP1* and *glgP2*, which both encode a glycogen/amylose phosphorylase, and *kojP*, which encodes a kojibiose phosphorylase (NB assigned functions are mostly based on predictions).

Four of the sugar transport and utilization genes which were up-regulated in the microarray analysis of *B. breve* UCC2003-*malR3*, are annotated to be and /or characterised as maltose/maltooligosaccharide associated utilisation genes (42, 64), and are located in close genetic vicinity to the *malR3* gene. Similarly, a number of genes, highlighted in blue in Table 5-5 and involved in central carbon metabolism, were shown to be differentially transcribed in the microarray analysis of *B. breve* UCC2003-*malR6*, and to a much lesser extent *B. breve* UCC2003-*malR2* and *B. breve* UCC2003-*malR5* (where it was solely the pyruvate kinase-encoding gene which was transcriptionally up-regulated in either of these mutants). Also, of interest was the differential transcription of a gene cluster encoding a cell surface sorting protein along with its co-expressed anchor proteins (Bbr_0113-Bbr_0115). Transcription of this cluster was shown to be up-regulated in *B. breve* UCC2003-*malR2*, while it was down-regulated in *B. breve* UCC2003-*malR5*. Inactivation of the *malR* TFs was furthermore shown to result in the differential transcription of several genes encoding eight other TFs and two distinct two-component regulatory systems, specifically in the case of *B. breve* UCC2003-*malR3*. Finally, of note was the down-regulation of the EPS2 cluster in a number of the transcriptomic analysis; 1.5, 1.9 and 5.5-fold for *B. breve* UCC2003-*malR5*, *B. breve* UCC2003-*malR6* and *B. breve* UCC2003-*malR3*, respectively. The possible link between EPS production and regulation of carbohydrate metabolism is currently not understood, though EPS production is expected to cause a drain on intracellular carbohydrate resources and may therefore be subject to transcriptional control.

The transcriptomic outputs for the *B. breve* UCC2003-*malR2* and *B. breve* UCC2003-*malR5* strains were found to have a lot of commonality. Out of the 29 and 37 differentially regulated genes for *B. breve* UCC2003-*malR2* and *B. breve* UCC2003-*malR5*, respectively, 19 were shown to be differentially regulated by both insertional mutants. For example, the gene associated with locus tag Bbr_1658 was shown to be 404.1-fold up-regulated in *B. breve* UCC2003-*malR5*, while 253.6-fold up-regulated in *B. breve* UCC2003-*malR2*, while a similar trend (up- or down-regulation) is seen in relation to the

genes associated with locus tags Bbr_0116, Bbr_0118, Bbr_0123, and Bbr_1847. In this context, transcription of a PTS system-encoding cluster (Bbr_1894-Bbr_1892) and an adjacent gene encoding a predicted GntR-type TF (Bbr_1891) is highly down-regulated, reminiscent of what had been observed by Alvarez-Martin *et al*. (65) for *B. breve* UCC2003 in which the SerR response regulator of the SerR/SerS two-component system was overexpressed (this also caused increased transcription of Bbr_1655-Bbr_1658). Further investigations are required to determine the regulatory links between the observed changes in the global gene expression of *B. breve* UCC2003-*malR2* and *B. breve* UCC2003-*malR5* mutants and *B. breve* UCC2003 overexpressing the SerR response regulator.

Also of note, two genes (Bbr_0114 and Bbr_1817) were found to exhibit differential transcription between the *B. breve* UCC2003-*malR2* and *B. breve* UCC2003-*malR5* mutant arrays (i.e. being transcriptionally up-regulated in *B. breve* UCC2003-*malR2* yet down-regulated in *B. breve* UCC2003-*malR5*).

Finally eleven of the gene/gene clusters which were predicted to be members of the the Khoroshkin *et al* (13) predicted MalR regulons (indicated in bold with an * in Table 5-5) were found to be differenitally regulated in the microarray analysis. The differential expression of these genes is in line with Khoroshkin *et al* (13) predictions, however due to the overlapping/interactive nature of the MalR regulons it is difficault to draw concrete conclusions from the microarray analysis alone.

Table 5-5. Heat-Map of Transcriptomic Analysis

Locus tag Bbr_	*malR2* Up	*malR2* Down	*malR3* Up	*malR3* Down	*malR5* Up	*malR5* Down	*malR6* Up	*malR6* Down	Function
0010			2.1						*lacZ1;* Beta-galactosidase
***0023**	2.1								*malR2;* LacI type transcriptional regulator
***0033**		2.1	1.9			1.8			Sucrose based uptake system
***0060**		6.1				4.8			*glgP1* Glycogen phosphorylase
0084			2.1						Glycosyltransferase
0105				2.3					*cldR;* LacI transcriptional regulator of cellobiose uptake system
0106	4.1				4.6			1.6	cldE-cldC Cellobiose/cellotriose binding protein (Bbr_0106-0109)
***0111**			3.1						*agl3;* Alpha-glucosidase
0114	2.1					2.9			Cell surface protein with Gram positive anchor and Cna protein B-type domain
***0116**		3.9	2.4			6.5			*malQ1;* 4-alpha-glucanotransferase
***0117**		2.1				3.2			*agl4* ; Alpha-glucosidase
***0118**		24.9	3.9			56.0			malE-Bbr_0121; Maltose/maltodextrin-binding protein (Bbr0118-0121)
***0123**		10.0				16.1			*apuB;* Amylopullulanase
0124		1.6	2.3			2.0			*dnaK;* Chaperone protein dnaK
0164	5.4				5.8				*nan-nag cluster;* ABC transport system for silaic acid, nanA and nanB (Bbr_0164-0169)
0171	4.2				3.3				Sialidase A
0243				2.2					*glnD;* uridylyltransferase, two component system
0285	6.4				5.9				Uncharacterised sugar symporter and Beta-galactosidase (Bbr_0285-284)
0303			2.5						Uncharacterised sugar uptake system with beta galactosidase (Bbr_0303-0305)
0366				2.7		1.6			Hypothetical protein with cell surface gram positive anchor domain (Bbr_0366-0369)
0372				2.2		1.8			Uncharacterised ATP binding protein of ABC transport system
0441								1.5	*eps1* ; Capsular polysaccharide biosynthesis protein (Bbr_0441-0445)
0442				5.5		1.5		1.9	*eps2* ; Capsular polysaccharide biosynthesis protein (Bbr_0441-0434)
0604				2.3			2.1		*rpsL- tuf;* SSU ribosomal protein S12P (Bbr_0604-0607)
0631							1.7		*efp;* Protein Translation Elongation Factor P (EF-P)
0725							1.7		*eno* ; Enolase
0757	1.9				1.7		1.5		*pyk;* Pyruvate kinase
0772							1.6		*pta;* Phosphate acetyltransferase
0776		1.5					1.9		Xylulose-5-phosphate/Fructose-6-phosphate phosphoketolase
0787							2.5		*pfl;* Formate acetyltransferase
0810				2.5					Two-component response regulator
0820			2.3		2.5				Bbr_0820-*araC;* Hypothetical protien and LacI type trancriptional regulator (Bbr_0820-0819)
0821			2.4						Na+ driven multidrug efflux pump
0845						2.2			*glgP2;* Glycogen phosphorylase
0938				2.5	3.6				Hypothetical membrane spanning protein
0994				2.0					*pgk;* Phosphoglycerate kinase
1003							2.0		*tkt-tal;* Transketolase/Transaldoase (Bbr_1003-1002)
1105							2.0		*rpsB;* SSU ribosomal protein S2P
1271			2.2						*lexA;* Transcriptional repressor
1273							1.6		*ldh2;* L-lactate dehydrogenase
1418							2.0		*rbsC1 -rbsB1;* Ribose transport system permease protein (Bbr_1418-1417)
1425	10.5		2.8		9.7				Sugar uptake system with ribokinase and phosphoglycolate hydrolase (Bbr_1425-1421)
1431	4.6				5.2				Sugar uptake system with Fructokinase (Bbr_1431-1426)
1435			2.1						Transcriptional regulator, MarR family
1442	2.3				2.0				*bgl2;* Beta-glucosidase
1475				3.4					Branched-chain amino acid transport ATP-binding protein
1574							1.8		*gpm2;* Phosphoglycerate mutase
1617							1.8		*rpsM;* SSU ribosomal protein
1619							2.6		*infA;* Bacterial Protein Translation Initiation Factor 1 (IF-1)
1631							1.7		*rplN-rplO;* 50S Ribosomal protein (Bbr_1631-1622)
1642			1.5				2.2		*rpsJ-rpsQ;* 30S Ribosomal protein (Bbr_1642-1632)
1649							2.1		*rplM-rpsI;* 50S/30 S Ribosomal proteins (Bbr_1649-1648)
1658	253.6				404.1			1.7	Uncharacterised Sugar uptake system with beta-glucosidase (Bbr_1658-1653)
1676							1.9		*rplJ-rplL;* Ribosomal proteins (Bbr_1676-1675)
1693	2.5				2.6				Sugar uptake system, kojibiose Phosphorylase, Beta-phosphoglucomutase (Bbr_1693-1691)
1696	3.7				3.8				Uncharacterised sugar uptake system (Bbr_1696-1694)
1719						2.3			*fas;* Type I multifunctional fatty acid synthase (Bbr_1719-1720)
1744	2.1				1.8				L-fucose uptake system and a short chain dehydrogenase (Bbr_1744 _1740)
1817	1.6		2.2			1.8			*oppB3 -oppA3* ; Oligopeptide transport system(Bbr_1817-1814)
1828			2.1						Transcriptional regulator, MarR family
1834			2.5			1.6			*rbsC2;* Ribose transport system permease protein (Bbr_1836-1831)
***1845**		10.8				11.4			Uncharacterised ABC type sugar uptake system (Bbr_1845-1843)
***1846**		2.1				2.5		2.6	*bifR2,* Transcriptional regulator, LacI family
***1847**		35.3				30.5			ABC transport system solute binding protein for sugars
1862					2.0				Uncharacterised ABC type sugar uptake system (Bbr_1862-1861)
1880	2.5				2.2			1.7	Glucose specicifc PTS system (Bbr_1880-1879)
1891		12.2				17.4			Transcriptional regulator, GntR family
1894		540.1				516.2			PTS system, IIA component (Bbr_1894-1892)
1901			2.5			2.3			*nrdH;* Glutaredoxin nrdH (Bbr_1901-1898)
1918			2.1						*trxB2;* Thioredoxin reductase

Transcriptome analysis of *B. breve* UCC2003-*malR2*, *B. breve* UCC2003-*malR3*, *B. breve* UCC2003-*malR5* *B. breve* UCC2003-*malR6* as compared with *B. breve* UCC2003 wt strain when grown on ribose. Colour intensity is derived from mean relative expression fold changes of dye swap results, increasing intensity green for up regulated, increasing intensity red for down-regulated and white for no significant change. Gene functions have been colour coded for ease and are as follows: yellow; carbohydrate uptake system, grey; carbohydrate metabolism associated, orange; transcription regulators, pink; eps related, green; ribosomal associated, brown; other uptake systems and white; other. Genes in bold with an * in front of them are predicted to be members of the MalR regulon (13).

Identification of the Effector Molecules for each TF

LacI-type TFs typically act by repressing transcription through binding to operator sequences that are either located down stream of or (partially) overlapping with the promoter(s) region. Small molecules known as effector molecules affect the binding ability of a given LacI-type TF, resulting in the increased (in most cases) or decreased transcription of the regulated genes. This interaction between LacI-type TFs and their corresponding effectors allows their action as couriers of external or internal molecular signals. Microarray and EMSA analyses were shown to be consistent with the predicted regulons of each of the MalR TFs, and in order to gain more insight into the molecular signals that control the activity of these TFs, EMSAs were carried out using a number of variety of possible (commercially available/relevant) effectors (at a concentration of 10 mM) listed in (Table S5-14).

The results obtained indicate that none of the effector molecules tested in this study (listed in Table S5-14), consistently decreased/increased MalR3 or MalR6 ability to bind to their identified TFBS (results not shown). However, from Figure 5-1 we can see that effector molecules were identified for MalR2 and MalR5. MalR2's ability to bind to a DNA target was shown to be inhibited in the presence of the three disaccharides turanose, palatinose and isomaltose. These three effector molecules were then tested for their ability to affect MalR2's ability to bind to its target at decreasing concentrations of the respective effector molecule (10, 5, 2.5, and 1 mM, including a control without carbohydrate addition; (Figure 5-1 Panel B (i-iii)). Interestingly, the three effector molecules showed varying capacities to decrease MalR2s ability to bind to its target. It was found that isomaltose had the least effect followed by palatinose, while turanose was shown to completely prevent MalR2 binding to its target even at the lowest concentration of effector molecule tested (1 mM). The effector molecule for MalR5 was shown to be galactose, which even at the lowest concentration tested (i.e. at a concentration of 1 mM) prevents MalR5 binding to its DNA target (Figure 5-1 Panel B (iv)).

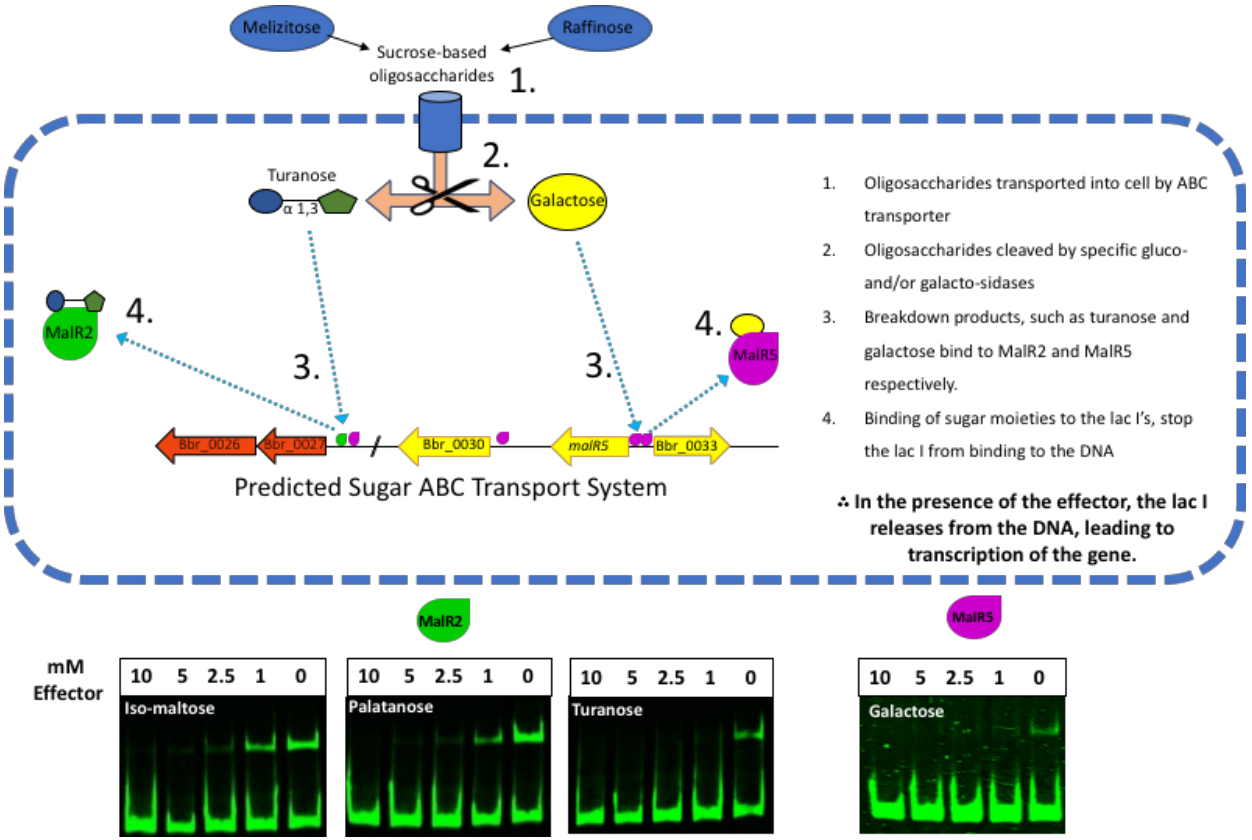


Figure 5-1 Effector Analysis:
Panel A Illustrates a schematic representation of the interaction of MalR2/MalR5 and their respective effector molecules (turanose and galactose).
Panel B (i)-(iii) EMSA of MalR2 (150 nM protein) incubated with 0.5 nM DNA (Bbr_0027 promoter region) and decreasing concentrations (10, 5, 2.5, 1, 0 mM) of the respective effector molecules (Iso-maltose, Palatanose and Turanose). (iv) EMSA of MalR5 (150 nM protein) incubated with 0.5 nM DNA (Bbr_0032 promoter region) and decreasing concentrations (10, 5, 2.5, 1, 0 mM) of its effector molecule galactose.

Precise mapping of MalR binding sites via fragmentation analysis

EMSA analysis was employed to determine the smallest DNA fragment that still contained a transcription factor binding site (TFBS) recognized by a given MalR TF. For this purpose, the DNA fragments that had been shown to be bound by a TF (and thus contains a TFBS for that TF) were dissected into smaller fragments and used for EMSA analysis. Fragmentation analysis was carried out on the following DNA regions Bbr_0032/Bbr_0033 for MalR5, Bbr_0112/Bbr_0113 for MlaR6 and Bbr_0112/Bbr_0113 and Bbr_0122/Bbr_0123 for both MalR2 and MalR3 (see Figure 5-2 Panel A). Fragmentation of these DNA regions was designed so as to either include or exclude the predicted Khoroshkin *et al* MalR TFBSs (13) from the respective DNA fragments in order to confirm the true position of the MalR TFBSs.

The promoter region of Bbr_0122/Bbr_0123 was divided into five fragments, and MalR2 and MalR3 were tested for their ability to bind to each fragment (Figure 5-2 Panel B Part (iii)). As mentioned above these DNA fragments were designed based on the location of the predicted MalR TFBS identified by Khoroshkin *et al* (13). From the obtained results it is clear that MalR3 and MalR2 bind to distinct DNA fragments and thus recognize different operator sequences or TFBSs. Both MalR2 and MalR3 were shown to bind to a fragment upstream of *apuB* (Bbr_0123) which contains the one of the predicted MalR TFBS, while MalR3 (and not MalR2) was shown to bind a DNA fragment just upstream of its encoding gene (Bbr_0122) (which also contained a predicted MalR TFBS). Similar fragmentation analysis was carried out with MalR2 and MalR3 protein purifications when incubated with the dissected promoter-containing region located between the *malR6* gene (Bbr_0112) and that of Bbr_0113 (Figure 5-2 Panel B Part (ii)). This region was fragmented to either include or exclude the predicted MalR TFBSs. In this analysis MalR2 (but not MalR3) was able to bind a DNA fragment located upstream of the *malR6* gene while both MalR2 and MalR3 could bind to a fragment corresponding to a DNA region upstream of Bbr_0113. Similarly, fragmentation analysis was carried out for MalR6 employing the promoter region upstream of its own gene (Bbr_0112) (Figure 5-2 Panel B Part (ii)), demonstrating that MalR6 bind to a specific sequence upstream of its own gene.

Finally, fragmentation analysis was carried out in a similar manner for MalR5 on an intergenic region located between its own encoding gene, *malR5*, and that of *malE2* (Bbr_0032 and Bbr_0033) (Figure 5-2 Panel B Part (i)). This analysis revealed that MalR5 could bind to two distinct fragments, one immediately upstream of *malR5* and a second fragment upstream of the *malE2* gene.

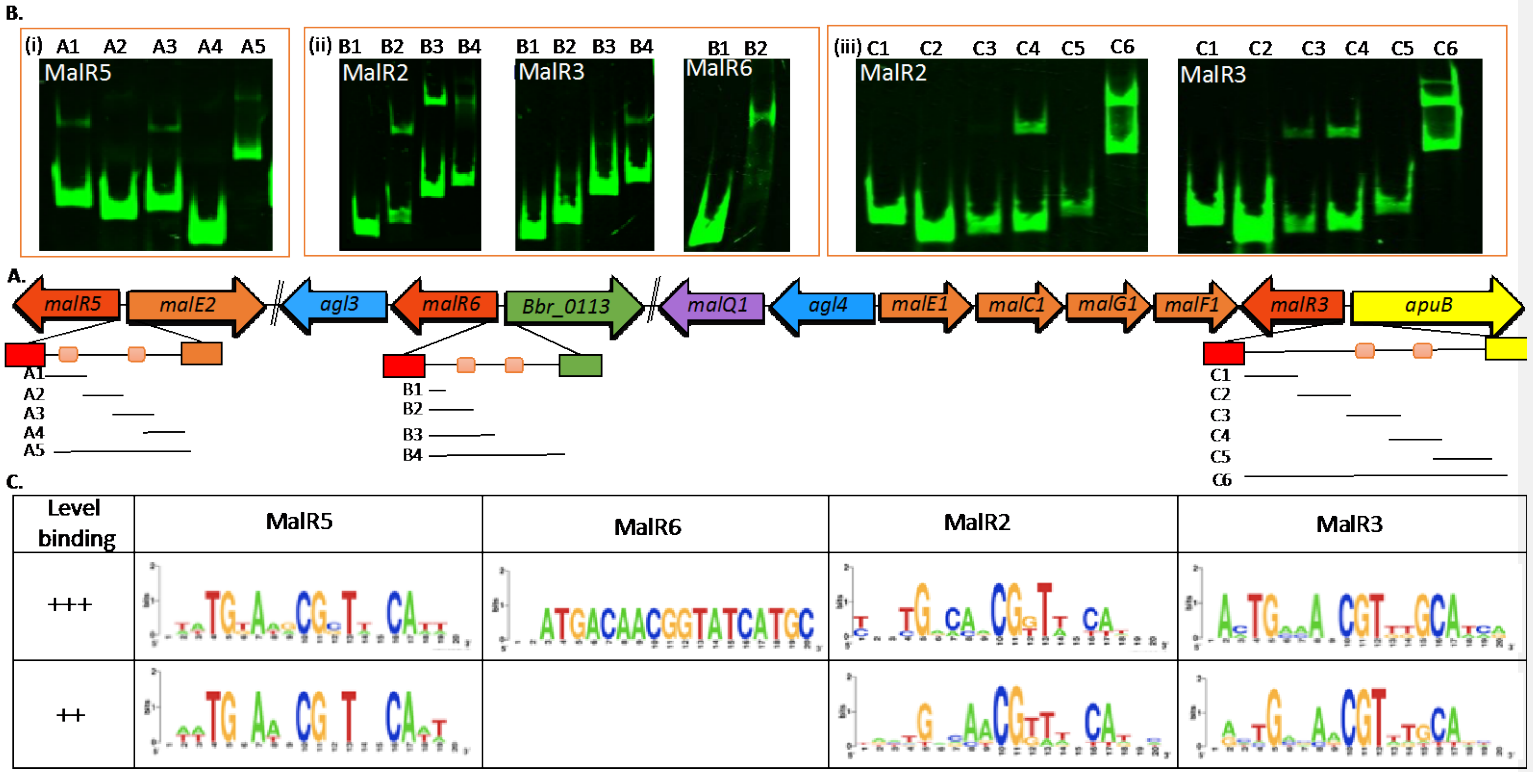


Figure 5-2 MalR TFBS Identification and Analysis
(A) Genetic organization of a number of genes in the regulons of MalR2, MalR3, MalR5 and MalR6 in *B. breve* UCC2003 and the corresponding DNA fragments used in EMSAs for the fragmentation of *malR5*/*malE2*, *malR6*/Bbr_0113 and *malr3*/*apuB* promoter regions.
(B) Part (i) EMSA of MalR5 when incubated with 0.5 nM DNA of each of the fragments A1-A5 of the *malR5*/*malE2* promoter region.
Part (ii) EMSA of MalR2, MalR3 and MalR6 respectively when incubated with 0.5 nM DNA of each of the fragments B1-B4 of the *malR6*/Bbr_0113 promoter region.
Part (ii) EMSA of MalR2 and MalR3 respectively when incubated with 0.5 nM DNA of each of the fragments C1-C6 of the *malr3*/*apuB* promoter region
(C) DNA Binding motifs of MalR2, MalR3, MalR5 and MalR6. Where the level binding is represented in the following non-linear scale; +++ represents genes in which each TF bond > than 40% of the DNA present reaction ++ represents genes in which each TF bond 10-40 % of the DNA present reaction (see EMSA Table 5-4).

Phenotypic analysis of *malR* mutations

Growth analysis of malR mutants on varying carbon sources

Transcriptomic and EMSA analyses had established/validated which genes are members of the MalR2, MalR3, MalR5 and MalR6 regulons ((13); see findings above). In order to gain a better understanding of the phenotypic implications of inactivation of the MalR TFs, strains *B. breve* UCC2003-*malR2, B. breve* UCC2003-*malR3, B. breve* UCC2003-*malR5* and *B. breve* UCC2003-*malR6* were tested for their ability to grow on a number of carbon sources (glucose, maltose, starch and cellobiose); these carbohydrates were chosen based on genes related to their utilisation which were differentially expressed in the microarrays. From this analysis it is clear that both *B. breve* UCC2003-*malR2* and *B. breve* UCC2003-*malR5* mutants were unable to grow in the presence of starch as a sole carbon source at 8 and 24 hours post-inoculation (Figure 5-3). *B. breve* UCC2003-*malR6* appeared to be impaired in growth on starch as a sole carbon source at 8 hours post inoculation although after 24 hours of growth it did reach a final cell density that was similar to the *wt* strain. In the case of *B. breve* UCC2003-*malR2* the observed phenotype is consistent with the EMSA results presented above as the MalR2 TF was found to be capable of binding to a number of genes which are associated with the degradation and utilisation of starch-like/-derived polymers. In other cases the growth deficiency on starch may not be easily explained due to the interactive connections between the various MalR regulators, and will thus require further investigations.

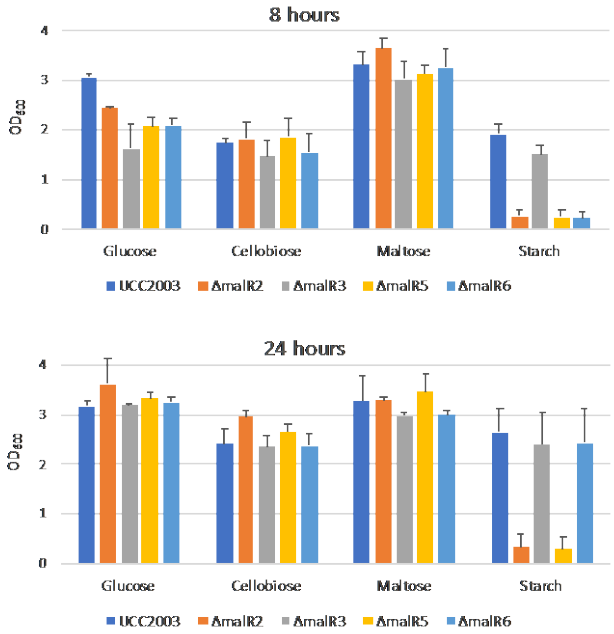


Figure 5-3 Carbohydrate Analysis MalR Mutants
Analysis of the growth: (OD_{600} readings) of *B. breve* UCC2003, *B. breve* UCC2003-*malR2*, *B. breve* UCC2003-*malR3*, *B. breve* UCC2003-*malR5* and *B. breve* UCC2003-*malR6* in the presence of varying carbon sources (glucose, cellobiose, maltose and starch) at 8 and 24hrs post inoculation. Error bars for each represent the standard deviation calculated from three replicates.

Survival of malR mutants in the presence of ox-gall bile

In order to investigate if any of the *malR* mutants is affected in its ability to withstand bile stress, survival assay in the presence of increasing concentrations of ox-gall bile were carried out (Figure 5-4). In short, *B. breve* UCC2003 and its derivatives *B. breve* UCC2003-*malR2*, *B. breve* UCC2003-*malR3*, *B. breve* UCC2003-*malR5* and *B. breve* UCC2003-*malR6* were spot plated on RCA agar supplemented with increasing concentration of ox-gall bile (0, 0.0625, 0.125 and 0.25 % (v/v)). From this analysis it is clear that both *B. breve* UCC2003-*malR2* and *B. breve* UCC2003-*malR5* had an increased resistance to ox-gall bile as compared with the *wt*, *B. breve* UCC2003-*malR3* or *B. breve* UCC2003-*malR6* insertional mutant strains.

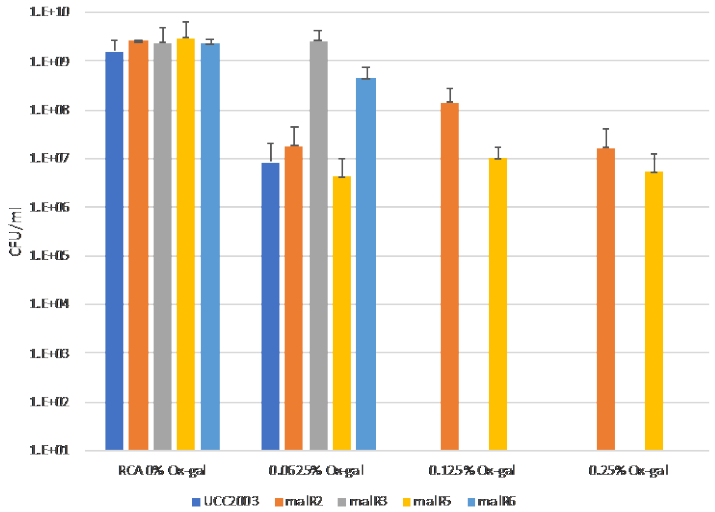


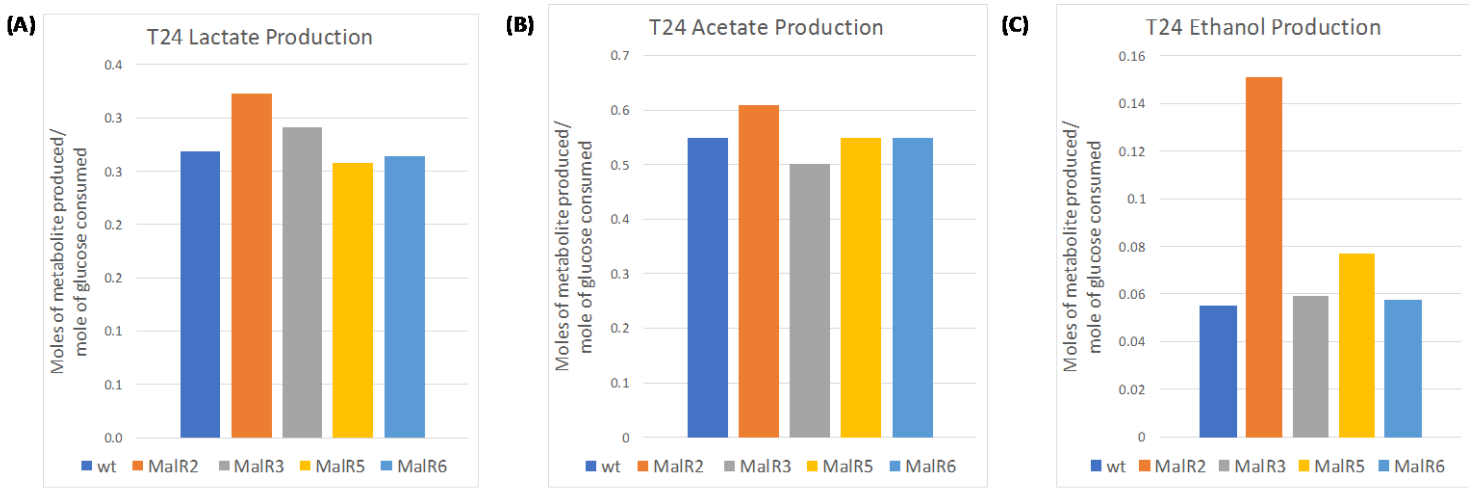
Figure 5-4 MalR Bile Survival Assays
Analysis of the survival of *B. breve* UCC2003 wt, *B. breve* UCC2003-malR2, *B. breve* UCC2003-malR3, *B. breve* UCC2003-malR5 and *B. breve* UCC2003-malR6, when overnight cultures were spot plated on RCA agar with increasing concentrations of ox-gall bile: 0, 0.0625%, 0.125% and 0.25% (v/v). Cell survival is expressed in CFU/ml). Error bars for each represent the standard deviation calculated from two replicates.

Metabolic Flux Analysis generated by malR mutants

HPLC analysis was carried out in order to investigate any impact of the *malR* insertional mutants on the production of metabolic end products (i.e. lactate, acetate, and ethanol). This analysis was carried out on *B. breve* UCC2003-*malR2*, *B. breve* UCC2003-*malR3*, *B. breve* UCC2003-*malR5* and *B. breve* UCC2003-*malR6* and compared with the *B. breve* UCC2003 *wt* strain when on the grown on mMRS medium supplemented with glucose with samples taken at 8 and 24 hours post inoculation (see Table S5-15 and Figure 5-5). This analysis found that all mutant strains produced similar levels of lactate and acetate as compared to the wt *B. breve* UCC2003 strain.

B. breve UCC2003-*malR2* was shown to produce a considerably higher level of ethanol (0.15 moles per mole of glucose; Fig.5-5, Panel C). Somewhat similar results were found for the *B. breve* UCC2003-*malR5* mutant (0.08 moles per mole of glucose consumed, Fig.5-5, Panel C), when compared to *wt B. breve* UCC2003 (0.05 moles per mole of glucose consumed) at 24 hours. The lactate to acetate ratios (Figure 5-5, Panel D) for the mutants were all shown to be skewed towards the production of lactate; the most

significant shift in this regard was observed for *B. breve* UCC2003-*malR2*, where the ratio is 1:1.7 as compared to a ratio of 1:2.6 for the WT. Further analysis will be necessary to validate the biological significance of these findings.



(D)

	Lactate : Acetate Ratio
WT	1 : 2.6
malR2	1 : 1.7
malR3	1 : 1.9
malR5	1 : 2.3
malR6	1 : 2.0

Figure 5-5 Metabolic end product analysis
Metabolic end product analysis by HPLC of *B. breve* UCC2003 (WT), *B. breve* UCC2003-*malR2* (MalR2), *B. breve* UCC2003-*malR3* (MalR3), *B. breve* UCC2003-*malR5* (MalR5) *and B. breve* UCC2003-*malR6* (MalR6). All strains were grown on glucose as the sole carbon source, and samples were taken after 24 hours. Results are presented as moles of metabolite detected 24 hours post inoculation. All values are calculated as per mole of glucose consumed.

Rate and Preference of Carbohydrate Uptake Analysis by HPAEC for B. breve UCC2003 and malR mutants

In order to determine if the MalR TFs are involved in the preferential uptake and utilisation of different carbohydrates, *B. breve* UCC2003-*malR2*, *B. breve* UCC2003-*malR3*, *B. breve* UCC2003-*malR5* and *B. breve* UCC2003-*malR6* were inoculated and grown in medium which contained a mix of different carbohydrate sources (glucose, LNnT, ribose, maltotriose, maltose, and cellobiose) (Figure 5-6). All mutants and the WT strain exhibited similar growth profiles when cultivated on this sugar mix (Figure 5-6). Samples of the spent medium were taken after 4, 6, 8, 10, 12 and 24 hours post-inoculation and analysed in order to measure the reduction in carbohydrate left in the media (All *malR* mutants, along with the *B. breve* UCC2003 WT strain take up the differing carbohydrate in the same order of preference, glucose, LNnT, ribose, maltotriose, maltose, and cellobiose (Figure 5-7)).

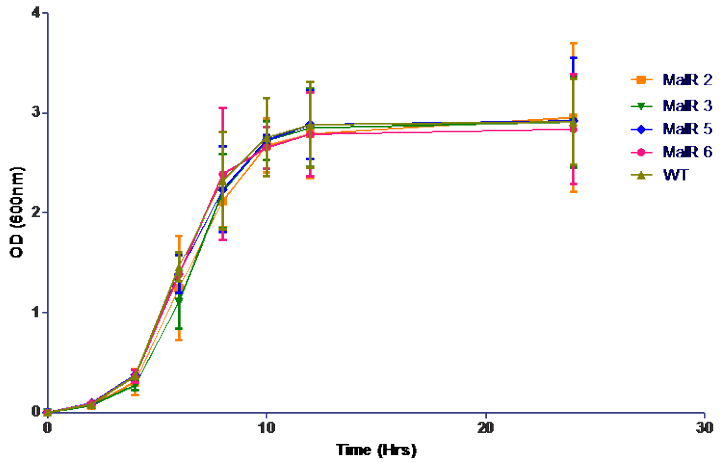


Figure 5-6 Growth Curve for Carbohydrate Uptake Analysis
Growth curve (OD_{600} readings) of *B. breve* UCC2003-*malR2*, *B. breve* UCC2003-*malR3*, *B. breve* UCC2003-*malR5 and B. breve* UCC2003-*malR6* as compared to the wt strain *B. breve* UCC2003 when grown on mMRS medium containing 0.1% of 6 different sugars; glucose, lnnT, cellobiose, maltose, maltotriose and ribose.

From this analysis we can see that at 4 hours post inoculation, glucose is the only sugar where there is a noticeable reduction in concentration levels in the spent medium, for all of the strains. At 6 hours post inoculation, there is no glucose remaining in the spent medium, and very little ribose remaining, again for all the strains. At 8 hours post-inoculation, there is no remaining LNnT and ribose in the media for all of the mutants

and the *wt* strain. In these samples, there is no remaining cellobiose in the *B. breve* UCC2003-*malR2*, *B. breve* UCC2003-*malR5* and *B. breve* UCC2003-*malR6* while the WT and *B. breve* UCC2003-malR3 mutant still have a portion of cellobiose which they have not internalised in their spent medium at this time point. Finally the *B. breve* UCC2003-*malR5* mutant is the only strain where there is some maltose and maltotriose (although at a very low concentration) remaining in the media at 8 hours post inoculation, so even though this mutant can take up maltose related sugars, it is at a reduced rate (see Figure 5-7). This slow rate of uptake may potentially contribute to the inability of *B. breve* UCC2003-*malR5* to grow on starch as the sole carbohydrate source (Figure 5-3). Finally, this analysis has revealed some differences between the malR mutants and the WT strains abilities to/speed at which they internalise carbohydrates from their surrounding environment. It would be beneficial to expand/repeat this experimental analysis to include a larger range of carbohydrates.

Commented [D4]: The differences are too small and without error bars so you can't conclude much from this. Also the inability to grow on starch may have different reasons (e.g. inability to produce ApuB). I would remove most of these statements.

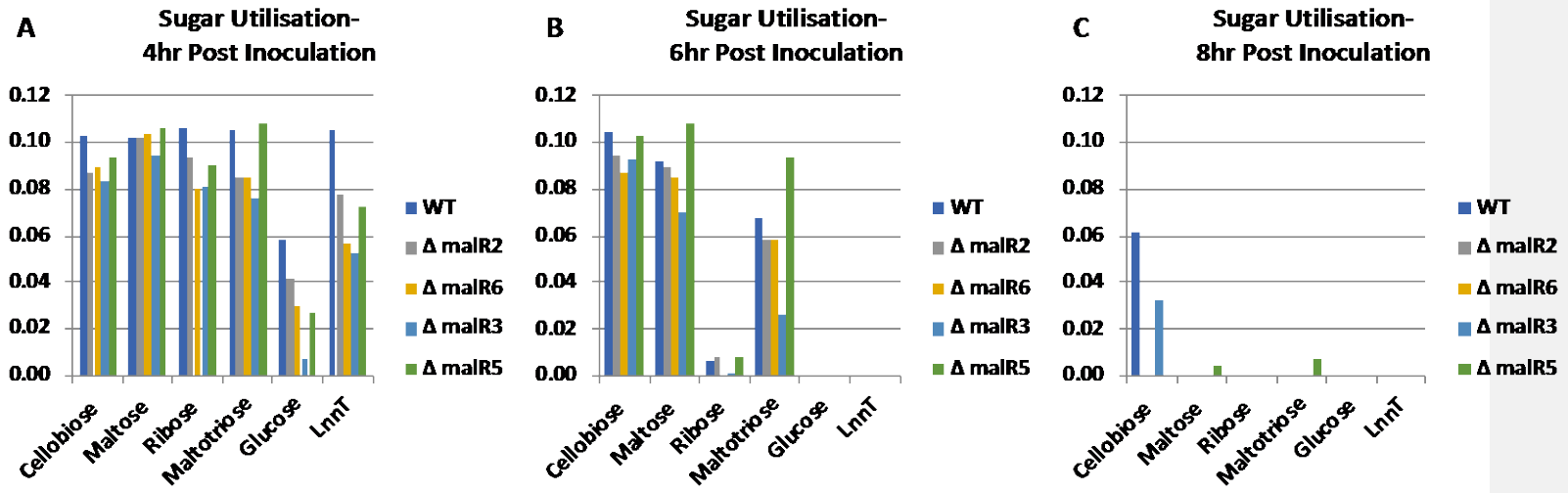


Figure 5-7 Carbohydrate Uptake Analysis
Analysis of uptake of differing carbohydrate sources from mMRS medium. 0.1 % of 6 different sugars (glucose, lnnT, cellobiose, maltose, maltotriose and ribose) was mixed and added to mMRS medium and used to culture *B. breve* UCC2003 *wt*, *B. breve* UCC2003-*malR2*, *B. breve* UCC2003-*malR3*, *B. breve* UCC2003-*malR5 and B. breve* UCC2003-*malR6.* Samples of the spent medium were taken at 4, 6, 8 and 10 hours post inoculation, and the concentration of sugar remaining in each sample was analysed by HPAEC-PAD. The concentration of each remaining carbohydrate was calculated by use of a standard curve for each carbohydrate based on the peak area (n*C) of the chromatograms. Values expressed on the Y-axis are % sugar reaming in the spent medium. Panel A, B and C represent HPAEC utilisation profiles at 4, 6 and 8 hours post inoculation respectively.

5.5 Discussion

The ability of bifidobacteria to detect and respond to environmental variation has received relatively little scientific attention. The distal gut does not supply bacteria with a single reliable carbon source; available carbohydrates are dependent on many factors such as host diet, other bacteria present in the GIT and the utilisation of carbon sources by the host. To overcome this carbohydrate insecurity bifidobacteria are able to utilise a variety of different carbon sources, and importantly can do so in an efficient yet versatile manner in order to compete with other bacteria resident in the gut. As for most living organisms, bifidobacteria conserve energy by only transcribing metabolic genes when necessary.

Several carbohydrate-associated LacI-type regulators have been characterised in *B. breve* UCC2003 (20-23). Generally, these TFs act locally and are involved in the regulation of a metabolic pathway that concerns just a single carbohydrate (e.g. galactose, ribose and melezitose). Often the genes involved in the metabolism of such a carbohydrate are located in close vicinity of the gene encoding the corresponding TF. In the current work we show that MalR5 and MalR6 are two such locally operating TFs, controlling a relatively small regulon and acting upon genes which are encoded close to their own genes on the genome. It had previously been predicted that the *B. breve* UCC2003-encoded MalR2, MalR3 and BifR2 regulators would be involved in the metabolism of α-glucosidic linked starch-like carbohydrates. However, it is now apparent that the regulatory scope of the MalR2 and MalR3 also includes the metabolism of additional carbohydrates such as β-glucosidic-linked cellobiose-like carbohydrates (Table 5-5 and Table 5-4). Regulatory networks have undergone much study in recent years (43, 44), and are thought to be made of up of modules which can be defined as a group of genes cooperating to achieve a particular physiological function. TFs have been classified within these modules in a hierarchical manner. Therefore, we postulate that MalR2 and MalR3 are placed in a higher hierarchical layer compared to MalR5 and MalR6 due to the number of genes in their respective regulons and due to functional diversity of these genes.

The regulon of a particular transcriptional repressor may be identified through microarray analysis of an insertional mutant in the repressor-encoding gene; transcription of genes under the direct regulation of that repressor should be increased compared to the wild

type. This study shows a shortcoming of this method, particularly when, as was the case here and other cases (2, 23, 45), many genes were down-regulated as compared to the wild type context. This down-regulation is unexpected and mostly difficult to explain, though it may be attributed to the overlapping nature of the MalR regulons or the subsequent transcription of another TF within that regulon, which then leads to the observed down-regulation.

Another confounding aspect of the transcriptomic data is the similarity between the transcriptomes of *B. breve* UCC2003-*malR2* and *B. breve* UCC2003-*malR5*, which do not seem to be consistent with corresponding EMSA analyses. These similarities included the drastic down-regulation of the PTS related gene Bbr_1894 in the transcriptomes of *B. breve* UCC2003-*malR2* and *B. breve* UCC2003-*malR5* (540.1 and 516.2, respectively), EMSA analysis revealed that neither MalR2 or MalR5 proteins is capable of binding upstream of said gene, or indeed, the promoter region of the nearby located gene encoding the GntR-type transcriptional regulator (Bbr_1891). This is not the first time that this phenomenon has been seen in microarray analysis, as it was also observed when microarray analysis was carried out on *B. breve* UCC2003 with SerR overexpressed, a two-component regulatory system (65), indicating that some of the global gene expression changes observed may in fact be due to indirect regulatory effects.

Bile exposure has been reported to cause changes in the glycolytic flux in bifidobacteria and two bile-resistant mutants of *Bifidobacterium animalis* subsp. *lactis* have been suggested to be due to mutations incurred in carbohydrate associated TFs/regulatory networks (46). *B. breve* UCC2003-*malR2* and *B. breve* UCC2003-*malR5* were shown to exhibit (compared to WT) increased resistance to ox-gall bile (Figure 5-4) and increased production of ethanol as an end product of fermentation (Figure 5-5). This phenomenon has also been reported by others in *Lactobacillus* and *Bifidobacterium* where it is proposed that increased ethanol production results in more regenerated NAD+ to combat oxidative stress effects of bile (47-50). The above-mentioned studies also reported that the bile resistant mutants of *Bifidobacterium* and *Lactobacillus* exhibited reduced exopolysaccharide (EPS) production (47, 51, 52). In addition, other studies have linked EPS production in *Bifidobacterium* to bile acid resistance (3, 53). Therefore, it is possible that the bile resistant phenotype of *B. breve* UCC2003-*malR2* and *B. breve* UCC2003-

malR5 is similarly linked to exopolysaccharide (EPS) production. This analysis also found that transcription of the EPS-associated gene clusters in *B. breve* UCC2003 is down-regulated in *B. breve* UCC2003-*malR5*, *B. breve* UCC2003-*malR3* and *B. breve* UCC2003-*malR6* (compared with the WT strain). Furthermore, it has been demonstrated for *B. longum* subsp. *longum* CRC 002, that EPS biosynthesis was modulated by the availability of differing sugar sources (54-57), and it is not unreasonable to think that (some of) the *malR* mutations affect carbohydrate availability for EPS biosynthesis.

Also of note, at 0.0625 % of ox-gall bile, *B. breve* UCC2003-*malR3* survives better than the WT and the other mutant strains. Although we do not have a direct explanation for this interesting phenotype, the observation is in line with *B. breve* UCC2003-*malR3's* ability to take up sugars at a higher rate compared to the WT strain and other *malR* mutant strains (Figure S5-7), with more carbohydrates within the cell there is the potential for more energy production to aid in the environmental bile response; however this would require further investigation.

As stated, the MalR TFs are involved in the uptake and utilisation of various carbohydrate sources. This analysis revealed that both the WT strain and the malR mutants internalised sugars in a similar order (ie: glucose, LNnT, ribose, maltotriose, maltose, and cellobiose in order of preference). However further investigation is required to understand why some of the MalR mutants internalised some of the carbohydrates (eg: cellobiose) at an accelerated rate as compared with the WT strain.

As a human-associated bacterium, *B. breve* UCC2003 faces the challenge of adaption to the ever-changing environment of the human gastrointestinal tract. This adaption requires the preservation of energy and this microbe must therefore regulate and control its metabolism precisely. Transcriptional regulation offers a fast acting and efficient method to govern metabolic pathways. Interestingly, not only is the transcription of carbohydrate utilisation systems dependent on available carbohydrate sources, but we propose that the transcription of these carbohydrate gene operons are also reacting to stresses such as bile stress, through the mechanism of lacI-type TFs. We have shown that (at least) four of these lacI-type TF's encoded by *B. breve* UCC2003 form a complex, overlapping and at times hierarchical network of gene regulation. This work represents one of the first steps

towards understanding this very complex and interactive regulatory network for carbohydrate metabolism in *B. breve* UCC2003. A good deal more work is required to fully comprehend and appreciate the intricacies of the MalR regulators and their impact on bifidobacterial physiology and survival in the gut.

5.6 Supplemental Tables and Figures

Table S5-6. *B. breve* UCC2003-*malR2* Microarray analysis (Up-regulation).
Global transcriptomic analysis of the fold up-regulation of *B. breve* UCC2003-*malR2* as compared with *B. breve* UCC2003 when grown on mMRS supplemented with ribose

Locus tag	Up	Gene name and/or predicted Function	P value
Bbr_0023	2.1	Transcriptional regulator, LacI family	9.25E-07
Bbr_0106	4.1	*cebE*, Cellobiose/cellotriose binding protein	0.00E+00
Bbr_0107	3.5	*cebF*, Cellobiose/cellotriose transport system permease protein	0.00E+00
Bbr_0108	2.7	*cebG*, Cellobiose/cellotriose transport system permease protein	0.00E+00
Bbr_0109	2.0	*bgl1*, Beta-glucosidase	1.51E-13
Bbr_0114	2.1	Cell surface protein with Gram positive anchor and Cna protein B-type domain	0.00E+00
Bbr_0160	2.1	Conserved hypothetical protein	6.27E-12
Bbr_0164	5.4	*oppA1*, Oligopeptide-binding protein	0.00E+00
Bbr_0165	4.8	*oppB1*, Oligopeptide transport system permease protein	0.00E+00
Bbr_0166	4.3	*oppD1*, Oligopeptide transport ATP-binding protein	0.00E+00
Bbr_0167	4.1	*oppF1*, Oligopeptide transport ATP-binding protein	0.00E+00
Bbr_0168	4.7	*dapA1*/*nanA*, Dihydrodipicolinate synthase/N-acetylneuraminate lyase	0.00E+00
Bbr_0169	4.1	*nagB1*, Glucosamine-6-phosphate isomerase	0.00E+00
Bbr_0171	4.2	Sialidase A	0.00E+00
Bbr_0284	6.0	Sugar/Sodium symporter	0.00E+00
Bbr_0285	6.4	lacZ2, Beta-galactosidase	0.00E+00
Bbr_0538	2.0	*cysK*, cysteine synthase	3.57E-12
Bbr_0539	2.5	*metB*, Cystathionine gamma-synthase	1.55E-15
Bbr_0757	2.0	*pyk*, Pyruvate kinase	1.71E-06
Bbr_1421	8.1	Conserved hypothetical membrane spanning protein	1.11E-16
Bbr_1422	6.8	*rbsK3*, Ribokinase	0.00E+00
Bbr_1423	6.9	*bdhA*, NADH-dependent butanol dehydrogenase 1	0.00E+00
Bbr_1424	7.7	*gph*, Phosphoglycolate phosphatase	0.00E+00
Bbr_1425	10.5	iunH2 Inosine-uridine preferring nucleoside hydrolase	0.00E+00
Bbr_1426	6.6	Fructokinase	0.00E+00
Bbr_1427	7.6	*trpF*, N-(5'-phosphoribosyl) anthranilate isomerase	0.00E+00
Bbr_1428	6.2	*CbiO1*, Cobalt transport ATP-binding protein	0.00E+00
Bbr_1429	4.9	*cbiO2*, Cobalt transport ATP-binding protein	0.00E+00
Bbr_1430	5.5	*cbiQ*, Cobalt transport protein	0.00E+00
Bbr_1431	4.6	Conserved hypothetical membrane spanning protein	5.51E-12
Bbr_1442	2.3	*bgl2*, Beta-glucosidase	4.44E-16
Bbr_1653	2.2	serA2, D-3-phosphoglycerate dehydrogenase	4.20E-14
Bbr_1655	62.9	bgl3, Beta-glucosidase	0.00E+00
Bbr_1656	91.3	Sugar ABC transporter, permease protein	0.00E+00
Bbr_1657	142.1	Sugar-binding protein of ABC transporter system	1.01E-14
Bbr_1658	253.6	Sugar-binding protein of ABC transporter system	5.29E-13
Bbr_1691	2.6	Beta-phosphoglucomutase	0.00E+00
Bbr_1692	2.1	Transporter, drug/metabolite exporter family	2.22E-16
Bbr_1693	2.5	*kojP*, Kojibiose phosphorylase	8.99E-15
Bbr_1694	2.0	Sugar-binding protein of ABC transporter system, permease	5.54E-12
Bbr_1695	3.0	Sugar-binding protein of ABC transporter system, permease	0.00E+00
Bbr_1696	3.7	Sugar-binding protein of ABC transporter system	0.00E+00
Bbr_1741	2.5	Conserved hypothetical protein	0.00E+00
Bbr_1742	2.5	L-fucose permease	0.00E+00
Bbr_1743	2.6	Short chain dehydrogenase	0.00E+00
Bbr_1744	2.1	Mandelate racemase	1.55E-15
Bbr_1879	2.3	PTS system, glucose-specific IIABC component	0.00E+00
Bbr_1880	2.5	PTS system, N-acetylglucosamine-specific IIBC component	0.00E+00

The level of expression is shown as a fold-value of increase in expression, with a cut-off of a minimum >1.9-fold increase in expression.

Table S5-7. *B. breve* UCC2003-*malR2* Microarray analysis (Down-regulation).
Global transcriptomic analysis of the fold down-regulation of *B. breve* UCC2003-*malR2* as compared with *B. breve* UCC2003 when grown on mMRS supplemented with ribose.

Locus tag	Down	Gene name and/or predicted Function	P value
Bbr_0033	2.1	Solute binding protein of ABC transporter system (MalE family)	1.06E-02
Bbr_0060	6.1	*glgP1*, Glycogen phosphorylase	1.11E-15
Bbr_0116	3.9	*malQ1*, 4-alpha-glucanotransferase	1.14E-07
Bbr_0117	2.1	*agl3*, Alpha-glucosidase	8.55E-10
Bbr_0118	24.9	*malE*, Maltose/maltodextrin-binding protein	0.00E+00
Bbr_0119	11.6	*malC*, Maltodextrin transport system permease protein	0.00E+00
Bbr_0120	7.9	*malG*, Maltose transport system permease protein	0.00E+00
Bbr_0121	3.6	Conserved hypothetical membrane spanning protein	0.00E+00
Bbr_0123	10.0	*apuB*, Amylopullulanase	0.00E+00
Bbr_0890	2.0	Conserved hypothetical protein	0.00E+00
Bbr_1843	5.1	Narrowly conserved hypothetical membrane spanning protein	0.00E+00
Bbr_1844	9.8	Permease protein of ABC transporter system for sugars	0.00E+00
Bbr_1845	10.8	Permease protein of ABC transporter system for sugars	0.00E+00
Bbr_1846	2.1	Transcriptional regulator, LacI family	0.00E+00
Bbr_1847	35.3	Solute binding protein of ABC transporter system for sugars	6.32E-12
Bbr_1891	12.2	Transcriptional regulator, GntR family	0.00E+00
Bbr_1892	38.4	PTS system, IIC component	4.44E-16
Bbr_1893	307.6	PTS system, IIB component	1.46E-10
Bbr_1894	540.1	PTS system, IIA component	1.00E-10

The level of expression is shown as a fold-value of increase in expression, with a cut-off of a minimum >2-fold increase in expression.

Table S5-8. *B. breve* UCC2003-*malR3* Microarray analysis (Up-regulation).
Global transcriptomic analysis of the fold up-regulation of *B. breve* UCC2003-*malR3* as compared with *B. breve* UCC2003 when grown on mMRS supplemented with ribose.

Locus tag	Up	Gene name and/or predicted Function	P value
Bbr_0010	2.1	*lacZ1*, Beta-galactosidase	6.81E-06
Bbr_0084	2.1	Glycosyltransferase	1.12E-10
Bbr_0111	3.1	*agl2*, Alpha-glucosidase	8.88E-16
Bbr_0116	2.4	*malQ1*, 4-alpha-glucanotransferase	9.27E-09
Bbr_0118	3.9	*malE*, Maltose/maltodextrin-binding protein	1.02E-08
Bbr_0119	2.6	*malC*, Maltodextrin transport system permease protein	0.00E+00
Bbr_0120	2.3	*malG*, Maltose transport system permease protein	0.00E+00
Bbr_0121	2.3	Conserved hypothetical membrane spanning protein	7.61E-12
Bbr_0122	2.3	Transcriptional regulator, LacI family	0.00E+00
Bbr_0124	2.3	dnaK Chaperone protein dnaK	3.52E-11
Bbr_0129	2.1	*fabG*, 3-oxoacyl-[acyl-carrier protein] reductase	4.44E-16
Bbr_0203	3.4	Conserved hypothetical membrane spanning protein	5.16E-12
Bbr_0211	2.1	Hypothetical protein	2.24E-05
Bbr_0229	2.4	Conserved hypothetical membrane spanning protein with similarity to phage infection protein	4.37E-14
Bbr_0231	3.0	Conserved hypothetical membrane spanning protein with DUF1113 domain	1.91E-13
Bbr_0302	2.1	Transposase	7.21E-13
Bbr_0303	2.5	Permease protein of ABC transporter system for sugars	2.51E-09
Bbr_0313	3.6	Hypothetical protein	1.80E-10
Bbr_0318	2.5	Predicted hypothetical protein	1.75E-07
Bbr_0362	2.2	Conserved hypothetical membrane spanning protein	7.42E-06
Bbr_0390	2.2	*ilvN*, Acetolactate synthase small subunit	0.00E+00
Bbr_0454	2.0	Conserved hypothetical protein	2.26E-07
Bbr_0539	3.2	*metB*, Cystathionine gamma-synthase	0.00E+00
Bbr_0540	2.8	*recQ*, ATP-dependent DNA helicase	3.40E-13
Bbr_0548	2.0	Conserved hypothetical protein	1.49E-14
Bbr_0560	2.0	*dppA1*, Solute-binding protein of ABC transporter system for peptides	2.64E-11
Bbr_0820	2.3	Conserved hypothetical protein	6.73E-12
Bbr_0821	2.4	Na+ driven multidrug efflux pump	0.00E+00
Bbr_0824	2.1	Fic family protein	2.00E-06
Bbr_0886	2.1	Permease protein of ABC transporter system	3.38E-13
Bbr_0891	3.3	Hypothetical membrane spanning protein	6.66E-16
Bbr_1091	2.4	Hypothetical protein	3.62E-14
Bbr_1271	2.2	*lexA*, LexA repressor	1.11E-15
Bbr_1425	2.8	*iunH2*, Inosine-uridine preferring nucleoside hydrolase	0.00E+00
Bbr_1435	2.1	Transcriptional regulator, MarR family	1.11E-16
Bbr_1441	2.8	Narrowly conserved hypothetical protein	4.03E-10
Bbr_1513	2.0	*parA2*, Chromosome partitioning protein	1.21E-07
Bbr_1515	2.1	ATP-binding protein of ABC transporter system	3.39E-06
Bbr_1523	2.1	*merR*, family regulatory protein	0.00E+00
Bbr_1525	2.1	Hypothetical protein	7.82E-09
Bbr_1528	2.1	Conserved hypothetical membrane spanning protein	2.78E-05
Bbr_1531	2.4	Narrowly conserved hypothetical protein	1.44E-06
Bbr_1536	2.8	*TraG/TraD* family	6.08E-07
Bbr_1537	3.6	Hypothetical protein	1.25E-10
Bbr_1543	2.8	Hypothetical protein	1.34E-07
Bbr_1544	2.2	Relaxase	8.37E-05
Bbr_1545	2.4	Mobilisation protein	1.20E-07
Bbr_1601	3.3	Narrowly conserved hypothetical membrane spanning protein	8.88E-16
Bbr_1815	2.1	*oppD3*, Oligopeptide transport ATP-binding protein	5.14E-06
Bbr_1817	2.2	*oppB3*, Oligopeptide transport system permease protein	4.71E-05
Bbr_1821	2.1	Narrowly conserved hypothetical protein	1.40E-08
Bbr_1822	2.0	Narrowly conserved hypothetical protein	4.20E-09

Bbr_1828	2.1	Transcriptional regulator, MarR family	2.27E-11
Bbr_1834	2.5	*rbsC2*, Ribose transport system permease protein	1.22E-06
Bbr_1900	2.4	*nrdI*, NrdI protein	0.00E+00
Bbr_1901	2.5	*nrdH*, Glutaredoxin	0.00E+00
Bbr_1909	2.0	Conserved hypothetical protein	0.00E+00
Bbr_1918	2.1	*trxB2*, Thioredoxin reductase	7.99E-15

The level of expression is shown as a fold-value of increase in expression, with a cut-off of a minimum >2-fold increase in expression.

Table S5-9. *B. breve* UCC2003-*malR3* Microarray analysis (Down-regulation).
Global transcriptomic analysis of the fold down-regulation of *B. breve* UCC2003-*malR3* as compared with *B. breve* UCC2003 when grown on mMRS supplemented with ribose.

Locus tag	Down	Gene name and/or predicted Function	P value
Bbr_0105	2.3	*cebR*, Cellobiose transport system transcriptional regulator	0.00E+00
Bbr_0243	2.2	*glnD*, [protein-PII] uridylyltransferase	2.25E-13
Bbr_0258	2.1	Hypothetical membrane spanning protein	1.11E-16
Bbr_0330	2.4	*atpC*, ATP synthase epsilon chain	0.00E+00
Bbr_0331	2.2	Conserved hypothetical protein with DUF91 domain	0.00E+00
Bbr_0366	2.7	Narrowly conserved hypothetical secreted protein with Gram positive anchor and Cna protein B-type domain	3.30E-09
Bbr_0367	5.7	Hypothetical protein	2.22E-16
Bbr_0368	2.7	Conserved hypothetical protein	1.63E-09
Bbr_0369	3.2	Narrowly conserved hypothetical membrane spanning protein	5.52E-10
Bbr_0372	2.2	ATP-binding protein of ABC transporter system	5.35E-14
Bbr_0443	5.5	Glycosyltransferase	4.11E-11
Bbr_0444	3.0	Membrane spanning polysaccharide biosynthesis protein	8.02E-04
Bbr_0445	7.2	Glycosyltransferase	8.77E-15
Bbr_0446	9.1	Acetyltransferase (cell wall biosynthesis)	1.00E-12
Bbr_0447	3.6	Conserved hypothetical protein	8.02E-13
Bbr_0448	6.6	Glycosyltransferase	1.63E-10
Bbr_0449	4.0	Hypothetical membrane spanning protein	0.00E+00
Bbr_0450	5.1	Membrane spanning protein involved in polysaccharide biosynthesis	5.70E-09
Bbr_0451	2.7	Acyltransferase	2.46E-11
Bbr_0452	2.2	Hypothetical protein	6.91E-08
Bbr_0604	2.3	*rpsL*, SSU ribosomal protein S12P	4.00E-06
Bbr_0810	2.5	Two-component response regulator	1.29E-14
Bbr_0872	2.0	Narrowly conserved hypothetical membrane spanning protein	4.63E-05
Bbr_0883	2.0	*Xpt*, Xanthine phosphoribosyltransferase	4.27E-13
Bbr_0938	2.5	Hypothetical membrane spanning protein	2.73E-05
Bbr_0963	2.2	Conserved hypothetical protein	3.50E-09
Bbr_0994	2.0	*pgk*, Phosphoglycerate kinase	1.11E-13
Bbr_1179	2.2	Ribosome-associated factor Y	1.07E-07
Bbr_1475	3.4	Branched-chain amino acid transport ATP-binding protein livF	1.16E-08
Bbr_1581	2.2	Narrowly conserved hypothetical membrane spanning protein	1.11E-16
Bbr_1582	2.1	Narrowly conserved hypothetical membrane spanning protein with PspC domain	1.10E-14
Bbr_1761	2.0	Permease protein of ABC transporter system for amino acids	8.91E-08
Bbr_1892	2.2	PTS system, IIC component	0.00E+00
Bbr_1898	4.1	*nrdF*, Ribonucleoside-diphosphate reductase beta chain	0.00E+00

The level of expression is shown as a fold-value of increase in expression, with a cut-off of a minimum 2>-fold increase in expression.

Table S5-10. *B. breve* UCC2003-*malR5* Microarray analysis (Up-regulation).

Global transcriptomic analysis of the fold up-regulation of *B. breve* UCC2003-*malR5* as compared with *B. breve* UCC2003 when grown on mMRS supplemented with ribose.

Locus tag	Up	Gene name and/or predicted Function	P value
Bbr_0106	4.6	*cebE*, Cellobiose/cellotriose binding protein	0.00E+00
Bbr_0107	3.1	*cebF*, Cellobiose/cellotriose transport system permease protein	0.00E+00
Bbr_0108	2.3	*cebG*, Cellobiose/cellotriose transport system permease protein	0.00E+00
Bbr_0109	2.2	*bgl1*, Beta-glucosidase	1.83E-14
Bbr_0164	5.8	*oppA1*, Oligopeptide-binding protein	0.00E+00
Bbr_0165	4.5	*oppB1*, Oligopeptide transport system permease protein	0.00E+00
Bbr_0166	3.7	*oppD1*, Oligopeptide transport ATP-binding protein	0.00E+00
Bbr_0167	3.8	*oppF1*, Oligopeptide transport ATP-binding protein	0.00E+00
Bbr_0168	3.4	dapA1/nanA, Dihydrodipicolinate synthase	0.00E+00
Bbr_0169	2.8	*nagB1*, Glucosamine-6-phosphate isomerase	0.00E+00
Bbr_0171	3.3	Sialidase A	0.00E+00
Bbr_0284	5.7	Sugar/Sodium symporter	0.00E+00
Bbr_0285	5.9	*lacZ2*, Beta-galactosidase	0.00E+00
Bbr_0453	2.2	Transposase	1.11E-15
Bbr_0454	2.0	Conserved hypothetical protein	3.13E-06
Bbr_0539	2.3	*metB*, Cystathionine gamma-synthase	9.99E-16
Bbr_0820	2.5	Conserved hypothetical protein	1.20E-12
Bbr_0938	3.6	Hypothetical membrane spanning protein	5.49E-07
Bbr_1024	2.3	Hydrolase (HAD superfamily)	0.00E+00
Bbr_1421	7.5	Conserved hypothetical membrane spanning protein	0.00E+00
Bbr_1422	7.2	*rbsK3*, Ribokinase	0.00E+00
Bbr_1423	6.7	*bdhA*, NADH-dependent butanol dehydrogenase	0.00E+00
Bbr_1424	8.6	*gph*, Phosphoglycolate phosphatase	0.00E+00
Bbr_1425	9.7	*iunH2*, Inosine-uridine preferring nucleoside hydrolase	0.00E+00
Bbr_1426	5.9	Fructokinase	0.00E+00
Bbr_1427	7.3	*trpF*, N-(5'-phosphoribosyl) anthranilate isomerase	0.00E+00
Bbr_1428	6.2	*cbiO1*, Cobalt transport ATP-binding protein	0.00E+00
Bbr_1429	4.9	*cbiO2*, Cobalt transport ATP-binding protein	0.00E+00
Bbr_1430	6.3	*cbiQ*, Cobalt transport protein	0.00E+00
Bbr_1431	5.2	Conserved hypothetical membrane spanning protein	4.60E-12
Bbr_1442	2.0	*bgl2*, Beta-glucosidase	3.59E-11
Bbr_1653	2.6	*serA2*, D-3-phosphoglycerate dehydrogenase	6.22E-15
Bbr_1655	107.4	*bgl3*, Beta-glucosidase	0.00E+00
Bbr_1656	145.4	Sugar ABC transporter, permease protein	4.56E-14
Bbr_1657	175.9	Sugar-binding protein of ABC transporter system	2.85E-14
Bbr_1658	404.1	Sugar-binding protein of ABC transporter system	2.60E-12
Bbr_1691	2.3	Beta-phosphoglucomutase	1.06E-11
Bbr_1692	2.0	Transporter, drug/metabolite exporter family	1.72E-11
Bbr_1693	2.6	*kojP*, Kojibiose phosphorylase	3.34E-10
Bbr_1694	2.5	Sugar-binding protein of ABC transporter system, permease	0.00E+00
Bbr_1695	2.7	Sugar-binding protein of ABC transporter system, permease	1.11E-16
Bbr_1696	3.8	Sugar-binding protein of ABC transporter system	0.00E+00
Bbr_1743	2.3	Short chain dehydrogenase	8.88E-16
Bbr_1862	2.0	Solute binding protein of ABC transporter system for sugars	1.71E-13
Bbr_1880	2.2	PTS system, N-acetylglucosamine-specific IIBC component	0.00E+00

The level of expression is shown as a fold-value of increase in expression, with a cut-off of a minimum >2-fold increase in expression.

Table S5-11. *B. breve* UCC2003-*malR5* Microarray analysis (Down-regulation).
Global transcriptomic analysis of the fold down-regulation of *B. breve* UCC2003-*malR5* as compared with *B. breve* UCC2003 when grown on mMRS supplemented with ribose.

Locus tag	Down	Gene name and/or predicted Function	P value
Bbr_0060	4.8	*glgP1*, Glycogen phosphorylase	0.00E+0
Bbr_0114	2.9	Cell surface protein with Gram positive anchor and Cna protein B-	0.00E+0
Bbr_0116	6.5	*malQ1*, 4-alpha-glucanotransferase	5.85E-08
Bbr_0117	3.2	*agl3*, Alpha-glucosidase	4.98E-10
Bbr_0118	56.0	*malE*, Maltose/maltodextrin-binding protein	0.00E+0
Bbr_0119	41.2	*malC*, Maltodextrin transport system permease protein	0.00E+0
Bbr_0120	19.0	*malG*, Maltose transport system permease protein	6.66E-16
Bbr_0121	8.1	Conserved hypothetical membrane spanning protein	0.00E+0
Bbr_0122	2.0	Transcriptional regulator, LacI family	0.00E+0
Bbr_0123	16.1	*apuB*, Amylopullulanase	0.00E+0
Bbr_0124	2.0	*dnaK*, Chaperone protein	0.00E+0
Bbr_0457	3.5	Transposase	5.37E-07
Bbr_0458	2.3	Hypothetical protein	2.04E-07
Bbr_0459	2.8	Conserved hypothetical protein	2.76E-08
Bbr_0460	3.8	Hypothetical membrane spanning protein	3.19E-11
Bbr_0461	16.4	Hypothetical protein	0.00E+0
Bbr_0610	2.4	Conserved hypothetical membrane spanning protein	0.00E+0
Bbr_0845	2.2	*glgP2*, Glycogen phosphorylase	7.68E-08
Bbr_1719	2.3	fas Type I multifunctional fatty acid synthase	2.22E-16
Bbr_1720	2.2	*accD*, Acetyl-/propionyl-CoA carboxylase beta chain	0.00E+0
Bbr_1781	2.1	*clpB*, ClpB protein	7.84E-13
Bbr_1843	7.6	Narrowly conserved hypothetical membrane spanning protein	0.00E+0
Bbr_1844	11.4	Permease protein of ABC transporter system for sugars	0.00E+0
Bbr_1845	11.4	Permease protein of ABC transporter system for sugars	0.00E+0
Bbr_1846	2.5	Transcriptional regulator, LacI family	0.00E+0
Bbr_1847	30.5	Solute binding protein of ABC transporter system for sugars	3.00E-15
Bbr_1891	17.4	Transcriptional regulator, GntR family	0.00E+0
Bbr_1892	122.8	PTS system, IIC component	4.22E-15
Bbr_1893	419.0	PTS system, IIB component	4.54E-12
Bbr_1894	516.2	PTS system, IIA component	2.08E-13
Bbr_1898	2.4	*nrdF*, Ribonucleoside-diphosphate reductase beta chain	0.00E+0
Bbr_1899	2.3	*nrdE*, Ribonucleoside-diphosphate reductase alpha chain	0.00E+0
Bbr_1900	2.3	*nrdI*, NrdI protein	0.00E+0

The level of expression is shown as a fold-value of increase in expression, with a cut-off of a minimum >2-fold increase in expression.

Table S5-12. B. breve UCC2003-malR6 Microarray analysis (Up-regulation).
Global transcriptomic analysis of the fold up-regulation of B. breve UCC2003-malR6 as compared with B. breve UCC2003 when grown on mMRS supplemented with ribose.

Locus tag	Up	Gene name and/or predicted Function	P value
Bbr_0256	1.5	Ribosomal protein	3.36E-04
Bbr_0328	1.6	*atpG*, ATP synthase gamma chain	5.70E-06
Bbr_0381	1.5	*rpsP*, SSU ribosomal protein S16P	3.30E-02
Bbr_0584	1.5	*purF*, Amidophosphoribosyltransferase	3.95E-12
Bbr_0589	1.6	Hypothetical protein	1.68E-09
Bbr_0604	2.1	*rpsL*, SSU ribosomal protein S12P	6.33E-07
Bbr_0605	2.0	*rpsG*, SSU ribosomal protein S7P	8.64E-06
Bbr_0606	1.6	*fusA*, Protein Translation Elongation Factor G (EF-G)	9.80E-04
Bbr_0607	2.2	*tuf*, *Protein* Translation Elongation Factor Tu (EF-TU)	2.61E-03
Bbr_0631	1.7	*Efp*, Protein Translation Elongation Factor P (EF-P)	2.24E-11
Bbr_0725	1.7	*eno*, Enolase	9.97E-04
Bbr_0757	1.5	*pyk*, Pyruvate kinase	2.71E-04
Bbr_0772	1.6	*pta*, Phosphate acetyltransferase	2.85E-11
Bbr_0776	1.9	Xylulose-5-phosphate/Fructose-6-phosphate phosphoketolase	2.50E-04
Bbr_0787	2.5	*pfl*, Formate acetyltransferase	2.34E-04
Bbr_0794	1.6	*clp1*, ATP-dependent Clp protease proteolytic subunit 1	8.35E-09
Bbr_0897	1.6	Narrowly conserved hypothetical secreted protein	7.33E-15
Bbr_0921	1.6	*fadD2*, Long-chain-fatty-acid--CoA ligase	4.88E-11
Bbr_0971	1.7	*bshB*, Choloylglycine hydrolase	8.79E-13
Bbr_0974	1.6	*pyrI*, Aspartate carbamoyltransferase	1.42E-13
Bbr_1002	1.6	*tal*, Transaldolase	5.06E-04
Bbr_1003	2.0	*tkt*, Transketolase	1.28E-05
Bbr_1040	1.5	*hisE*, Phosphoribosyl-ATP pyrophosphatase	2.80E-14
Bbr_1102	1.5	*frr*, Ribosome Recycling Factor (RRF)	2.05E-12
Bbr_1105	2.0	*rpsB*, SSU ribosomal protein S2P	2.13E-05
Bbr_1156	1.6	Peptidyl-prolyl cis-trans isomerase	2.84E-13
Bbr_1204	1.7	*oppB2*, Oligopeptide transport system permease protein	9.76E-08
Bbr_1205	1.7	*oppA2*, Oligopeptide-binding protein	6.73E-06
Bbr_1230	1.6	Bacterial Protein Translation Initiation Factor	2.93E-07
Bbr_1233	1.9	Glyceraldehyde 3-phosphate dehydrogenase	3.93E-03
Bbr_1273	1.6	*ldh2*, L-lactate dehydrogenase	6.34E-06
Bbr_1377	1.6	*purB*, Adenylosuccinate lyase	3.77E-14
Bbr_1381	1.5	Conserved hypothetical protein with DUF797 domain	2.66E-14
Bbr_1417	1.8	*rbsB1*, D-ribose-binding protein	3.37E-03
Bbr_1418	2.0	*rbsC1*, Ribose transport system permease protein	1.36E-02
Bbr_1463	1.5	*map*, Methionine aminopeptidase	1.03E-08
Bbr_1482	1.6	*rpmE2*, LSU ribosomal protein L31P	4.95E-04
Bbr_1574	1.8	*gpm2*, Phosphoglycerate mutase	1.46E-04
Bbr_1619	2.6	Bacterial Protein Translation Initiation Factor	3.74E-04
Bbr_1620	1.5	*adk*, Adenylate kinase	6.72E-06
Bbr_1649	2.1	*rbsC1*, Ribose transport system permease protein	3.53E-03
Bbr_1667	1.6	*rpmG*, LSU ribosomal protein L33P	5.26E-05
Bbr_1675	1.9	*rplL*, LSU ribosomal protein L12P (L7/L12)	7.08E-03
Bbr_1676	1.9	*rplJ*, LSU ribosomal protein L10P	5.74E-05

The level of expression is shown as a fold-value of increase in expression, with a cut-off of a minimum >1.5-fold increase in expression.

Table S5-13. *B. breve* UCC2003-*malR6* Microarray analysis (Down-regulation).
Global transcriptomic analysis of the fold down-regulation of *B. breve* UCC2003-*malR6* as compared with *B. breve* UCC2003 when grown on mMRS supplemented with ribose.

Locus tag	Down	Gene name and/or predicted Function	P value
Bbr_0041	1.6	Conserved hypothetical membrane spanning protein	8.54E-02
Bbr_0047	1.5	Conserved hypothetical protein	2.41E-13
Bbr_0048	1.7	Conserved hypothetical membrane spanning protein	3.28E-10
Bbr_0049	1.6	Conserved hypothetical protein containing diguanylate cyclase/phosphodiesterase (EAL/GGDEF) domain	4.93E-12
Bbr_0106	1.6	*cebE*, Cellobiose/cellotriose binding protein	3.52E-13
Bbr_0107	1.9	*cebF*, Cellobiose/cellotriose transport system permease protein	1.68E-12
Bbr_0108	1.7	*cebG*, Cellobiose/cellotriose transport system permease protein	7.71E-12
Bbr_0129	1.5	*fabG*, 3-oxoacyl-[acyl-carrier protein] reductase	3.72E-02
Bbr_0154	1.5	Permease protein of ABC transporter system	1.54E-12
Bbr_0155	1.6	Permease protein of ABC transporter system	4.44E-16
Bbr_0158	1.5	Hypothetical protein	6.78E-12
Bbr_0174	1.5	Conserved hypothetical protein	1.20E-11
Bbr_0439	1.7	Capsular polysaccharide biosynthesis protein	1.44E-15
Bbr_0440	1.5	Polysaccharide biosynthesis protein	2.43E-12
Bbr_0442	1.9	Capsular polysaccharide biosynthesis protein	1.23E-13
Bbr_0443	1.7	Glycosyltransferase	7.77E-16
Bbr_0444	1.6	Membrane spanning polysaccharide biosynthesis protein	3.00E-14
Bbr_0445	1.5	Glycosyltransferase	5.55E-16
Bbr_0446	1.5	Acetyltransferase (cell wall biosynthesis)	2.49E-12
Bbr_0447	1.5	Conserved hypothetical protein	7.56E-11
Bbr_0469	2.1	Conserved hypothetical membrane spanning protein	0.00E+00
Bbr_1209	1.5	Narrowly conserved hypothetical protein	2.39E-01
Bbr_1656	1.6	Sugar ABC transporter, permease protein	8.06E-14
Bbr_1657	1.7	Sugar-binding protein of ABC transporter system	4.35E-14
Bbr_1762	1.5	Conserved hypothetical protein	1.55E-14
Bbr_1790	1.8	Phosphoglycerol transferase	3.73E-12
Bbr_1791	1.9	Phosphoglycerol transferase	1.33E-15
Bbr_1792	1.6	Glycosyltransferase	2.40E-12
Bbr_1794	1.7	Permease protein of ABC transporter systemfor polysaccharides	6.04E-14
Bbr_1795	1.5	Alpha-L-Rha alpha-1,2/1,3-L-rhamnosyltransferase	1.06E-11
Bbr_1846	2.6	*bifR1*, Transcriptional regulator, LacI family	4.68E-12
Bbr_1879	1.7	PTS system, glucose-specific IIABC component	2.40E-12
Bbr_1880	1.5	PTS system, N-acetylglucosamine-specific IIBC component	8.80E-08
Bbr_1882	1.5	*spoU*, rRNA methylase family protein	2.84E-12
Bbr_1920	1.8	*parA3*, Chromosome partitioning protein	4.46E-13
Bbr_1921	1.6	*gidB*, Methyltransferase (Glucose inhibited division protein B)	6.62E-14

The level of expression is shown as a fold-value of increase in expression, with a cut-off of a minimum >1.5-fold increase in expression.

Table S5-14. Effector molecules tested.

Effector Molecule
Maltose
Maltotriose
Maltulose
Isomaltose
Lactose
Glucose
Galactose
Sucrose
Trehalose
Glucose 6-phosphate
Glucose 1- phosphate
Pyruvic acid
Sodium Acetate
Fructose 6- phosphate
Cellobiose
Palatinose
Turanose
DL-Glyceraldehyde 3-phosphate
Acetyl coenzyme A sodium salt
Phosphoenolpyruvate
Acetyl Phosphate
D-Sedoheptulose-7-phosphate
D-Ribose 5-phosphate disodium salt
D-(-)-3-Phosphoglyceric acid disodium salt
Butyrate
Lactate
Propioante
Acetate
Acetyl Aldehyde
1,2 Propanediol
D-erythrose-4-Phosphate
Oxaloacetic acid
Cyclic-AMP
Succinic acid
D-Ribulose 5-phosphate disodium salt

Table S5-15. Summary metabolic end product analysis.

HPLC Analysis				
Time Point	**Strains**	**Lactate***	**Acetate***	**Ethanol***
8 hrs	WT	0.226	0.550	0.046
	malR2	0.147	0.400	0.063
	malR3	0.205	0.501	0.040
	malR5	0.235	0.526	0.030
	malR6	0.190	0.467	0.037
24 hrs	WT	0.269	0.555	0.055
	malR2	0.323	0.549	0.151
	malR3	0.292	0.551	0.059
	malR5	0.258	0.595	0.077
	malR6	0.265	0.541	0.058

Metabolic end product analysis by HPLC of *B. breve* UCC2003 (WT), *B. breve* UCC2003-*malR2 (malR2)*, *B. breve* UCC2003-*malR3 (malR3)*, *B. breve* UCC2003-*malR5 (malR5) and B. breve* UCC2003-*malR6 (malR6)*. All strains were grown on glucose as the sole carbon source, and samples were taken at 8 and 24 hours post inoculation. Results are presented as moles of metabolite detected at either 8 hours or 24 hours post inoculation. * All values are calculated as per mole of glucose consumed.

MalR2

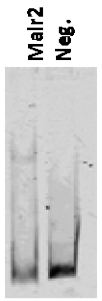
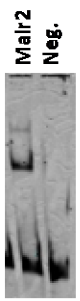
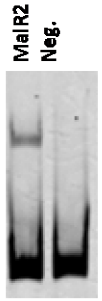
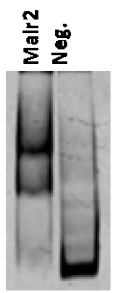
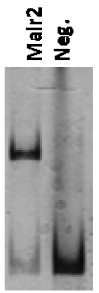
Gene Name Locus tag	*malR5* Bbr_0032	Bbr_1841	*malQ* Bbr_0116	*agl4/malE* Bbr_0117/118	*glgP* Bbr_0060	*malR3/apuB* Bbr_0122/123	*malR6/113* Bbr_0112/113	*malG2* Bbr_0027
EMSA Image								
Percentage binding	-	-	+	+	++	++	+++	+++

Figure S5-8. MalR2 Example EMSA.
All EMSA analysis illustrated in the above table were carried out with 150nM protein (Malr2) or 0nM protein (Neg.) incubated with 0.5nM Ird labelled DNA fragments encompassing the promoter region of the specified gene. Binding affinity was calculated based on the total percentage DNA bound – is representative of no binding, + is representative of an ability to bind 5%-15% of the total DNA, ++ is representative of an ability to bind 15%-40% of the total DNA present and +++ is representative of an ability to bind greater than 40% of the DNA present in the reaction.

Commented [D5]: Similar to the previous chapter, the last row of the figure should say Binding level as opposed to binding percentage

MalR3

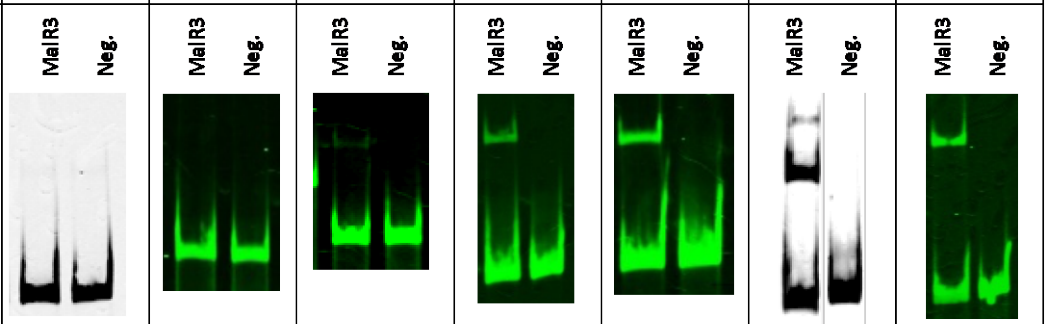
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Locus tag	Bbr_0106	Bbr_0116	Bbr_1845/1846	Bbr_0060	Bbr_0122/123	Bbr_0112/113	Bbr_0117/118
EMSA Image							
Percentage binding	-	-	+	++	++	+++	+++

Figure S5-9. MalR3 Example EMSA.
All EMSA analysis illustrated in the above table were carried out with 150nM protein (Malr3) or 0nM protein (Neg.) incubated with 0.5nM Ird-labelled DNA fragments encompassing the promoter region of the specified gene. Binding affinity was calculated based on the total percentage DNA bound – is representative of no binding, + is representative of an ability to bind 5%-15% of the total DNA, ++ is representative of an ability to bind 15%-40% of the total DNA present and +++ is representative of an ability to bind greater than 40% of the DNA present in the reaction.

Commented [D6]: See previous comment

MalR5

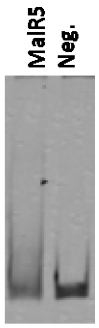
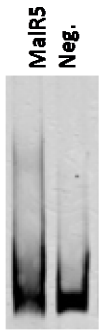
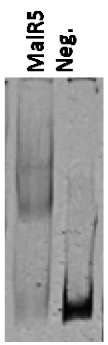
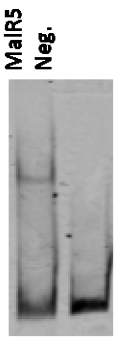
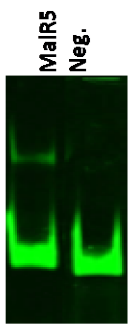
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Locus tag	Bbr_0105	Bbr_0122/123	Bbr_0106	Bbr_0116	Bbr_0117/118	Bbr_0032
EMSA Image						
Percentage binding	-	-	++	++	+++	+++

Figure S5-10. MalR5 Example EMSA.
All EMSA analysis illustrated in the above table were carried out with 150nM protein (Malr5) or 0nM protein (Neg.) incubated with 0.5nM Ird-labelled DNA fragments encompassing the promoter region of the specified gene. Binding affinity was calculated based on the total percentage DNA bound – is representative of no binding, + is representative of an ability to bind 5%-15% of the total DNA, ++ is representative of an ability to bind 15%-40% of the total DNA present and +++ is representative of an ability to bind greater than 40% of the DNA present in the reaction.

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MalR6

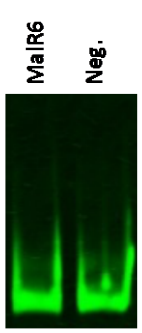
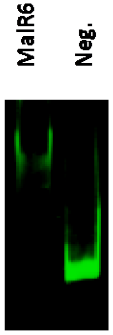
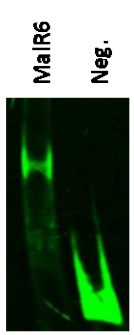
Gene Name Locus tag	*malG2* Bbr_0027	*malR3/apuB* Bbr_0122/123	*agl3* Bbr_0111	*malR6* Bbr_0112
EMSA Image				
Percentage binding	-	-	+++	+++

Figure S5-11. MalR6 Example EMSA.
All EMSA analysis illustrated in the above table were carried out with 150nM protein (Malr6) or 0nM protein (Neg.) incubated with 0.5nM Ird-labelled DNA fragments encompassing the promoter region of the specified gene. Binding affinity was calculated based on the total percentage DNA bound – is representative of no binding, + is representative of an ability to bind 5%-15% of the total DNA, ++ is representative of an ability to bind 15%-40% of the total DNA present and +++ is representative of an ability to bind greater than 40% of the DNA present in the reaction.

Commented [D8]: See previous comment

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Commented [D9]: Just check abbreviations of journals and consistency in the abbreviations

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Chapter 6 General Discussion and Future Perspectives

6.1 Discussion and Overall Conclusions

Several members of the genus *Bifidobacterium* are used as so-called probiotics, which are defined as live bacteria that confer a positive health benefit on their host when administered in adequate amounts (41-47). Such probiotic bifidobacteria are purported to elicit a range of beneficial effects upon their host, although the precise molecular details underlying any such health-promoting activity are still obscure. In order to understand the mechanism(s) of action of these health benefits, and to fully exploit such probiotic activities for the betterment of human health it is important to study the mechanisms which underlie the ability of bifidobacteria to colonise and survive in the gut environment. Bifidobacteria, like other gut-colonizing commensals, face many challenges during their transit through, colonisation of and persistence in the gastrointestinal tract (GIT). The work in this thesis describes experimental approaches intended to obtain a focused view of some of these challenges.

Iron is a micronutrient which is essential (with some known exceptions among *Lactobacillus* and *Borrelia species*, which require very little if any iron) for growth and proliferation of both commensal and pathogenic bacteria alike, and within the gastrointestinal tract dietary iron has been found to elicit a clear influence on microbiota composition (1-4). Clear shifts have been observed in the gut microbiota composition in hosts that are nourished by either iron-restrictive or iron-fortified diets. Prolonged consumption of iron-fortified biscuits has been associated with increased abundance of faecal enterobacteria and a decrease in the relative abundance of bifidobacteria and lactobabilli (5). These observations make this an area which warrants further investigation, particularly in relation iron-fortified, probiotic-containing foods. Furthermore, one of the mechanisms by which probiotic bacteria have been found to benefit their host is through the ability to compete for micronutrients, such as iron, thereby restricting access of such micronutrients to pathogenic bacteria (6). Bifidobacteria are thought to be efficient scavengers of iron and have been found to be capable of growth even under low iron conditions. This notion is corroborated by the finding that certain bifidobacterial species are found in greater numbers under low luminal iron conditions as compared with normal or high luminal iron conditions (7).

Much of the relevant literature to date focuses on the ability of bifidobacteria to internalise and sequester iron, and to proliferate in either high or low concentrations of iron (8-11). Chapter 2 was aimed at uncovering the genes and encoded systems responsible for iron uptake and metabolism in *B. breve* UCC2003, as a prototypical representative of its genus. Phenotypic screening of a *B. breve* UCC2003-derived random mutant library combined with transcriptomic analyses led to the identification of a number of genes, which were of diverse (predicted) function, and which were shown to be important for *B. breve* UCC2003 survival under iron-limiting conditions. The range of identified genes and their functional divergence appears to reflect the importance of iron to cellular maintenance. Among the identified genes were *bfeUO* and *sifABCDE*, which are predicted to encode a ferrous and a ferric iron uptake system, respectively. The ubiquitous presence of *bfeUO* and *sifABCDE* homologs across the *Bifidobacterium* genus indicates that bifidobacteria utilise both ferrous and ferric iron depending upon its availability in the gastrointestinal tract.

Recent publications have also identified these iron uptake systems as being essential for normal growth (under laboratory conditions) of *B. breve* UCC2003 utilising a Tradis mutant library approach (50). This is in accordance with the work of this thesis, which demonstrates that these iron uptake systems are essential for growth under *in vitro* iron-limiting conditions, but not under *in vivo* conditions, in which *B. breve* UCC2003-*bfeU* and *B. breve* UCC2003-*sifA* mutants were able to colonise a healthy murine GIT as efficiently as the *B. breve* UCC2003 WT strain. Similarly, *B. breve* UCC2003-*bfeU* and *B. breve* UCC2003-*sifA* mutants were found to colonize the GIT of nematodes with equal efficiency as the *B. breve* UCC2003 WT strain (12). Interestingly, these authors also found that the *B. breve* UCC2003-*bfeU* and *B. breve* UCC2003-*sifA* mutants exhibit a significantly decreased ability to confer protection to *Salmonella*-infected nematodes as compared to the *B. breve* UCC2003 WT strain in this nematode model (12). Therefore, we postulate that either iron is available in the gut in a form which is not taken up by *bfeUO* or *sifABCDE*, or that these iron uptake systems only play an important role when iron is limiting in the GIT (for example as a result of gastrointestinal infection), perhaps as a result of a phenomenon known as nutritional immunity (6). Nutritional immunity was originally associated with the ability of a higher organism to withhold trace elements such as iron, consequently reducing the proliferation of pathogenic organisms (13).

However, this mechanism of nutritional immunity is not exclusive to vertebrates, bacteria such as members of the genus *Bifidobacterium* may provide protection to their host/themselves by reducing the availability of iron within the GIT which results in the decreased ability of certain pathogens to proliferate (13, 14). Furthermore, iron sequestration via these two iron uptake systems possibly reduces free radical production in the gut, a phenomenon that has been associated with inflammation and increased risk of colorectal carcinoma via the induction of DNA damage within the GIT (15).

Chapter 3 expands on the investigation on iron availability and the effect of chronic iron starvation on *B. breve* UCC2003. Our investigations revealed that *B. breve* UCC2003's transcriptomic responses to chronic iron starvation as opposed to immediate, yet severe iron limitation as studied in Chapter 2 are distinct. When these transcriptomic responses are compared we can see that *B. breve* UCC2003 initially activates the expression of multiple (bivalent) cation uptake systems as an immediate reaction to iron-limiting conditions; however, when exposed to long-term iron starvation *B. breve* UCC2003 mainly expresses two iron uptake systems namely *bfeUO* and *sifABCDE*. This indicates that these uptake systems are critically important for the survival of *B. breve* UCC2003 (and probably other bifidobacteria, given the conserved nature of these two gene clusters) under both iron-limiting and -starvation conditions, at least under the conditions tested.

As mentioned above, bifidobacteria must withstand various challenges during their transit through the upper intestinal tract and then during their colonisation of and persistence in the lower parts of the gastrointestinal environment. Of particular interest to this thesis is bile stress, bile is an aqueous substance which plays a vital role in digestion and lipid absorption (16, 17). Bile salts, representing an important component of bile with strong bactericidal properties, are strongly lipophilic in nature and act as antimicrobial compounds by disorganising the bacterial cellular membrane (18). The ability of gut-residing (bifido)bacteria to resist bile-imposed stress has been the focus of much research. Such previous studies have suggested that bile salt hydrolase (BSH) activity is involved in resisting the toxic bactericidal effects exerted by bile stress (19, 20). Various research groups have in fact linked the ability of probiotic bacteria to modify primary bile acid, which in humans is mostly composed of glycine- or taurine-conjugated cholic and chenodeoxycholic acids, with positive effects on the host (48, 49). These health benefits

range from influencing weight gain, lipid metabolism and cholesterol levels (21). However, much confusion still remains with regards to the function of the BSH-encoding gene in members of the *Bifidobacterium* genus and the factors which either induce or repress the expression of this gut-specific and possible probiotic activity. *In vitro* experiments in a number of bifidobacterial species, including *B. longum*, *B. animalis* and *B. breve*, suggest that the BSH-encoding gene is constitutively transcribed, rather than being induced by the presence of bile salts (22-25). However, a study carried out on the proteome of *B. longum* in the GIT of rabbits found that transcription of the *bsh* gene was increased under *in vivo* conditions, with the authors suggesting that BSH expression in this strain may be responding to factors other than bile (26).

Chapter 3 describes investigations on the environmental factors inducing the expression of the BSH-encoding gene in *B. breve* UCC2003 (designated as *bshB*) as a model species of its genus. Our findings indicate that *B. breve* UCC2003 transcribes *bshB* under ferrous and/or ferric iron starvation conditions, that *bshB* encoded on the genome of *B. breve* UCC2003 indeed encodes BSH activity and that this ability appears to confer protection against bile stress. These results indicate that iron limitation may act as an environmental signal to induce *bshB* transcription, consequently providing *B. breve* UCC2003 with increased resistance to bile salts. These findings greatly add to the knowledge regarding BSH activity, the factors which induce its activity, and the mechanisms employed by BSH-expressing bifidobacateria to survive in the gut environment.

As mentioned above, Chapters 2 and 3 contain experimental descriptions on the identification and characterization of genes that are important for survival of bifidobacteria under iron limitation and its associated impact on BSH expression and bile stress adaptation. Chapters 4 and 5 relate to investigations pertaining to the regulatory mechanisms employed by bifidobacteria to control its arsenal of carbohydrate uptake systems and its unique central carbohydrate metabolic pathway. The distal gut does not supply bacteria with reliable carbon sources; available carbohydrates are dependent on many factors such as host diet and other (competing) bacteria present in the GIT. This carbohydrate transience deems it necessary for bifidobacteria to utilise a multitude of carbon sources, and importantly to be capable of doing so swiftly and efficiently, making sure energy generation and biosynthetic demands are in balance, so as to (out)compete

(with) other GIT resident bacteria. Consequently, the regulation of carbohydrate uptake and central metabolism is of vital importance to the metabolic flexibility of bifidobacteria in the gastrointestinal tract and can be one mechanism by which bifidobacteria increase their competitiveness and survival in the gastrointestinal tract.

All members of the *Bifidobacteriaceae* family utilise a particular pathway for the metabolism of carbohydrates (the so-called phosphoketolase pathway or bifid shunt), typified by the presence of a phosphoketolase, called X5P/F6P phosphoketolase (XFPK), which is unique in exhibiting comparable affinities for both xylulose 5-P (X5P) and fructose 6-P (F6P) (27). In addition, this pathway and its superior ATP yield has been accredited with the ability of bifidobacteria to thrive in the highly competitive gastrointestinal environment. Recent studies have revealed that the XFPK enzyme, which is the keystone enzyme of this bifidobacterial pathway, is related to an enzyme produced by members of the *Coriobacteriales*. It has also been suggested that the XFPK-encoding gene was horizontally transferred between these two groups (28-30). Chapter 4 describes the characterisation of two LacI-type transcription factors (TFs), designated BifR1 and BifR2. The regulons controlled by these TFs were found to be very similar, and in particular include various genes that encode components of the bifid shunt. The dual ability of particular LacI-type TFs to either enhance or repress activity of their target promoters (based on the position of the TFBS) has also been reported in *Corynebacterium glutamicum*, which is also a Gram-positive, high GC-content organism (31). This study has revealed that BifR1 and BifR2 repress transcription of genes related to sugar uptake and activate transcription of genes related to central carbon metabolism, the opposite has been noticed for two LacI-type TFs in *C. glutamicum* (32). This regulatory mechanism governing central carbon metabolism employed by *Bifidobacterium* also differs from the carbon catabolite control systems reported for *E. coli* and *Bacillus* (33-35).

The findings reported in Chapter 4 reveal an apparently unique regulatory mechanism, perhaps as a result of its distinctive central carbohydrate metabolic pathway, in *Bifidobacterium* and possibly other related taxa such as *Corynebacterium*. Additionally, BifR1 and BifR2 were also linked with the ability of bifidobacteria to resist bile stress, thereby connecting control of central carbon flux to perhaps an enhanced energy demand for increased survival in the gastrointestinal environment.

Chapter 5 takes a focused view on four LacI-type TFs (designated MalR2, Malr3, MalR5 and MalR6) in *B. breve* UCC2003. These four TFs along with BifR2 had previously been predicted to be involved in the uptake, catabolism and subsequent cytoplasmic catabolism of various α-glucosidic-linked starch-like carbohydrates (36). Our EMSA and transcriptome analyses involving these four LacI-type regulators revealed a complex, interactive regulatory system in which *Bifidobacterium* (*breve*) apperas to control a variety of different carbon sources including β-glucosidic-linked carbohydrates and maltose.

One major conclusion that can be drawn from the work described in this thesis is that while these regulatory mechanisms may never have been explored without their initial bioinformatical investigation, experimental investigation and validation of these bioinformatical predictions remains essential to fully understand the precise function of regulatory mechanisms and their individual players in *Bifidobacterium* and other bacteria alike. Furthermore, experimental validation and exploration can bring to light interesting phenotypic implications of these regulatory mechanisms which could not have been predicted or may not have been observed otherwise. One obvious example of this is the increased resistance to bile stress which was reported for the *B. breve* UCC2003-*malR2* and *B. breve* UCC2003-*malR5* mutants (compared to WT). This increased resistance to bile stress was associated with an increased production of ethanol as an end product of fermentation. This again links the ability of bifidobacteria to control its central carbon flux with an increased ability to survive in the gut environment. This phenomenon is not unique to *B. breve* UCC2003 and has also been reported in *Lactobacillus* and *Bifidobacterium* where it is proposed that increased ethanol production results in more regenerated NAD+ to combat oxidative stress effects of bile (37-40).

Throughout this work *B. breve* UCC2003 has demonstrated its highly resourceful and metabolically flexible nature, coupled to its diverse and complex range of strategies to survive and compete in the gastrointestinal environment. Iron uptake and metabolism in the context *Bifidobacterium*, still remains an area of study in which many questions remain unanswered. The involvement of *bfeUO* and *sifABCDE* in conferring pathogen protection against *Salmonella* has been demonstrated in a nematode model, and

confirmation of this potential probiotic activity in a murine model would be beneficial. A number of genes have been identified to be important under iron limitation and starvation, examples of which are the two DPS proteins encoded on the *B. breve* UCC2003 genome. It would be particularly interesting to characterise these two enzymes in relation to their predicted ability to act as intracellular iron stores. Carbohydrate uptake and metabolism has enjoyed much research scrutiny in recent years, though regulation of the associated metabolic processes including its peculiar central carbon metabolism had as yet not been investigated. This work revealed that *B. breve* UCC2003 employs two LacI-type TFs, BifR1 and BifR2, which together govern the carbon flux through the central carbohydrate pathway. In addition, they appear to regulate various other local TFs (including MalR2, MalR3, MalR5 and MalR6) which in turn are involved in the uptake and utilisation of carbohydrate resources. This work has helped to reveal some of the mechanisms involved in the regulation of carbohydrate uptake and metabolism in *Bifidobacterium*. Nonetheless, many questions still remain and include, among others, 'what environmental signals are these TF responding to?', and 'are these signals distinct for both BifR1 and BifR2?', and 'why are two very similar LacI-type regulators required?'.

Together these results lead to a better understanding of *B. breve* UCC2003 response to the ever-changing conditions of the gastrointestinal tract and help to shed light into the complex network of gene regulation which *B. breve* UCC2003 utilises.

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